Conventional Measurements of Sulfur Dioxide (SO_2) in Red Wine Overestimate SO_2 Antimicrobial Activity

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Abstract: Conventional approaches to measuring sulfur dioxide (SO_2) in wine, such as aeration-oxidation, iodometric titration, and flow-injection analysis, are known to overestimate molecular SO_2, particularly in red wine because of the dissolution of weak anthocyanin-bisulfite complexes during analysis. Methods for determining molecular SO_2 without perturbing anthocyanin-bisulfite complexes and other weak adducts exist, e.g., headspace gas-detection tube measurements. However, it is unclear whether SO_2 values achieved through conventional methods ("Molecular SO_2") or nonperturbing methods (molecular SO_2) provide a better measure of antimicrobial activity. In our work, white and pseudo-red wines were prepared with varying additions of SO_2; the red wine was produced by spiking the white wine with an anthocyanin extract. "Molecular SO_2" and molecular SO_2 concentration in white wines were well correlated, but "Molecular SO_2" was significantly higher in red wines. Wines were inoculated with *Saccharomyces cerevisiae* strain EC1118 (Lallemand), and viability and culturability were evaluated at regular intervals. Both culturable and viable cell counts decreased significantly for treatments with 0.5 to 2.0 mg/L molecular SO_2 in the white and red wines, and for 0.5 to 2.0 mg/L "Molecular SO_2" in the white wine, but concentrations >2.0 mg/L "Molecular SO_2" were necessary to decrease cell counts in the red wine. These results indicate that anthocyanin-bisulfite complexes have negligible antimicrobial activity, and that conventional approaches to measuring "Molecular SO_2" are poorly suited to predicting the microbial stability of red wines.

Key words: apparent SO_2, challenge study, flow cytometry, sulfur dioxide, true SO_2, wine stability

Sulfur dioxide (SO_2) has been used since at least the end of the 18th century as a wine preservative because of its antioxidant and antimicrobial effects (McGovern 2003). Although trace amounts of SO_2 are produced by yeast during fermentation, the majority of SO_2 in wine is intentionally added by winemakers. Because the first logarithmic acid dissociation constant of SO_2 (pK_a = 1.81 in H_2O at 20°C) is low compared with wine pH, the major SO_2 species at wine pH is bisulfite (HSO_3^-), and less than 5% of SO_2 typically exists in its neutral, so-called *molecular* form (Waterhouse et al. 2016b). The sum of HSO_3^- and molecular SO_2 is referred to as *free* SO_2 (Waterhouse et al. 2016b). A portion of HSO_3^- in wine will also exist in the form of covalent adducts with wine nucleophiles, e.g., acetaldehyde. These *bound* SO_2 forms have diminished preservative activity, but are counted along with free SO_2 as part of *total* SO_2. The antimicrobial activity of SO_2 is primarily due to its molecular form, putatively because this neutral species can readily diffuse across the cell membrane (Divol et al. 2012).

Because the molecular SO_2 species is volatile and perceived as "burning" or "irritating" at high concentrations (sensory threshold = 2 mg/L) (Waterhouse et al. 2016b), and because total SO_2 concentrations are regulated in most winemaking countries, defining appropriate minimum molecular SO_2 concentrations is of importance to winemakers. Specific recommendations vary with challenge study conditions and microorganism, and have been summarized elsewhere (Boulton et al. 1999). For example, for *Saccharomyces* (a major spoilage risk for sweet wines), decreasing cell counts from 5×10^4 cfu/mL to 1 or fewer cfu/mL after 24 hr at room temperature requires 1.41 to 1.74 mg/L molecular SO_2 across *Saccharomyces cerevisiae* strains, whereas decimal (10-fold) reduction requires 0.24 to 0.32 mg/L molecular SO_2, respectively (King et al. 1981). This previous work was performed in growth media, and other recommendations include 0.825 mg/L molecular SO_2 for *S. cerevisiae* control, according to studies in model wine (10% ethanol) (Beech et al. 1979), or up to 1.55 mg/L molecular SO_2 to reduce *Saccharomyces bayanus* to undetectable levels in white wines supplemented with 36 g/L sugars and adjusted to different pH levels and ethanol concentrations (Sudraud and Chauvet 1985).

Because of the range of different literature values, wine production textbooks may give a range of values, for example, 0.5 to 0.8 mg/L (Margalit and Crum 2004), with higher values recommended for wines at greater risk for spoilage, such as sweet wines. A complication to determining appropriate molecular SO_2 recommendations is that molecular SO_2 is not

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Acknowledgments: The authors gratefully thank ETS Laboratories, St. Helena, CA, for collaboration and analytical support. Additional thanks to Scott Laboratories (Petaluma, CA) for the donation of yeast and bacteria and to Polyphenolics (Madera, CA) for the donation of anthocyanin powder. Funding was from the Peter and Tacie Saltonstall Endowment and the New York Wine & Grape Foundation. The authors have no conflicts of interest.

Supplemental data is freely available with the online version of this article at www.ajevonline.org.

Manuscript submitted Mar 2017, revised Oct 2017, Jan 2018, accepted Feb 2018

doi: 10.5344/ajev.2018.17037

directly measured. Instead, free SO_2 and pH are measured, and molecular SO_2 is calculated using a modified Henderson-Hasselbalch equation (Waterhouse et al. 2016b). One problem with this approach is that the pK_a value of SO_2 depends on ethanol concentration, temperature, and ionic strength of the wine. These factors can have considerable consequences, as typical table wines with 11 to 14% alcohol by volume will have a pK_a = 1.9 to 2.1 at 20°C, compared with 1.81 in pure water (Usseglio Tomasset and Bosia 1984).

A potentially larger issue to establishing appropriate SO_2 targets is that conventional analytical approaches to free SO_2 measurement, such as flow-injection analysis, aeration-oxidation (A-O), and iodometric titration, all involve an initial acidification and dilution step. These approaches can result in disruption of weakly bound bisulfite adducts, resulting in artifactually high concentrations of free SO_2, and therefore molecular SO_2. This effect is particularly noticeable in red wines because of dissociation of anthocyanin-bisulfite complexes (Burroughs 1975). Although formation of adducts between the cationic form of anthocyanins (flavylium ion) and HSO_3^- is strongly favored (with a low dissociation constant of $K_d = 1 \times 10^{-5}$ for malvidin-3-glucoside) (Brouillard and El Hage Chahine 1980), the half-life for dissociation is on the order of minutes (Brouillard and El Hage Chahine 1980), comparable to the time necessary for conventional SO_2 analyses (Coelho et al. 2015). In a recent study, Waterhouse et al. (2016a) referred to the sum of free SO_2 and weakly bound SO_2 measured by conventional analyses as "Free SO_2", in quotes, to distinguish it from the true amount of free SO_2. We will adopt this convention in this paper, along with the analogous concept of "Molecular SO_2" as compared with molecular SO_2.

