Persistence of Elemental Sulfur Spray Residue on Grapes during Ripening and Vinification

Misha T. Kwasniewski,[1,3]* Gavin L. Sacks,[1] and Wayne F. Wilcox[2]

Abstract: Elemental sulfur (S^0) is commonly used to control powdery mildew in vineyards, but S^0 residues in musts have been correlated with increased hydrogen sulfide (H_2S) and sulfurous off-aroma formation during fermentation. As a consequence, S^0 is often used sparingly late in the season, but defining appropriate preharvest intervals for S^0 sprays has been challenging due to limited data on S^0 persistence in vineyards and during prefermentation operations. Using a new quantification method, S^0 residues were monitored in the vineyard over three years of field studies. Treatments varied in commercial formulation, application rate, and timing of the last application before harvest, all of which affected S^0 concentrations on the fruit at harvest. Residue concentrations generally were lower for a wettable powder versus a micronized formulation applied at the same rate and timing and increased proportionally to the application rate when timing and formulation were constant. In all years, ceasing application ≥35 days prior to harvest resulted in S^0 residues below the 10 µg/g concentration associated with increased H_2S production in several previous studies. S^0 residues >1 µg/g correlated with increased H_2S production in our current work and were observed on all fruit sprayed within 56 days of harvest. However, clarification decreased S^0 in must by >95% prior to fermentation in all treatments. Furthermore, fermentation on treated skins increased H_2S formation nearly 3-fold over fermentations without skin contact. Collectively, these results indicate that S^0 residues are likely of low concern in white winemaking, whereas residue concentrations in red fermentations can exceed those associated with increased H_2S production when some S^0 sprays are applied within eight weeks of harvest.

Key words: pesticide, fungicide, reduced off-aromas, quantification, powdery mildew

Various commercial formulations of elemental sulfur (S^0) are used to control the most common disease of grapes worldwide, powdery mildew (PM), caused by the fungus *Erysiphe necator* (syn. *Uncinula necator*) (Gadoury et al. 2011). The advantages of S^0 over alternatives include its low cost, good efficacy, low risk of resistance development, and its acceptability within various "organic" and "biological" production systems, where it is arguably the most efficacious material available for control of PM (Savocchia et al. 2011). However, S^0 residues remaining at harvest can be reduced to hydrogen sulfide (H_2S) during fermentation, and its use in the vineyard has long been tied to reduced sulfur characters in some finished wines made from treated grapes (Rankine 1963, Acree et al. 1972, Schutz and Kunkee 1977). While the aforementioned studies indicate that increased H_2S production occurs when must S^0 concentrations exceed 10 mg/L (or ~10 µg/g of harvested fruit when fermented with skins), there is disagreement as to the impact of S^0 residues at lower concentrations, with some concentrations as low as 1 mg/L significantly increasing H_2S production (Thoukis and Stern 1962, Wenzel et al. 1980). The S^0 concentration necessary to cause problems is not well agreed upon, in part because H_2S production is affected by factors other than S^0 concentration. H_2S is produced during fermentation as a byproduct of amino acid synthesis during normal yeast (*Saccharomyces cerevisiae*) metabolism (Jiranek et al. 1995), and this pathway can lead to differences in H_2S production in the absence of S^0 residues, related to differences in juice nutrient status (Ugliano et al. 2009), must turbidity, yeast strain (Rankine 1963), and fermentation temperature (Schutz and Kunkee 1977).

Unfortunately, there are few data available concerning the persistence of S^0 in the vineyard or during prefermentation vinification practices, and the limited number of studies that have attempted to quantify S^0 residues following field treatments show conflicting data. For example, Thomas et al. (1993b) working in California found that applications of 10 to 17 kg/ha of S^0 formulated as dust resulted in residues <14 µg/g on fruit one day after application; that residues had declined to <4 µg/g within two additional weeks; and that final residue concentrations at harvest (six weeks after the last application) were 1 to 3 µg/g. In contrast, Wenzel et al. (1980) working in Germany found residue concentrations as high as 8 µg/g at harvest when applications of a sprayable S^0 formulation ceased seven weeks beforehand, although application rates were not disclosed. The same group also demonstrated

[1]Department of Food Science and [2]Department of Plant Pathology and Plant-Microbe Biology, NYS Agricultural Experiment Station, Cornell University, Geneva, NY 14456; and [3]present address: Grape and Wine Institute and Department of Food Science, University of Missouri, Eckles Hall, Columbia, MO 65211.

*Corresponding author (kwasniewskim@missouri.edu; tel: 573-882-6656)

Acknowledgments: The authors gratefully acknowledge the assistance of Herb Cooley, Dr. Olga Padilla-Zakour, Luann Preston-Wilsey, Pam Raes, and Duane Riegel. This work was supported in part by Federal Formula Funds and the NY Farm Viability Institute.

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

Manuscript submitted Feb 2014, revised Jul 2014, accepted Jul 2014

doi: 10.5344/ajev.2014.14027

that clarification of white wine must can greatly lower the S^0 concentration therein, leading to lower H_2S production during fermentation (Wenzel and Dittrich 1978, Wenzel et al. 1980). As a result of these conflicting observations, growers and winemakers cannot objectively assess the risk that late season applications will yield deleterious residues on berries, sometimes resulting in arbitrary commercial restrictions and conflicting recommendations regarding late-season sulfur use. A poor understanding of this relationship increases the likelihood of economic losses resulting from (1) an unnecessary overreliance on more expensive alternatives to S^0, which also increases the probability of compromised disease control following the eventual development of pathogen resistance to many of the substituted materials, or (2), at the other extreme, the production of faulted wine as a result of S^0 application too close to harvest.

A major impediment to studies requiring quantification of S^0 residues has been the lack of an affordable technology to do so in complex matrices such as grape juice and must, as standard elemental analysis techniques measure total sulfur, including not only S^0 but also sulfur from endogenous sulfates, S-amino acids, and other compounds. Thomas et al. (1993b) circumvented this limitation by washing sulfur dust residues from the surface of intact clusters and measuring total S in the rinsate. We were unable to apply this technique successfully in our own initial field studies, as the sprayable formulations of S^0 used in many regions (and which are standard in humid climates such as New York) left visible residues on the fruit after repeat washings and measured S concentrations in the rinsate were unexpectedly low. However, we recently reported the development of a rapid, inexpensive technique for measuring S^0 in complex matrices, based upon its quantitative reduction to H_2S in situ and simultaneous colorimetric quantification using commercially available detection tubes (Kwasniewski et al. 2011). The present report details the subsequent use of this technique to study the effect of fungicide formulation, rate, and application timing on the persistence of S^0 residues on grape clusters in the field and their transfer to the must after harvest and crushing. Additionally, we report on the influence of vinification factors such as whole-cluster pressing, length of skin contact, and must clarification on the proportion of S^0 transferred into the must.

