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Impact of Nitrogen and Elemental Sulfur on Formation of Volatile Sulfur Compounds during Fermentation of Pinot Noir Grapes

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Abstract: The influence of yeast assimilable nitrogen (YAN) and elemental sulfur (S^0) on the formation of volatile sulfur compounds (VSCs) during fermentation was investigated. Pinot noir fermentations were performed using *Saccharomyces cerevisiae* strain UCD522 or P1Y2 with an addition of 0, 5, or 15 µg/g elemental sulfur. H_2S production during fermentation was measured using lead acetate tubes and additional VSCs measured by GC-PFPD. The addition of S^0 resulted in H_2S formation during alcoholic fermentation regardless of which yeast strain was used. H_2S production was greater in fermentations performed by UCD522 with increasing amounts of S^0 resulting in increased production of H_2S. Higher S^0 resulted in wines containing higher concentrations of methyl thioacetate and glutathione disulfide. Additional experiments examined the impact of nitrogen composition and S^0. The addition of diammonium phosphate (DAP) resulted in an increase in H_2S formation during fermentation whereas the addition of amino acids did not, whether S^0 was added or not. Fermentations where DAP and S^0 were both added produced a higher concentration of H_2S compared to fermentations where S^0 or DAP additions were made individually. VSCs in the wine were also impacted by the addition of nitrogen and/or S^0 with the addition of S^0 and nitrogen (DAP or amino acids) resulting in elevated concentrations of methyl thioacetate in the wines.

Keywords: elemental sulfur; yeast assailable nitrogen; *Saccharomyces cerevisiae*; hydrogen sulfur; volatile sulfur compounds

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1. Introduction

Hydrogen sulfide (H_2S) and other volatile sulfur compounds (VSCs) produced during the winemaking process can be extremely detrimental to the quality of a wine due to their offensive aromas, described as rotten egg, onion, cabbage, and rubber [1]. Although a number of studies have investigated the formation of VSCs [2–5] during wine production, the underlying causes driving their formation are still not well elucidated. Of all of the VSCs, H_2S is the most widely studied. H_2S can be produced by the fermentative yeast *Saccharomyces cerevisiae* as part of the formation of sulfur-containing amino acids methionine and cysteine [1,5,6] via the sulfate reduction sequence (SRS). H_2S can also be formed by enzymatic catabolism of the S-containing amino acids, cysteine and methionine, as yeast scavenge for sulfur during fermentation. In addition, glutathione (GSH), a tripeptide containing cysteine, has also been proposed as a possible precursor to H_2S and other VSCs [7]. Finally, H_2S can also be formed via the non-enzymatic reduction of elemental sulfur (S^0) [8] that can be present on grapes due its use as a fungicide in the vineyard.

Yeast strains vary greatly in their production of H_2S with some yeasts, such as *S. cerevisiae* UCD522 (Montrachet), that are reported as high producers of H_2S [9,10] whereas other strains such as EC1118 are noted as low producers [11]. Recently, strains have been developed that purportedly produce no H_2S due to allele differences in the *MET10* gene

which encodes for catalytic subunits in sulfite reductase, a key enzyme in the SRS [9]. Factors such as nitrogen content, elemental sulfur residues, vitamins, and excessive amounts of sulfite [4,5,8,12] can also impact H_2S formation. One of the most important of these is nitrogen content. The nitrogen content of a grape must or juice is typically expressed as yeast assimilable nitrogen (YAN) and is composed of nitrogen from ammonia and nitrogen from amino acids containing a primary amine group. Sufficient YAN is required during fermentation for yeast cell growth and biomass production so that the yeast can successfully complete the fermentation [4] and the recommended minimum value is 140 mg/L YAN [13]. If YAN supplementation is required, the addition of diammonium phosphate (DAP) is most common due to its low cost and high yield of ammonia nitrogen [4].

The concentration and composition of YAN can influence the production of H_2S during fermentation. Jiranek et al. [4] reported that most amino acids, with the exception of threonine, proline, and cysteine, were successful at reducing H_2S formation. A number of researchers have also reported that additions of DAP can reduce H_2S production [4,14]; although, others have noted that moderate DAP supplementation increases H_2S formation [15,16]. Additions of DAP have also been shown to increase methanethiol (MeSH) and ethanethiol (EtSH) content in wines [16] and Fang et al. [17] reported that foliar or soil nitrogen application significantly increased the contents of H_2S and methanethiol (MeSH) in Pinot noir wines. It has also been suggested that the type of nitrogen (ammonia versus amino acid nitrogen) as well as concentration can impact the formation of H_2S produced during fermentation [18]. In addition, although the impact of YAN on H_2S has received considerable study, little is known about the impact of YAN on the formation of other VSCs or possible VSC precursors such as cysteine, methionine, and glutathione.

H_2S can also be formed by the reduction of S^0 during fermentation [8]. The reductive environment established during fermentation is thought to be sufficient for this non-enzymatic conversion to occur [8] although alternative mechanisms of H_2S formation from S^0 have been proposed [19]. S^0 is frequently used as a fungicide to inhibit the growth of powdery mildew, one of the most common problems in viticulture. Early studies reported large concentrations of H_2S being produced in fermentations with levels of 10–20 ug/g of S^0 as well as an observed lack of connection between fermentative capacity and H_2S production [2,8]. Typically, the application of S^0 in the vineyard is halted well before harvest so as to minimize the risk of residual S^0 being present on the grapes at harvest. However, there is debate over how far this period of time should be based on how long S^0 persists on the grapes as well as the concentrations of S^0 that would increase production of H_2S during fermentation. For example, Thomas et al. [20] observed that application of dusting sulfur up to, but not after, veraison resulted in 1–3 ug/g S^0 on the fruit at harvest. Thomas et al. [21] noted this concentration did not cause a significant increase in H_2S production during fermentation. However, others have noted that concentrations as low as 1 ug/g may cause H_2S problems [22].

Determining what concentration of S^0 will result in problematic H_2S production is complicated by the fact that yeast also produces H_2S during fermentation. Neither Thomas et al. [21] nor Kwasniewski et al. [22] used yeast strains that could not produce H_2S, so it is difficult to separate yeast-produced H_2S from H_2S generated from S^0 reduction. The yeast strain used, nitrogen concentration and composition, and fermentation conditions will all impact yeast-produced H_2S and must be considered when studying the role of S^0 in H_2S production during fermentation. In addition, the role of S^0 in the formation of other VSCs, particularly their formation post-fermentation, has not been well studied. This may be important to the formation of VSCs post-fermentation as in non-wine systems, reactions between S^0 and glutathione [23] or cysteine [8] have generated H_2S. Furthermore, Jastrzembski et al. [19] reported that S^0 residues on grapes can form latent precursors of H_2S that may be released during aging of the wine.

Because of the importance of YAN concentration and composition and the potential role of elemental sulfur in the production of H_2S and other VSCs, the objective of this study was to determine the impact of increasing amounts of elemental sulfur on the formation of

H_2S and other VSCs during the fermentation of Pinot noir grapes as well as to investigate how YAN concentration and composition impact the formation of VSCs.