The measurement of molecular SO_2 instead of "Molecular SO_2" can be achieved by using nonperturbing methods that avoid changes to wine composition. For example, gas-detection tubes (GDT) can be used to measure headspace SO_2 concentrations in an equilibrated sample, and molecular SO_2 can then be calculated using Henry's law (Coelho et al. 2015). Molecular SO_2 concentrations measured by headspace GDT (HS-GDT) in red wines were, on average, only 32% that of molecular SO_2 measured by A-O. The discrepancy between the two analytical methods could be modeled as a function of anthocyanin concentration and pH. By comparison, GDT measurements of white and rosé wines yielded molecular SO_2 concentration that averaged 86% of A-O values (Coelho et al. 2015). Similar conclusions have been reached using other nonperturbing methods such as headspace gas chromatography (Davis et al. 1983).

"Free SO_2" measurements of red wines are still fit for purpose if the weakly bound SO_2 has antimicrobial activity comparable to that of free SO_2. This assumption has justification, since strongly bound acetaldehyde-bisulfite complexes are reported to have activity against lactic acid bacteria (Wells and Osborne 2012), either because the adducts will release free HSO_3^- following metabolism of acetaldehyde, or because the acetaldehyde-bisulfite complex is itself inhibitory. However, similar antimicrobial effects of strongly bound SO_2 on yeast are less pronounced; for example, acetaldehyde-bisulfite (160 mg/L as SO_2) showed no effect on *Brettanomyces bruxellensis* viability (measured by direct epifluorescence filter technique) and survival (by plate counts) in a red wine over a two day period (Du Toit et al. 2005).

To our knowledge, an evaluation of the antimicrobial activity of anthocyanin-bisulfite adducts has been reported only once in the literature. Usseglio-Tomasset and colleagues reported that the presence of anthocyanin-bisulfite complexes (30 mg/L as SO_2) delay fermentation by *S. cerevisiae*, *S. bayanus*, *Saccharomyces uvarum*, and *Saccharomycodes ludwigii* compared with an unsulfited control, but this delay is less pronounced than that observed with additions of free SO_2; the authors concluded that anthocyanin-bisulfite adducts retain some antiseptic activity (Usseglio-Tomasset et al. 1982). However, survival was not evaluated, for example, by growth on selective media. Moreover, molecular (or "Molecular") SO_2 concentrations were not measured during the fermentation, so it is unclear whether initial differences in SO_2 among treatments persisted.

In summary, current literature reports are ambiguous as to whether anthocyanin-bisulfite adducts possess antimicrobial activity and, thus, whether inadvertent measurement of these adducts in conventional "Free SO_2" measurements is justified. In this study, we compared the validity of conventional (A-O) and nonperturbing (HS-GDT) measurements of molecular SO_2 for predicting *S. cerevisiae* viability in challenge studies using wines with varying SO_2 additions, and in the presence or absence of anthocyanins.

Materials and Methods

Chemicals. Potassium metabisulfite (97% [w/w]) and ethanol (95% [v/v]) were obtained from Acros Organics. 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. A nominally 25% phosphoric acid solution was prepared as a 2.38:1 dilution of 294 mL phosphoric acid (85%) with 700 mL deionized water. Hydrochloric acid (36.5% [w/w]) was obtained from BDH Merck.

SO_2 working standards. SO_2 stock solutions at nominal concentrations of 10 g/L as SO_2 were prepared weekly by dissolution of potassium metabisulfite in a solution of 10% (v/v) ethanol in water to avoid SO_2 autooxidation. Analysis by A-O was used to confirm the concentration in the stock and working solutions (Iland 2004).

SO_2 measurements by A-O. During the experiments, conventional analyses of "Free SO_2" and total SO_2 were performed by A-O, as described elsewhere (Iland 2004). To compare "Molecular SO_2" with the HS-GDT molecular SO_2 value, "Free SO_2" for each wine was converted to "Molecular SO_2" using a pK_a value (2.09) calculated on the basis of the alcohol content (11.8%), HS-GDT measurement temperature (23°C), and an assumed wine ionic strength of 0.05 M, using equations described elsewhere (Usseglio Tomasset and Bosia 1984). The reported detection limit for "Free SO_2" by A-O is 2 mg/L (Iland 2004), which equates to a "Molecular

SO_2" detection limit of 0.06 mg/L at the pK_a value of the tested wines.

SO_2 measurements by HS-GDT. HS-GDT measurements of headspace SO_2 concentrations were performed using Gastec 5Lb GDT tubes (Gastec Corporation), and free SO_2 concentrations were calculated, based on a protocol described elsewhere (Coelho et al. 2015). Two hundred mL of headspace was sampled for each analysis. According to the minimum detectable length of stain detectable on the GDT (0.3 mm), the limit of detection was estimated to be 0.03 mg/L molecular SO_2, which would equate to a free SO_2 detection limit of 1 mg/L for the pK_a value (2.09) of the tested wines.

Wines. A 2013 commercial, "no sulfites added" white wine (45% Riesling, 24% Müller Thurgau, 17% Muscat Canelli, and 14% Chenin blanc; all grapes were *Vitis vinifera*, Columbia Valley AVA, WA) was used in all trials. Wines with identical lot numbers and bottling dates were used within each biological replicate. To produce a "red wine" with a basic composition similar to that of white wine, 2 g/L of grape anthocyanin powder (21.2% anthocyanin by weight) (Polyphenolics) was added to the control white wine. Basic wine compositional parameters are reported in Table 1 and were measured at ETS Labs (St. Helena, CA) by ISO 17025–accredited methods. Analyses of monomeric anthocyanins, polymeric anthocyanins, and tannins were performed by high-performance liquid chromatography (HPLC) using methods described elsewhere (Waterhouse et al. 1999).

Table 1 Compositional data for the original white and "red" wines produced by addition of commercial anthocyanin powder.

Wine component	White wine	"Red wine"
Ethanol (% [v/v] at 20°C)	11.8[a]	
Total SO_2 (mg/L)	<5	7
"Free SO_2" (mg/L)	<2	
pH	3.56	
Titratable acidity (g/L as tartaric acid)	4.9	
Malic acid (g/L of malic)	<0.05	
Volatile acidity (g/L as acetic acid)	0.34	
Glucose + fructose (g/L)	2.2	
Monomeric anthocyanins (mg/L as malvidin-3-glucoside)	n.d.[b]	458
Tannins (mg/L as catechin)	n.d.	299

[a]In rows with a single value, the variable did not significantly differ between the "red" and white wines.
[b]n.d.: not detectable.

Experiment A: challenge experiment with varying SO_2 additions. An overview of this experiment is depicted in Figure 1. Prior to SO_2 adjustments, both white and "red" wines were sterile-filtered by 0.2 µm polyethersulfone Nalgene Rapid-Flow disposable filter units (Thermo-Fisher). Total SO_2 was then adjusted by addition of the 10 g/L stock SO_2 solution. For both wines, SO_2 was added to 1 L samples to yield the following concentrations: 0 (control), 22, 32, 44, 56, and 67 mg/L. For "red wines", an additional five 1 L treatments were prepared at the following SO_2 levels: 78, 92, 104, 114, and 128 mg/L. An additional 67 mg/L sample was prepared for both wines for use as an uninoculated control. "Free" and "Molecular SO_2" were determined in each wine by A-O, and free and molecular SO_2 were determined by HS-GDT, as described above.