Materials and Methods

S^0 persistence following field applications. Three years of field trials were conducted in test vineyards at the New York State Agriculture Experiment Station in Geneva, NY (lat: 42°52'43"; long: -77°00'56"), to determine the effect of preharvest spray interval, product formulation, and application rate on S^0 persistence. In 2009 and 2010, these trials were conducted on vines of *Vitis vinifera* cv. Chardonnay, and in 2011 on *V. vinifera* cv. Riesling. All vines were planted in 2004 on 3309C rootstock and were trained to a vertical shoot-positioned system with 3 m row spacing and 2 m vine spacing. Vines were sprayed and fertilized according to typical commercial practices for the region, except that no S^0 sprays were applied other than those in the variable treatment regimens. S^0 treatments were applied to test vines using a custom-built, over-the-row, hooded boom sprayer operating at 2070 kPa pressure and delivering 935 L/ha through seven hollow cone nozzles on each side of the boom. Cumulative temperature and rainfall data for each intervening period between S^0 applications and between the final application and harvest were determined (Table 1).

Two commercial elemental sulfur products were applied over the course of this study: a micronized formulation (Microthiol Disperss 80DF, Cerexagri Inc., King of Prussia, PA) and a wettable powder formulation (Yellow Jacket Wettable Sulfur, Georgia Gulf Sulfur Corp., Valdosta, GA). Particle size for these formulations was quantified using a Mastersizer 2000 (Malvern Instruments, Worcestershire, UK). The median particle diameter of the micronized formulation was 4.7 µm with 90% of particles between 2.6 and 8.4 µm, and the median particle diameter of the wettable powder was 32.0 µm with 90% of particles between 9.0 and 73.5 µm.

In 2009, a single application of the micronized formulation was made either 68, 40, or 12 days preharvest, at a rate of either 2.69 or 5.38 kg/ha S^0. Each of the seven treatments, including a control in which no S^0 was incorporated, was applied to six replicate four-vine panels arranged in a randomized complete block design. Fruit was harvested 14 Oct.

In 2010, all treatments were initiated on 12 Aug (veraison), with additional sprays applied at approximately two-week intervals and continuing until either 50, 35, 22, or 8 days before harvest (1 Oct 2010), depending on the treatment. Vines in the 50-day preharvest treatment received only a single application of micronized sulfur at a rate of 2.69 kg/ha S^0, whereas those

Table 1 Accumulation of heat units and precipitation during the periods between sulfur applications in field experiments, 2009 to 2011.

Treatment date (PHI)[a]	Degree days (10 C)[b]	Precipitation (mm)[c]
2009		
7 Aug (68)	–	–
4 Sep (40)	522	69.0
2 Oct (12)	267	145.0
Harvest	28	33.0
2010		
11 Aug (50)	–	–
26 Aug (35)	272	96.0
9 Sep (22)	263	13.2
23 Sep (8)	127	34.8
Harvest	88	25.7
2011		
16 Aug (54)	–	–
1 Sep (38)	305	103.4
14 Sep (25)	236	57.7
27 Sep (12)	136	32.3
Harvest	102	63.0

[a]PHI = preharvest interval (days).
[b]Accumulated degree days (base 10°C) since previous sulfur application.
[c]Accumulated precipitation (mm) since previous sulfur application.

in the latter three timing regimens received applications of either wettable sulfur at 2.69 or 5.38 kg/ha S^0 or micronized sulfur at 5.38 kg/ha S^0. Individual plots consisted of two consecutive four-vine panels for each of the 11 treatments (including control), arranged in a randomized complete block design with three replications. For each treatment, five clusters were randomly sampled for S^0 residue analysis from all panel replicates at 32, 30, 28, 24, 20, 16, 7, 2, and 0 days before harvest.

In 2011, individual plots again consisted of two consecutive four-vine panels with the 11 treatments (including control) arranged in a randomized complete block design with three replications. Vines received 4.48 kg/ha S^0 in either micronized or wettable powder formulation, beginning on 10 Aug and continuing at approximately two-week intervals until 54, 38, 25, or 12 days before harvest (16 Oct) for a maximum of five possible applications. An additional treatment was included that received micronized sulfur at 4.48 kg/ha in the first applications and 2.24 kg/ha in the final two applications, 54 and 38 days before harvest. For all treatments, five clusters were randomly sampled for S^0 residue analysis from each of the two-panel plots in each of the three replicate blocks at 62, 53, 47, 40, 31, 24, 17, 9, and 0 days before harvest. In all experiments, S^0 residues were determined as described below, and treatment means were first compared within a given sampling date using two-way ANOVA, followed by parametric testing within a sampling period using Tukey HSD.

Quantification of S^0 residues. The method described in Kwasniewski et al. (2011) was followed for S^0 residue quantification. Briefly, for grape samples from the field, a whole cluster (fresh or frozen) was first blended with an equal weight of water using an immersion blender; juice and must samples obtained after pressing were used without initial preparation. Each sample was heated in PEG 400 (Fisher Scientific, Pittsburgh, PA) to disperse S^0, diluted with water, and subsequently de-aerated and adjusted to pH 6 by adding a pharmaceutical antacid tablet (Alka-Seltzer, Bayer Healthcare, Morristown, NJ). The 2.95 g antacid tablets consisted of 0.32 g acetylsalicylic acid, 1.63 g sodium hydrogen carbonate, 0.97 g citric acid anhydrous, and <0.04g of the following: povidone, dimeticone, calcium silicate, docusate sodium, sodium benzoate, and sodium saccharin. Following de-aeration, dithiothreitol (Fisher Scientific) was added to reduce S^0 to H_2S, and the H_2S was sparged through either a Gastec 4L or 4LL model H_2S gas detection tube (Fisher Scientific) via sequential addition of two additional antacid tablets. The S^0 concentration was determined by relating the distance of color change on an H_2S detection tube to that observed for calibration standards.

Vinification. All wines were vinified in triplicate using the following procedure commonly applied to white wines, unless otherwise noted. Grapes from a given treatment were hand harvested, crushed and destemmed, then pressed in a hydraulic basket press. The collected juice was treated with 50 mg/L SO_2 and allowed to settle for 24 hr. Following settling, juice was inoculated with *Saccharomyces cerevisiae* strain DV10 (Lallemand, Petaluma, CA) previously rehydrated in 10 mg/L GoFerm (Lallemand) according to the manufacturer's instructions. Nutrient analysis was conducted and soluble solid content was determined by refractometry. Ammonia and amino acid were quantified enzymatically prior to inoculation using Unitab reagents and a ChemWell multiscanner (Unitech Scientific, Hawaiian Gardens, CA). If necessary, nutrients were added at inoculation to raise yeast available nitrogen to 300 mg/L. Additions were in the form of Fermaid K (Lallemand), to a maximum concentration of 25 mg/L, with the reminder provided as $(NH_4)_2HPO_4$. Wines were fermented at 10°C to dryness as determined by Clinitest (Bayer), cold stabilized at -4°C, and bottled under Stelvin closures (Waterloo Container, Waterloo, NY). Following primary fermentation, wine transfers (racking and bottling) were made under N_2 gas.

In 2009, the vinification procedures described above were amended due to berry desiccation from powdery mildew development. Water was added at a rate of 200 mL/L must to reduce the soluble solids and titratable acidity from 30.4 ± 0.5 Brix and 14.8 ± 0.3 g/L, respectively, to 24.6 ± 0.5 Brix and 11.4 ± 0.2 g/L, respectively. Nitrogen concentrations were tested and adjusted following amelioration.

