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 Ripper titration and aeration-oxidation (A-O) are widely used 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 solid phase microextraction (SPME) yielded poor precision in our experiments, 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 SO_2, or ~1 mg/L free SO_2 in wine at pH 3.5). A-O, Ripper, HS-GC-SCD, and HS-GDT methods were compared on a diverse set of wine samples. Results from HS-GC were correlated with those from the HS-GDT method ($r^2 = 0.92$) and achieved higher precision (relative standard deviation = 3.7%). HS-GC was highly correlated with A-O in white wines ($r^2 = 0.85$, slope = 0.90) but had weaker correlation for red wines ($r^2 = 0.71$, slope = 0.44). The flexibility of GC for other procedures as well as its stability and low operating costs per sample make it an attractive option, and headspace methods have been shown to be better for predicting microbial stability in red wines.

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

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; and (5) it reversibly binds aldehydes and ketones produced by oxidation or during fermentation, rendering them non-odorous (Waterhouse et al. 2016).

SO_2 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 molecular (SO_2), bisulfite (HSO_3^-), or sulfite (SO_3^{2-}) forms. In the typical pH range of wine (3.0 to 4.0), the dominant species is the bisulfite anion, which acts as an antioxidant and participates in various binding/complexing reactions. Molecular SO_2, the main antimicrobial form of SO_2, is present at only a small fraction (<5%) of the HSO_3^- concentration at wine pH. SO_3^{2-} is present at even a smaller fraction of the HSO_3^- concentration (<0.1%) at wine pH; thus, its influence on wine stability is likely negligible. SO_2 in wine is further divided into two classes: free and bound. Free SO_2 is defined as the sum of molecular and bisulfite forms and is the class with antimicrobial, antioxidant, and enzyme-inhibiting properties. Bound SO_2 comprises the bisulfites that react (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). 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, which do not have these properties. Common analytical methods for free SO_2 in wineries include iodometric titration (Ripper method) and aeration-oxidation (A-O) method (Iland et al. 1993, Urbano-Cuadrado et al. 2004). These standard methods utilize an

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initial acidification step to 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 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).

This artefactual overestimation of free SO_2 can be avoided by measuring the headspace SO_2 concentration of an equilibrated wine sample. This concentration of headspace SO_2 can 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, 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 an approximately three-fold overestimation of free SO_2 in red wines 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 for prediction of 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, including 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 magnitude in red wines, although none of the methods 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 in 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 those analyzed by other analytical approaches for measuring SO_2. In this work, we reported development of an HS-GC-SCD method for "truly" free SO_2, and compared it to other methods for measuring SO_2 (HS-GDT, A-O, Ripper). We also compared the differences between headspace methods and conventional methods (A-O, Ripper) for measurement of substances that might form metastable bound SO_2 that could be released during conventional analyses to evaluate a basis for the discrepancy between the headspace and conventional methods.

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. L-Tartaric acid (99%) was obtained from Fisher Scientific. Ethanol (anhydrous, ≥99.5%) was obtained from Decon Laboratories. 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 Enartis Vinquiry. Ethyl methyl sulfide (1000 µg/mL) was obtained from SPEX CertiPrep.

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 and 10% ethanol and adjusted with NaOH solution to a pH of 3.50. The ethanol concentration was verified using an Alcolyzer Wine M. The true pKa (pK_M) for SO_2 in each batch of model wine was determined using the following calculations, and the concentration of each of the calibration standards was calculated using the Henderson-Hasselbalch equation.

Estimation of pK_{a1} (pK_M) of SO_2 and calculation of free SO_2 from molecular SO_2. The following equations were built from a multiple linear regression model using XLSTAT (Addinsoft) 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, °C), the following equation was used (Equation 1).

Estimation of pK_T.

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

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

Estimation of A constant.

$$A = 0.482724 + (0.000883782 * T) + (0.00443752 * Alc.) + (0.00000595973 * T^2) + (0.0000489638 * Alc.^2) \quad \text{Eq. 2}$$

Estimation of B constant.

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

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) was determined by Equation 4 below.

Estimation of pK_M.

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

Because the measurement of ionic strength (I) is complex and labor intensive, a typical ionic strength of 0.056 M can be assumed (the typical range for ionic strength in wine is 0.016 M to 0.100 M) and was used in the calculation of pK_M (Berg and Keefer 1958, 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 SO_2 as a function of pH.

Modified Henderson-Hasselbalch equation.