Yeast/mold media. Yeast/mold (YM) broth media (Difco-BD) were prepared from 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, and 10 g/L dextrose; YM agar was prepared identically to the YM broth, except that 21 g/L agar

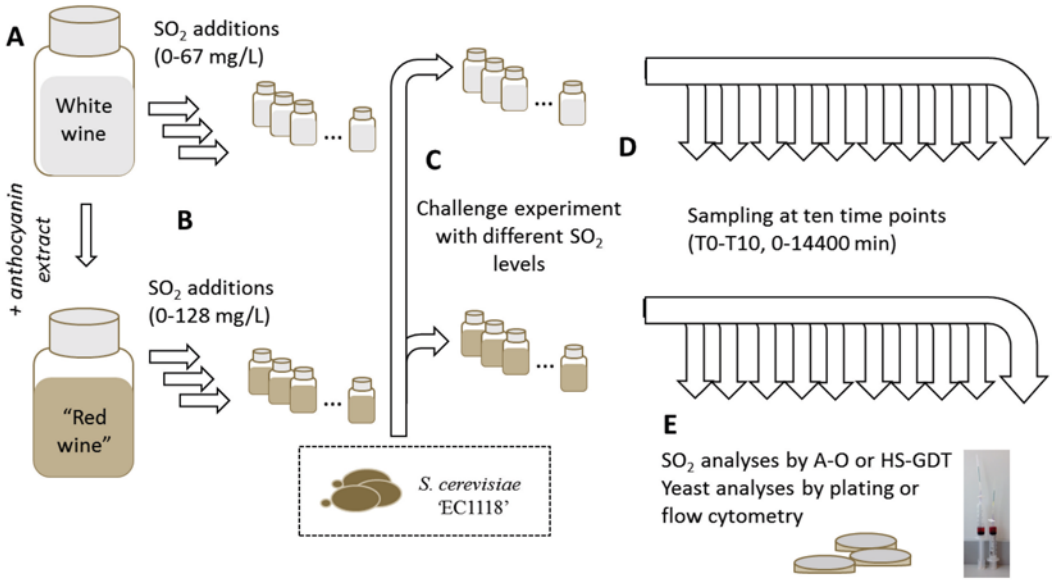

Figure 1 Overview of the experiment A design. **(A)** A commercial white wine with no detectable SO_2 was spiked with an anthocyanin extract to produce a "red wine". **(B)** Both wine types were spiked with varying amounts of SO_2 to generate wines encompassing a range of SO_2 concentrations. **(C)** Wines were then inoculated with *Saccharomyces cerevisiae* EC1118 as part of a challenge experiment. **(D)** Samples were collected at regular intervals **(E)** to determine yeast viability and survival (plating, flow cytometry) and SO_2 (total by aeration-oxidation [A-O], "Free" by A-O, and molecular by headspace gas-detection tubes [HS-GDT]). Subsequently, "Molecular SO_2" was calculated from "Free SO_2", and free SO_2 from molecular SO_2 using the Henderson-Hasselbalch equation. "Red" and white wine samples with similar molecular SO_2 or with similar "Molecular SO_2" are listed in Table 3.

(Difco-BD) was also added, and heat was used to facilitate dissolution. Both broth and agar media were sterilized by autoclaving for 15 min.

SO_2 challenge experiments. Ethanol-conditioned yeasts were prepared twice, that is, before each set of replicate challenge experiments. One gram of *S. cerevisiae* strain EC1118 (Scott Laboratories) was added to 10 mL sterile water at 40°C for 30 min, with gentle agitation by hand after 15 min. The rehydrated sample was added to 125 mL sterilized 100% strength YM broth and agitated by platform rotary shaker at 20 to 25°C for 4 to 12 hr. This protocol was repeated three times, using broth with increasing alcohol concentrations (4, 8, and 12% [v/v]) and decreasing YM concentrations (76, 38, and 19% of full-strength YM broth). Yeast were collected by centrifugation for 10 min at 6000 rpm, washed twice with 0.1% peptone, consolidated into one tube, and refrigerated at an approximate concentration of 10^8 cfu/mL under peptone until use.

Ethanol-conditioned yeasts were then inoculated into each 1 L wine treatment at target rates of 10^6 cells/mL and mixed by gentle agitation and inversion; flasks were loosely capped. The flasks were stored in the dark except during sampling. The challenge experiment took place at room temperature (~22°C). Aliquots of each treatment were sampled prior to inoculation (T0) and at 10 time points (T1 to T10) after inoculation throughout the experiment for microbial analysis, and in some cases SO_2 analyses, out to a maximum of 14,400 min (10 days) (Table 2). The same bottles were used and repeatedly sampled throughout the experiment. At each of the sampling times (T0-T10), 62 mL was drawn, for a total of 682 mL removed. The total liquid volume was 1 L at the start of fermentation, with ~100 mL headspace at T0, and the final headspace volume was ~782 mL after the T10 sampling. As discussed below, the increase in headspace may have resulted in nonenzymatic loss of SO_2 by the last time point (T10). After sampling, SO_2 was removed or inactivated, as previously described (Johnston et al. 2002). One mL of sample was added to 9 mL of 0.1% peptone (resulting in pH = 3.9), and SO_2 activity was quenched by the combined effects of dilution and pH shift.

Table 2 Sampling time points.[a]

Time point	Minutes after inoculation	SO_2 analyses	Microbial survival (plating) or viability (flow cytometry)
T0	0 (just prior)	X	X
T1	8	X	X
T2	16		X
T3	24		X
T4	60		X
T5	120		X
T6	240		X
T7	480	X	X
T8	960		X
T9	1920	X	X
T10	14,440	X	X

[a]An "X" indicates that either SO_2 or microbial analyses were performed at the time point.

Survival assessed by plating on YM agar. Treatments were mixed prior to sampling by vigorous shaking. For the first biological replicate, a 4 mL sample was transferred directly into 16 mL of 0.1% peptone for a 5-fold dilution and serially repeated for 200- and 2000-fold dilutions. Samples were vortexed for 5 sec between dilutions or prior to plating. Plating was done using the drop-plate method (Herigstad et al. 2001), with half a dilution series (10^0 and 10^1, or 10^2 and 10^3) duplicated once per plate. Samples were incubated at 25°C and read between 48 and 72 hr. Counts between 10 and 200 cfu per 100 µL were used for calculating cell concentrations in the original treatment.

Viability assessed by flow cytometry. Flow cytometer staining buffers were prepared with 0.2% Pluronic F68 (BASF Corporation) and 1 mmol/L EDTA (Sigma) in phosphate-buffered saline (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, and 0.24 g/L KH_2PO_4) tablets (Sigma-Aldrich), adjusted to pH 7.4 with HCl, and filtered on Nalgene Rapid-Flow sterile, disposable filter units through a 0.2 µm polyethersulfone membrane (Thermo-Fisher). Thiazole orange (TO, "live/dead" stain) at 42 µmol/L in dimethyl sulfoxide and propidium iodide (PI, "dead" stain) at 4.3 mmol/L in water were obtained from Becton, Dickinson and Company. One hundred µL of sample was added to 400 µL of staining buffer, with 5 µL of each TO and PI (for a final concentration of 420 nmol/L TO and 42 µmol/L PI), mixed, and allowed to develop for 10 min in the dark.