In 2010, clusters with visible late season Botrytis bunch rot problems were removed prior to crushing and destemming. Soluble solids and titratable acidity of juice produced from sorted fruit were 20.8 Brix (±0.4) and 8.4 g/L (±0.3), respectively, with a mean pH value of 3.35 ± 0.1. Due to poor yield resulting from a combination of late spring frost events and losses due to sorting, there was insufficient fruit to vinify all treatments. Thus, triplicate 1 L fermentations were made with fruit from all timings of the 5.38 kg/ha micronized sulfur treatments and from the other treatments that ceased eight days before harvest.

No amendments were necessary prior to fermentation in 2011. Each treatment yielded triplicate 20 L batches, which were fermented to dryness. H_2S production was monitored daily using detection tubes as described above. S^0 residues were measured on the intact fruit prior to processing and in the juice prior to and at various points during the prefermentation settling process. In 2011, juice clarity was determined by measuring the turbidity of must samples taken 30 cm below the surface with a wine thief, using a Hach 2100Q Turbidimeter (Hach Company, Loveland, CO). All clarified musts obtained a turbidity of <20 NTU after 24 hr of settling and racking. After racking, the sediment fraction consisted of the 2 L left in the carboy after removing the clarified must. In earlier years, the determination of final clarity prior to fermentation was made visually.

In 2010 and 2011, H_2S produced during fermentation was monitored daily by measuring the escaping gas with a Gastec 4H or 4HH model H_2S detection tube (Fisher Scientific) fitted into the fermentation airlock as described (Park 2000, Ugliano and Henschke 2010). In these years, H_2S was also quantified in duplicate 80 mL samples of all wines produced, using the apparatus described above for elemental sulfur quantification. For this purpose, two antacid tablets were used for carrier gas generation (Kwasniewski et al. 2011), and H_2S was quantified using H_2S gas detection tubes as described by Park (2008).

Effects of skin contact time on S^0 persistence and H_2S production. In 2010, a trial was conducted to investigate the effect of skin contact duration prior to or during fermentation on S^0 persistence into fermentation and attendant H_2S production. Fruit was sourced from a commercial vineyard of Cabernet franc located near Geneva, NY (lat: 42°50'40"; long: -77°0'13"), which was established in 2005 on 3309C rootstock with 3 m row spacing and 2 m vine spacing. Following cessation of the grower's standard fungicide program, on 22 Sep all test vines received a single application of micronized sulfur, providing 2.69 kg/ha S^0, using the spray equipment and technique described above. Fruit was harvested by hand on 3 Oct and processed the following day.

Five different vinification treatments were imposed in triplicate upon this single source of fruit, as follows: (1) whole-cluster pressed; (2) crushed, destemmed, and pressed; (3) crushed, destemmed, and pressed following 24 hr skin contact; (4) crushed, destemmed, and pressed following a one week maceration on the skins; or (5) crushed, destemmed, and pressed following a two week maceration on the skins. The basic winemaking protocol described above was used except for the changes described below. The whole-cluster treatment (1) was imposed on ~20% of the fruit from each of five harvest bins, which was removed immediately upon arrival from the field and pooled, separated into vinification replicates (n = 3), pressed, and settled for 24 hr before racking and inoculation. The remaining fruit was homogenized, crushed, and destemmed; then it was divided among twelve 60 L stainless-steel tanks to accommodate three replicates of each of the four remaining treatments, with 30 kg macerate per tank. The macerate in treatment (2) was pressed immediately after crushing and destemming, while that in treatment (3) was allowed to remain in contact with the skins at 4°C for 24 hr before pressing. Following pressing, vinification of treatments (2) and (3) proceeded according to the basic protocol above. Treatments (4) and (5), simulating typical red wine fermentation conditions, were inoculated following crushing and destemming and division into fermentation replicates. The macerate for each replicate of treatments (4) and (5), was placed into an individual 25 L plastic pail with an airtight lid, and the pails remained closed during the ensuing 7- or 14-day maceration period while the skins were integrated by swirling. After the given period of maceration, the wines were hand pressed through cheesecloth and transferred into a glass carboy. Yeast inoculum for all treatments was *S. cerevisiae* strain ICV-GRE (Lallemand).

S^0 residue levels were quantified in the juice before and after settling and in wine postfermentation and in the lees. H_2S produced during fermentation and remaining in the finished wines thereafter was quantified as described above.

Statistics. JMP version 9.0.2 (SAS Institute Inc., Cary, NC) and Minitab 17 were used for statistical analyses. An assessment of equal variance by Levene's test was first conducted on Minitab. When the assumption of equal variance was met, one-way or two-way ANOVA was conducted (setting $p < 0.05$ for both) followed by parametric mean testing using Tukey HSD on JMP. When equal variance was not determined, two measures were taken to guard against type-I error: (1) a Welch's ANOVA was used in one-way testing ($p < 0.05$) or the p-value required in two-way ANOVA analysis was lowered to $p < 0.01$ and (2) parametric comparisons were conducted by Games-Howell, using Minitab. Linear regressions were conducted using JMP.

Results

Residue concentrations on grapes at harvest. In 2009, applications of S^0 continuing until 12 days before harvest resulted in residues more than 10-fold greater than those on berries last treated four or eight weeks earlier (Figure 1). Applications that ceased 40 days preharvest resulted in residues significantly higher than those on the control vines (no measurable residues), but at an order of magnitude below the concentration of 10 mg/L demonstrated to increase H_2S production in fermentations (Acree et al. 1972). Only fruit treated until 12 days before harvest resulted in residues above this threshold. S^0 was detectable on some samples from the 68-day preharvest interval (PHI) treatment, but the mean concentration could not be differentiated statistically ($p > 0.05$) from that of the control. A two-way ANOVA showed that the timing of the S^0 application was a contributor to the variance ($p < 0.0001$) but the application rate (2.69 or 5.38 kg/ha S^0) was not.

In 2010, both the S^0 treatment (formulation-rate) and PHI impacted final residue concentrations ($p < 0.001$) (Figure 1). All treatments applied until eight days before harvest resulted in residues exceeding 10 µg/g, although concentrations following applications of S^0 at 2.69 kg/ha in a wettable powder (WP) formulation were only about one-third the concentration of those following applications at 5.38 kg/ha in a micronized form. Residues following applications at this higher rate of the WP formulation were intermediate between those of the two other treatments and all three means were significantly different from one another ($p < 0.05$). When sprays ceased 22 days before harvest, residues resulting from applications of the WP formulation at the lower rate averaged 6.4 ± 2.6 µg/g, whereas applications of either formulation at the higher rate resulted in significantly higher levels ($p < 0.05$), well in excess of 10 µg/g. At 35-day PHI, all three S^0 treatment residues were below 10 µg/g (0.6 to 4.6 µg/g), and at 50-day PHI, the mean residue level on the one treatment imposed (the lower rate of the micronized formulation) was <0.5 µg/g.

In 2011, both the duration of the PHI and the S^0 formulation affected residue concentrations on grapes at harvest. For both the wettable and micronized formulations applied at a constant S^0 rate of 4.48 kg/ha, residues were inversely proportional to the length of the PHI, with the exception that there was no significant ($p < 0.05$) difference between the 38- and 54-day PHI for the micronized form (Figure 1). Residues were above 1 µg/g for all treatments and near or well above 10 µg/g when either formulation was applied until either 25 or 12 days before harvest; those resulting from the micronized formulation were significantly ($p < 0.05$) greater than those from the wettable powder given the shorter PHI, whereas the converse was true for the longer PHI.

