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Research Article

Determination of Molecular and "Truly" Free Sulfur Dioxide in Wine: A Comparison of Headspace and Conventional Methods

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Abstract: Conventional methods such as the Ripper titration and Aeration-Oxidation (A-O) are employed widely for the analysis of sulfur dioxide (SO_2) in wine. However, the free SO_2 reported by these procedures is overestimated due to dissociation of weakly bound SO_2 forms during the analysis, particularly from anthocyanin-bisulfite complexes. "Truly" free SO_2 in wine can be determined from the headspace SO_2 concentration of an equilibrated wine sample. A headspace SO_2 method based on gas detection tubes (HS-GDT) was recently described but is not readily automated. While SPME gave poor precision in our hands, our new method, based on static headspace gas chromatography and sulfur chemiluminescence detection (HS-GC-SCD), is readily automated, and achieves high precision (<5%) and low limits of detection (0.033 mg/L molecular

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SO_2, or approximately 1 mg/L free SO_2 in wine at pH 3.5). When A-O, Ripper, HS-GC-SCD, and HS-GDT methods were compared on a diverse set of wine samples, the HS-GC method correlates to the HS-GDT method (r^2 = 0.92) and achieves higher precision (RSD = 3.7), whereas HS-GC correlates well with A-O on white wines (r^2 = 0.85, slope = 0.90) but was found to have a weaker correlation for red wines (r^2 = 0.71, slope = 0.44). The GC's flexibility for other procedures, stability, and low operating costs per sample make it attractive, and headspace methods have been shown to be better at predicting microbial stability in red wines.

Key words: gas chromatography, headspace, sulfur chemiluminescence, sulfur dioxide, "truly" free SO_2, wine analysis

Introduction

Sulfur dioxide (SO_2) is the oldest and arguably one of the most important additives used in winemaking. When present in sufficient concentration, SO_2 has five major effects in wine/musts: (1) SO_2 is a strong antimicrobial agent, and provides a protection against a wide array of detrimental microorganisms; (2) it is an effective antioxidant that consumes oxidants such as hydrogen peroxide or quinones formed during the course of wine/must oxidation; (3) it can inhibit polyphenol oxidase enzymes present in grapes; (4) it reversibly binds and bleaches wine pigments, particularly monomeric anthocyanins; (5) it reversibly binds aldehydes and ketones produced by oxidation or during fermentation, rendering them non-odorous (Waterhouse, et al., 2016).

Sulfur dioxide gives a weak, diprotic acid in aqueous solution (pK_{a1} = 1.81, pK_{a2} = 7.20 in H_2O at 20°C) and can exist in one of three forms: molecular (SO_2), bisulfite (HSO_3^-), and sulfite (SO_3^{2-}). In the typical pH range of wine (between 3.0 and 4.0) the dominant species is the bisulfite

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anion, which acts as an antioxidant, in addition to participating in various binding/complexing reactions. Molecular sulfur dioxide (SO_2), the main antimicrobial form of sulfur dioxide, is present at only a small fraction of the HSO_3^- concentration (<5%) at wine pH. Sulfite (SO_3^{2-}) is present at even a smaller fraction of the HSO_3^- concentration (<0.1%) at wine pH, and its influence on wine stability is thus likely negligible. Sulfur dioxide in wine is further divided into two classes: free and bound. Free sulfur dioxide is defined as the sum of molecular and bisulfite forms, and is the class with antimicrobial, antioxidant, and enzyme inhibiting properties. Bound sulfur dioxide comprises the bisulfites which have reacted (both weakly and strongly) with other molecules within the wine matrix and do not exhibit those protective properties, with some exceptions (Wells and Osborne, 2011). Finally, the sum of the free and bound sulfites defines the "total" sulfite concentration (Buechsenstein and Ough, 1978).

To obtain enologically useful information, analytical methods for SO_2 must distinguish between the free form, with its protective properties, and the bound forms, without these properties. Common methods for free SO_2 in wineries include iodometric titration (Ripper method) and aeration-oxidation (Urbano-Cuadrado, et al.) (Iland, et al., 1993). These standard methods utilize an initial acidification step to either avoid interferences from phenolics (Ripper) or to favor the molecular SO_2 species prior to a separation step (A-O and related flow injection or segmented flow analysis methods). This acidification step, coupled with consumption of free SO_2 during the course of analysis, can result in release and subsequent measurement of some weakly bound SO_2, particularly from anthocyanin-bisulfite complexes. As a result, standard measurement approaches will overestimate free SO_2 particularly in red wines (Coelho, et al., 2015).

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This artefactual overestimation of free SO_2 can be avoided by measuring the headspace SO_2 concentration of an equilibrated wine sample. This headspace SO_2 concentration can then be related to the aqueous molecular SO_2 concentration by its Henry's Law coefficient (*H*), which can then be related to the concentration of "truly" free SO_2 by pK_{a1} and the Henderson-Hasselbalch equation. To calculate free SO_2, the ethanol concentration, pH, and temperature of the wine sample must be accurately known to establish the correct values of pK_{a1} and *H*. A recently described approach used a syringe to create an equilibrated enclosed headspace above a wine sample, and then expel the headspace through a commercial gas detection tube (GDT). The GDTs contain a colorimetric, SO_2-selective reagent, such that length of discoloration on the GDT is proportional to the analyte concentration. The HS-GDT technique does not involve pH shifts, sample dilution, and/or temperature changes and thus avoids disturbances in SO_2 equilibria in wine or contributions from weakly bound SO_2 (Coelho, et al., 2015). The authors observed that A-O resulted in ~3-fold overestimation of free SO_2 in red wines as compared to HS-GDT. The HS-GDT approach was later shown to yield more accurate predictions of yeast survivability and viability during challenge tests, suggesting that "truly" free SO_2 measurements may be of greater relevance to predicting antimicrobial activity(Howe, et al., 2018).

Although easy to implement, one drawback of the HS-GDT approach is that it is not readily automated. Other potentially more automatable approaches for indirect and direct measurement of "truly" free SO_2 have been described, such as capillary electrophoresis (CE), and headspace gas chromatography (HS-GC) coupled to an electrolytic conductivity detector (ECD). These studies have come to similar conclusions that free SO_2 may be overestimated by up to an order of

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magnitude in red wines, although none appear to be widely used (Davis, et al., 1983, Boulton, et al., 1996, Collins and Boulton, 1996).

The coupling of HS-GC with a sulfur chemiluminescence detector (SCD) for analysis of SO_2 and other volatile sulfur compounds on unadjusted wine samples was recently described (Ontanon, et al., 2019). HS-GC-SCD is readily automated and has excellent selectivity for sulfur compounds. Because samples were not adjusted or heated prior to or during analysis, the SO_2 measured by HS-GC-SCD should be proportional to the "truly" free SO_2. However, this earlier report did not compare results for wines analyzed by HS-GC-SCD to other SO_2 analytical approaches. In this work, we report development of an HS-GC-SCD method for "truly" free SO_2, and its comparison against other SO_2 methods (HS-GDT, A-O, Ripper). We also compare the differences between headspace methods and other methods (A-O, Ripper) versus a number of substances that might form metastable bound SO_2 that could be released during conventional analyses, to evaluate a basis for the discrepancy.