Samples from the unsulfured white control wines were used as yeast controls. Dead yeast controls were produced through two methods. Heat-killed samples were made using 1 mL of live culture subjected to 10 min in a 90°C water bath. Alcohol-killed samples were produced by adding 1 mL of culture to 3.0 mL of 95% ethanol (final concentration 71%) for 10 min. Additionally, high level SO_2-treated samples were made by adding 1 mL of live culture to 1 mL of 1 g/L SO_2 solution (for a final concentration of 500 mg/L) for 10 min.

Cell counts and cell viability were then measured on an Accuri C6 flow cytometer (BD Biosciences) with a 488 nm laser and equipped with a 24 sample plate. A forward scatter threshold was set at fewer than 80,000 arbitrary units. Ultrapure HPLC-grade water (Alpha Aeser) with bacteriostatic solution (BD Biosciences) was used as sheath fluid. CFlow Plus software (BD Biosciences) was used for collection and evaluation. Samples were gated on yeast from the unsulfured white wine sample, using forward and side scatter, and viability was evaluated using FL1 (530 BP) and FL3 (670 LP) after staining with TO (emission 530 nm) and PI (emission at 617 nm), using killed yeast samples to confirm dead gates. No color compensation was used. Sample size varied from 25 to 100 µL, depending on culture concentration. Viability was determined in replicate at each time point.

Experiment B: confirmation experiment. To confirm the results of experiment A, a follow-up challenge experiment with a limited number of SO_2 treatments was performed to generate a range of "Molecular" and molecular SO_2 concentrations. Sterile-filtered wines were prepared as

described above, and SO_2 was added at the following levels: 0 mg/L (white, control), 74 (white), 0 (red), 74 (red), and 142 (red). "Free" and "Molecular SO_2" were determined in each wine by A-O, and free and molecular SO_2 were determined by HS-GDT, as described above. *S. cerevisiae* EC1118 inocula were prepared as described above, with the exception that the ethanol-acclimated yeast was stored in 0.2 µm-filtered, sulfite-free control white wine until inoculation. After inoculation, the wines were stored at room temperature for 960 min (equivalent to T8 in experiment A). To quench the effect of SO_2 prior to plating analyses, 50 mL of each sample was centrifuged for 10 min at 6000 rpm, the supernatant wine sample carefully removed, and the pellet washed and resuspended in 50 mL of 0.2 µm-filtered control white wine (Johnston et al. 2002). Survival was then evaluated by duplicate plating, as described above, with the following exception of the dilution step size. In this experiment, 1 mL of sample was added to 9 mL of 0.1% peptone for a 10-fold dilution, and serially repeated for 10^2 and 10^3 dilutions.

Calculating log reductions and death curves. Viability counts in cfu/mL for plating (Vp) and in events/mL for flow cytometry (V_{fc}) at each time point (T) were converted to log values. The log values for replicates of both wine controls (no SO_2 added) were averaged at each time point, and this value was subtracted from the sample value to generate a log reduction value for each sample (Equation 1).

$$\text{Log reduction} = \log(\text{Sample V})_T - \overline{\log(\text{Control V})_T} \quad \text{Eq. 1}$$

Statistical analyses. Minitab v. 16 (Minitab Inc) was used for statistical analyses. Paired Student's *t* tests were used to evaluate whether differences existed in survival or viability between the molecular SO_2 and "Molecular SO_2" measurements for each wine type. Student's *t* tests were also used to evaluate whether different levels of molecular SO_2 or "Molecular SO_2" (low, medium, or high) resulted in significant decreases in survival compared with the unsulfited control. Statistical significance was defined as $p < 0.05$.

Results

An initial goal of this study was to generate two wines for challenge studies with near-identical compositions and molecular SO_2, but differing in "Molecular SO_2", i.e., the apparent molecular SO_2 based on conventional SO_2 measurements that include some weakly bound SO_2 forms. To create control and treated wines differing in weak SO_2 binders, we selected a base white wine with negligible "Free SO_2" and total SO_2 (<2 and <5 mg/L, respectively, Table 1) and spiked it with a commercial anthocyanin extract. Both wines were then analyzed for monomeric anthocyanin, polymeric anthocyanin, and tannin by an HPLC method. Monomeric anthocyanins were not detectable in the white wine, but were present in the "red wine" at 458 mg/L as malvidin-3-glucoside equivalents. The monomeric anthocyanin concentration in our "red wine" was higher than in typical commercial red wines, which would have undergone aging and lost monomeric anthocyanins through formation of polymeric pigment and other reactions, but were within the ranges observed in newly fermented wines (Monagas et al. 2006). Tannins were detectable only in the "red wine" (299 mg/L catechin equivalents). The presence of tannins in this wine presumably resulted from impurities in the anthocyanin extract, but the tannin concentration was still at the low end of a typical commercial red wine (Harbertson et al. 2008). "Free SO_2" was still undetectable after the anthocyanin addition, but total SO_2 (7 mg/L) exceeded the detection limit, possibly because of trace amounts of bound SO_2 in the anthocyanin extract.

SO_2 was then added at varying concentrations to each wine to generate wines with "Molecular SO_2" (determined with A-O) and molecular SO_2 (determined with HS-GDT) ranging from undetectable to at least 1.0 mg/L. The resulting "red" and white wines had similar total SO_2 concentrations for a given SO_2 addition (Supplemental Figure 1A). Determination of "Molecular SO_2" involved initial measurement of "Free SO_2", and subsequent calculation of "Molecular SO_2". Our calculations accounted for effects of temperature, ionic strength, and ethanol on the acid dissociation constant, resulting in pK_a values of 2.09, compared with the more common value of 1.81, based on the pK_a in water at 20°C.

Challenge studies were performed in which a commercial wine yeast (*S. cerevisiae* strain EC1118) was inoculated into each of the SO_2-adjusted wines and the control. Samples were taken at 10 time points over 10 days, with microbial analyses performed for all time points and SO_2 analyses performed for some time points (Table 2). No significant change in total, "Free", or free SO_2 (Supplemental Figure 1A to 1E) occurred over the first 32 hrs (up to T9) for any treatment level, but total SO_2 for a given SO_2 addition level was significantly lower at 10 days for "red wine" with ≥32 mg/L SO_2 (Tukey's test, $p < 0.05$). Because the decrease in total SO_2 was not significantly different between the 67 mg/L challenge treatment and the uninoculated control, this decrease was attributed to chemical oxidation, likely resulting from repeated opening of bottles for sampling. To avoid confounding effects of the loss of SO_2 during the experimental course, data on yeast viability and survival at t = 10 days (T10) were not used.