2. Materials and Methods

2.1. Effect of Elemental Sulfur on Volatile Sulfur Compounds

Pinot noir grapes (clone 777 grafted to 101-14 rootstock) were harvested from Oregon State University's Woodhall Vineyard (Alpine, OR, USA) in September 2014. After arrival at the OSU research winery, grapes were kept in a temperature-controlled room overnight at 4 °C and then destemmed with a Velo DPC 40 destemmer/crusher (Altivole, Italy). The initial residual S^0 content of the Pinot noir grapes was measured (in triplicate) utilizing the method outlined by Kwasniewski [24]. No S^0 was detected on the grapes. After destemming, grapes were pooled and aliquoted (1.5 kg) into 4L microfermentors equipped with plungers for cap management as described by Takush and Osborne [25]. The basic grape parameters were 22.5 °Brix, pH 3.20, 0.84 g/100 mL titratable acid (as tartaric acid) and 80 mg/L YAN. °Brix was measured using an Anton Paar DMA 35N density meter (Graz, Austria), titratable acidity was determined by titration with 0.1 N sodium hydroxide solution and YAN was quantified as the sum of primary amino acids and ammonia in units of mg/L N. Primary amino nitrogen was measured according to Dukes and Butzke [26] and ammonia was measured using an enzymatic test kit (R-Biopharm, Darmstadt, Germany). Because YAN levels were low, a 0.125 g/L addition of the yeast nutrient Fermaid K (Lallemand, Montreal, QC, Canada) and 0.25 g/L DAP (Scott Laboratories, Petaluma, CA, USA) was made to all treatments to raise the YAN content to approximately 150 mg/L. Elemental colloidal sulfur (S^0) (>99% purity, Sigma-Aldrich, St. Louis, MO, USA) was added in different amounts to each treatment. In one set of fermenters, no S^0 was added; to another set of fermenters, 5 µg/g of S^0 was added, and to a third set of fermenters, 15 µg/g of S^0 was added. No SO_2 additions were made.

Microfermenters were modified so that the original fermentation lock was replaced with a fermentation lock where fermentation gases were forced to pass through a lead acetate H_2S detection tube (Gastec, Ayase, Japan). Both 4M and 4H tubes were used. Tubes were monitored multiple times throughout the day and replaced with fresh tubes when needed. H_2S concentration was determined by relating the distance of color change on the detection tube to that observed for calibration standards [27]. Calibration curves were prepared by addition of a known amount of sodium sulfide nonahydrate (Sigma-Aldrich, St. Louis, MO, USA) to an acidic environment. The hydrogen sulfide gas generated was passed through the detection tube with the aid of Alka-Seltzer tablets (Bayer, Morristown, NJ, USA) as described by Kwasniewski et al. [24] and discoloration of the tube was measured and correlated with concentration of sodium sulfide nonahydrate.

Two different yeast strains, *S. cerevisiae* UCD522 (Lallemand, Montreal, QC, Canada) or P1Y2 (Phyterra, Napa, CA, USA) were used to conduct fermentation. In one set of fermenters (0, 5, 15 µg/g S^0), *S. cerevisiae* P1Y2 was inoculated and in another set of fermenters, *S. cerevisiae* UCD522 was inoculated. Yeasts were hydrated according to the manufacturer's specifications prior to inoculation at a rate of 0.25 g/L. All treatments were performed in triplicate. Fermentations were conducted in a temperature-controlled room at 27 °C. °Brix was monitored twice per day using an Anton Paar DMA 35N density meter (Graz, Austria) that was rinsed repeatedly with 70% ethanol between sampling to prevent cross-contamination. H_2S production was assessed using lead acetate tubes as previously detailed. Completion of fermentation was confirmed with Clinitest tablets (Bayer, Morristown, NJ, USA). Once all fermentations had reached dryness (<0.5 g/L reducing sugars), wines were drained and the pomace pressed using a small custom basket press modified with a pressure gauge to apply a constant pressure of 0.1 MPa for 5 min. Replicates were pressed into 500 mL Schott bottles (VWR, Radnor, PA, USA), settled at 4 °C for 48 h, and samples were taken and frozen at −80 °C until needed for analysis.

2.2. Impact of Nitrogen Composition and Concentration, and Elemental Sulfur on Volatile Sulfur Compounds

Pinot noir grapes (clone 777 grafted to 101-14 rootstock) were harvested in September 2015 from Oregon State University's Woodhall Vineyard (Alpine, OR, USA). After arrival at the OSU research winery, grapes were kept in a temperature-controlled room at 4 °C overnight and then destemmed with a Velo DPC 40 destemmer/crusher (Altivole, Italy). The initial residual S^0 content of the Pinot noir grapes was measured (in triplicate) utilizing the method outlined by Kwasniewski et al. [24]. No S^0 was detected on the grapes. Basic grape parameters were 24.8 °Brix, pH 3.42, 0.64 g/100 mL titratable acid and 110 mg/L YAN. After destemming, grapes were pooled and aliquoted (1.5 kg) into 4 L fermenters equipped with plungers for cap management and lead acetate tubes for H_2S monitoring as previously described. The following treatments were applied: (1) control, (2) addition of 10 ug/g S^0 (Sigma-Aldrich), (3) addition of DAP (Scott Laboratories), (4) addition of DAP and 10 ug/g S^0, (5) addition of amino acid mixture, (6) addition of amino acid mixture and 10 ug/g S^0. DAP additions were made to raise YAN to approx. 250 mg/L. The amino acid mixture used was based on Wang et al. [10] with adjustments to mimic the relative amino acid composition of Pinot noir grape juice as reported by Lee and Schreiner [28] (Table S1). All amino acids were purchased from Sigma-Aldrich. The quantity of amino acids used was calculated to raise the free amine nitrogen content to a concentration that would boost YAN to approx. 250 mg/L. All treatments were performed in triplicate.

Treatments were inoculated with *S. cerevisiae* UCD522 at 0.25 g/L after rehydration according to the manufacturer's recommendations. After inoculation, fermenters were placed in a temperature-controlled room at 27 °C. °Brix was monitored daily using a density meter (DMA) and Gastec H_2S detection tubes were regularly changed out during the course of the alcoholic fermentation in order to determine the production of H_2S over time. Once all fermentations had reached dryness (<0.5 g/L reducing sugars as measured by Clinitest), wines were pressed using a modified basket press with an applied constant pressure of 0.1 MPa for 5 min. Treatments were pressed into 500 mL Schott bottles (VWR, Radnor, PA, USA), settled at 4 °C for 48 h, and samples were taken for analysis. Samples were frozen at −80 °C until needed for analysis.

2.3. Volatile Sulfur Compounds Analysis

VSCs in the wines were analyzed by gas chromatography as detailed by Fang and Qian [29]. In brief, analysis was performed using a Varian CP-3800 gas chromatography equipped with a pulsed-flame photometric detector (PFPD) (Varian, Walnut Creek, CA, USA) operating in sulfur mode. Two milliliters of filtered (0.45 µm) wine was placed in a 20 mL autosampler vial and then diluted with eight milliliters of Milli-Q water. A 100 µL aliquot of internal standard solution (500 ppb ethylmethyl sulfide (EMS) and 2 ppb diisopropyl disulfide) and 50 µL 5% acetaldehyde (*w*/*v*) were added to each vial. Duplicate analysis was performed for each wine sample.