Persistence and accumulation in the vineyard. In 2010 and 2011, vines subjected to an S^0 treatment with the same formulation and application rate but designated for different preharvest withholding periods had experienced identical spray regimes previously (Figure 2, Figure 3). Therefore, for the following data summation, residue values were pooled for all treatments that received undifferentiated S^0 applications up to a particular sampling time. Furthermore, although samples from control panels in which no S^0 was applied were quantified at every time point in both years, residue concentrations were always below the limit of detection (0.01 µg/g); hence, no additional data are presented for the control treatment.

In 2010, S^0 residues 32 days before harvest (i.e., three days after the most recent application) averaged 27 µg/g for all

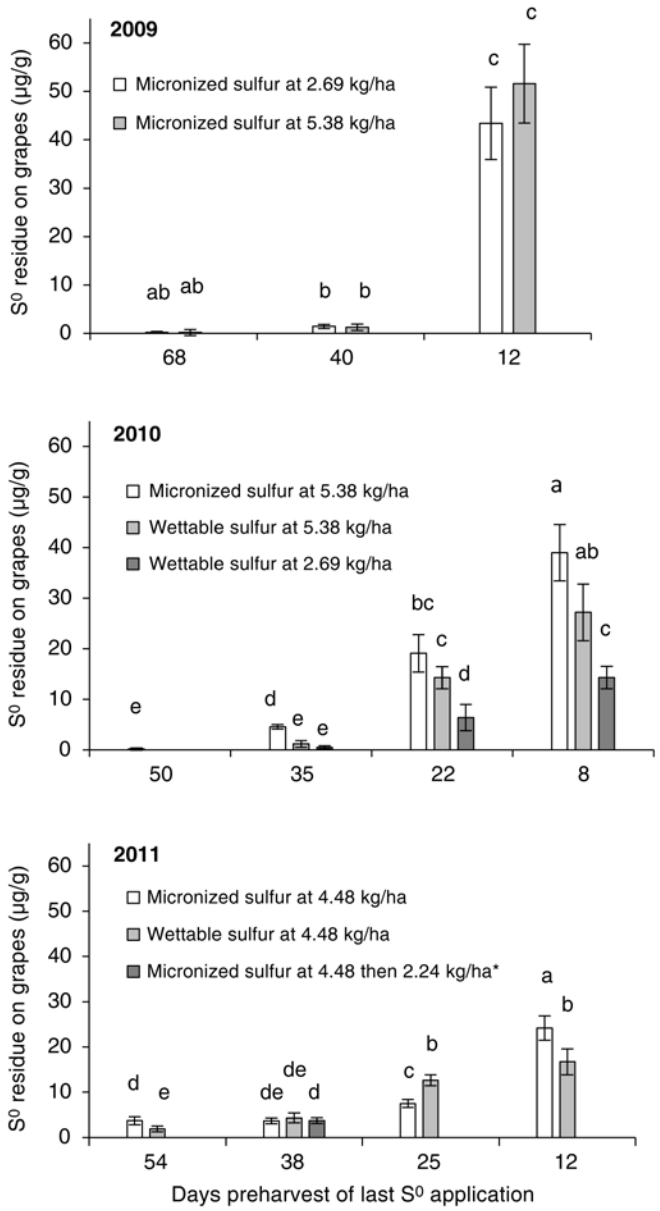

Figure 1 S^0 residues on Chardonnay (2009 and 2010) and Riesling (2011) clusters at harvest. Data are grouped by days before harvest of the final S^0 application, with each bar representing the mean value for a five-cluster sample taken from each of six treatment sampling units (2/replicate plot). Means not labeled with a common letter are significantly different ($p < 0.05$). No residue was detected on any samples from the control treatment (no S^0 applied, data not shown). The * denotes a treatment which received micronized sulfur at 4.48 kg/ha in the first three applications and 2.24 kg/ha in the final two.

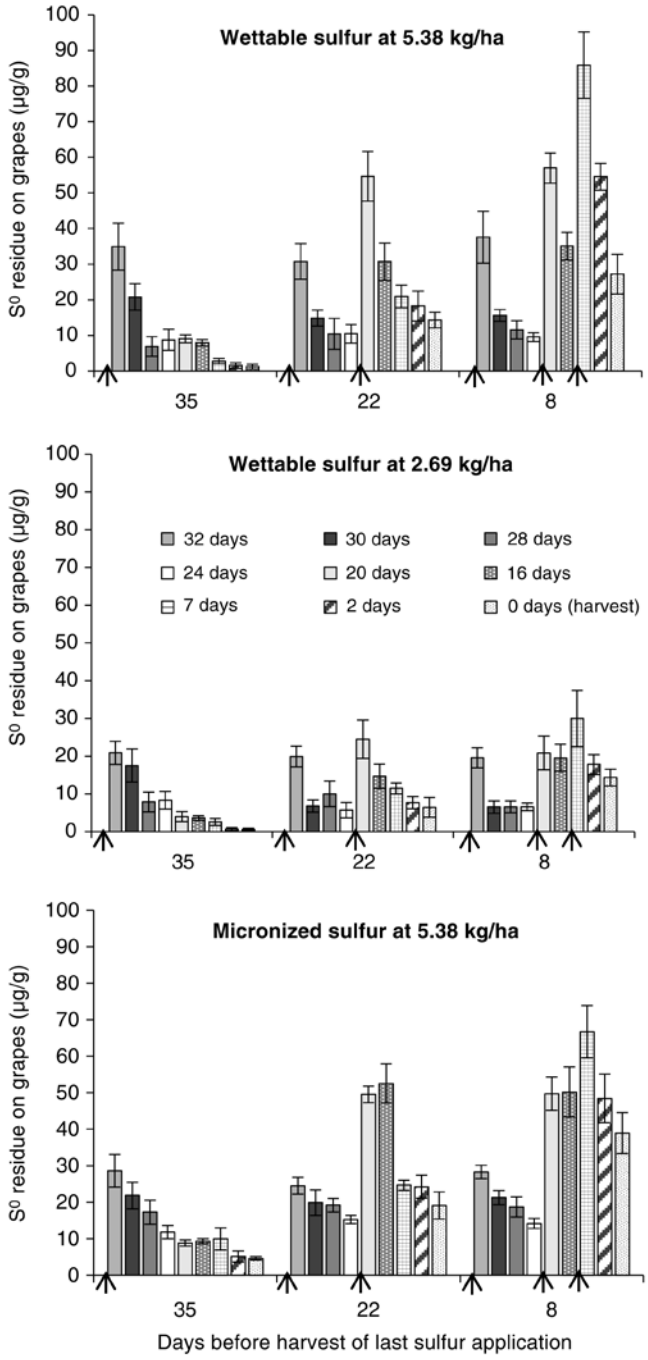

Figure 2 Elemental sulfur (S^0) residue on Chardonnay grape clusters sampled throughout the 2010 growing season. Sequential sprays of commercial sulfur formulations were applied starting 50 days before harvest and continuing at ~2-week intervals, ceasing a variable number of days before harvest on designated vines as denoted on the x-axis. S^0 residue data are grouped by sulfur treatment, with each bar representing the mean value for a five-cluster sample taken from each of six treatment sampling units (2/replicate plot). The legend denotes the number of days before harvest that samples were obtained, and arrows signify a S^0 application within the sampling period for each group of vines. No residue was detected on samples from the control treatment (no S^0 applied, data not shown).