"Molecular SO_2" and molecular SO_2 for the T0 to T9 time points in the white wine as a function of SO_2 addition are shown in Figure 2 (left plot). The proportional increase in "Molecular SO_2" and "Free SO_2" for the lowest addition level (22 mg/L) was smaller than the increase observed with subsequent additions, presumably because of formation of adducts with SO_2 binders present in the original wine, e.g., acetaldehyde or other carbonyls (Waterhouse et al. 2016b). "Molecular SO_2" and molecular SO_2 did not significantly differ for SO_2 additions <50 mg/L, but molecular SO_2 was significantly greater for the highest addition levels (56 and 67 mg/L). For the white wine, molecular and "Molecular" SO_2 >0.8 mg/L could be achieved through addition of 67 mg/L SO_2 (Figure 2), with similar results observed for "Molecular SO_2" in the "red wine". However, greater additions of SO_2 (114 mg/L or more) were necessary to achieve >0.8 mg/L molecular SO_2 in the "red wine" at T0 (Figure 2). The largest absolute difference between molecular and "Molecular" SO_2 in the

"red wine" was observed for an addition of 92 mg/L SO_2, which resulted in molecular SO_2 = 0.38 mg/L and "Molecular SO_2" = 1.78 mg/L. Similarly, SO_2 additions of 44 mg/L to the "red wine" were necessary to have >0.6 mg/L "Molecular SO_2" (a typical winemaking target), compared with an SO_2 addition of 104 mg/L to have >0.6 mg/L molecular SO_2. "Molecular SO_2" was also slightly higher (average = 0.2 mg/L "Molecular SO_2", or ~5 mg/L "Free SO_2") in the "red wine" than in the white wine for SO_2 additions of 22 to 67 mg/L (paired t test, $p < 0.05$). The reason for this was unclear, but this possibly occurred because the added anthocyanins partially reacted with other SO_2-binding nucleophiles in the time between addition and the SO_2 measurement.

An analogous discrepancy was observed for "Free SO_2" (Supplemental Figure 1C) and free SO_2 (Supplemental Figure 1E), in that higher SO_2 additions were necessary for the "red wine" than the white wine to achieve similar free SO_2 levels, but no differences were observed among wine "Free SO_2" values for the same SO_2 addition. Total SO_2 did not significantly differ between red and white wines at a given SO_2 addition level (Supplemental Figure 1A).

Yeast survival at T0 to T9 (0 to 1920 sec) during the challenge study were determined by serial dilution and plating onto YM media. Changes in yeast counts (log cfu/mL) with respect to the 0 mg/L SO_2 control were then calculated, and these changes were plotted as a function of time for each SO_2 addition level (Figure 3). Data from T0 to T4 (0 to 63 sec) highly varied because of difficulties in quenching the SO_2 quickly, so these data were averaged prior to plotting. SO_2 additions were less effective in decreasing survival in "red wines" then in white wines. For example, in the white wine (Figure 3, left), decimal decreases (1 log or greater) were observed for all time points at or after 240 sec (T6) for SO_2 additions ≥56 mg/L, and for ≥44 mg/L at the final time point (1920 sec, T9). However, in the "red wine" (Figure 3, right), SO_2 additions of at least 104 mg/L were necessary to observe a decimal reduction for any time point. We observed an increase in viability for the 104 and 114 mg/L (but not 128 mg/L) "red wine" treatments between 480 and 1920 min, presumably because the yeast were initially rendered nonviable, but then recovered and grew at these intermediate SO_2 concentrations.

Using the data in Figure 2, we identified red and white treatments with similar molecular SO_2 and "Molecular SO_2" (Table 3). For example, the 44 mg/L SO_2 addition to white wine and 92 mg/L SO_2 addition to "red wine" both yielded ~0.37 mg/L molecular SO_2 and were treated as one pair, and the 44 mg/L SO_2 addition to white wine and 32 mg/L SO_2 addition to "red wine" yielded ~0.43 mg/L "Molecular SO_2" as a different pair.

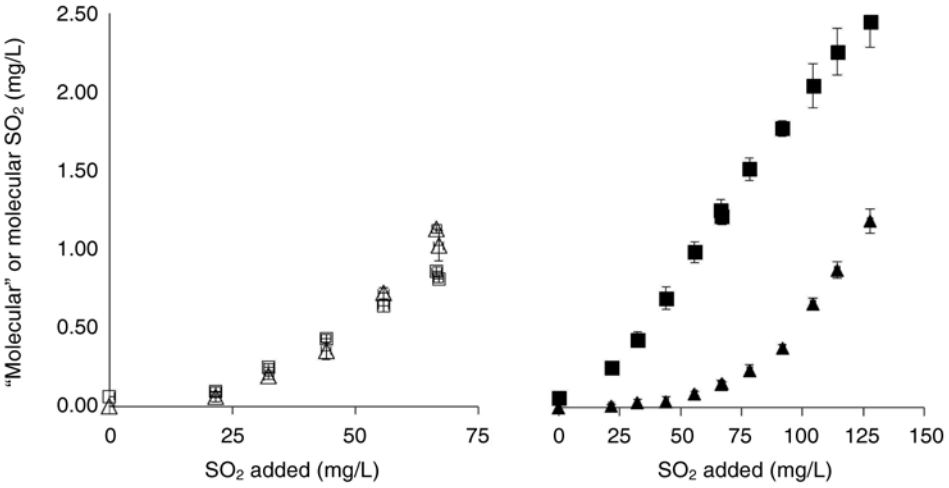

Figure 2 Experiment A. "Molecular SO_2" (measured by aeration-oxidation, squares) and molecular SO_2 (measured by headspace gas-detection tubes, triangles) as functions of added SO_2 for the white wine (left plot, open markers) and "red wine" (right plot, shaded markers). Values are averages over 10 time points (T0 to T9); error bars represent one standard deviation.

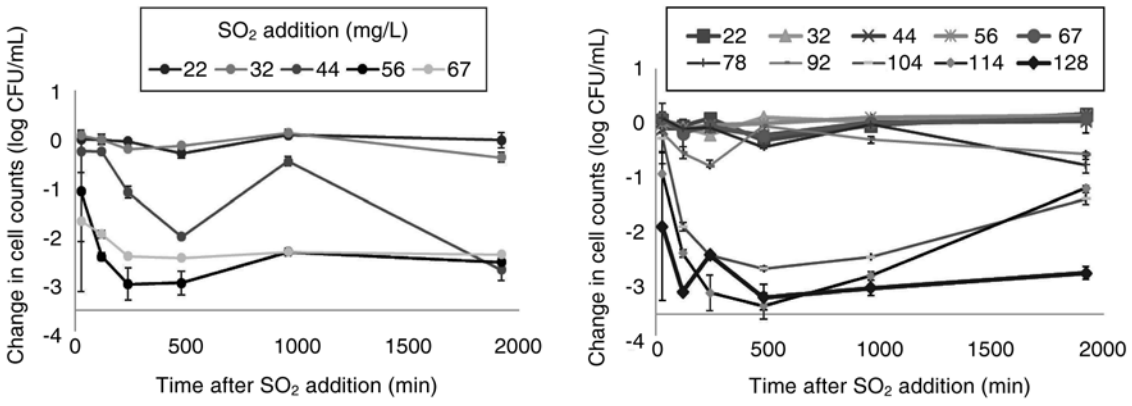

Figure 3 Experiment A. Change in cell counts (log cfu/mL) over time relative to the unsulfited control for different levels of SO_2 addition to white (left) and "red" wines (right).

The maximum decrease in yeast survival occurred at the final time points, T6 to T9 (240 to 1920 sec, Figure 3). To evaluate whether differences in SO_2 addition effects in red and white wines were statistically different, average log reduction at T6 to T9 was calculated for each pair listed in Table 3, and plotted as a function of either molecular SO_2 or "Molecular SO_2" (Figure 4). Pairs with similar molecular SO_2 (Figure 4, left) did not differ significantly in survival between red and white wines, but pairs with similar "Molecular SO_2" did differ significantly (paired *t* tests, $p > 0.05$). For example, a 2-log reduction in survival was observed for the 0.67 mg/L molecular SO_2 red and white wine pair. The 0.67 mg/L "Molecular SO_2" white wine also had a more than 2-log reduction in survival, but the "red wine" with 0.69 mg/L "Molecular SO_2" had no significant decrease in survival.