After extraction, the SPME fiber was directly injected into the GC injection port using the splitless mode at 300 °C and kept for 7 min. Separation was performed using a DB-FFAP capillary column (30 m × 0.32 mm I.D., 1 µm film thickness, from Agilent Technologies, Inc., Santa Clara, CA, USA). The oven temperature was programmed as follows: 35 °C (initial hold 3 min), ramp at 10 °C/min to 150 °C (hold for 5 min), and then ramp at 20 °C/min to 220 °C (final hold 3 min). The carrier gas was nitrogen with a constant flow rate of 2 mL/min. The temperature of the detector was 300 °C, and the detector was supplied with 14 mL/min hydrogen, 17 mL/min air 1, and 10 mL/min air 2. The detector voltage was 500 V, the gate delay for sulfur compounds was 6 ms, and the gate width was 20 ms. All sulfur compounds were identified by comparing their retention times with those of the pure standards. The sulfur responses of specific compounds were calculated using the square root of peak area.

2.4. Cysteine and Methionine Analysis

Cysteine and methionine were analyzed by high performance liquid chromatography (HPLC) using a Hewlett-Packard/Agilent Series 1100 (Palo Alto, CA, USA) equipped with HP ChemStation software and a photodiode array detector (DAD). Analysis was performed according to Henderson [30] with some modifications as described by Kraft [31]. In brief, wine samples were centrifuged for 10 min at 6000 rpm with an Allegra X-22 centrifuge (Beckman Coulter, Brea, CA, USA) and then filtered through 0.45 um syringe filters (Pall Corporation, Port Washington, NY, USA). Before injection, inline derivatizations with o-phthaldehyde (OPA) were performed to react primary amino acids into fluorescent products. The HPLC was fitted with a Zorbax Eclipse AAA analytical column (150 mm × 4.6 mm, 5 um, Agilent Technologies) and guard column (12.5 mm × 4.6 mm, 5 um, Agilent Technologies). Mobile phase A was a 40 mM sodium phosphate solution, adjusted to pH 7.8 with 6 N sodium hydroxide solution. Sodium phosphate stock solution was made with Milli-Q water (Millipore, Bedford, MA, USA) and monobasic sodium phosphate (Avantor Performance Materials, Center Valley, PA, USA). Mobile phase B was acetonitrile, methanol, and water (45:45:10 $v/v/v$). Mobile phase B solvents were from EMD Millipore (Billerica, MA, USA). Gradients of mobile phase A and mobile phase B were applied as follows: 0% B (2.0 mL/min) from 0 to 1.9 min, 0–57% B linear (2.0 mL/min) from 1.9 to 18.1 min, 57 to 100% B linear (2.0 mL/min) from 18.1 to 18.6 min, static at 100% B (2.0 mL/min) from 18.6 to 22.3 min, 100–0% B (2.0 mL/min) from 22.3 to 23.2 min, and static at 0% B (2.0 mL/min from 23.2 to 26 min to re-equilibrate the column to initial conditions. Identification and quantification of cysteine and methionine was determined from calibration curves using amino acid standards (Sigma-Aldrich) with five points ranging from 0–1 nmol/mL. Additional single amino acid solutions of cysteine and methionine were used for peak identifications using pure cysteine and methionine (Sigma-Aldrich) dissolved in a solution of 50% 1 N HCl and 50% Milli-Q water. OPA solution was prepared as follows: 2.5 mg OPA was dissolved in 500 uL of methanol, 4.5 ml of borate buffer (pH of 10.8), and 21 uL of 3-mercaptoproprionic acid (3-MPA). OPA and 3-MPA were obtained from Agilent Technologies.

2.5. Glutathione Analysis

Glutathione (GSH) and glutathione disulfide (GSSG) were quantified at Oregon State University Central Labs using liquid chromatography-MS/MS. The technique was adapted from a method for analysis of GSH in blood [32]. Samples were centrifuged for 10 min at 6000 rpm and supernatant was decanted into a fresh 1.5 mL microcentrifuge tube. An aliquot of 100 µL of each sample was collected and added to 180 µL of precipitating solution (24 mM NEM +20 mM EDTA) in a blood Eppendorf tube. Then, 20 µL of internal standard (100 µM of GSH $^{13}C_2$, ^{15}N) was added before vortexing for 3 s. Next, 20 µL of 20% SSA was added before vortexing vigorously for 10 s. Finally, samples were incubated for 20 min at room temperature (20 °C). Following this derivatization, samples were centrifuged for 5 min at 14,000 rpm at room temperature. Supernatant (approximately 150 µL) was transferred to mass spectrometry vials for either immediate LC-MS/MS analysis or storage at −80 °C until analysis.

LC-MS/MS was performed on an Applied Biosystems 4000 QTRAP hybrid linear ion trap-triple quadrupole instrument (AB Sciex, Concord, ON, Canada) operated at a source temperature of 400 °C with an ion spray voltage of 5000 kV in positive ion mode. Instrument parameters for GSH and GSSG were as follows: declustering potential (40 for GSH, 60 for GSSG), collision energy (30), entrance potential (8), curtain gas (30) and collision cell exit potential (3). A Shimadzu Prominence HPLC system (Shimadzu, Columbia, MD, USA), consisting of two LC-20AD pumps, a DQU-20A_5 degasser, and an SIL-HTC autosampler were used for all chromatography. Chromatographic separations of GSH-NEM and GSSG were achieved on a Hypercarb column (Thermo Scientific, Waltham, MA, USA) (2.1 mm × 100 mm × 5 um) at room temperature. Mobile phase A was water containing 0.1% formic acid, and mobile phase B was 100% acetonitrile containing 0.1% formic acid.

The run consisted of isocratic delivery of 5% phase B for 0.3 min, a linear gradient of 5–40% phase B over 3.3 min, a second linear gradient of 40–95% phase B over 0.9 min, isocratic delivery of 95% phase B for 1.5 min, and a final wash with 100% phase B over 2 min. Parent → product ion transitions for SRM were developed using standards. SRM transitions used for quantitation included: 433.3 → 304.3 for GSH-NEM, 436.3 → 307.3 for GSH-$^{13}C_2$,^{15}N-NEM, and 613.4 → 355.2 for GSSG.

2.6. Statistical Analysis

Statistical tests consisted of one- or two-way analysis of variance (ANOVA) tests performed using R Studio version 3. 4. 4 (Boston, MA, USA). Tukey's HSD multiple comparison was performed to test least squares means of treatment effects at the $p < 0.05$ significance level.

3. Results

3.1. Elemental Sulfur and Pinot Noir Fermentation

The impact of elemental sulfur on the formation of volatile sulfur compounds post-fermentation was determined. An addition of elemental sulfur was made to Pinot noir grapes which were then fermented by either a high (UCD522) or low (P1Y2) H_2S-producing *S. cerevisiae* strain. The amount of elemental sulfur added (either 5 or 15 μg/g) was based on a recent publication [24] that reported that levels of residual sulfur on grapes in the Finger Lakes area (NY) ranged from 0–40 μg/g depending on harvest year and the date of last spray application. Fermentations proceeded quickly in all treatments and were completed after 168 h (Figure 1A). UCD522 fermentations, particularly those with 15 ug/g of elemental sulfur added, fermented slightly slower (Figure 1A). H_2S production peaked during the first 48–72 h after inoculation for all treatments with the highest rate of production occurring in treatments containing 15 μg/g S^0 and fermented by UCD522 (Figure 1B). As expected, wines fermented by UCD522 released H_2S at a higher rate during fermentation than the non-H_2S-producing strain P1Y2. No H_2S was measured during fermentations conducted by P1Y2 when no S^0 was added. However, H_2S was measured in fermentations conducted by P1Y2 when S^0 was added (Figure 1B). For example, fermentations conducted by P1Y2 with the addition of 15 μg/g S^0 had the second highest rate of H_2S production (Figure 1B).