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

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 (Coelho et al. 2015) were all performed in triplicate for 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 SCD (Agilent Technologies). 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 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 sec. The temperature program for the final method started at 50°C, which was maintained for 2.5 min, increased at a rate of 50°C/min to 220°C, and held at 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 and an air flow rate of 40 mL/min. The SCD pressure was 6 Torr with the controller at 200 Torr.

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) for each analysis. 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 hr before running the procedure. HS-GC-SCD analyses were then performed as described above. Because a headspace volume of 50 mL at room temperature has already been shown to take a minimum of 5 min 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 in Table 1.

Monomeric anthocyanins by high performance liquid chromatography (HPLC). The separation of the monomeric anthocyanins was conducted with reverse-phase HPLC using an Agilent 1100 series (Agilent Technologies) 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; the pumps were adjusted to 70% A and 30% B at 15 min; to 50% A and 50% B at 30 min; to 40% A and 60% B at 35 min; and to 94% A and 6% B at 41 min (end of analysis). After a 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-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 HPLC. Acetaldehyde, 2-ketoglutarate, and pyruvate were determined by HPLC after derivatization reaction with DNPH reagent (Sigma-Aldrich) as reported (Han et al. 2015). Briefly, aliquots of wine samples (100 µL) were dispensed into a vial, followed by the addition of 20 µL of freshly prepared 1120 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 was 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,

Table 1 Method figures of merit.

Parameters	Analytical parameter
Correlation coefficient	0.997
Linear range (molecular SO_2 mg/L)	0.067-2.00
Limit of detection (molecular SO_2 mg/L)	0.033
Limit of quantification (molecular SO_2 mg/L)	0.067
RSD, %[a]	3.72

[a]Based on triplicate analysis of 27 different wines. RSD, relative standard deviation.

SB-C18 column (1.8 µm, 4.6 × 100mm^2; Agilent Technologies) was used for separation. Separation was obtained using a flow rate of 0.75 mL/min and column temperature of 35°C, and the mobile phase solvents were (A) 0.5% formic acid (Sigma Aldrich ≥95%) in water milli-Q and (B) acetonitrile (Sigma Aldrich ≥99.9%). The gradient elution protocol was as follows: 35% B to 60% B (0 to 8 min); 60% B to 90% B (9 to 13 min); 90% B to 95% B (14 to 15 min, 2-min hold); and 95% B to 35% B (16 to 20 min, 4-min hold), with a total run time of 20 min. Eluted peaks were measured at 365 nm 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).

pH. The pH of all wine samples and model wines was measured using an Orion 5 Star (Thermo Scientific). The pH probe was calibrated daily using buffers of 2.00, 4.01, and 7.00 pH standards. Slopes of each calibration ranged from 96 to 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.

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

Sample ID	Wine	Wine type
BLAU	2015 Paso Robles Blaufränkisch	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	NV Brut Sparkling[a]	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 Viognier	White
VIO 2	2015 Central Coast Viognier	White
WHITE	2014 Central Coast White Blend	White

[a]NV: Non-vintage.

Results and Discussion

In our initial work, we evaluated the use of solid-phase microextraction (SPME) followed by separation on a porous layer open tubular (PLOT) GC column, since similar methods have been used for analysis of other volatile sulfur compounds in wine. This initial approach used short SPME exposure times to avoid perturbation of equilibria but was determined to be unacceptable due to poor precision and excessive peak broadening on the PLOT column that was difficult to analyze (data not shown). We then evaluated static headspace injection with different columns (DB-Sulfur, DB-WAX-ETR, and 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% relative standard deviation [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 using ethyl methyl sulfide (EMS) as the internal standard (Carrascon et al. 2017). By design, the use of an SCD as opposed to a mass spectrometer detector was intended to improve sensitivity and selectivity to SO_2. The selected column does degrade with time from the SO_2 exposure and should be replaced after ~200 injections.

With the DB-WAX-UI column and corresponding GC parameters for this method, the elution time for the SO_2 peak and EMS internal standard was ~3.2 and 1.8 min, respectively, and neither of these co-eluted with any other potentially interfering compounds typically found in wine. A representative chromatogram of a 2014 Central Coast Viognier 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 performed in triplicate to assess and compare the precision of the methods.