SO_2 treatments were then divided into categories of low (<0.5 mg/L molecular SO_2 or "Molecular SO_2"), medium (0.5 to 2.0 mg/L), or high (>2.0 mg/L). For both low molecular and "Molecular SO_2", we observed no significant decrease in the average survival at T6 to T9 for either wine type. For the medium SO_2 levels, we observed a significant decrease ($p < 0.05$, *t* test) in survival compared with that in the control for the white wine based on either molecular or "Molecular SO_2", and for the "red wine" based on molecular SO_2, but no significant decrease based on "Molecular SO_2". None of the treatments had molecular SO_2 in the high category, but "red wines" with high "Molecular SO_2" gave significantly lower survival than the control.

SO_2-treated wines were also characterized by flow cytometry to selectively measure viable cells (as opposed to surviving cells measured by plating). Results for matched pairs of red and white wines were similar to those seen with survival, in that pairs with similar molecular SO_2 gave similar decreases in viable cells compared with the untreated control, but pairs with similar "Molecular SO_2" did differ significantly in viability (data not shown). SO_2 treatments were again divided into

Table 3 SO_2-treated red and white wines organized as matching pairs with similar concentrations of either molecular SO_2 (left four columns) or "Molecular SO_2" (right four columns).[a]

Molecular SO_2, category	Nominal molecular SO_2 for paired comparison	White wine (added SO_2, molecular SO_2)	Red wine (added SO_2, molecular SO_2)	"Molecular SO_2", category	Nominal "Molecular SO_2" for paired comparison	White wine (added SO_2, "Molecular SO_2")	Red wine (added SO_2, "Molecular SO_2")
Control	0.00	0, 0.00	22, 0.01	Control	n/a	0, 0.06	
Low	n/a[b]		32, 0.03	Low	0.08	22, 0.10	0, 0.06
Low	0.05	22, 0.06	44, 0.04	Low	0.25	32, 0.25	22, 0.26
Low	n/a		56, 0.09	Low	0.43	44, 0.43	32, 0.43
Low	n/a		67, 0.15	Medium	0.67	56, 0.64	44, 0.70
Low	0.22	32, 0.20	78, 0.24	Medium	0.93	67, 0.86	56, 0.99
Low	0.37	44, 0.36	92, 0.38	Medium	n/a		67, 1.26
Medium	0.69	56, 0.72	104, 0.66	Medium	n/a		78, 1.52
Medium	n/a		114, 0.88	Medium	n/a		92, 1.78
Medium	1.16	67, 1.13	128, 1.19	High	n/a		104, 2.05
				High	n/a		114, 2.27
				High	n/a		128, 2.46

[a]Treated wines were also categorized according to molecular or "Molecular" SO_2 concentration: low (<0.5 mg/L), medium (0.5 to 2.0 mg/L), or high (>2.0 mg/L).
[b]n/a: no matching pair for the row.

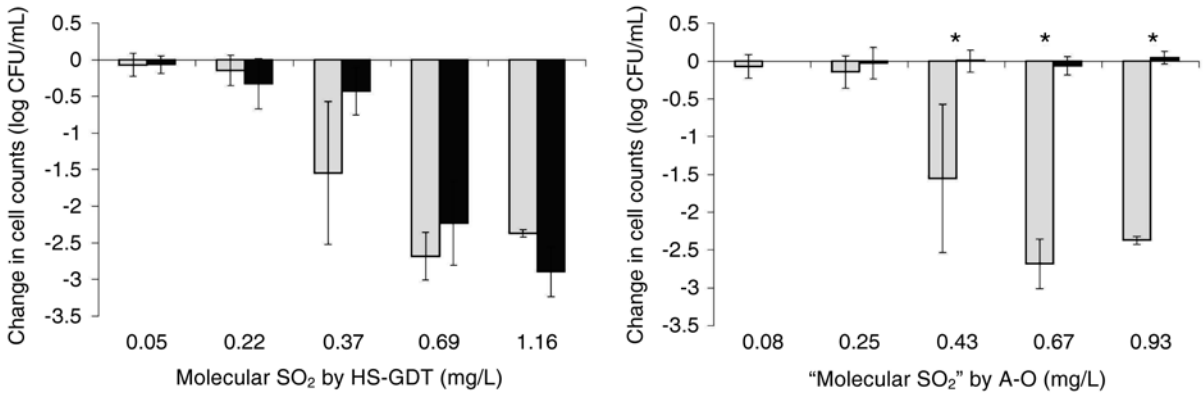

Figure 4 Experiment A. Change in cell counts (log cfu/mL) relative to SO_2-free control for pairs of white wines (light bars) and "red" wines (dark bars). The treatment pairs were matched for either molecular SO_2 (left) or "Molecular SO_2" (right), as described in Table 3. Cell counts were calculated as the average of the time points T6 to T9; error bars represent standard deviations of these measurements. *: significant difference in cell count change between "red" and white wines at a given SO_2 level ($p < 0.05$). HS-GDT, headspace gas detection tubes; A-O, aeration-oxidation.

categories (low, medium, and high), and average decreases at T6 to T9 in viable cells are shown in Figure 4 (right). Medium levels of molecular SO_2 levels significantly decreased viability ($p < 0.05$, t test) in both red and white wines, but no significant decrease was observed for "red wines" with medium "Molecular SO_2".

Finally, we performed a confirmation challenge experiment with selected SO_2 treatments (experiment B). The SO_2 treatments were chosen to create the following wines:

- Medium "Molecular SO_2", medium molecular SO_2 white wine (74 mg/L SO_2 added, 1.20 mg/L "Molecular", 1.19 mg/L molecular).
- High "Molecular SO_2", medium molecular SO_2 "red wine" (142 mg/L SO_2 added, 2.56 mg/L "Molecular", 1.59 mg/L molecular).
- Medium "Molecular SO_2", low molecular SO_2 "red wine" (74 mg/L SO_2 added, 1.33 mg/L "Molecular", 0.27 mg/L molecular).
- A control "red wine" and a control white, with no added SO_2.

The challenge experiment lasted 960 min, and yeast survival was again determined by plating of serial dilutions. The effects of the treatments were determined by comparing changes in surviving cells (log cfu/mL) in a treatment with the unsulfited white control. The results were similar to those of the original experiment (data not shown): molecular SO_2 >0.5 mg/L in either wine type, or "Molecular SO_2" in the white wine >0.5 mg/L was sufficient to decrease survival compared with the control, but survival at molecular SO_2 <0.5 mg/L, i.e., the "red wine" with 74 mg/L added SO_2, was not significantly different from that in the control.