plots that received the micronized formulation at 5.38 kg/ha, 34 µg/g for WP at this same rate, and 20 µg/g for WP at 2.69 kg/ha. At 30 days before harvest, the mean concentrations for these three treatments had decreased to 21, 17, and 10 µg/g, respectively; at 28 days they were 28, 10, and 8 µg/g, respectively; and at 24 days, they were 14, 10, and 7 µg/g, respectively (Figure 2). Differences among rates and formulations were more pronounced immediately following an application and appeared to be cumulative over time. For example, across all vines treated 22 days before harvest, residues on fruit sampled two days later averaged 50 µg/g for the micronized formulation applied at 5.38 kg/ha, 56 µg/g for the WP formulation applied at this same rate, and 28 µg/g for the WP at 2.69 kg/ha. One day following the subsequent application (as shown on the 8-day PHI vines), these values were 67, 86, and 30 µg/g, respectively (Figure 2). However, differences between the two S^0 formulations were inconsistent in 2011: residues were higher for the micronized formulation shortly after the final treatment and at harvest when applications ceased 12 days before harvest, whereas the converse was true on vines in the 25-day PHI treatment. As in 2010, residue concentrations typically spiked immediately after treatment, declining by about one-half after ~1 week (Figure 3). Detailed data on 2009 to 2011 S^0 residue concentrations are provided in supplemental data available online (Supplemental Tables 1–3).

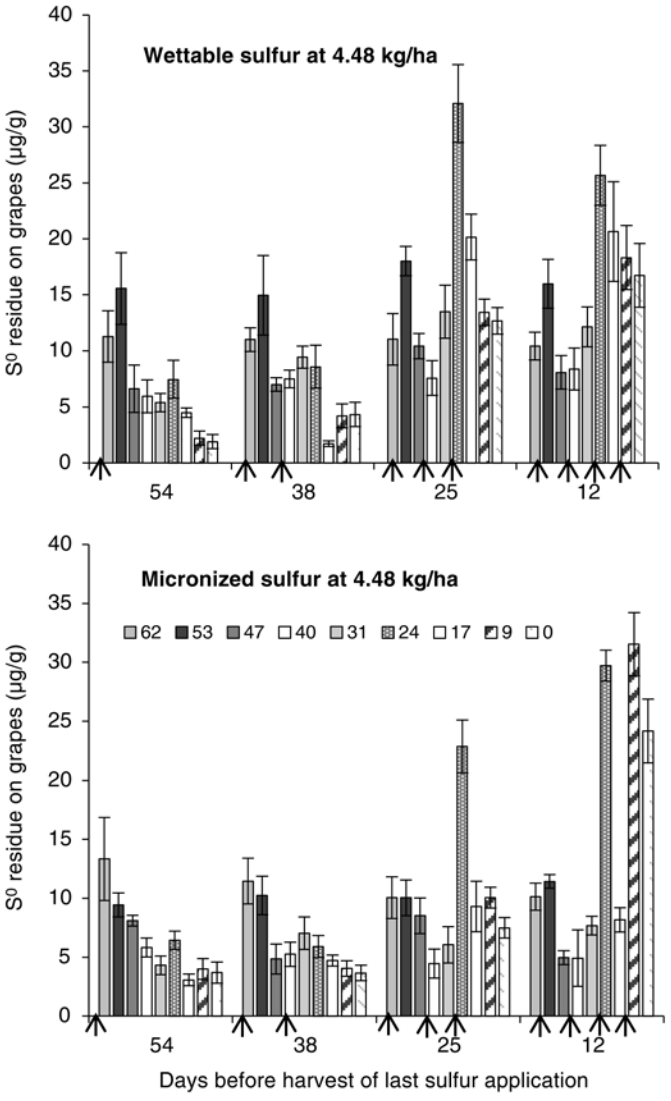

Figure 3 S^0 residues on Riesling grape clusters sampled throughout the 2011 growing season. Sequential sprays of commercial sulfur formulations were applied starting 81 days before harvest and continuing at ~2-week intervals, ceasing a variable number of days before harvest on designated vines as denoted on the x-axis. S^0 residue data are grouped by sulfur treatment, with each bar representing the mean value for a five-cluster sample taken from each of six treatment sampling units (2/replicate plot). The legend denotes the number of days before harvest that samples were obtained, and arrows signify a S^0 application within the sampling period for each group of vines. No residue was detected on samples from the control treatment (no S^0 applied, data not shown).

Residue fate during prefermentation operations. In 2009, there was a dramatic reduction in S^0 residues measured in the clarified musts from those on the harvested fruit. Residues were ~10 to 25% of those on the fruit, and the greatest absolute reductions occurred in treatments with the highest initial concentrations. The H_2S concentration was measured only in the finished wines that year, with all concentrations below the sensory threshold of 1 µg/L (Siebert et al. 2009) and no significant differences among treatments.

S^0 residue concentrations were compared among spray treatments on whole berries and in both unclarified and clarified juice in 2010 and 2011; they also were monitored at various times during cold settling in 2011. In 2010, mean residue concentrations for all treatments decreased from a range of 4.6 to 60.8 µg/g on the harvested grapes to 1.5 to 15.5 µg/g in the unclarified juice immediately after pressing. S^0 residues in the juice declined substantially further after settling, to between 0.43 and 1.75 µg/g. Following clarification, the majority of the S^0 residues resided in the sediment fraction, which contained substantially greater concentrations of 23.9 to 174.1 µg/g. The S^0 residue concentrations on the grapes correlated well with those in unclarified juice ($R^2 = 0.90$, $p = 0.014$; Figure 4), but not with those in the clarified juice ($R^2 = 0.37$, $p = 0.28$; data not shown). Similarly, S^0 residues on the harvested grapes did not correlate well with the amount of H_2S produced during fermentation ($R^2 = 0.45$, $p = 0.21$; data not shown), whereas S^0 concentrations in the settled must were good predictors of total H_2S production during its subsequent fermentation ($R^2 = 0.69$, $p < 0.001$; Figure 5).

A similar pattern of the fate of S^0 residue on grapes following crushing and pressing was observed in 2011, with residue concentrations on grapes again being a good predictor of those in the unsettled must ($R^2 = 0.74$, $p = 0.002$; Figure 4). Initial S^0 residues in the musts ranged from a mean of 1.52 to 12.82 µg/g across S^0 application treatments, but declined to 0.14 to 0.28 µg/g after they had settled to a turbidity below 20 NTU (Figure 6). There was no relationship between these low S^0 concentrations after settling and H_2S production during subsequent fermentation ($p = 0.64$, Figure 5). Thus, S^0 residues in grapes, unclarified juice, and clarified juice were not good predictors of H_2S formation in the 2011 fermentations of clarified juice.