Materials and Methods

Chemicals. Potassium metabisulfite (97%), acetaldehyde (99%), 2-ketoglutaric acid (99%), pyruvic acid (99%), 2,4-dinitrophenylhydrazine (DNPH), ammonium dihydrogen phosphate (≥95%), formic acid (≥95%), methanol (≥99.9%), and acetonitrile (≥99.9%) were obtained from Sigma-Aldrich (St. Louis, MO). L-Tartaric acid (99%) was obtained from Fisher Scientific (Waltham, MA). Ethanol (anhydrous, ≥99.5%) was obtained from Decon Laboratories (King of Prussia, PA). Hydrogen peroxide (30% w/v), sodium hydroxide (10%, 0.1N, and 0.01N), *o*-phosphoric acid (85%), sulfuric acid (25%), starch (1%), and iodine (0.02N) were obtained from

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Enartis Vinquiry (Windsor, CA). Ethyl methyl sulfide (1000 µg/mL) was obtained from SPEX CertiPrep (Metuchen, NJ).

SO_2 working standards. SO_2 stock solutions at nominal concentrations of 6000 mg/L as SO_2 were prepared weekly by dissolving potassium metabisulfite in a 10% (v/v in water) solution of methanol to avoid SO_2 autooxidation. Working standards were then prepared as needed by adding an appropriate volume of a stock SO_2 solution to model wine. Model wine solution was prepared in ultrapure water containing 4 g/L of tartaric acid, 10% ethanol, and adjusted with NaOH solution to a pH of 3.50. The ethanol concentration was verified using an Alcolyzer Wine M (Graz, Austria). The true pKa (pK_M) for SO_2 in each batch of model wine was determined using the following calculations. Then the concentration of each of the calibration standards could be calculated using the Henderson-Hasselbalch equation.

Estimation of pK_{a1} (pK_M) of sulfur dioxide and calculation of free SO_2 from molecular SO_2. The following equations were built from a multiple linear regression model using XLSTAT (Addinsoft, Paris, France, 2018) to predict the pK_a values contained in published tables (Usseglio-Tomasset and Bosia, 1984).

To estimate the value of the thermodynamic constant, pK_T, for various alcohol concentrations (Alc., %v/v) and temperatures (T, ºC), the following equation is used, equation 1.

Estimation of pK_T.

$$pK_T = 0.655664 + (0.0698386 * T) + (0.02015 * Alc.) - (0.000621693 * T^2) \quad (Eq. 1)$$

To estimate the value of the coefficients A and B, for various alcohol concentrations and temperatures, the following two equations are used (Equations 2 and 3).

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Estimation of A constant.

$$A = 0.482724 + (0.000883782 * T) + (0.00443752 * Alc.) + (0.00000595973 * T^2) + (0.0000489638 * Alc.^2) \qquad (Eq. 2)$$

Estimation of B constant.

$$B = 1.61645 + (0.000935347 * T) + (0.00479931 * Alc.) + (0.00000492357 * T^2) + (0.0000315093 * Alc.^2) \qquad (Eq. 3)$$

Finally, the value of the mixed dissociation constant, pK_M, as a function of pK_T, the coefficients A and B, and the ionic strength (I), is determined by equation 4 below.

Estimation of pK_M.

$$pK_M = pK_T - \frac{(A\sqrt{I})}{(1 + B\sqrt{I})} \qquad (Eq. 4)$$

Since measurement of ionic strength (I) is complex and labor intensive, a typical ionic strength of 0.056M can be assumed (the typical range for ionic strength in wine is 0.016M to 0.100M) and is used in the calculation of pK_M (Berg and Keefer, 1958, Berg and Keefer, 1959, Ough, et al., 1982, Abgueguen and Boulton, 1993) without resulting in significant error in estimation of free SO_2 (Coelho, et al., 2015).

The value of pK_M can then be used in the Henderson-Hasselbalch equation (Equation 5) to determine the molecular and free species of sulfur dioxide as a function of pH.

Modified Henderson-Hasselbalch equation.

$$[Molecular\ SO_2] = \frac{[Free\ SO_2]}{1 + 10^{(pH - pK_M)}} \qquad (Eq. 5)$$

SO_2 measurements using previously described approaches: A-O, Ripper, and HS-GDT. SO_2 analysis by A-O (Iland, et al., 1993), Ripper (Vahl and Converse, 1980), and HS-GDT

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(Coelho, et al., 2015) were all performed in triplicate on each wine. The Ripper method was also used to measure total SO_2.

SO_2 measurement by HS-GC-SCD. Analysis of molecular and free SO_2 were performed with an Agilent 7890B gas chromatograph coupled with an Agilent 8355 sulfur chemiluminescence detector (Agilent Technologies, Santa Clara, CA, USA). The capillary column used was an Agilent DB-WAX-UI (30 m × 0.25 mm i.d. × 0.25 μm film thickness). The autosampler was a PAL3 RSI from CTC analytics (Zwingen, Switzerland) operated in static headspace mode. The 2.5 mL gas-tight syringe was heated to 40 °C to prevent condensation of the headspace sample in the syringe. Injections were split (4:1 ratio) at an injector temperature of 200 °C. Before and after injection, the syringe was purged with pure He for 90 s. The temperature program for the final method started at 50 °C, kept for 2.5 min, and then raised at 50 °C min^{-1} to 220 °C and holding this temperature for 2 min. The complete chromatogram took 7.9 min, with a total GC cycle time of 10.5 min between injections. The carrier gas was He (44.2 cm/sec) in constant flow mode. The SCD burner temperature was 800 °C with a hydrogen flow rate of 100 mL min^{-1} and an air flow rate of 40 mL/min. The SCD pressure was 6 Torr with the controller at 200 Torr.

For each analysis, immediately after opening a bottle, 15 mL of room temperature wine (23 °C) was transferred into a 20 mL amber crimp top headspace vial and spiked with 50 μL of internal standard (30 μg/mL ethyl methyl sulfide in methanol). The vials were then capped with magnetic crimp seals with PTFE/silicone septa. If not already equilibrated to room temperature, the samples were equilibrated for 1 hour before running the procedure. HS-GC-SCD analyses were

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then performed as described above. Since it was already shown that a headspace volume of 50 mL at room temperature takes a minimum time of 5 minutes to fully equilibrate, the equilibration time for the GC vials with 5 mL of headspace volume was assumed to be equivalent or less (Coelho, et al., 2015). The analytical characteristics of the method are summarized below in Table 1.

Monomeric anthocyanins by high performance liquid chromatography. The separation of the monomeric anthocyanins was conducted with a reverse-phase high performance liquid chromatography (HPLC) using an Agilent 1100 series (Agilent Technologies, Santa Clara, CA) modular HPLC system based on the method described elsewhere (Ritchey and Waterhouse, 1999). The HPLC system included a system controller, G1379A degasser, G1311A quaternary pump, G1313A autosampler, G1316A column compartment, and a G1315A DAD/UV-vis detector. Data was processed using ChemStation version B.04. Separation of anthocyanins was performed with a LiChrospher 100 RP-18 column (4 × 250 mm, 5 µm particle size; Agilent Technologies); a guard column of the same material was also installed, and column temperature was maintained at 40°C.