Discussion

We have recently reported that "Molecular SO_2" of wines measured by conventional analytical approaches (e.g., A-O) that rely on initial acidification and dilution of the sample are often much higher than molecular SO_2 values measured by headspace methods, e.g., HS-GDT, that do not perturb the sample (Coelho et al. 2015). This discrepancy also has been reported by other authors (Burroughs 1975, Davis et al. 1983), and is particularly severe for red wines. For example, we previously observed that molecular SO_2 averaged only 32% of "Molecular SO_2" values across a range of red wines. The different values measured between methodologies are likely due to dissociation of weakly bound anthocyanin-bisulfite adducts over the time course of a conventional SO_2 analysis (Coelho et al. 2015). The key question investigated by our group was whether this weakly bound anthocyanin-bisulfite fraction has antimicrobial activity and, hence, whether conventional "Molecular SO_2" analyses are more fit for purpose than nonperturbing molecular SO_2 analyses. In principle, it could be possible to evaluate the efficacy of SO_2 in a set of commercial white and red wines, but such a study could be confounded by differences in other parameters (e.g., pH, acetic acid, residual sugar).

To control for these differences, a "red wine" was prepared by adding a commercial anthocyanin extract to the white wine. Although other weak SO_2 binders exist in wine, our recent work has indicated that the discrepancy between "Molecular" and molecular SO_2 analyses can be largely explained by differences in monomeric anthocyanin concentration (Coelho et al. 2015). The resulting "red wine" had elevated monomeric anthocyanins at concentrations representative of a young red wine, and had low levels of tannins, but otherwise resembled the white wine (Table 1). SO_2 addition resulted in nearly identical increases in "Molecular SO_2" in both wine types, but in much lower levels of molecular SO_2 in the "red wine" than in the white wine (Figure 2). Because the wines were of similar composition, besides higher anthocyanins and tannins, and because anthocyanins are well known to bind SO_2, this difference in SO_2 activity was presumed to result from binding of SO_2 by anthocyanins. In support of this hypothesis, 78 mg/L SO_2 needed to be added to the "red wine" to significantly increase molecular SO_2 levels, compared with 32 mg/L to significantly increase "Molecular SO_2", a difference of 46 mg/L total SO_2 (0.72 mM). This concentration represents 78% of the monomeric anthocyanin concentration (458 mg/L as malvidin-3-glucoside, or 0.92 mM), which is squarely within the range of monomeric anthocyanins reported to be bound in typical red wines (70 to 85%) (Usseglio-Tomasset et al. 1982).

Interestingly, the SO_2 addition (56 mg/L) that led to a typical winemaking target "Free SO_2" (~30 mg/L, Supplemental Figure 1C) resulted in a "red wine" with molecular SO_2 below the analytical detection limit (<0.05 mg/L, Supplemental Figure 1D); thus, these concentrations were far below any typical winemaking recommendation. Although not tested in the current report, we would expect similar "Free SO_2" for other methods that perturb equilibrium, e.g., the Ripper iodometric titration. An earlier study reported that free SO_2 measured by capillary electrophoresis were up to an order of magnitude lower than "Free SO_2" measured by the Ripper method or A-O (Bogren 1996). The use of a modified Ripper method to account for other nonreducing substances (Zoecklein et al. 1999) is not expected to improve agreement with free SO_2 methods, since the initial acidification step in Ripper would still perturb equilibrium.

S. cerevisiae was used for the challenge studies in sweet wines because of the well-known importance of using SO_2 to prevent refermentation, and because there are multiple literature reports on recommended SO_2 levels for *S. cerevisiae* control in both wine and defined media (Beech et al. 1979, King et al. 1981, Sudraud and Chauvet 1985). The EC1118 strain was selected because of its widespread use in international winemaking (Hornsey 2007). The results of our challenge studies with *S. cerevisiae* on the "red" and white wines demonstrate that although yeast survival decreases with increasing total SO_2 level, the efficacy of SO_2 is lower in "red wine" than in white wine for the same total SO_2 addition (Figure 3). Using conventional SO_2 analysis approaches (A-O), we could not easily explain these differences in survival, since apparent "Molecular SO_2" at a given total

SO_2 was similar in both the red and white wines (Figure 2). However, the molecular SO_2 concentration determined by HS-GDT was much lower in the "red wine" for a given total SO_2 addition (Figure 2). We used two statistical approaches to evaluate whether molecular SO_2 was a better predictor of antimicrobial activity than "Molecular SO_2". First, we identified pairs of "red" and white wines similar in either molecular SO_2 or "Molecular SO_2". No significant difference was observed in survival between molecular SO_2 pairs, but a significant difference was observed for "Molecular SO_2" pairs (Figure 4). Similar results were observed for viability measured by flow cytometry.

Second, we classified the SO_2 treatments according to the measured molecular or "Molecular" SO_2, and compared antimicrobial activity among these treatments. These categories were based on the broad range of "target" molecular SO_2 levels suggested in the literature. One report states that ~1.5 mg/L molecular SO_2 was required for 4-log reductions in *S. cerevisiae* in media after 24 hr (King et al. 1981), another paper recommends at least 0.9 mg/L to prevent yeast growth in sweet wines (Sudraud and Chauvet 1985), and yet another recommends a range of 0.5 to 0.9 mg/L for controlling yeast spoilage (Beech et al. 1979). On the basis of these reports, we categorized wines with 0.5 to 2.0 mg/L molecular or "Molecular" SO_2 as having "medium" levels that covered the broad range suggested in the literature, with wine treatments having <0.5 mg/L or >2.0 mg/L SO_2 classified as "low" or "high", respectively. We observed that molecular SO_2 >0.5 mg/L results in a significant and greater than 2-log reduction in both viable and culturable cells in "red" and white wines (Figure 5). This result was in good agreement with the minimal concentrations suggested in the previous reports. By comparison, to yield an antimicrobial effect, high concentrations of "Molecular SO_2" were necessary in the "red wine". A follow-up confirmation challenge study (experiment B) gave results similar to those in experiment A: a red wine with "Molecular SO_2" >0.5 mg/L, but molecular SO_2 <0.5 mg/L, showed no decrease in yeast survival, but the red and white wines with both "Molecular SO_2" and molecular SO_2 >0.5 mg/L had a 2-log decrease in survival.

Taken together, the data from experiments A and B suggest that anthocyanin-bisulfite complexes measured by conventional SO_2 analyses like A-O have little (if any) antimicrobial effect. To our knowledge, this is the first demonstration that the anthocyanin-bisulfite adducts have negligible activity in wine. Our results contradict the observations of Usseglio-Tomasset and colleagues, who reported that anthocyanin-bisulfite complexes (30 mg/L as SO_2) delayed fermentation by *S. cerevisiae* and other yeasts almost as much as free SO_2 (Usseglio-Tomasset et al. 1982). Because molecular (or "Molecular") SO_2 concentrations were not measured after addition or during fermentation in this earlier work, it is unclear whether the SO_2 stayed in the anthocyanin-bisulfite complexes, or whether molecular SO_2 increased because of reactions that would consume anthocyanins.