Table 4 tabulates other basic chemistry parameters of the wine samples analyzed in this set. The estimates of true pKa

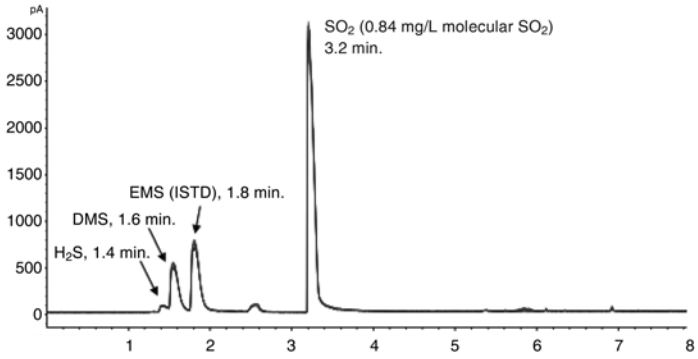

Figure 1 Chromatogram of a 2014 Central Coast Viognier. Column: DB-WAX-UI. Sampling method: Static headspace. DMS, dimethyl sulfide; EMS, ethyl methyl sulfide; ISTD, internal standard (EMS).

based on alcoholic strength, temperature, and ionic strength are also shown. For SO_2 measured 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 measured by HS-GDT, a related approach was used in Coelho et al. (2015) for estimating the true pKa.

Because the temperatures of the HS-GC and HS-GDT analyses were not controlled beyond the prevailing ambient room temperature (18 to 21°C), a few comparative analyses were conducted at non-equivalent temperatures. Specifically, analysis of the 2015 Paso Robles Blaufränkisch (BLAU), 2015 California Cabernet Sauvignon (CAB), and 2014 Napa Valley Chardonnay (CHA 1) by HS-GC and HS-GDT was at a 2°C differential, with the HS-GC analysis performed at 20°C and the HS-GDT analysis performed at 18°C. Analysis of the 2014 Central Coast White Blend (WHITE), 2015 Central Coast Viognier (VIO 2), and 2014 Central Coast Chardonnay (CHA 3) samples also occurred at a 2°C differential, with the HS-GC analysis performed at 25°C and the HS-GDT analysis performed at 23°C. While the respective formulas for calculating the true pKa have built-in functions that account for the difference in temperature, it is unclear whether 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 ± 1°C. Given the uncertainty, these data points were excluded from the statistical analysis.

The full comparison of analytical methods indicated that analytical precision of the A-O and Ripper methods were comparable and satisfactory, as both methods had RSDs (%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. 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, which was likely due to dissolved CO_2 that carried over into the peroxide trap used in the A-O procedure and resulted in an over-titration and subsequent over-reporting of apparent free SO_2. Interestingly, the overall RSD for the Ripper method (3.79%)

Table 3 Results of free SO_2 in the test wines using aeration-oxidation (A-O), Ripper, headspace gas chromatography (HS-GC), and headspace gas detection tube (HS-GDT) methods.[a]

		"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)	<LD	<LD
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)	<LD
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)	<LD
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	<LD	6.4 (0.8)	<LD	<LD
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[b]		4.60%	3.79%	3.72%	11.83%

[a]Standard deviation is shown in brackets. LD: limit of detection.
[b]RSD: Relative standard deviation.

was lower than that for the A-O method (4.60%). This finding is in contrast to findings from older studies that reported RSDs 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 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, with the maximum absolute difference 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 measured by A-O. This effect may be due to over-titration beyond 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 interfering compounds such as 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 a maximum absolute difference of 6.3 mg/L. The HS-GC and HS-GDT methods had average standard deviations of 0.4 and 1.4 mg/L free SO_2, respectively. In terms of analytical precision, the HS-GC technique had an RSD (3.72%) that was appreciably lower than the RSD for the HS-GDT method (11.83%). The lower precision of the HS-GDT method was likely due to the difficulty in reproducibly identifying 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 analysis of wines by the HS-GC and HS-GDT methods was performed at the same temperature ± 1°C. The bottled wine samples were equilibrated at room temperature (23°C) for a minimum of 24 hrs prior to analysis. Temperature of the wine samples was recorded at the time of each batch of HS-GDT analysis. For the HS-GC analysis, the heating element of the sample agitator was turned off because 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 to 3°C for comfort but is not regulated to ± 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

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) (Usseglio-Tomasett and Bosia 1984)	True pKa (pK_M) (Coelho et al. 2015)
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[a]	2.01[a]
CAB	Red	13.83	3.73	51.2	1.99[a]	2.02[a]
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[a]	2.02[a]
WHITE	White	14.43	3.04	23.3	2.21[b]	2.03[b]
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[b]	2.03[b]
CHA 3	White	13.50	3.26	86.7	2.19[b]	2.01[b]

[a]Temperature of analysis between headspace gas chromatography (HS-GC) and headspace gas detection tube (HS-GDT) differed by 2°C (HS-GC: 20°C, HS-GDT: 18°C).

