The Effect of Fungicide Residues and Yeast Assimilable Nitrogen on Fermentation Kinetics and H$_2$S Production during Cider Fermentation

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Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science
In
Food Science and Technology

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31 March 2016
Blacksburg, VA

Keywords: Apple cider, fermentation, yeast assimilable nitrogen (YAN), hydrogen sulfide, amino acids, fungicides, sensory evaluation

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ABSTRACT

The Virginia cider industry has grown rapidly in the past decade, and demands research-based recommendations for cider fermentation. This study evaluated relationships between the unique chemistry of apples and production of hydrogen sulfide (H_2S) in cider fermentations. Yeast assimilable nitrogen (YAN) concentration and composition and residual fungicides influence H_2S production by yeast during fermentation, but these factors have to date only been studied in wine grape fermentations. This study surveyed 12 Virginia-grown apple cultivars and found that the majority were severely deficient in YAN. The effects of three fungicides on cider fermentation were investigated; elemental sulfur, fludioxonil and fenbuconazole. Fenbuconazole adversely impacted fermentation kinetics. Sulfur and fludioxonil marginally impacted fermentation kinetics. Sulfur increased H_2S production, but fludioxonil and fenbuconazole did not affect H_2S production. There was no difference in fermentation kinetics and H_2S between nitrogen sources arginine (approximating grape), asparagine (approximating apple) and ammonium (YAN supplement). Supplementation with methionine resulted in increased fermentation rate and decreased H_2S production. The detrimental effects of fenbuconazole and beneficial effects of methionine were diminished with increasing total YAN. Contrary to previous findings, the most H_2S was formed at 153 mg/L YAN which is above the generally recommended minimum to prevent H_2S formation. These results indicate that apple juice chemistry may influence yeast metabolism during cider fermentation, in ways that have not been previously studied in grape fermentation. Our findings indicate the need for and contribute to the development of targeted fermentation management practices for cidermaking.

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GENERAL AUDIENCE ABSTRACT

The Virginia cider industry has recently grown rapidly, with growth generating demand for cider fermentation management strategies. The goal of this study was to evaluate potential causes for the occurrence of sulfur off-aromas (H_2S) in cider fermentation. Cider fermentation practices are largely based on white winemaking techniques, although there are differences between apple chemistry and grape chemistry. Our experiments assessed: (1) yeast assimilable nitrogen in 12 apple varieties used in cidermaking, (2) the effect of fungicides used in orchards and (3) the effect of juice nitrogen composition on cider fermentation health and H_2S production. Yeast assimilable nitrogen (YAN), an essential yeast nutrient, is a major contributor to cider quality. The majority of apple varieties investigated contained insufficient YAN to conduct healthy fermentations. Three fungicides were examined in this study. Fludioxonil did not affect fermentation performance. Sulfur increased total H_2S but did not affect fermentation health. Fenbuconazole adversely affected fermentations but did not affect H_2S formation. When comparing nitrogen sources, arginine (the predominant grape amino acid), asparagine (the predominant apple amino acid), and ammonium (a common nitrogen supplement) there was no difference in fermentation health or H_2S formation. Adding the nitrogen source methionine which is found in grapes, and may inhibit H_2S, decreased H_2S at all concentrations added. However, when additional YAN was added, the adverse effects of fenbuconazole and the positive effects of methionine were eliminated. The highest H_2S production was at moderate YAN concentrations. These results indicate cider quality can be impacted by juice chemistry, aiding in the development of fermentation management practices for cidermaking.

Acknowledgements

I would like to thank my advisor, Dr. Amanda Stewart, for all of her direction, help and support while conducting this research. The advice she gave me while also allowing me to grow independently as a researcher is invaluable for my career and future endeavors.

I would also like to thank my committee members, Dr. Sean O'Keefe and Dr. Greg Peck for all of their advice, input and support during this project.