Briefly, the procedure used two mobile phase solutions for analysis. The solvents were (A) 50 mM ammonium dihydrogen phosphate (Sigma-Aldrich ≥ 95%) adjusted to pH 2.6, and (B) 20% Mobile A + 80% acetonitrile (v/v) (Sigma-Aldrich ≥ 99.9%). The gradient used was: zero-time conditions were 94% A and 6% B; after 15 min the pumps were adjusted to 70% A and 30% B; at 30 min to 50% A and 50% B; at 35 min to 40%, and 60% B; at 41 min (end of analysis), to 94% A and 6% B. After 10-min equilibrium period the next sample was injected.

The concentration of total monomeric anthocyanins was determined by the summation of the peak areas measured at 520 nm for delphinidin 3-glucoside, pelargonidin, cyanidin 3-

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glucoside, pelargonidin 3-glucoside, delphinidin, malvidin 3-glucoside, and malvidin. The concentration was expressed as mg/L of malvidin-3-glucoside equivalents.

Free and SO_2-Bound Wine Carbonyls by High Performance Liquid Chromatography. Acetaldehyde, 2-ketoglutarate, and pyruvate were determined by HPLC after derivatization reaction with 2,4-dinitrophenylhydrazine (DNPH) reagent (Sigma-Aldrich) as reported by (Han, et al., 2015). Briefly, wine sample aliquots (100 µL) were dispensed to a vial, followed by the addition of 20 µL of freshly prepared 1,120 mg/L SO_2 solution, 20 µL of 25% sulfuric acid, and 140 µL of 2 g/L DNPH reagent. After mixing, the solution was allowed to react for 15 min at 65°C and then promptly cooled to room temperature in a water bath. Carbonyl hydrazones were analyzed by HPLC using the system described above. In the chromatographic system, a ZORBAX Rapid Resolution HT, SB-C18 column (1.8 µm, 4.6 x 100mm^2, Agilent Technologies) was used for separation. Separation was obtained using a flow rate of 0.75 mL/min, column temperature of 35°C; mobile phase solvents were: (A) 0.5% formic acid (Sigma Aldrich ≥ 95%) in water milli-Q and (B) acetonitrile (Sigma Aldrich ≥ 99.9%); gradient elution protocol was: 35% B to 60% B (8 min); 60% B to 90% B (13 min); 90% B to 95% B (15 min, 2-min hold); 95% B to 35% B (16 min, 4-min hold); total run time, 20 min. Eluted peaks were measured at 365nm and were compared with derivatized acetaldehyde, 2-ketoglutarate, and pyruvate standards (Sigma-Aldrich).

Analysis of Alcohol, pH, and Temperature. *Alcohol.* The ethanol content of all wine samples and model wines was determined using an Alcolyzer Wine M (Anton-Paar, Ashland, VA).

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pH. The pH of all wine samples and model wines was measured using an Orion 5 Star (Thermo Scientific, Boston, MA). The pH probe was calibrated daily using buffers of 2.00, 4.01, and 7.00 pH standards. Slopes of each calibration were between 96% and 100%.

Temperature. Sample temperature was measured using VWR® Traceable® Lollipop™ Water-Resistant Thermometers.

Wine Samples. Table 2 shows the identity of the wines used to compare the four methods. Various wines (n = 27) covering a range of varieties, vintages, and appellations were donated from Constellation Brands.

Results and Discussion

In initial work, we evaluated the use of solid phase micro-extraction (SPME) followed by separation on a porous layer open tubular (PLOT) GC column; similar methods have been used for analysis of other volatile sulfur compounds in wine. The approach used short SPME exposure times to avoid perturbation of equilibria. This approach was determined to be unacceptable due to poor precision and excessive peak broadening on the PLOT column that were difficult to analyze (data not shown). We then evaluated static headspace injection with different columns: (DB-Sulfur, DB-WAX-ETR, DB-WAX-UI). We selected the DB-WAX-UI column because it could achieve rapid separation of SO_2 with gaussian peak shape, excellent peak precision (2.8% RSD), and a low limit of detection. The final GC parameters used were similar to a reported method, with the exceptions of eliminating the SO_2 preconcentration step in favor of drawing a 0.500 mL sample directly from the headspace vial with the autosampler, and use of ethyl methyl sulfide (EMS) as the internal standard (Carrascon, et al., 2017). By design, the use of a sulfur chemiluminescence

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detector as opposed to a mass spectrometer detector was intended to offer improved sensitivity and selectivity to SO_2 specifically. The selected column does degrade with time from the SO_2 exposure and should be replaced after approximately 200 injections.

With the DB-WAX-UI column and corresponding GC parameters for this method, the elution time for the SO_2 peak is approximately 3.2 minutes, and the ethyl methyl sulfide internal standard is approximately 1.8 minutes, neither of which co-elute with any other potentially interfering compounds typically found in wine. A representative chromatogram, of a 2014 Central Coast Viogner is shown in Figure 1.

Table 3 presents free SO_2 (mg/L) on a set of California wines as measured by the A-O, Ripper, HS-GDT, and HS-GC methods. The results of the A-O and Ripper methods will be referred to as 'apparent' free SO_2, and the free SO_2 measured using the HS-GDT and HS-GC techniques will be referred to as "truly free" SO_2. All analyses on each wine using each method were done in triplicates so that the precision of the methods could be assessed and compared.

Table 4 tabulates other basic chemistry parameters of the wine samples analyzed in this set. The estimates of true pKa based on alcoholic strength, temperature, and ionic strength are also shown. For SO_2 by HS-GC, the formula for estimating the truly free SO_2 is based on the Usseglio-Tommaset calculations (Usseglio-Tomasset and Bosia, 1984). For SO_2 by HS-GDT, a related approach was used in Coelho 2015 for estimating the true pKa.

Since the temperature of the analysis by both HS-GC and HS-GDT was not controlled beyond the prevailing ambient room temperature (18-21 °C), there were a few comparative analyses that were conducted at non-equivalent temperatures. Specifically, analysis of the BLAU, CAB, and CHA 1 by HS-GC and HS-GDT was at a 2ºC differential, with the HS-GC analysis

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occurring at 20ºC and the HS-GDT analysis occurring at 18ºC. Analysis of the WHITE, VIO 2, and CHA 3 samples also occurred at a 2ºC differential, however, the HS-GC analysis occurred at 25ºC and the HS-GDT analysis occurred at 23ºC. While the respective formula for calculating the true pKa have built in functions that account for the difference in temperature, it is not clear if these are sufficient to overcome instances when analysis is done at a non-standard temperature or can account for slight variations in analysis temperature beyond +/- 1ºC. Given the uncertainty, these data-points were excluded from the statistical analysis.