[b]Temperature of analysis between HS-GC and HS-GDT differed by 2°C (HS-GC: 25°C, HS-GDT: 23°C). All other samples were analyzed at 23°C by HS-GC and HS-GDT.

analysis temperatures. Moreover, the imprecision of the end point determination with the GDTs may have amplified the apparent differences. Better temperature control should be possible to improve the correlation and precision. In practice, precise management of GDT temperatures would be difficult in a small-scale operation.

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

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

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

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

For example, for a liquid concentration 1.8×10^{-5} M molecular SO_2 at 23°C, the K_H value is 1.38 M/atm. Using Henry's Law, the vapor pressure of SO_2 above the liquid would be 1.3×10^{-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.

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

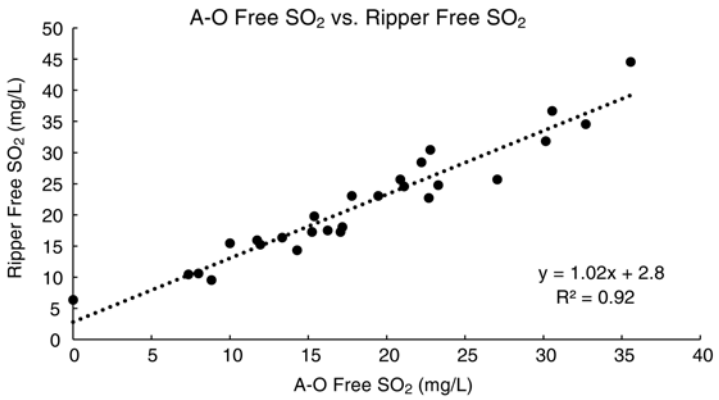

Figure 2 Correlation of free SO_2 values measured by aeration-oxidation (A-O) and Ripper methods.

Further calculations showed that under these conditions, ~1% of the SO_2 in the sample was present in the 5 mL of headspace in the GC vial that contained 15 mL of sample, a small fraction that 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, including monomeric anthocyanins, acetaldehyde, pyruvate, and 2-ketoglutarate, which are all candidate compounds for metastable bisulfite complexes (Table 5). Monomeric anthocyanins were evaluated by HPLC and expressed as mg/L of malvidin-3-glucoside equivalents. Concentrations of acetaldehyde, pyruvate, and 2-ketoglutarate in the wine samples were determined by HPLC after derivatization reaction with DNPH reagent (Han et al. 2015). We also calculated "metastable bisulfite" as the difference between the A-O and HS-GC methods and performed linear regressions for metastable SO_2 binders (monomeric anthocyanins acetaldehyde, pyruvate, and 2-ketoglutarate) against the concentration of metastable bisulfite complexes observed in each wine.

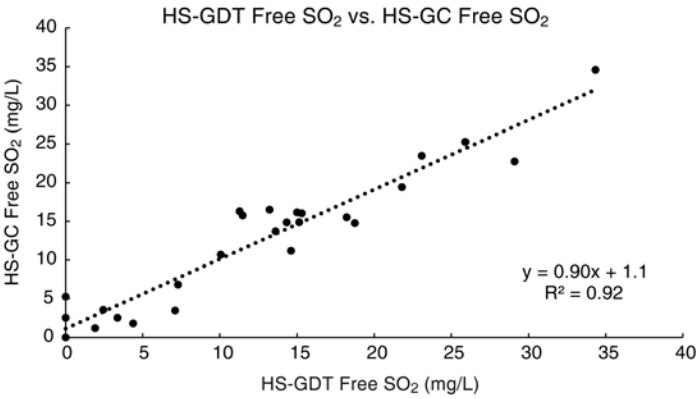

Figure 3 Correlation of free SO_2 values measured by the headspace gas detection tube (HS-GDT) and headspace gas chromatography (HS-GC) methods.