Skin contact effect on S^0 persistence and H_2S production. At harvest, Cabernet franc clusters used in the

vinification trials had S^0 residue concentrations of 11.4 ± 1.2 µg/g. By the time of inoculation, mean must S^0 levels ranged from 0.05 to 0.20 µg/g in those treatments that were pressed and settled first, whereas those undergoing an initial one or two week maceration had S^0 concentrations of 10.8 and 11.1 µg/g, respectively (Table 2). Subsequent fermentation on the skins produced mean concentrations of H_2S 2- to 3-fold greater than those for treatments where juice was pressed off the skins and settled before inoculation.

Discussion

Several reports have shown that ≥10 µg/g S^0 in must results in increased H_2S production during fermentation (Rankine 1963, Acree et al. 1972, Schutz and Kunkee 1977). However, less work has gone into understanding S^0 persistence in the vineyard and defining application regimes that will avoid excess residues in the fermentation. Two previous studies quantified S^0 that could be rinsed from intact clusters using either a water-detergent mixture (Thomas et al. 1993b) or petroleum ether (Wenzel et al. 1980), although neither approach appears to have been validated using recovery experiments. During method development, we found the former technique to be inadequate for quantitative removal under our experimental conditions. We did not explore petroleum ether extraction, as it is a poor solvent for S^0 (Chen et al. 1973). Instead, we opted to blend whole cluster samples for subsequent quantification with a newly validated assay that allows quantification of S^0 in the presence of other sulfur-containing compounds (Kwasniewski et al. 2011). These methodological differences may explain why we observed residue concentrations as high as 86 µg/g berry weight on some clusters immediately after application of S^0, whereas Thomas et al. (1993b) reported maximum concentrations <14 µg/g immediately postapplication when using rates ~two to four times greater than those we employed. Wenzel et al. (1980) observed a maximum S^0 residue of 5.37 µg/g immediately after a single application (rate not specified), declining

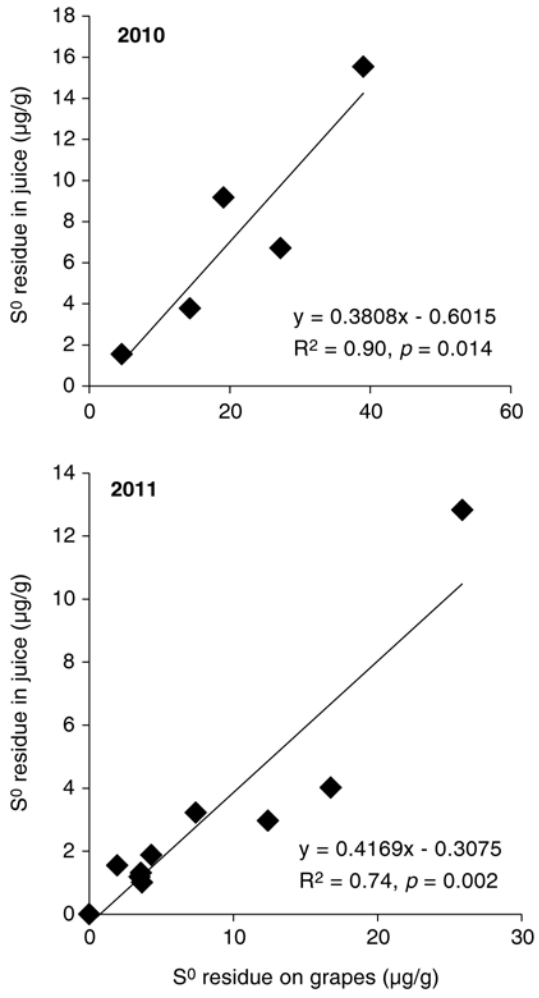

Figure 4 S^0 residues in unsettled Chardonnay (2010) and Riesling (2011) juice after pressing as a function of residues measured on grapes at harvest. Each data point represents the mean value for six replicate measures of grape residues and three replicate measure of residues in the expressed juice, per treatment.

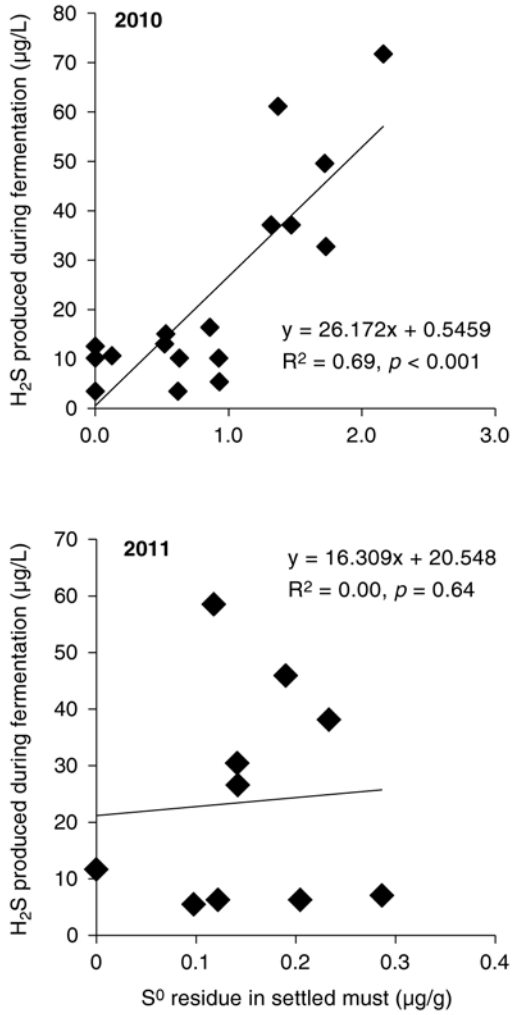

Figure 5 Hydrogen sulfide production during fermentation as a function of S^0 residues in the initial, settled Chardonnay (2010) and Riesling (2011) musts. In 2010, fermentation replicates were racked for the same period of time but turbidity was not measured and residue concentrations differed among fermentation replicates; data points represent values for individual replicates. In 2011, all samples were settled to a turbidity of >20 NTU and fermentation replicates were divided after racking; data points represent averages for three fermentation replicates per treatment.

to 0.83 µg/g at harvest 51 days after treatment, with the greatest concentration, 3.89 µg/g, at harvest following eight sequential S^0 applications that concluded 51 days earlier. Although the difference in S^0 formulation used by Thomas et al. (1993b) relative to our study (dusting versus sprayable, respectively) may have contributed to the differences in our findings, Wenzel et al. (1980) used a colloidal formulation similar to ours and also found far lower concentrations at harvest than we report. These differing results, consistent with our initial inability to remove all visible residues with a dilute detergent solution in preliminary experiments, may reflect an underreporting of the total S^0 on fruit when only the residue in rinsate is quantified. Additional research is needed to ascertain whether the increased S^0 concentrations that we report from blended clusters versus those reported by previous workers from rinsate may be due at least in part to incomplete recovery of S^0 using the latter technique, resulting from its adsorbance to the waxy cuticle of the fruit.