Wines fermented by UCD522 with an addition of 15 μg/g S^0 produced more than triple the amount of H_2S than any other treatment, totaling nearly 2800 μg/L over the course of the fermentation (Table 1). Wines fermented by P1Y2 with an addition of 15 μg/g S^0 produced significantly more H_2S than other P1Y2 treatments and totaled more than 600 ug/L (Table 1). Additionally, both 15 μg/g S^0 treatments were still producing measurable H_2S at the end of fermentation whereas the other treatments did not. For example, after fermentations had reached 1 °Brix, UCD522 and P1Y2 fermented wines with 15 μg/g S^0 produced an additional 221.8 and 136.8 μg/L of H_2S, respectively (Table 1). In the case of P1Y2 wine, this accounted for approx. 22% of the total H_2S produced during fermentation. Concentrations of the sulfur-containing compounds methionine, cysteine, glutathione, and glutathione disulfide in the Pinot noir wines after pressing were measured. Wines fermented by P1Y2 with 0 μg/g S^0 contained the highest methionine concentrations (Table 2) whereas there was no significant difference between methionine concentrations in any of the other wines. Cysteine concentrations were similar for wines produced with varying S^0 (Table 2) as were glutathione (GSH) concentrations (Table 2). In contrast, significant differences were observed in the concentration of glutathione disulfide (GSSG). For wines fermented by UCD522, an increase in the concentration of S^0 added resulted in an increase in the concentration of GSSG in the wines (Table 2). A similar trend was seen for P1Y2 fermented wines where an addition of 15 μg/g S^0 to the grapes prior to fermentation resulted in a significantly higher GSSG concentration in the wine (Table 2). Overall, the highest concentration of GSSG was present in wines fermented by UCD522 with an addition of 15 μg/g S^0.

Ten VSCs were assessed in the wines using gas chromatography post pressing and settling. VSC concentrations in the wine samples are presented in Table 2. Concentrations of CS_2 were low in all wines (Table 3) whereas concentrations of MeSOAc were significantly higher in wines with S^0 added (Table 3). The highest concentration of MeSOAc was present in wines fermented by P1Y2 where 15 µg/g S^0 was added. In addition, wines made from grapes with 15 µg/g S^0 added contained significantly more MeSOAc if fermented by P1Y2 rather than UCD522 (Table 3).

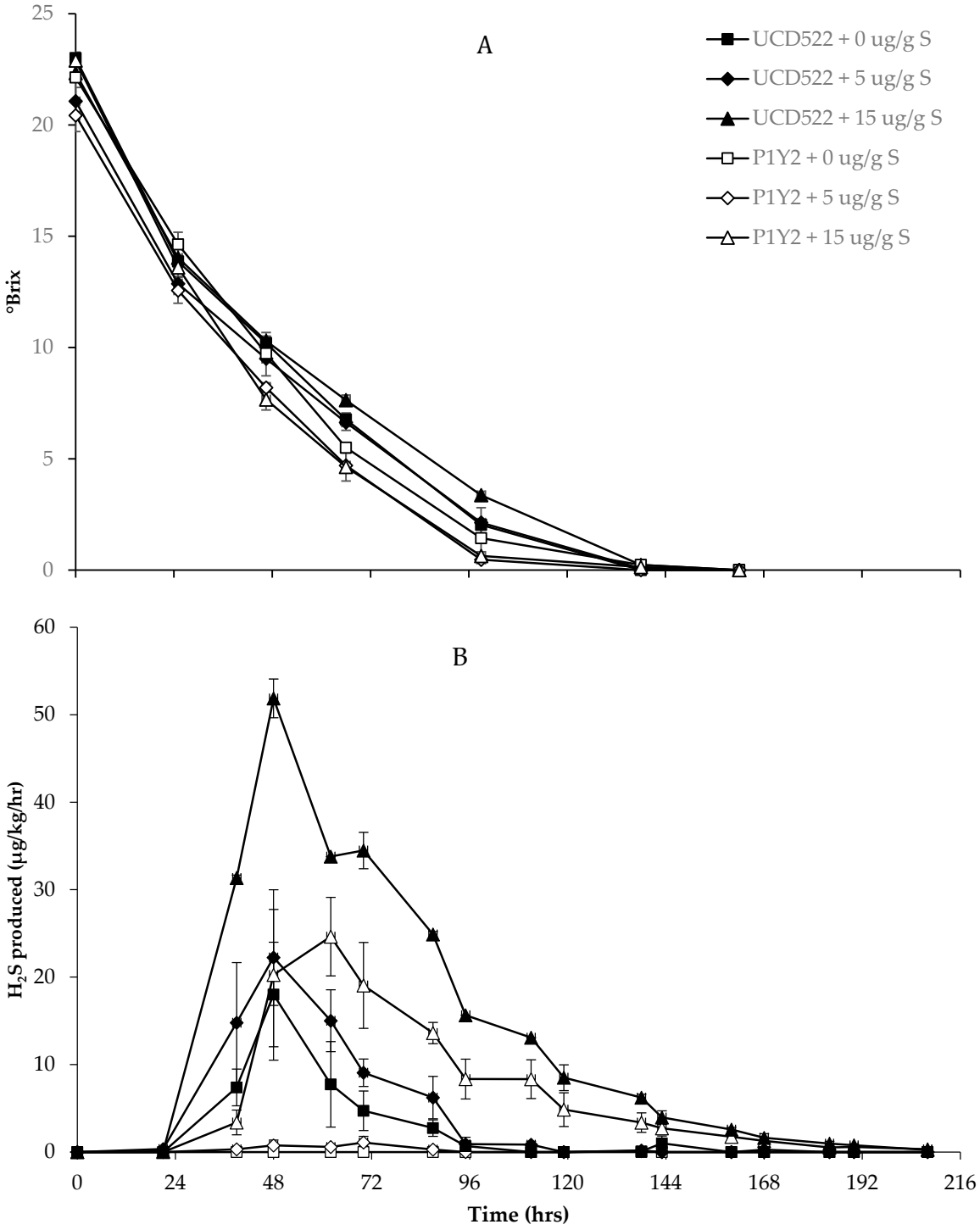

Figure 1. Change in Brix (**A**) and production (**B**) of H_2S by *S. cerevisiae* strain UCD522 (closed symbols) or strain P1Y2 (open symbols) during fermentation of Pinot noir grapes with the addition of 0, 5, or 15 µg/g elemental sulfur.

Table 1. Total H_2S produced and H_2S produced during late stages (<1 °Brix) of fermentation of Pinot noir grapes by *S. cerevisiae* strain UCD522 or P1Y2 with the addition of 0, 5, or 15 μg/g elemental sulfur (S^0).