The results of the full comparison of analytical methods indicate that the A-O and Ripper methods are comparable and satisfactory in terms of analytical precision as both methods had relative standard deviation (%RSD) below 5%. Both the A-O and Ripper methods had similar average standard deviations across the 27 wines analyzed, 0.8 and 0.7 mg/L free SO_2 respectively. A graphical comparison of the Free SO_2 results of the 27 wines by A-O and Ripper analysis is shown in Figure 2, and the methods showed good agreement based on a regression analysis (slope = 1.02, intercept = 2.8, r^2 = 0.92, Figure 2). The highest standard deviation in free SO_2 measurement by A-O was observed in the analysis of the non-vintage Brut Sparkling wine. The high standard deviation in this specific case was likely due to dissolved CO_2 that carried over into the peroxide trap used in the A-O procedure, resulting in an over-titration and subsequent over-reporting of apparent free SO_2. Interestingly, the overall relative standard deviation for the Ripper method (3.79%) was lower than the relative standard deviation for the A-O method (4.60%), which is in contrast with findings some older studies that reported relative standard deviations ranging as high as 9.5% to 12% for the Ripper method, (Buechsenstein and Ough, 1978, Vahl and Converse, 1980) but in agreement with more recent results from interlaboratory proficiency testing that

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observed little variation in precision between the two methods (Howe, et al., 2015). The average absolute difference in free SO_2 between the two methods was 3.3 mg/L of free SO_2, with the maximum absolute difference being 9.0 mg/L. In most cases, the free SO_2 results measured by Ripper were 0.7 to 5.8 mg/L higher than the free SO_2 as measured by A-O. This effect may be due to over-titration past the true end-point by the operator to reach a visually detectable end-point, especially in darkly pigmented samples, or due to the presence of interferences like reducing sugars or ascorbic acid (Iland, et al., 1993).

With respect to the headspace techniques for measuring free SO_2 (after mathematical conversion from molecular SO_2), good linear agreement was observed between the HS-GC and HS-GDT methods (slope = 0.90, intercept = 1.1, r^2 = 0.92, Figure 3). The average absolute difference in free SO_2 between the two methods was 2.1 mg/L of free SO_2, with the maximum absolute difference being 6.3 mg/L. Both the HS-GC and HS-GDT methods had varying average standard deviations across the 27 wines analyzed, 0.4 and 1.4 mg/L for free SO_2 measurements respectively. In terms of analytical precision, the HS-GC technique had a relative standard deviation (3.72%) which was appreciably lower than the relative standard deviation for the HS-GDT method (11.83%). The lower precision of the HS-GDT method is likely due to the difficulty in reproducibly determining the start and stop points of tube staining.

Since partitioning of SO_2 in the headspace is governed by Henry's Law, effort was made to ensure that wines analyzed by the HS-GC and HS-GDT method were analyzed at the same temperature +/-1°C. The bottled wine samples were equilibrated at room-temperature (23°C) for a minimum of 24 hours prior to analysis. Temperature of the wine samples was recorded at the

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time of each batch of HS-GDT analysis. For the HS-GC analysis, the heating element of the sample agitator was turned off since precise temperature control was not available under 30°C, therefore, samples in the GC vials were at the prevailing room temperature at the day and time of analysis. The laboratory is temperature controlled within 2-3 °C but that control is for comfort and is not regulated +/-1 °C. Despite those efforts, the difference in results between the HS-GC and HS-GDT methods could be due to slight differences (>1°C) in analysis temperatures. Moreover, the imprecision of the endpoint determination of the GDTs may have amplified the apparent differences. It should be possible to improve the correlation, as well as precision, with better temperature control. In practice, it would be difficult to precisely manage GDT temperatures in a small-scale operation.

For red wines, free SO_2 by the A-O method was higher than free SO_2 measured by the HS-GC method in all red wines (range = 5-20 mg/L, Table 3). On average, HS-GC free SO_2 values for red wines were only 39% of A-O values (61% lower). Better agreement between the HS-GC and A-O free SO_2 values was observed for white wines. On average, the free SO_2 values for white wines were 87% of the value for free SO_2 (13% lower) determined by the A-O method for the same wines. Correlations between the HS-GC and A-O methods were also better for white wines (r^2=0.85, Figure 4) than red wines (r^2=0.71, Figure 4). These results are comparable to previous work comparing HS-GDT and A-O, which reported 51% lower values for HS-GDT than A-O for red wines, and 13% lower values for white wines (Coelho, et al., 2015).

To determine the possible magnitude of the error contributed by the volatilization of SO_2 into the 5mL of headspace in the amber headspace vial the following calculations were carried out.

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To estimate K_H as a function of temperature (in ºC), the following equation (6) was used, the temperature correction for Henry's Law volatility constant K_H:

$$K_H = 2.775\ x10^{-5} \exp\left(\frac{3203}{T + 273.16}\right) \qquad (Eq. 6)$$

For example, for a liquid concentration 1.8 x 10^{-5} M molecular SO_2 at 23 ºC, the K_H value is 1.38 M/atm and using Henry's Law the vapor pressure of SO_2 above the liquid would be 1.3 x 10^{-5} atm. The concentration of SO_2 (in g/L) in the headspace is calculated using the following equation (7) and the known vapor pressure.

Calculation of headspace SO_2 concentration at equilibrium.

$$SO_2\ (g/L) = \frac{VP_{SO_2}(atm) * 64.06\ g/mol}{22.4\ L/atm} \qquad (Eq. 7)$$

Further calculations show that under these conditions, approximately 1% of the SO_2 in the sample is present in the 5 mL of headspace in the GC vial containing 15 mL of sample, a small fraction which should not significantly disrupt the free SO_2 equilibrium.

To evaluate the hypothesis that the discrepancies between the two analysis methods (HS-GC and A-O) could be explained by dissociation of metastable bisulfite complexes during analysis, the 27 wines used in the study were analyzed for the concentrations of major SO_2 binders: monomeric anthocyanins, acetaldehyde, pyruvate, and 2-ketoglutarate, all candidate compounds for such complexes (Table 5). Monomeric anthocyanins were evaluated by HPLC and expressed as mg/L of malvidin-3-glucoside equivalents. Acetaldehyde, pyruvate, and 2-ketoglutarate concentration in the wine samples were determined by HPLC after derivatization reaction with 2,4-dinitrophenylhydrazine (DNPH) reagent (Han, et al., 2015). We also calculated "metastable

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bisulfite" as the difference between the A-O and HS-GC methods. Linear regressions were then performed for metastable SO_2 binders (monomeric anthocyanins acetaldehyde, pyruvate, and 2-ketoglutarate) against the concentration of metastable bisulfite complexes observed in each wine.

We observed a significant correlation between monomeric anthocyanins and metastable bisulfite ($r^2 = 0.42$, Figure 5); no correlation was observed between metastable bisulfite complexes and either acetaldehyde, alpha-ketoglutarate, or pyruvate ($r^2<0.1$; plots not shown) in either red or white wines, suggesting that these compounds are not related to the discrepancies between methods, as also reported by others (Bisson, 1999). Similar correlations were observed to explain the discrepancies in measurements using the Ripper technique and HS-GDT technique (data not shown). Anthocyanin-bisulfite complexes likely contribute to A-O and Ripper measurements of free SO_2 due to their rapid dissociation (first order rate constant for the dissociation of anthocyanin-bisulfite adducts = 0.2 min^{-1}) (Brouillard and Elhagechahine, 1980). By comparison, the first order rate constant glucose-bisulfite complex dissociation is 3.7×10^{-4} min^{-1} (Vas, 1949). There are also some derived anthocyanin pigments that are known to also bind SO_2 or respond to pH changes (Zimman and Waterhouse, 2004) which are not quantified by this method. If a different method had been used for total anthocyanin content, such as SO_2 bleaching, a higher correlation would likely have been found, as in Coelho et al, 2015.