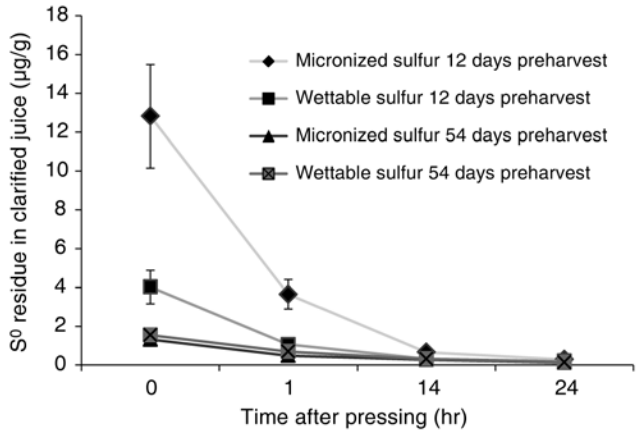

Figure 6 Elemental sulfur (S^0) residue present in juice pressed from fruit that received sequential applications of two commercial formulations (4.48 kg/ha S^0) during 2011, ceasing either 54 or 12 days preharvest. Samples were obtained from 30 cm below the juice surface in a 20-L carboy, at the postpressing time intervals indicated. Data points represent the means for three replicate fermentations per treatment, with error bars indicating standard deviation. All means for 38- and 25-day PHI treatments were intermediate between those for the 12- and 54-day extremes but are omitted from the graph for clarity.

Table 2 Transfer of S^0 from Cabernet franc clusters into must and subsequent evolution of H_2S during fermentation, as affected by vinification method.

Treatment[a]	S^0 before settling[b] Mean ± SD (µg/g)	S^0 at inoculation[c] Mean ± SD (µg/g)	H_2S produced during fermentation Mean ± SD (ng/mL)
Whole-cluster pressed	1.24 ± 0.2 b[d]	0.2 ± 0.1 b	70.5 ± 5.1 a
Crushed-destemmed	0.6 ± 0.0 a	0.05 ± 0.0 a	67.8 ± 3.2 a
24-hr skin contact	1.92 ± 0.2 c	0.18 ± 0.1 b	75.6 ± 8 a
1-week maceration	N/A[e]	10.8 ± 0.8 c	140.6 ± 9.4 b
2-week maceration	N/A[e]	11.1 ± 1.1 c	179.2 ± 35 b

[a]Grapes for all vinification treatments received an application of micronized sulfur at a rate of 2.69 kg/ha 10 days before harvest, resulting in S^0 residues of 11.4 ±1.2 µg/g on harvested clusters; variable treatments were imposed on a single lot of fruit in the winery.
[b]Samples were obtained immediately after pressing; treatments fermented on the skins had not been pressed at this time.
[c]For treatments processed as white wines, must concentrations were determined at the time of inoculation after pressing, settling, and racking.
[d]Values represent the means of three replicate vinifications per treatment. Means within a column not followed by a common letter are significantly different ($p < 0.05$) according to Games–Howell analysis, following Welch's ANOVA.
[e]Red wine style fermentations were not settled prior to inoculation.

Of the limited studies on S^0 persistence in the vineyard, Thomas et al. (1993b) determined that residues would not exceed concentrations ultimately detrimental to wine quality if applications ceased by the time that fruit had reached veraison. This developmental stage was chosen as a point to cease application based on the then-current belief that berries lose their susceptibility to new PM infections soon thereafter. Although it is now known that berries are resistant to new infections far before this point of development, continued control of PM after veraison may nevertheless be necessary as the rachis and new shoot growth remain susceptible (Gadoury et al. 2003). In our studies, S^0 residues did not exceed 4.6 µg/g when applications ceased by 35 to 38 days before harvest and were typically near or below the value of 3 µg/g previously shown to provide no increase in H_2S production during fermentation (Thomas et al. 1993a). However, residues consistently exceeded the 10 µg/g threshold when S^0 was applied within 25 days of harvest, and in all three years only those treatments ceasing ≥50 days from harvest were below 1 µg/g. In addition to the timing of the final application, S^0 formulation and application rate also affected residue concentrations and persistence, both at harvest and throughout the season. For example, in 2010, applications of the WP formulation at 2.69 kg/ha with a 22-day PHI resulted in residue concentrations at harvest comparable to those for the same material applied at a rate of 5.38 kg/ha with a 33-day PHI. Furthermore, concurrent applications of WP versus a micronized formulation at the same rate of S^0 typically resulted in higher residues for the latter treatment. Thus, limiting the application rate and using a WP rather than micronized formulation in later sprays may help minimize the PHI necessary to attain a given concentration of residue on harvested fruit.

While vineyard treatments can have a significant influence on S^0 residue levels on fruit, prefermentation decisions involving factors such as skin contact and settling time will exert a strong influence on S^0 concentrations in must. In both 2010 and 2011, S^0 residues on harvested Chardonnay and Riesling clusters, respectively, were a good predictor of S^0 residues in unclarified juice following crushing and destemming but did not correlate well with S^0 residues in clarified juice. Examination of the postclarification sediment fraction produced from these trials and from a separate trial involving Cabernet franc vinified as a white wine indicated that most of the S^0 present in the unclarified must could be found in the sediment. Considering that over 95% of residues were removed during settling, achieving must S^0 concentrations >10 µg/g following settling would require initial S^0 residues of >200 µg/g, a concentration far exceeding any residues detected immediately after spraying. Thus, in agreement with

the finding by Wenzel et al. (1980), highly clarified musts (<20 NTU) appear to be at minimal risk of containing S^0 residues sufficient to produce increased H_2S during fermentation. However, because the current work looked at only a single target turbidity, we have not established general guidelines for the relationship between NTU and S^0 residue loss.

In the preceeding discussion, must S^0 concentrations of ≥10 µg/g were used as a threshold for increased H_2S production during fermentation. However, some authors have reported increased H_2S with S^0 residues as low as 1 µg/g (Thoukis and Stern 1962, Wenzel et al. 1980), whereas another group reported that residues as high as 3.0 µg/g generally had no effect while also noting an interaction among S^0 concentration, fermentation medium, and yeast strain on H_2S (Thomas et al. 1993a). In vinifications of Chardonnay from the 2010 spray treatments, S^0 residue concentrations (<0.01 to 2.2 µg/g) correlated linearly with the quantity of H_2S produced during fermentation, whereas there was no such correlation within a lower range of S^0 residues (<0.01 to 0.3 µg/g) from the Riesling treatments in 2011 (Figure 5). Thus, under these particular fermentation conditions, results here agree with previous reports that S^0 residues above 1 µg/g can increase H_2S production (Thoukis and Stern 1962, Wenzel et al. 1980). However, at low S^0 concentrations, other factors such as juice nutrient status (Ugliano et al. 2009) likely have a larger role in explaining differences in H_2S production. Additionally, yeast strain will affect not only H_2S production, but also the conversion efficiency of S^0 to H_2S (Acree et al. 1972).