Treatment		Total H_2S (ug/L)	<1 °Brix H_2S (ug/L)
P1Y1	0 μg/g S^0	ND †	ND †
	5 μg/g S^0	35.7 ± 8.8 ^{c}	2.0 ± 3.5 ^{c}
	15 μg/g S^0	619.9 ± 98.6 ^{b}	136.8 ± 84.1 ^{b}
UCD522	0 μg/g S^0	489.9 ± 68.9 ^{b}	6.8 ± 8.4 ^{c}
	5 μg/g S^0	870.9 ± 342.2 ^{b}	5.4 ± 6.2 ^{c}
	15 μg/g S^0	2796.5 ± 124.4 ^{a}	221.8 ± 37.9 ^{a}
Source of variation			
Yeast		***	**
S^0		***	***
Yeast x S^0		***	*

$^{a–c}$ Means within a column with different superscripts are significant at $p \leq 0.05$, $n = 3$. † ND, not detected; significance of source of variation and interactions are denoted as *** $p \leq 0.0001$, ** $p \leq 0.001$, * $p \leq 0.05$.

Table 2. Concentration of methionine (Met), cysteine (Cys), glutathione (GSH), and glutathione disulfide (GSSG) in Pinot noir wine fermented by *S. cerevisiae* strain UCD522 or P1Y2 with the addition of 0, 5, or 15 μg/g elemental sulfur (S^0).

Treatment		Met (mg/L)	Cys (mg/L)	GSH (μg/L)	GSSG (μg/L)
UCD522	0 μg/g S^0	2.6 ± 1.2 ^{b}	2.0 ± 0.2	0.5 ± 0.2	296.3 ± 153.2 ^{d}
	5 μg/g S^0	1.9 ± 0.9 ^{b}	1.9 ± 0.4	0.9 ± 0.1	1009.6 ± 46.8 ^{c}
	15 μg/g S^0	1.2 ± 0.2 ^{b}	2.2 ± 0.1	0.6 ± 0.1	2159.7 ± 319.1 ^{a}
P1Y2	0 μg/g S^0	6.7 ± 2.7 ^{a}	2.7 ± 0.8	0.3 ± 0.3	1047.5 ± 360.4 ^{c}
	5 μg/g S^0	1.9 ± 0.3 ^{b}	3.3 ± 0.1	0.6 ± 0.6	1149.8 ± 249.4 ^{c}
	15 μg/g S^0	2.7 ± 0.8 ^{b}	2.0 ± 0.6	0.8 ± 0.3	1529.6 ± 239.4 ^{b}
Source of variation					
Yeast		ns	ns	ns	*
S^0		*	ns	ns	**
Yeast x S^0		ns	ns	ns	**

$^{a–d}$ Means within a column with different superscripts are significant at $p \leq 0.05$, $n = 3$. Significance of source of variation and interactions are denoted as ns (not significant), ** $p \leq 0.001$, * $p \leq 0.05$.

Table 3. Concentration (mg/L) of volatile sulfur compounds in Pinot noir wines produced from grapes containing 0, 5, or 15 μg/g elemental sulfur (S^0) and fermented by *S. cerevisiae* strain P1Y2 or UCD522.

		H_2S	MeSH	CS_2	DMS	DES	MeSOAc	DMDS	EtSOAc	DEDS	DMTS
UCD522	0 μg/g S^0	ND †	ND	0.09 ± 0.01 ^{ab}	ND	ND	ND	ND	ND	ND	ND
	5 μg/g S^0	ND	ND	0.06 ± 0.01 ^{bc}	ND	ND	2.06 ± 1.02 ^{c}	ND	ND	ND	ND
	15 μg/g S^0	ND	ND	0.08 ± 0.02 ^{b}	ND	ND	8.02 ± 0.15 ^{b}	ND	ND	ND	ND
P1Y2	0 μg/g S^0	ND	ND	0.04 ± 0.01 ^{c}	ND	ND	ND	ND	ND	ND	ND
	5 μg/g S^0	ND	ND	0.08 ± 0.01 ^{b}	ND	ND	2.31 ± 0.17 ^{c}	ND	ND	ND	ND
	15 μg/g S^0	ND	ND	0.11 ± 0.01 ^{a}	ND	ND	11.89 ± 0.93 ^{a}	ND	ND	ND	ND
Source of variation											
Yeast		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
S^0		ns	ns	*	ns	ns	**	ns	ns	ns	ns
Yeast x S^0		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

† ND, not detected; $^{a–c}$ Means within a column with different superscripts are significant at $p \leq 0.05$, $n = 3$. Significance of source of variation and interactions are denoted as ns (not significant), ** $p \leq 0.001$, * $p \leq 0.05$. H_2S = Hydrogen sulfide, MeSH = Methanethiol, CS_2 = carbon disulfide, DMS = dimethyl sulfide, DES = Diethyl sulfide, MeSOAc = methyl thioacetate, DMDS = dimethyl disulfide, EtSOAc = ethyl thioacetate, DEDS = diethyl disulfide, DMTS = dimethyl trisulfide.

3.2. Impact of Nitrogen Composition and Concentration, and Elemental Sulfur on Volatile Sulfur Compounds

The influence of elemental sulfur, nitrogen composition and YAN concentration on wine VSCs was investigated by supplementing Pinot noir grapes with DAP or amino acids with or without the addition of 10 µg/g S^0. Although all fermentations completed to dryness (<0.5 g/L), fermentations where no nitrogen additions were made to the grapes took the longest to complete (Figure 2A). Fermentations finished quickest in the treatments where only amino acids were added. The addition of nitrogen and/or S^0 also impacted the production of H_2S during fermentation. H_2S production peaked between 48 and 72 h after inoculation for all treatments with the highest rate of production occurring in treatments containing 10 µg/g S^0 and an addition of DAP (Figure 2B). Total H_2S production during the fermentation was also impacted by the amount and type of nitrogen present as well as S^0 (Table 4). The lowest amount of H_2S produced was in fermentations where no additions were made (controls) or in fermentations where an addition of amino acids had been made. The addition of either 10 µg/g S^0 or DAP resulted in significantly higher production of H_2S than the control, whereas the combined addition of DAP and S^0 resulted in the highest production of H_2S during fermentation for any treatment (Table 4). For two treatments (DAP with S^0 addition and amino acids with S^0 addition), H_2S was still being produced during the final stages of fermentation. During fermentation of the final one °Brix of sugar, fermentations with an addition of DAP and 10 µg/g S^0 produced 241.5 ug/L of H_2S, whereas ferments with an addition of amino acids and 10 µg/g S^0 produced 122.4 ug/L of H_2S (Table 4). This accounts for approx. 12 and 15% of the total amount of H_2S produced during fermentation.

Table 4. Total H_2S produced during alcoholic fermentation conducted by *S. cerevisiae* strain UCD522 and H_2S produced during late stage (<1 °Brix) of fermentation of Pinot noir grapes with the addition of 10 µg/g elemental sulfur (S^0), diammonium phosphate (DAP) or amino acids (AA).