Reports that have attempted to establish appropriate molecular SO_2 levels for control of *S. cerevisiae* spoilage using white wines, model wines, or media would not have observed any effect due to anthocyanin binding of SO_2. However, several studies on SO_2 inhibition of *B. bruxellensis* in red wine are reported in the literature. *Brettanomyces* control is of considerable interest to the wine industry because of its high ethanol tolerance and low nutrient requirements, and because it can convert vinylphenols to more potent and malodorous ethylphenols (yielding "Band-Aid", "horse", or "spicy" odor) (Smith and Divol 2016). *Brettanomyces* can be inhibited by molecular SO_2 (Smith and Divol 2016), but requisite concentrations appear to vary not only by strain, but also between media and red wine. For example, challenge studies in red wines evaluating multiple *Brettanomyces* strains have reported that most of these strains are culturable at 0.4 mg/L "Molecular SO_2", and sometimes up to 1.0 mg/L (Barata et al. 2008, Zuehlke and Edwards 2013). However, a large survey of SO_2 tolerance among *Brettanomyces* strains grown in media revealed that most strains could grow only at below 0.2 mg/L "Molecular SO_2" (Vigentini et al. 2013). A separate

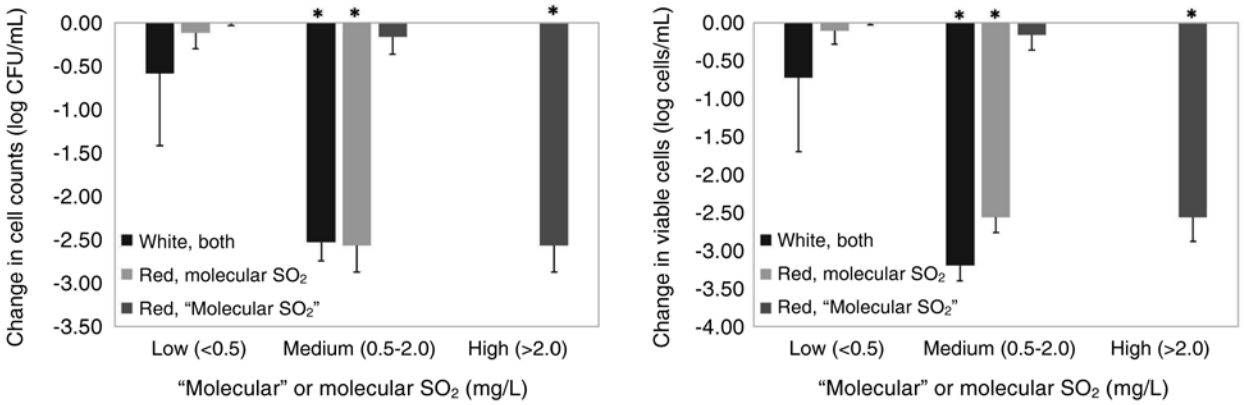

Figure 5 Experiment A. Change in cell counts by plating (left) or viable cells by flow cytometry (right) relative to SO_2-free controls as a function of SO_2 concentration. Values are averages for the time points T6 to T9. Wines are categorized as low, medium, or high on the basis of molecular SO_2 or "Molecular SO_2". No wines with molecular SO_2 >2.0 mg/L were prepared. Because white wines were categorized similarly by molecular SO_2 and "Molecular SO_2" measurements, only a single bar is shown. SO_2 addition treatments included in each category are listed in Table 3. Values represent the average value within an SO_2 category; error bars represent standard deviations. *: significant decrease in cells for an SO_2 category compared with the SO_2-free control ($p < 0.05$).

survey showed that the majority of *Brettanomyces* strains did not survive (>4-log reduction) at 0.4 mg/L "Molecular SO_2" (Curtin et al. 2012). Another report using model wines indicated growth inhibition at 0.3 mg/L "Molecular SO_2" and 10% ethanol for all but one of 18 *Brettanomyces* strains (Duckitt 2012).

Although direct comparisons among results are complicated by varying methodologies for evaluating SO_2 antimicrobial activity, the higher levels of "Molecular SO_2" necessary for *Brettanomyces* control in red wines as compared to model wine or media reported in the literature could be explained by the fact that conventional "Molecular SO_2" analyses overestimate the true molecular SO_2 fraction. In a recent survey of commercial wines, we observed that "Molecular SO_2" ranged from 0.2 to 1.2 mg/L (Coelho et al. 2015). In the same work, using HS-GDT, we observed that only two of nine red wines had molecular SO_2 >0.2 mg/L. On the basis of the slope of the regression line for HS-GDT versus A-O data (0.32) (Coelho et al. 2015), we can surmise that on average, the molecular SO_2 value was only 32% of the "Molecular SO_2" value in red wines. By comparison, 17 of 18 white and blush wines in this previous study had molecular SO_2 >0.2 mg/L, and the molecular SO_2 was only 82% (slope of regression line = 0.82) of the "Molecular SO_2" value. As demonstrated in our current work, molecular SO_2 is a much better predictor of antimicrobial activity against *Saccharomyces* than "Molecular SO_2". *Brettanomyces* spoilage is much more common in barrel-aged red wines than barrel-aged white wines, a phenomenon often attributed to the lower pH of white wines favoring the molecular SO_2 form (Oelofse et al. 2008). However, our work suggests that an additional issue is that conventional "Molecular SO_2" measurements overestimate the amount of active molecular SO_2 in red wines. This hypothesis was not evaluated in our current work, but should be considered in future studies.

As a caveat, previous reports that used red wines in microbial challenge studies did not report the monomeric anthocyanin concentration of the wines used. Monomeric anthocyanin concentrations in red wines decrease to near undetectable concentrations within the first few years after fermentation because anthocyanins react with other wine components, leading to formation of a range of pigmented and nonpigmented products, e.g., pyranoanthocyanins and tannin-anthocyanin adducts, that are less able to bind SO_2 (Waterhouse et al. 2016a). Thus, in older red wines, as opposed to young red wines or "red wines" produced from white wines spiked with anthocyanins, we expect that "Molecular" and molecular SO_2 concentrations should be closer to each other, and a reasonable prediction of SO_2 antimicrobial activity can be achieved based on conventional "Molecular SO_2" analyses.

Our current study evaluated the antimicrobial activity of molecular SO_2 compared with that of "Molecular SO_2". However, in winemaking, free SO_2 in the form of HSO_3^- also serves as an antioxidant, capable of reacting with major oxidation products (acetaldehyde and related carbonyls, quinones, or H_2O_2) (Waterhouse et al. 2016b). Potentially, "Free SO_2" measurements may also overestimate the antioxidant activity of wines, analogous to the situation with "Molecular SO_2". Waterhouse et al. (2016a) recently observed that lees-aged wines exposed to large amounts of air during bottle storage develop oxidized aromas despite having ~10 mg/L "Free SO_2" (measured by iodometric titration) at the end of the storage period. By comparison, wines aged without lees had lower "Free SO_2", but did not have oxidized aromas. The authors speculated that this may have been due to the presence of lees-derived weak SO_2 binders that were detected as "Free SO_2", but did not prevent formation of oxidized off-aromas. Based on our current work, this is a reasonable hypothesis to investigate.

Conclusion

Using a "red wine" produced by spiking a white wine with anthocyanin extract, we have demonstrated that anthocyanin-bisulfite complexes have negligible antimicrobial activity. These complexes are measured as part of conventional "Free" and "Molecular SO_2" analyses, such as A-O and flow-injection analysis. Our results indicate that conventional approaches to measuring "Molecular SO_2" are ill-suited for predicting the microbial stability of red wines, particularly of younger red wines that are rich in monomeric anthocyanins. Future work is necessary to determine whether conventional SO_2 analyses also overestimate protection against other spoilage organisms (e.g., *Brettanomyces*) and oxidation in red wines.

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