I would like to thank Sihui Ma, Nick Patrick, Ken Hurley, Anne Sandbrook, Melissa Wright, Dr. Susan Duncan, Dr. Mizuho Nita, Kemia Amin, Kayla Moberg Andrew Smithson, and the staff of the Alson H. Smith, Jr. Agricultural Research and Extension Center for all of their help with the experimental design and analyses.

Thanks to all the faculty and staff of the Virginia Tech Department of Food Science and Technology for the guidance and education I gained here at the university. Thanks to all my fellow graduate students who made my experience at Virginia Tech a wonderful part of my life.

And finally, thank you to Rachel Metras for all of your love and support during my master's research, and for bringing such happiness into my life without which none of this would be possible.

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Chapter 1: Introduction

In the past decade, Virginia cider production has grown immensely as the national cider market has grown more popular for consumers. Cider sales grew 65% from 2011 to 2012 alone[2], and in 2015 there were 126 new cidermakers in the United States, the largest growth of US cideries to date[3]. Therefore, it has been increasingly important for cider makers to employ optimized fermentation management strategies to consistently meet product stylistic goals at increasing production volumes for production of high quality cider.

The ability to predict and manage fermentation kinetics is essential in wine and cider production. Fermentation kinetics can be defined as fermentation rate and fermentation duration. Fermentation rate and duration will influence the rate of CO_2 production during fermentation and sugar concentrations upon completion of fermentation. There are many factors including temperature, yeast strain, aeration and others which influence fermentation kinetics, one of the most important being yeast assimilable nitrogen (YAN) concentration and composition [4]. This study aims to determine how YAN concentration and composition and the presence of fungicide residues influence fermentation kinetics during cider fermentation.

The formation of hydrogen sulfide (H_2S) during alcoholic fermentation is a prominent issue impacting final product quality, aroma and flavor in hard cider as well as wine [5]. This off-aroma has been vigorously studied and analyzed in viticultural and enological conditions, and many root causes and sources have been determined and identified and great effort is targeted at prevention of its formation during normal fermentative conditions [5, 6]. This problem, however, is anecdotally considered more prevalent in hard cider production as compared to wine production. The overall goal of this thesis project is to evaluate the impact of two probable causes of H_2S production in

cider fermentation (YAN composition and concentration, and the presence of fungicide residues) and provide recommendations based on our findings for prevention of H_2S production in cider fermentation.

One cause of H_2S production during fermentation is deficiency in Yeast Assimilable Nitrogen (YAN). YAN is one of the most important factors determining optimal fermentation health. YAN also promotes complete fermentation and optimal yeast metabolism without the production of off-aromas. All of these factors contribute to the successful production of wines and ciders. YAN is defined as the nitrogen components of the juice which are readily assimilated and metabolized by yeast. Deficiency in YAN in grape and apple juice used for wine and hard cider production is widely known to lead to slow or stuck fermentations[4]. Insufficient YAN has also been known to increase undesirable volatile aroma compounds such as higher alcohols while simultaneously reducing the production of desirable esters and long-chain fatty acids [4]. Though most of the research in this area has been conducted regarding the influence of YAN on wine fermentation from grape must, due to differences in amino acid composition between grape and apple, the same total YAN concentration may impact apple fermentation in a significantly different manner, which may alter fermentation conditions and final cider quality. Few studies been published linking the total YAN concentration to fermentation performance in hard ciders[7], and the body of research on cider fermentation is limited.

There are many known factors influencing H_2S production by *Saccharomyces cerevisiae* in alcoholic fermentations, including yeast strain[8] and YAN concentration and composition[4]. One notable problem is late-season fungicide residues which remain in contact with the fruit post-harvest. These fungicides are applied to prevent the growth of fungal pathogens in the orchard or vineyard. If not washed or settled from the juice, fungicide residues can inhibit the optimal growth