To determine the limit of detection and quantification, model wine solutions containing known trace amounts of molecular SO_2 were analyzed with the described HS-GC-SCD method. The signal to noise ratio of each of the SO_2 peaks were determined using the ChemStation software (version C.01.07 SR2 [255]). Limit of detection was calculated as the amount of molecular SO_2 required to attain a signal to noise ratio of 3, and the limit of quantification was calculated as the

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amount of molecular SO_2 required to attain a signal to noise ratio of 10. It was determined that the limit of detection and limit of quantification for the method are 0.033 mg/L and 0.067 mg/L molecular SO_2, respectively. The similar HS-GC-SCD method published in Ontañón et al. 2019 claims an even lower limit of detection (0.46 µg/L molecular SO_2), however it is not clear how these values were calculated, making direct comparison difficult.

The improved winemaking significance of headspace methods versus conventional with regard to microbial stability have been shown (Howe, et al., 2018). Conventional methods detected significant molecular SO_2 that should suppress yeast, but no suppression was seen in red wine. On the other hand, the headspace method properly predicted suppression at approximately 0.8 mg/L molecular SO_2.

Conclusion

Based on a gas detection tube method, an analytical procedure using headspace gas chromatography (HS-GC) coupled with sulfur chemiluminescence detection (SCD) has been developed which can rapidly and precisely quantify molecular and free sulfur dioxide in wine. The method requires minimal sample preparation and involves no chemical reagents (with the exception of a trace internal standard). At room temperature (23ºC), the method can successfully detect levels of molecular sulfur dioxide at concentrations as low as 0.033 mg/L. The total chromatographic time for the method is 8 minutes and, provided that information on the alcohol concentration and pH is readily available, the molecular and free sulfur dioxide concentrations for the sample can be rapidly calculated using simple formulae. The HS-GC method offers a high degree of precision, with a coefficient of variation of 3.72%.

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In comparing SO_2 analysis methods on a large set of wine samples, the HS-GC method further confirms that conventional SO_2 methods systematically overestimate the molecular and free SO_2 in red wines, largely due to the presence of anthocyanins. It appears that the presence of anthocyanins in wine leads to the formation of metastable complexes with bisulfite which are inadvertently released during conventional analysis methods, leading to inflated, misleading results. Since headspace analysis of sulfur dioxide in wine has been shown to better predict microbial stability, the adoption of headspace-based methods may provide a better prediction of wine stability.

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Table 1 Method figures of merit.

Parameters	Analytical parameter
Correlation coefficient	0.997
Linear Range (*mg/L)	0.67 - 2.00
Limit of detection (*mg/L)	0.033
Limit of quantification (*mg/L)	0.067
RSD, % **	3.72

* Molecular SO_2.
** Based on triplicate analysis of 27 different wines.

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Table 2 Wines used for the comparison of methods and respective sample codes.

Sample ID	Wine	Wine Type
BLAU	2015 Paso Robles Blaufrankisch	Red
CAB	2015 California Cabernet Sauvignon	Red
MER 1	2014 Napa Valley Merlot	Red
MER 2	2015 Central Coast Merlot	Red
MER 3	2013 Paso Robles Merlot	Red
PIN 1	2016 Monterey County Pinot Noir A	Red
PIN 2	2016 Monterey County Pinot Noir B	Red
PIN 3	2015 Central Coast Pinot Noir	Red
PORT	2012 Napa Valley Port	Red
RED	2015 California Red Blend	Red
ZIN 1	2014 Sonoma County Zinfandel	Red
ZIN 2	2013 Alexander Valley Zinfandel	Red
ZIN 3	2013 California Zinfandel	Red
ROSE	2016 Central Coast Rose	Rose
BRUT	N.V. Brut Sparkling	White
CHA 1	2014 Napa Valley Chardonnay	White
CHA 1	2015 Napa Valley Chardonnay	White
CHA 2	2015 California Chardonnay	White
CHA 3	2014 Central Coast Chardonnay	White
MOSC 1	2014 California Moscato	White
MOSC 2	2015 Napa Valley Moscato	White
MOSC 3	2016 Sonoma County Moscato	White
SAB 1	2015 Alexander Valley Fume Blanc	White
SAB 2	2015 California Sauvignon Blanc	White
VIO 1	2014 Central Coast Viogner	White
VIO 2	2015 Central Coast Viogner	White
WHITE	2014 Central Coast White Blend	White

N.V.: Non-Vintage.

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Table 3 Results of free SO_2 on the test wines using four methods. Aeration-Oxidation (A/O), Ripper, Headspace Gas Chromatography (HS-GC), Headspace Gas Detection Tube (HS-GDT).

		“Apparent” Free SO_2 (mg/L)		“Truly” Free SO_2 (mg/L)	
Sample ID	Wine Type	A/O	Ripper	HS-GC	HS-GDT
RED	Red	35.6 (1.0)	44.6 (1.0)	14.9 (0.2)	14.3 (1.6)
ZIN 1	Red	22.7 (1.0)	22.7 (1.0)	3.5 (0.0)	7.1 (0.9)
PIN 1	Red	30.6 (1.0)	36.7 (0.7)	14.9 (0.8)	15.1 (0.0)
BLAU	Red	13.3 (0.9)	16.3 (0.7)	1.2 (0.1)	1.9 (0.0)
CAB	Red	15.4 (0.0)	19.8 (1.5)	3.6 (0.2)	2.4 (0.0)
ZIN 2	Red	11.9 (0.5)	15.2 (0.4)	1.8 (0.1)	4.4 (2.2)
MER 1	Red	10.0 (2.0)	15.5 (0.8)	< L.D.	< L.D.
SAB 1	White	16.2 (1.0)	17.5 (0.8)	22.7 (0.2)	14.6 (0.0)
MER 2	Red	17.8 (0.5)	23.0 (0.4)	10.7 (0.4)	10.1 (1.2)
PIN 2	Red	22.8 (0.5)	30.4 (0.4)	16.3 (1.5)	11.3 (2.4)
MER 3	Red	11.7 (0.5)	15.9 (0.6)	5.3 (0.6)	< L.D.
ROSE	Rose	21.1 (1.0)	24.6 (1.0)	14.8 (0.1)	18.8 (2.3)
MOSC 1	White	8.8 (0.0)	9.5 (0.0)	2.5 (0.0)	3.4 (0.0)
PIN 3	Red	22.2 (1.0)	8.4 (1.5)	16.2 (0.1)	15.0 (2.0)
ZIN 3	Red	8.0 (0.0)	10.6 (0.4)	2.5 (0.1)	< L.D.
CHA 1	White	20.9 (0.0)	25.7 (0.4)	15.5 (0.5)	18.2 (2.5)
CHA 2	White	30.1 (0.5)	31.8 (0.4)	11.2 (0.2)	29.1 (1.4)
BRUT	White	27.1 (2.0)	25.7 (1.4)	25.3 (0.9)	25.9 (1.3)
CHA 1	White	17.2 (1.6)	18.1 (0.0)	16.0 (0.6)	15.3 (2.2)
WHITE	White	14.3 (1.6)	14.3 (0.7)	13.7 (0.5)	13.6 (0.9)
MOSC 2	White	17.1 (1.0)	17.3 (0.8)	16.5 (0.7)	13.2 (2.4)
MOSC 3	White	7.4 (1.0)	10.5 (0.8)	6.8 (0.5)	7.3 (1.5)
VIO 1	White	19.5 (0.9)	23.1 (0.9)	19.4 (0.2)	21.8 (2.7)
PORT	Red	< L.D.	6.4 (0.8)	< L.D.	< L.D.
SAB 2	White	23.3 (0.9)	24.8 (0.4)	23.5 (1.2)	23.1 (7.1)
VIO 2	White	15.2 (1.0)	17.3 (0.8)	15.8 (0.4)	11.5 (1.2)
CHA 3	White	32.7 (0.5)	34.5 (0.8)	34.6 (0.7)	34.3 (0.8)
	Average Std. Dev. (mg/L)	0.8	0.7	0.4	1.4
	Average % RSD	4.60%	3.79%	3.72%	11.83%