Treatment	Total H_2S (ug/L)	<1 °Brix H_2S (ug/L)
Control	595.2 ± 107.1 ^{c}	ND †
DAP	1146.1 ± 67.92 ^{b}	6.7 ± 1.1 ^{d}
AA	642.9 ± 46.8 ^{c}	13.6 ± 1.2 ^{c}
10 µg/g S^0	1302.7 ± 108.2 ^{b}	6.8 ± 0.7 ^{d}
DAP + 10 µg/g S^0	2068.1 ± 339.8 ^{a}	241.5 ± 14.5 ^{b}
AA + 10 µg/g S^0	812.9 ± 56.2 ^{c}	122.4 ± 4.9 ^{a}

$^{a–d}$ Means within a column with different superscripts are significant at $p \leq 0.05$, $n = 3$. † ND, not detected.

After pressing and settling, Pinot noir wines were assessed for VSCs content (Table 5). Methanethiol (MeSH), carbon disulfide (CS_2), and methyl thioacetate (MeSOAc) were present in all wines no matter the treatment (Table 5). No H_2S was detected in the control wine or wines produced with an addition of DAP. Wines fermented with an addition of amino acids contained the highest concentration of H_2S (Table 5). No significant differences in methanethiol concentrations were noted, whereas there were only minor differences in MeSOAc. Although there were only small differences in many of the VSCs measured, significant differences in MeSOAc were measured (Table 5). The addition of DAP resulted in 11.76 µg/L compared to the control which contained 2.88 µg/L, whereas wines made with an addition of amino acids contained 15.09 µg/L MeSOAc. The addition of S^0 resulted in an increase in MeSOAc when DAP was also added but not when amino acids were added (Table 5).

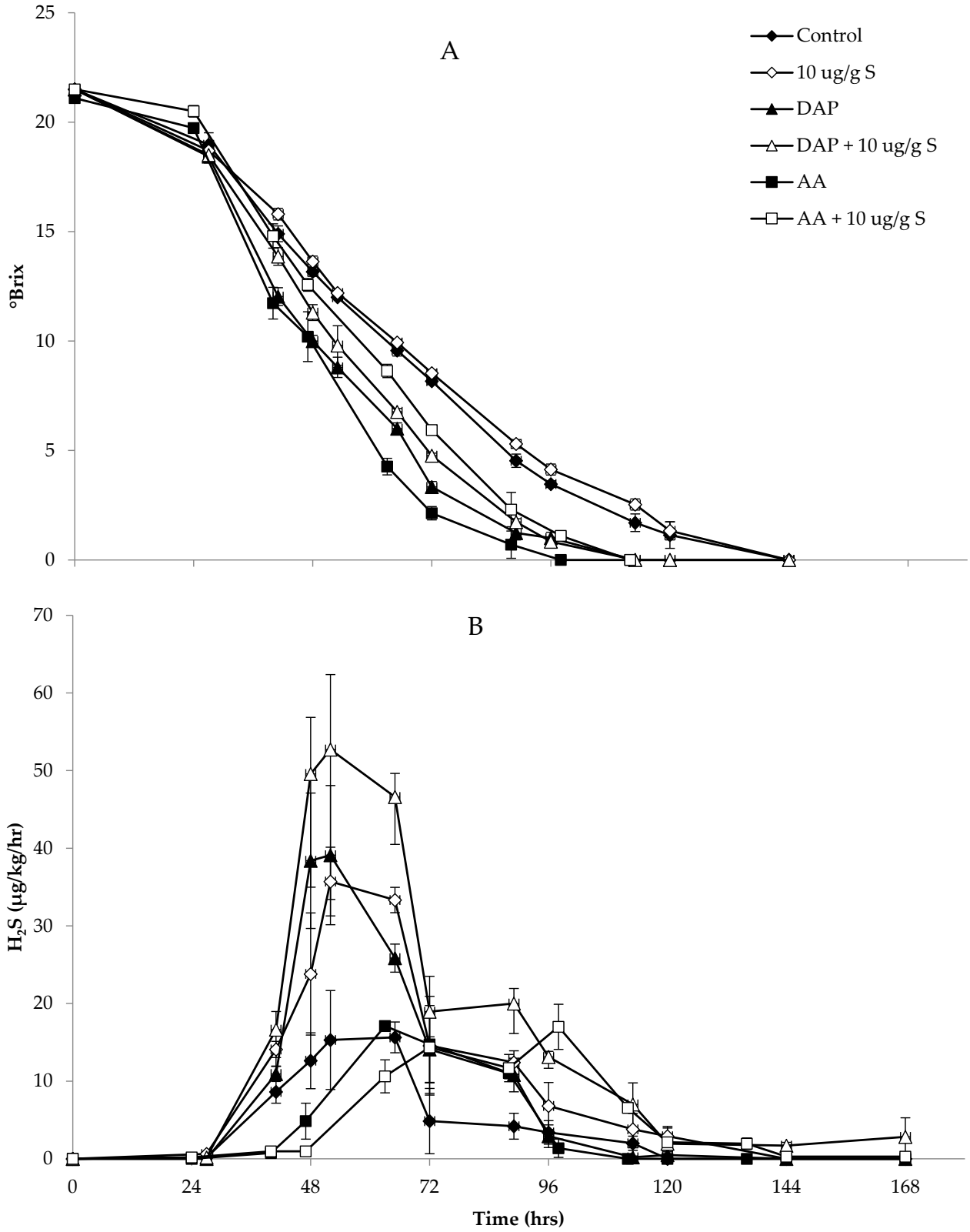

Figure 2. Change in Brix (**A**) and H_2S production (**B**) during fermentation of Pinot noir grapes by *S. cerevisiae* UCD 522 with the addition of 10 µg/g of elemental sulfur (S), diammonium phosphate (DAP) and/or amino acids (AA). Error bars are standard error (n = 3).

Table 5. Concentration (μg/L) of volatile sulfur compounds in Pinot noir wines fermented by *S. cerevisiae* strain UCD 522 with the addition of 10 μg/g elemental sulfur (S^0), diammonium phosphate (DAP), or amino acids (AA).

	H_2S	MeSH	CS_2	DMS	DES	MeSOAc	DMDS	EtSOAc	DEDS	DMTS
Control	ND †	0.83 ± 0.72	0.16 ± 0.03 ^{b}	ND	ND	2.88 ± 1.34 ^{d}	ND	ND	ND	ND
S^0	1.25 ± 2.17 ^{c}	0.53 ± 0.91	0.24 ± 0.03 ^{ab}	ND	ND	6.83 ± 1.81 ^{c}	ND	ND	ND	ND
DAP	ND	1.54 ± 0.24	0.16 ± 0.03 ^{b}	ND	ND	11.76 ± 2.62 ^{b}	ND	ND	ND	ND
DAP & S^0	2.60 ± 2.68 ^{bc}	1.75 ± 0.23	0.15 ± 0.02 ^{b}	ND	ND	16.08 ± 0.56 ^{a}	ND	0.71 ± 0.19 ^{ab}	ND	ND
AA	8.55 ± 1.01 ^{a}	1.97 ± 0.23	0.17 ± 0.04 ^{b}	ND	ND	15.09 ± 1.17 ^{a}	ND	0.35 ± 0.30 ^{b}	ND	ND
AA & S^0	5.70 ± 2.09 ^{b}	1.66 ± 0.41	0.26 ± 0.06 ^{a}	ND	ND	16.50 ± 2.52 ^{a}	ND	0.93 ± 0.06 ^{a}	ND	ND

† ND, not detected; $^{a–d}$ Means within a column with different superscripts are significant at $p \leq 0.05$, $n = 3$. H_2S = Hydrogen sulfide, MeSH = Methanethiol, CS_2 = carbon disulfide, DMS = dimethyl sulfide, DES = Diethyl sulfide, MeSOAc = methyl thioacetate, DMDS = dimethyl disulfide, EtSOAc = ethyl thioacetate, DEDS = diethyl disulfide, DMTS = dimethyl trisulfide.