Fermentation treatments on Chardonnay and Riesling grapes simulated typical white winemaking conditions in which fruit is pressed and the resulting juice clarified prior to fermentation. To evaluate the effects of using typical red versus white winemaking practices, different prefermentation treatments were applied to Cabernet franc clusters. Pressing and settling prior to fermentation resulted in negligible S^0 residues (0.05 to 0.2 µg/g), even when a 24-hr cold soak was introduced. However, skin-fermented treatments (involving one and two week macerations to simulate typical red winemaking conditions) had prefermentation S^0 must concentrations nearly identical to residue concentrations on the intact berries, i.e., one to two orders of magnitude greater than those in clarified musts from the same lot of fruit. Skin-fermented Cabernet franc treatments also produced 2- to 3-fold more H_2S during fermentation than treatments pressed prior to fermentation. It should be noted, however, that control treatments with undetectable S^0 residues were not included and that we cannot exclude the possibility that differences in H_2S production resulted from some other unknown factor associated with skin fermentation rather than variable S^0 residues.

Lastly, this study did not attempt to determine the impact of potential variables that might influence S^0 loss in the vineyard, including temperature, precipitation, spray application technique, or canopy management and variety. Further work is needed to understand what roles these factors may play in S^0 accumulation and persistence, perhaps leading to an improved ability to predict S^0 residues at harvest. However, monitoring S^0 residue concentrations with the assay used in this study is a viable option for producers looking to inform their viticultural and vinification decisions relative to this factor.

Conclusion

S^0 plays an important role in powdery mildew management due to its cost, efficacy, low resistance risk, and cachet as a natural product, but the development of guidelines for preharvest withholding periods has been hindered by a paucity of data relating vineyard use patterns to residue concentrations on harvested fruit and their potential contribution to increased H_2S production during fermentation. Ceasing sprays no later than 35 days before harvest resulted in S^0 residues on harvested fruit below 10 µg/g, a concentration consistently shown in previous literature to increase H_2S production when present at inoculation. A more conservative threshold for S^0 residue in must (1 µg/g) was exceeded even with a 56-day preharvest interval in some treatments. Although S^0 residue concentrations in unclarified musts correlated strongly with those on the grapes prior to crushing, prefermentation clarification reduced residues in the juice by >95%, such that S^0 contamination should be of concern only for skin-fermented wines (i.e., in red winemaking) under most circumstances. Because S^0 persistence on fruit in the vineyard was affected by application rate and formulation as well as vintage, an accurate determination of vineyard residues is best determined by measuring samples from a given site, which is relatively easy and inexpensive using the newly described methodology. Potentially, this information could also be useful in determining when S^0 needs to be reapplied or to evaluate the selectivity of a sprayer for targeting the canopy versus the fruit. Finally, future work could attempt to better link the kinetics of S^0 disappearance to weather phenomena, with the goal of generating predictive models that will negate the need for growers to individually measure S^0. Expanding beyond the single site used in this study, to survey studies of S^0 residues across multiple sites with known spray schedules, could be used to construct confidence intervals for recommended S^0 spray cessation times to ensure grapes are at safe concentrations to avoid potential wine defects at harvest.

Literature Cited

Acree, T.E., E.P. Sonoff, and D.F. Splittstoesser. 1972. Effect of yeast strain and type of sulfur compound on hydrogen sulfide production. Am. J. Enol. Vitic. 23:6-9.

Bell, S.J., and P.A. Henschke. 2005. Implications of nitrogen nutrition for grapes, fermentation and wine. Aust. J. Grape Wine Res. 11:242-295.

Chen, K.Y., M. Moussavi, and A. Sycip. 1973. Solvent extraction of sulfur from marine sediment and its determination by gas chromatography. Environ. Sci. Technol. 7:948-951.

Gadoury, D.M., L. Cadle-Davidson, W.F. Wilcox, I.B. Dry, R.C. Seem, and M.G. Milgroom. 2011. Grapevine powdery mildew (*Erysiphe necator*): A fascinating system for the study of the biology, ecology and epidemiology of an obligate biotroph. Molec. Plant Pathol. 13:1-16.

Gadoury, D.M., R.C. Seem, A. Ficke, and W.F. Wilcox. 2003. Ontogenic resistance to powdery mildew in grape berries. Phytopathology 93:547-555.

Jiranek, V., P. Langridge, and P. Henschke. 1995. Regulation of hydrogen sulfide liberation in wine-producing *Saccharomyces cerevisiae* strains by assimilable nitrogen. Appl. Environ. Microbiol. 61:461-467.

Kwasniewski, M.T., R.B. Allison, W.F. Wilcox, and G.L. Sacks. 2011. Convenient, inexpensive quantification of elemental sulfur by simultaneous in situ reduction and colorimetric detection. Anal. Chim. Acta 703:52-57.

Park, S.K., R.B. Boulton, and A.C. Noble. 2000. Formation of hydrogen sulfide and glutathione during fermentation of white grape musts. Am. J. Enol. Vitic. 51:91-97.

Park, S.K. 2008. Development of a method to measure hydrogen sulfide in wine fermentation. J. Microbiol. Biotech. 18:1550-1554.

Rankine, B.C. 1963. Nature, origin and prevention of hydrogen sulphide aroma in wines. J. Sci. Food Agric. 14:79-91.

Savocchia, S., R. Mandel, P. Crisp, and E.S. Scott. 2011. Evaluation of 'alternative' materials to sulfur and synthetic fungicides for control of grapevine powdery mildew in a warm climate region of Australia. Australas. Plant Path. 40:20-27.

Schütz, M., and R.E. Kunkee. 1977. Formation of hydrogen sulfide from elemental sulfur during fermentation by wine yeast. Am. J. Enol. Vitic. 28:137-144.

Siebert, T., B. Bramley, and M. Solomon. 2009. Hydrogen sulfide: Aroma detection threshold study in white and red wines. AWRI Tech. Rev. 183:14-16.

Thomas, C.S., R.B. Boulton, M.W. Silacci, and W.D. Gubler. 1993a. The effect of elemental sulfur, yeast strain, and fermentation medium on hydrogen sulfide production during fermentation. Am. J. Enol. Vitic. 44:211-216.

Thomas, C.S., W.D. Gubler, M.W. Silacci, and R. Miller. 1993b. Changes in elemental sulfur residues on Pinot noir and Cabernet Sauvignon grape berries during the growing season. Am. J. Enol. Vitic. 44:205-210.

Thoukis, G., and L.A. Stern. 1962. A review and some studies of the effect of sulfur on the formation of off-odors in wine. Am. J. Enol. Vitic. 13:133-140.

Ugliano, M., B. Fedrizzi, T. Siebert, B. Travis, F. Magno, G. Versini, and P.A. Henschke. 2009. Effect of nitrogen supplementation and *Saccharomyces* species on hydrogen sulfide and other volatile sulfur compounds in Shiraz fermentation and wine. J. Agric. Food Chem. 57:4948-4955.

Ugliano, M., and P.A. Henschke. 2010. Comparison of three methods for accurate quantification of hydrogen sulfide during fermentation. Anal. Chim. Acta 660:87-91.

Wenzel, K., and H. Dittrich. 1978. Zur Beeinflussung der Schwefelwasserstoff-Bildung der Hefe durch Trub, Stickstoffgehalt, molekularen Schwefel und Kupfer bei der Vergärung von Traubenmost. Wein-Wissens. 33:200-213.

Wenzel, K., H. Dittrich, H. Seyffardt, and J. Bohnert. 1980. Schwefelrückstände auf Trauben und im Most und ihr Einfluss auf die H_2S-Bildung. Wein-Wissen. 35:414-420.