* SO_2 data are in mg/L and standard deviation is shown in brackets. L.D.: limit of detection.
RSD: Relative standard deviation.

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Table 4 Standard enological data and calculated pK_a values on the tested wines.

Sample ID	Wine Type	Alcohol (% v/v)	pH	Total SO_2 (mg/L)	True pKa (pK_M) (UT)	True pKa (pK_M) (Coelho)
RED	Red	13.81	3.59	108.9	2.11	2.02
ZIN 1	Red	15.10	3.65	80.0	2.14	2.04
PIN 1	Red	13.81	3.68	76.9	2.11	2.02
BLAU	Red	13.26	3.63	28.8	1.98*	2.01*
CAB	Red	13.83	3.73	51.2	1.99*	2.02*
ZIN 2	Red	15.26	3.78	43.3	2.14	2.04
MER 1	Red	15.38	3.63	87.6	2.14	2.04
SAB 1	White	13.86	3.32	78.3	2.12	2.02
MER 2	Red	13.66	3.49	76.9	2.11	2.02
PIN 2	Red	13.90	3.50	61.5	2.12	2.02
MER 3	Red	13.87	3.71	46.7	2.12	2.02
ROSE	Rose	11.81	3.15	54.5	2.08	1.99
MOSC 1	White	8.44	3.57	103.3	2.01	1.95
PIN 3	Red	13.80	3.64	73.8	2.11	2.02
ZIN 3	Red	13.77	3.72	43.3	2.11	2.02
CHA 1	White	14.16	3.53	76.7	2.12	2.02
CHA 2	White	14.05	3.13	78.3	2.12	2.02
BRUT	White	11.57	3.44	156.7	2.07	1.99
CHA 1	White	13.86	3.37	83.2	1.99*	2.02*
WHITE	White	14.43	3.04	23.3	2.21**	2.03**
MOSC 2	White	8.00	3.35	113.3	2.00	1.94
MOSC 3	White	7.37	3.23	84.3	1.99	1.93
VIO 1	White	14.67	3.35	73.3	2.13	2.03
PORT	Red	18.57	3.82	22.7	2.21	2.08
SAB 2	White	13.53	3.23	76.7	2.11	2.02
VIO 2	White	14.30	3.45	46.7	2.20**	2.03**
CHA 3	White	13.50	3.26	86.7	2.19**	2.01**

* Temperature of analysis between HS-GC and HS-GDT differed by 2ºC (HS-GC: 20ºC, HS-GDT: 18ºC).
** Temperature of analysis between HS-GC and HS-GDT differed by 2ºC (HS-GC: 25ºC, HS-GDT: 23ºC).
All other samples were analyzed at 23ºC by HS-GC and HS-GDT.

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Table 5 Evaluation of metastable bisulfite complexes, total monomeric anthocyanins (mg/L malvidin-3-glucoside equivalents), acetaldehyde, 2-ketoglutarate, and pyruvate on California wines (n = 27).

Sample ID	Wine Type	* Metastable Bisulfite Complexes (mg/L)	Monomeric Anthocyanin (mg/L)	Acetaldehyde (mg/L)	2-Ketoglutarate (mg/L)	Pyruvate (mg/L)
RED	Red	20.7	107.19	11.1	43.3	15.6
ZIN 1	Red	19.2	42.89	22.5	67.2	12.7
PIN 1	Red	15.7	81.98	3.1	35.6	10.5
BLAU	Red	12.1	48.40	4.6	10.1	9.9
CAB	Red	11.8	37.71	6.1	55.7	11.0
ZIN 2	Red	10.1	22.48	9.5	67.0	8.6
MER 1	Red	10.0	40.16	20.1	76.1	15.4
SAB 1	White	7.4	0.00	27.5	26.6	12.3
MER 2	Red	7.1	40.16	12.2	28.5	17.7
PIN 2	Red	6.5	83.05	10.8	39.3	11.8
MER 3	Red	6.5	28.09	3.1	6.1	13.8
ROSE	Rose	6.4	6.32	21.2	38.9	10.5
MOSC 1	White	6.3	0.00	66.0	30.2	39.6
PIN 3	Red	6.1	49.52	9.8	41.4	17.7
ZIN 3	Red	5.5	19.96	8.6	91.9	8.7
CHA 1	Red	5.4	0.00	43.9	39.4	31.2
CHA 2	White	5.0	0.00	49.5	30.9	14.1
BRUT	White	1.8	0.00	81.9	33.6	46.8
CHA 1	White	1.2	0.00	54.2	37.9	15.7
WHITE	White	0.6	0.00	39.4	22.7	14.7
MOSC 2	White	0.6	0.00	24.7	42.2	13.1
MOSC 3	White	0.5	0.00	47.4	0.0	18.2
VIO 1	White	0.1	0.00	46.8	22.7	16.6
PORT	Red	0.0	11.11	13.3	56.8	46.0
SAB 2	White	-0.2**	0.00	40.1	29.5	19.1
VIO 2	White	-0.5**	0.00	27.5	26.6	13.1
CHA 3	White	-1.9**	0.00	42.5	36.2	14.6

* Metastable bisulfite complexes calculated from the difference between Free SO_2 by A-O and Free SO_2 by HS-GC.
** Artefact of percent recovery greater than 100%.