4. Discussion

Many factors are known to impact the formation of VSCs during alcoholic fermentation. In the present study, the influence of yeast strain, YAN concentration and composition, and the presence of elemental sulfur (S^0) was investigated. Various amounts of S^0 were added to Pinot noir grapes and fermented by a low/no or high H_2S-producing yeast strain. All fermentations proceeded rapidly and no differences in time to complete fermentation were noted. H_2S formation did not occur until sugar levels were rapidly decreasing and no H_2S was produced during the first 24 h of fermentation despite a reduction of at least 7 °Brix in all treatments. This finding is in contrast to the findings of Acree et al. [2] and may be due to the rapid fermentation in the present study or the need for the development of a reductive environment in the fermentation to induce reduction of S^0 to H_2S. No H_2S was measured during fermentations conducted by P1Y2 when S^0 was not added, reinforcing the manufacturer's claims that it is a "no-H_2S"-producing yeast strain.

Formation of H_2S during fermentation peaked mid-fermentation between 48 and 72 h after yeast inoculation. This finding is in agreement with Thomas et al. [21], who reported that the peak formation of H_2S occurred early-to-mid fermentation. Thomas et al. [21] also noted that a second peak of H_2S production occurred late in fermentation. In agreement, we observed that H_2S formation continued late in fermentations where 15 μg/g of S^0 had been added. This late formation of H_2S is more problematic as H_2S is more likely to be retained in the wine as the sparging effect of yeast-produced CO_2 would be reduced near the end of fermentation [33]. Increasing amounts of S^0 in the fermentation resulted in higher concentrations of H_2S produced during the fermentation. When the amount of S^0 added tripled from 5 to 15 μg/g, the amount of additional H_2S produced increased by more than ten times in P1Y2 fermentations and by more than five times in UCD522 fermentations. This finding is in contrast with Schutz and Kunkee [8], who reported a linear response of S^0 to H_2S produced. This difference may be due to the fact that Schutz and Kunkee [8] used significantly higher amounts of S^0 during fermentation. Whereas in the present study concentrations of 5 and 15 μg/g S^0 were used, Schutz and Kunkee [8] used 25, 50, 75, and 100 mg/L S^0 to measure the response of S^0 concentration to H_2S formation. It is possible that at lower levels, a linear relationship between S^0 and H_2S does not exist.

Because a yeast strain that cannot produce H_2S via the SRS pathway was used, it was possible to compare the amount of H_2S produced by *S. cerevisiae* via the SRS pathway versus the amount of H_2S formed due to the addition of S^0. In this study, the addition of S^0 impacted the formation of H_2S due to the reduction of S^0 as well as the production of H_2S by UCD522. For example, the addition of 15 μg/g S^0 to fermentations conducted by P1Y2 resulted in the production of 619 μg/L H_2S. This H_2S produced was due to the addition of S^0 only as P1Y2 did not produce any H_2S if S^0 was not added. If no S^0 was added to UCD522 fermentations, an average of 490 μg/L was produced, likely primarily via the SRS pathway. These two H_2S values combined equal 1109 ug/L H_2S and represent the theoretical amount of H_2S production you would expect in fermentations conducted by UCD522 with the addition of 15 μg/g S^0 if the addition of S^0 did not impact yeast-produced H_2S. However, a

significant interaction between yeast strain and S^0 was observed resulting in fermentations conducted by UCD522 with the addition of 15 µg/g S^0 producing 2796 µg/L of H_2S. This is over double the amount of H_2S production expected if H_2S production by UCD522 was not impacted by S^0.

The production of H_2S from S^0 during alcoholic fermentation and the influence of yeast strain is not well understood. Acree et al. [2] noted that yeast strain may impact the production of H_2S from S^0 but why this occurs is unknown. Jastrzembski et al. [19] offered a potential pathway for the production of H_2S from S^0 due to the non-enzymatic reduction of S^0 to H_2S with glutathione (GSH). This would lead to the formation of glutathione disulfide (GSSG) and glutathione trisulfide (GSSSG). As evidence, Jastrzembski et al. [19] measured an increase in concentrations of these compounds in red wines fermented with the addition of S^0. The increase in GSSG due to fermenting with S^0 also occurred in the present study where wines with the highest addition of S^0 contained the highest concentration of GSSG post-fermentation. However, Jastrzembski et al. [19] also noted that the reported concentrations of GSH typically present in juice or wine may not be sufficient to induce the release of H_2S from S^0 and that the release of H_2S from S^0 is most likely enzymatic unless the reductive environment during fermentation helped regenerate GSH. Clearly, additional study is required to better elucidate the pathway or pathways by which H_2S is formed from S^0 during alcoholic fermentation. Although there is consensus that increasing concentrations of S^0 result in higher production of H_2S, how this occurs is unclear, as is the influence of the *S. cerevisiae* strain conducting the fermentation.

The amount of S^0 necessary in a fermentation to cause production of H_2S at concentrations that could negatively impact wine aroma is ill defined. Thomas et al. [21] reported that 3.4 µg/g of S^0 was not sufficient to reliably increase H_2S production during fermentation, whereas Kwasniewski et al. [22] reported that concentrations as low as 1 µg/g S^0 correlated with increased H_2S production. Neither Thomas et al. [21] nor Kwasniewski et al. [22] used yeast strains that could not produce H_2S via the SRS pathway, so it is difficult to separate yeast-produced H_2S from H_2S generated from the addition of S^0. In the present study, the presence of 5 µg/g of S^0 was sufficient to produce measurable amounts of H_2S in fermentations conducted with P1Y2 yeast. As this yeast cannot produce H_2S via the SRS pathway, the formation of H_2S was due to the addition of S^0. Additional fermentations at lower S^0 concentrations should be conducted using a non-H_2S-producing yeast strain such as P1Y2 to better understand the concentrations of S^0 that result in increased H_2S formation independent of yeast-produced H_2S. In addition, factors such as the oxidation–reduction state of the medium/fermentation should be considered when determining what S^0 concentrations could be problematic as it is known that under more reductive conditions, the formation of H_2S is accelerated [8]. Given that the present study utilized small micro-fermenters, the results from S^0 additions may differ if larger fermenters are used as the rate of change in redox potential during a fermentation in a small vessel will probably differ from that of a larger fermentation vessel.