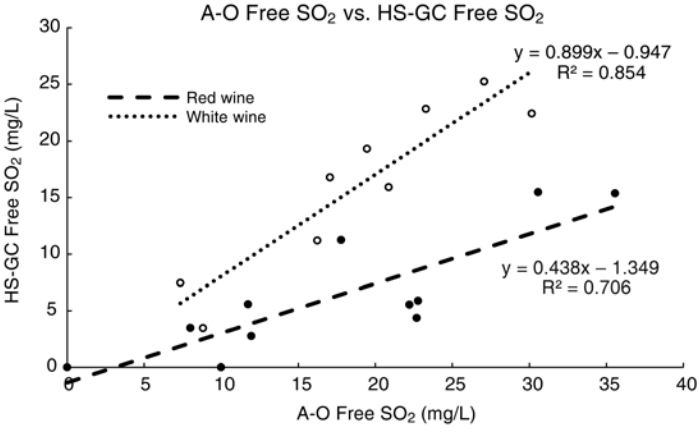

Figure 4 Correlations between aeration-oxidation (A-O) and headspace gas chromatography (HS-GC) methods for red and white wines.

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 acetaldehyde, alpha-ketoglutarate, or pyruvate ($r^2 < 0.1$; plots not shown) in red or white wines, suggesting that these compounds were not related to the discrepancies between methods, similar to findings reported by others (Bisson 1999). Similar correlations were observed to explain the discrepancies in measurements using the Ripper and HS-GDT techniques (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) (Brouillard and Elhagechahine 1980). By comparison, the first-order rate constant glucose-bisulfite complex dissociation is 3.7×10^{-4}/min (Vas 1949). Some derived anthocyanin pigments that are known to also bind SO_2 or respond to pH changes (Zimman and Waterhouse 2004) are not quantified by the method presented here. As demonstrated in Coelho et al. (2015), a higher correlation would likely have been found if a different method, such as SO_2 bleaching, had been used for total anthocyanin content.

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 ratios 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

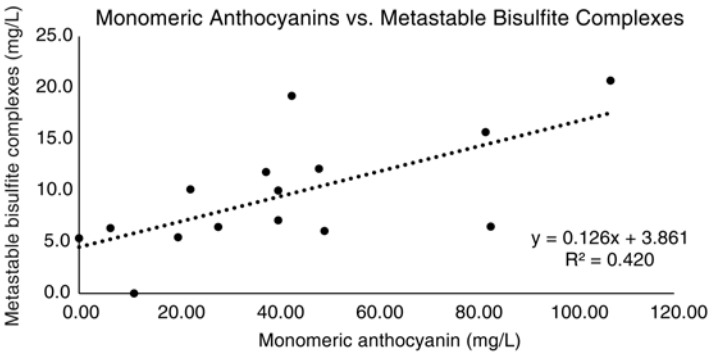

Figure 5 Correlation between metastable bisulfite complexes and anthocyanin concentration (red wines only). Metastable bisulfite complexes were calculated as the difference between free SO_2 by aeration-oxidation (A-O) and free SO_2 by headspace gas chromatography (HS-GC).

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[a] (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[b]	0.00	40.1	29.5	19.1
VIO 2	White	-0.5[b]	0.00	27.5	26.6	13.1
CHA 3	White	-1.9[b]	0.00	42.5	36.2	14.6

[a]Metastable bisulfite complexes calculated from the difference between free SO_2 by aeration-oxidation and free SO_2 by headspace gas chromatography.
[b]Artefact of percent recovery greater than 100%.

amount of molecular SO_2 required to attain a signal to noise ratio of 3, and the limit of quantification was calculated as the amount of molecular SO_2 required to attain a signal to noise ratio of 10. For the HS-GC-SCD method, the limits of detection and quantification were 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) reported an even lower limit of detection (0.46 µg/L molecular SO_2); however, it is not clear how these values were calculated, which makes direct comparison difficult.

The increased significance of headspace versus conventional methods with regard to microbial stability in winemaking has been demonstrated (Howe et al. 2018). Conventional methods detected significant molecular SO_2 that should suppress yeast, but no suppression was observed in red wine. By contrast, the headspace method properly predicted suppression at ~0.8 mg/L molecular SO_2.

Conclusion

Based on a gas detection tube method, we developed an analytical procedure using HS-GC coupled with SCD that can rapidly and precisely quantify molecular and free SO_2 in wine. The method requires minimal sample preparation and involves no chemical reagents (with the exception of a trace internal standard). At room temperature (23°C), the method can successfully detect levels of molecular SO_2 at concentrations as low as 0.033 mg/L. The total chromatographic time for the method is eight minutes and, provided that information on the alcohol concentration and pH is readily available, the molecular and free SO_2 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%.

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 formation of metastable complexes with bisulfite that are inadvertently released during conventional analysis methods, leading to inflated and misleading results. Since headspace analysis of SO_2 in wine has been shown to predict microbial stability better than conventional methods, the adoption of headspace-based methods may improve prediction of wine stability.

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