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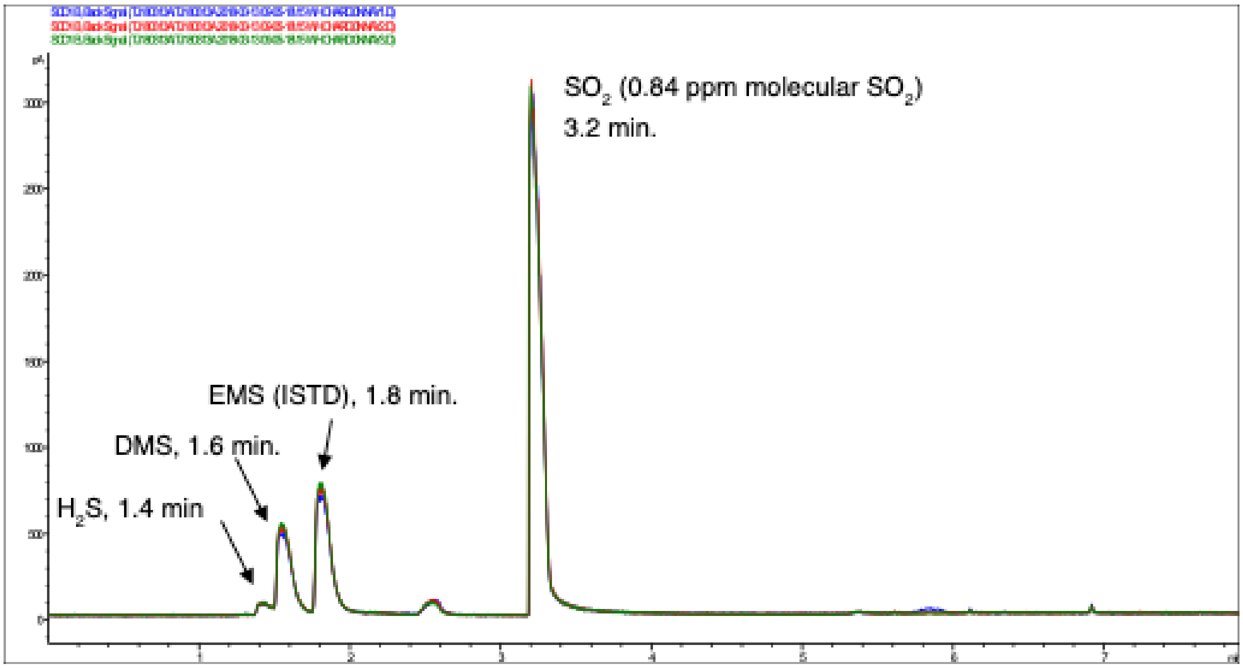

Figure 1 Chromatogram of a 2014 Central Coast Viogner. Column: DB-WAX-UI. Sampling method: Static headspace.

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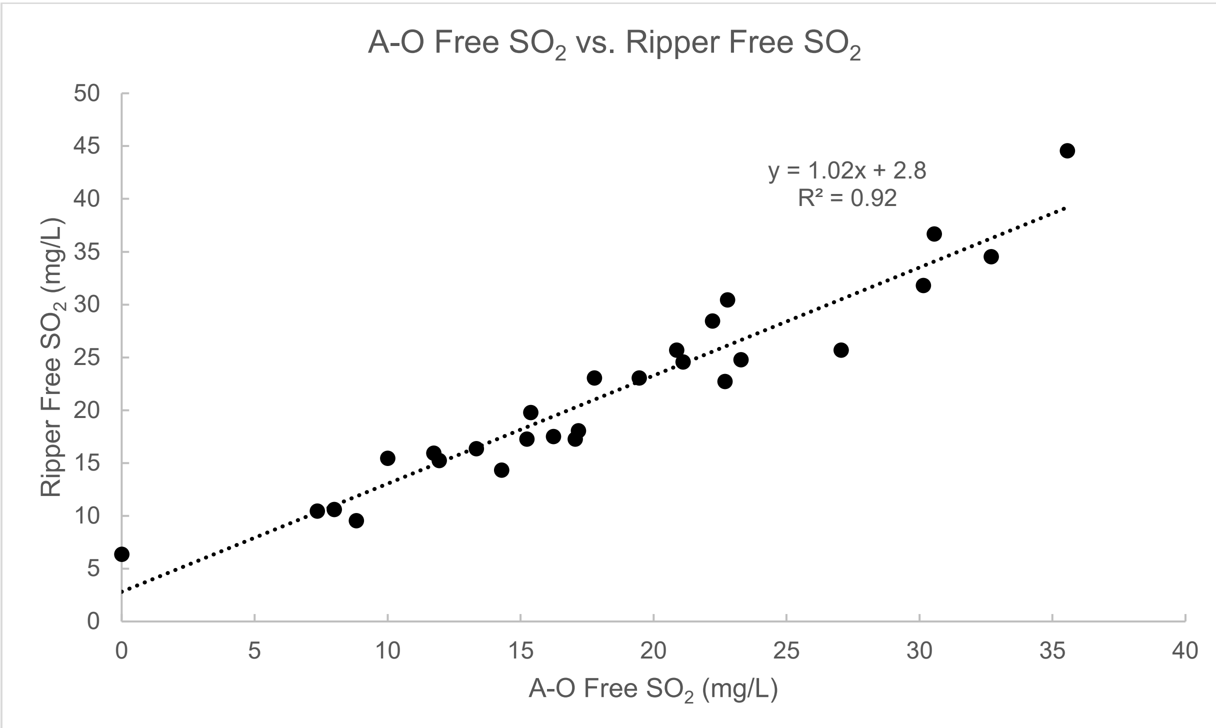

Figure 2 Correlation of free SO_2 values between aeration-oxidation and Ripper methods.

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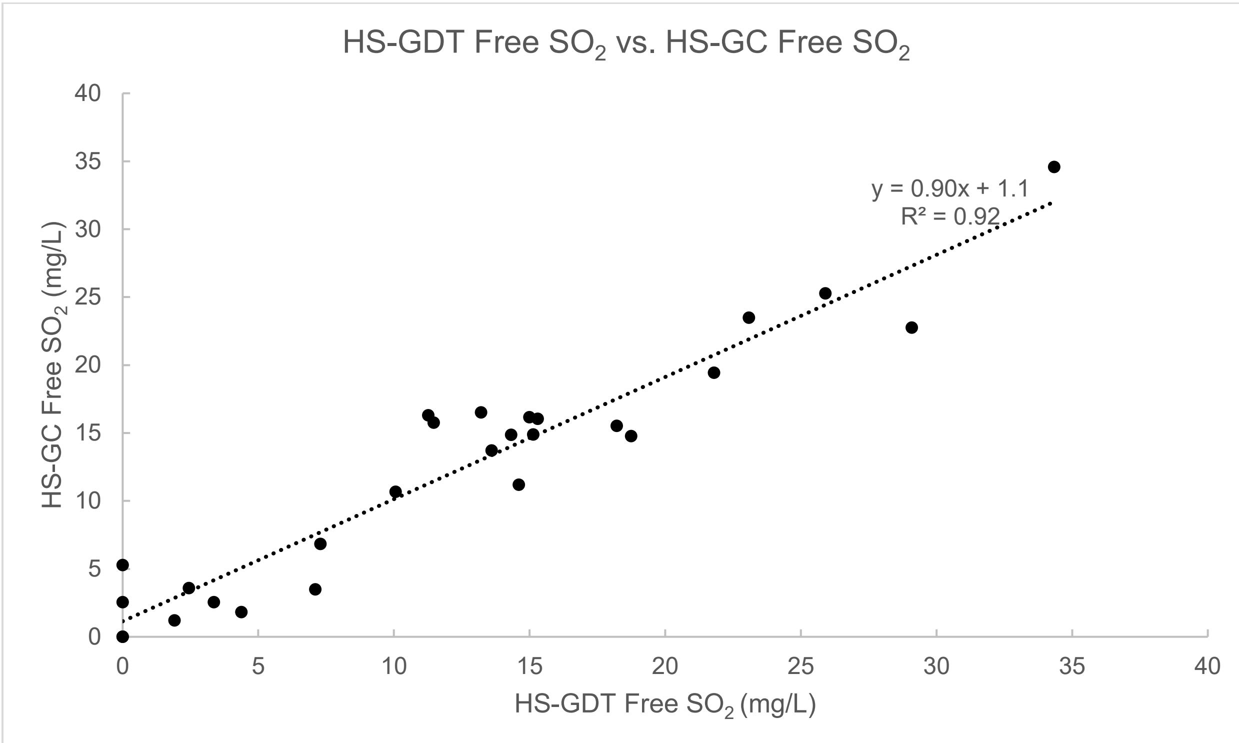