Although large amounts of H_2S were produced during the course of the fermentation, no H_2S was detected in the wines. This phenomenon has been noted by others [16,18] where total H_2S production during fermentation does not correlate with the final H_2S concentration in the wine. This is likely due to the high volatility of H_2S and the sparging effect of CO_2 produced by the yeast during fermentation [14]. However, the lack of H_2S in the wines was still surprising, as fermentations where 15 µg/g S^0 had been added produced relatively high amounts of H_2S late in fermentation. One possibility is that some of the H_2S formed during fermentation may have reacted to form more complex VSCs [8,16,18] or formed a 'bound' form of H_2S such as polythionates as reported by Franco-Luesma and Ferreira [34] and Jastrzembski et al. [19]. MeSH is another possible sulfur compound that may be formed from H_2S produced during fermentation. However, MeSH was not present in any of the wines at detectable levels.

In contrast to MeSH, MeSOAc was detected in the wines and was significantly affected by S^0 as it was only present in wines where 15 µg/g S^0 had been added. In addition,

significantly more MeSOAc was present in P1Y2 fermented wines where 15 μg/g S^0 had been added than in the corresponding UCD522 fermented wine. MeSOAc is thought to be produced via the reaction of MeSH with acetyl-CoA and elevated formation of MeSOAc has been correlated with the production of high levels of H_2S by some yeast strains [1]. However, in the present study, the correlation between MeSOAc and high H_2S production during fermentation was not observed. For example, wines fermented with P1Y2 and 15 μg/g S^0 produced <20% as much H_2S as wines fermented by UCD522 with 15 μg/g S^0 yet contained significantly higher MeSOAc. Of interest, is that in treatments where 15 μg/g S^0 was added, significantly higher concentrations of H_2S were produced late in fermentation. Potentially, this late formation of H_2S may have resulted in elevated concentrations of MeSOAc in the wines. Although the concentrations of MeSOAc in the wines were below the reported sensory threshold (50 μg/L in beer), this compound can be hydrolyzed to form MeSH during aging in a reductive environment [35,36]. MeSH has an extremely low sensory threshold (1–3 μg/L) [1].

Additional sulfur-containing compounds (cysteine, methionine, and glutathione), which may play a role in VSC formation during wine fermentation [2,37,38], were also assessed in this study. Small differences in cysteine and methionine concentrations were noted between some treatments, but there was no clear relationship with increasing additions of S^0 to the fermentations. Furthermore, the concentrations of these amino acids were relatively low. Similarly, only minor differences in GSH were noted and the concentration of GSH measured in the wines was relatively low compared to the few reports of GSH in red wine and very low compared to the concentrations found in many white wines which can exceed 10 mg/L [14,39]. The concentration of GSSG was impacted by the addition of S^0 to the fermentations. The significance of this finding relative to the formation of volatile sulfur compounds is unknown and requires additional investigation.

The impact of both S^0 and nitrogen concentration and composition was examined as production of H_2S during fermentation has frequently been linked to YAN concentration [4,10]. Low YAN concentration is often noted to be a major cause of H_2S production by yeast during fermentation [4]. However, it has also been reported that high H_2S production can also occur under high YAN conditions [10,15,16]. For example, Mendes-Ferreira et al. [15] reported that low YAN (66 mg/L) resulted in the lowest amount of H_2S produced, whereas the highest levels of H_2S were produced in juice with 267 YAN mg/L. Ugliano et al. [16] also reported that high DAP supplementation to a final YAN of 250 or 400 mg/L during fermentation yielded higher H_2S production during alcoholic fermentation. The findings of the present study support these reports as significantly higher amounts of H_2S were produced when YAN was raised by the addition off DAP to approx. 250 mg/L compared to the control. Ugliano et al. [16] also noted that ferments supplemented with high amounts of DAP had late formation of H_2S, a finding also observed in the present study. Ugliano et al. [16] noted that the higher cell biomass in the high DAP treatment may have resulted in greater H_2S production and may explain the higher H_2S observed in the DAP addition treatment in the present study.

The source of nitrogen (ammonia nitrogen from DAP vs. amino acid-derived) also impacted the production of H_2S during fermentation. The lowest amount of H_2S produced was in fermentations where amino acids had been added, whereas the highest amount measured was in fermentations where DAP and S^0 had been added. Although it was not surprising that the addition of S^0 resulted in elevated H_2S production, the large increase in H_2S production if DAP was also added was unexpected and this interaction has not been reported previously. Furthermore, the amount of H_2S produced in ferments containing S^0 was lowered if amino acids were added instead of DAP. Suppression of H_2S production by the addition of amino acids has been noted in previous studies [4]. However, the reduced H_2S production measured when amino acids were added to fermentations containing S^0 (compared to S^0 only) was unexpected and the reason unknown. It should be noted that the amino acid mix added to the fermentations did not contain cysteine or methionine, amino acids that can induce or suppress the production of H_2S [4,40] in a concentration-

dependent manner that is also impacted by nitrogen concentration [5]. The amino acid mix did contain threonine, an amino acid reported to increase H_2S production due to suppression of homoserine biosynthesis [4]. However, in the present study, the addition of this amino acid as part of the amino acid mix did not increase H_2S production compared to the control. Our results suggest that it was not just the increase in YAN that led to the higher amount of H_2S, but also the type of nitrogen added. For example, the addition of amino acids to approx. the same YAN as DAP supplementation resulted in lower H_2S production. This has implications for nitrogen supplementation of grape musts as both the type of nitrogen as well as the quantity must be considered if H_2S production is to be minimized.

The type of nitrogen added (ammonium vs. amino acid) had less of an impact on MeSOAc concentration than the increase in YAN. All treatments where YAN had been increased resulted in significantly higher MeSOAc concentrations. As noted before, the relative differences in MeSOAc concentrations between treatments did not necessarily match the relative production of H_2S. For example, although there were significant differences in the production of H_2S between the DAP + 10 μg/g S^0 treatment and the amino acids + 10 μg/g S^0 treatment, there was no significant difference in the production of MeSOAc. The high concentration of MeSOAc in the treatment with only an amino acid addition was also unusual and difficult to explain as late production of H_2S was not seen in this treatment which could explain the higher MeSOAc. Clearly, the interaction between VSCs production and nitrogen concentration and composition is complex. Understanding these relationships will be important to allow further refinement of recommendations for nitrogen supplementation to prevent VSCs from odors in wines.

5. Conclusions

The formation of VSCs in wine during and after fermentation is a complex process impacted by many factors including yeast strain, nitrogen content, and elemental sulfur. In this study, increasing concentrations of S^0 resulted in increasing production of H_2S during fermentation even when a 'no-H_2S' yeast strain, P1Y2, was used. However, significantly more H_2S was produced by strain UCD522 at each level of S^0 added, demonstrating a significant interaction between yeast strain, S^0, and H_2S production. The concentration of VSCs other than H_2S in the wines post-fermentation was not necessarily associated with the relative amount of H_2S produced during fermentation with MeSOAc in particular being more associated with wines where there was late production of H_2S during fermentation. Nitrogen concentration and composition must also be accounted for when assessing the risk of formation of VSCs with or without the presence of S^0. In particular, DAP additions led to increased H_2S formation during fermentation whereas high YAN resulted in elevated MeSOAC in the wines post-fermentation. Future work should investigate the interactions between lower concentrations of elemental sulfur and nitrogen type and the formation of VSCs should be tracked during aging as the present study only assessed VSCs at the end of alcoholic fermentation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9100904/s1, Table S1: Composition and concentration of amino acids added to Pinot noir fermentations.

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