Figure 3 Correlation of free SO_2 values between the HS-GDT and HS-GC methods.

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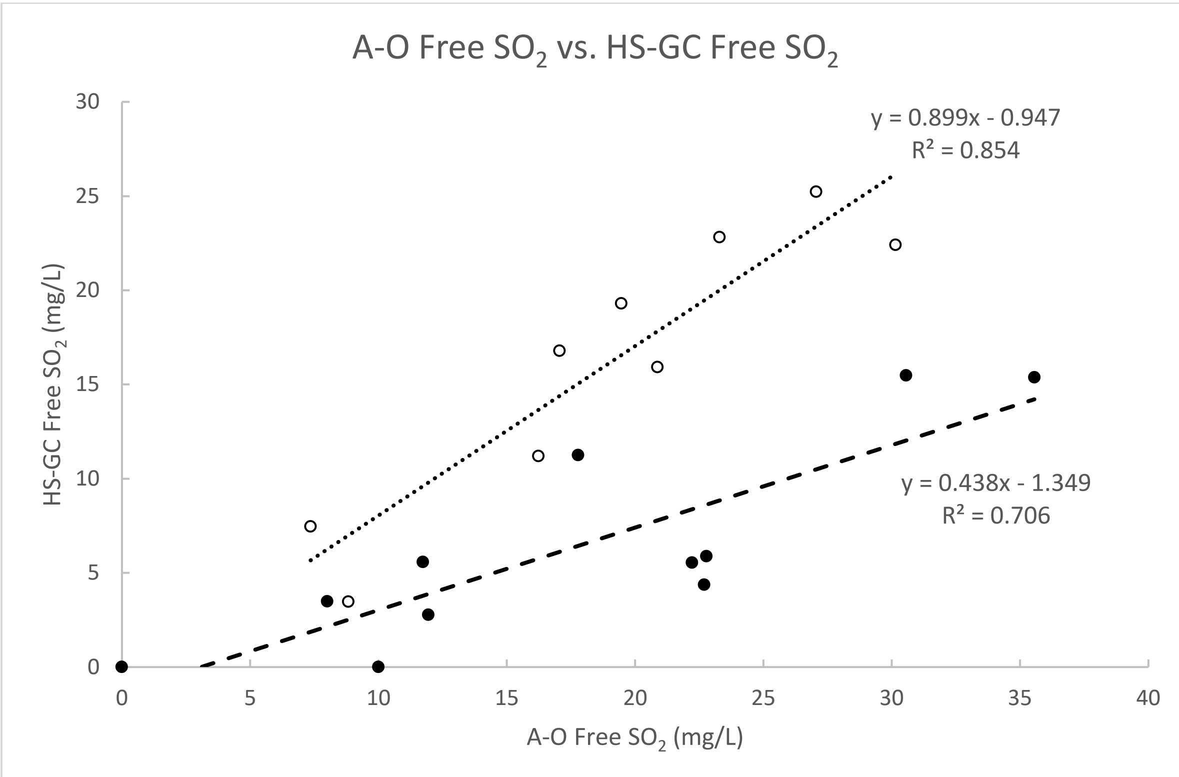

Figure 4 Correlations between A-O and HS-GC methods for red (---) and white (···) wines.

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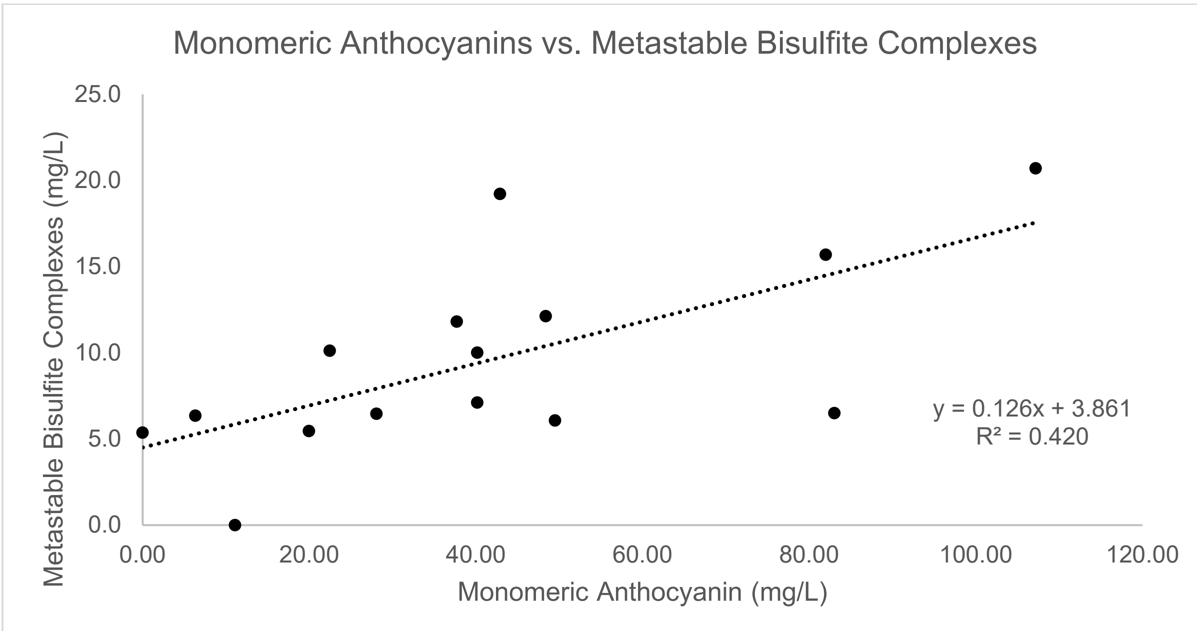

Figure 5 Correlation between metastable bisulfite complexes and anthocyanin concentration (red wines only). * Metastable bisulfite complexes by from the difference between Free SO_2 by A-O and Free SO_2 by HS-GC.