of *Saccharomyces cerevisiae*, which are also fungi, and cause fermentative stress which can lead to increased H_2S production. Though research has been conducted on the effect of sulfur spray residues in viticultural and enological conditions, no such investigation has been conducted in hard cider production. Furthermore, very little research has been conducted investigating the effect of fungicides used in orchards on apple fermentation conditions and final cider quality. Fungicides used in orchards may differ significantly as compared to those used in vineyards, however prior studies of the effects of fungicides residues on fruit fermentation have been limited to those used in vineyards. Orchard fungicides, such as fenbuconazole, may adversely affect cider fermentations. However, as nearly all of the prior research on the effect of fungicide residues on fruit fermentation performance have been conducted with vineyard-applied fungicides, the effect of fungicides used in orchards but not in vineyards on fermentation has not been investigated. Additionally, because of the agricultural practice of storing apples for long periods before processing, many apples are sprayed with a post-harvest fungicide to prevent deterioration during storage. These fungicides, such as the commonly-used fludioxonil, could present challenges during cider fermentation. These factors, in combination with the differences in apple and grape chemistry, may pose challenges in cider fermentation that are not faced in wine fermentation.

Complete fermentation and the prevention of H_2S production during fermentation are critical for consistent successful production of hard ciders and the continued growth, expansion and acclaim of the industry. Therefore, this study aims to identify causes of adverse fermentation kinetics and target the causes of H_2S production during hard cider fermentations in order to develop fermentation management recommendations for cider makers in Virginia.

Chapter 2: Review of Literature

Cidermaking in the United States

In the United States, the term "cider" is most often attributed to unfiltered non-alcoholic apple juice. However, in the United Kingdom the term "cider" refers to a fermented beverage made from apple juice, which is usually termed "hard cider" in the United States. Other fermented apple beverages, especially those with higher alcohol per volume, have been called "apple wine" in some European countries as well as the US and UK. Recently, the growth of the US cider industry has made the term "cider" more recognizable as the alcoholic beverage rather than its unfermented counterpart. In the present work, "cider" will be used to describe the fermented apple juice product.

Cider has been a popular beverage in the US since at least the 18th century[2]. Virginia in particular has been an ideal orchard-growing and cidermaking location, with Thomas Jefferson famously making cider at his home in Monticello[2]. Though the cider market declined drastically in the 19th and 20th centuries, but has once again risen in the past two decades[3]. The cider market has grown 10-fold in the last decade, with 126 cideries opening in the US in 2015 alone[3]. Cider growth has outpaced both beer and wine growth nationally in the same period[2]. Therefore, it has become increasingly important to investigate methods to optimize cider fermentation for ideal cider quality.

Yeast Assimilable Nitrogen and Fermentation

Nitrogen is an essential nutrient for yeast growth and metabolism during the fermentation of alcoholic beverages. Notably, deficiencies in nitrogen concentration can lead to slow, sluggish or stuck fermentations, which is defined as the slowing or stopping of fermentation due to limited nutrients in the juice or must [7, 9-18]. Other than a carbon source (usually fermentable sugars),

nitrogen is the most important yeast nutrient required to ensure complete and successful fermentations [4].

Deficiencies in nitrogen can lead to several adverse fermentative conditions. As mentioned above, low YAN is widely known to result in decreased fermentation vigor and increased overall duration of fermentation, low yeast biomass production, and ultimately slow and sluggish fermentations [7, 9-18]. Also, the fermentative stress caused by low YAN can lead to increased levels of volatile thiols, including hydrogen sulfide, which can be detrimental to product flavor [4]. Elevated levels of undesirable higher alcohols can also be produced, while lower levels of beneficial flavor compounds such as ester and long-chain fatty acids will be produced as compared to nitrogen-sufficient musts [4].

Though nitrogen deficiencies in grape juice or must have been the subject of considerable research, excess YAN can also lead to several adverse fermentative conditions. These include the production of detrimental flavor and aroma compounds such as ethyl acetate, acetic acid and other volatiles[1]. Most notably, excess YAN can lead to an increased risk for growth and metabolic health of unwanted microbes such as *Botrytis cinerea* and *Brettanomyces bruxellensis* in the finished wine or cider[4].

Yeast Assimilable Nitrogen Composition

Yeast Assimilable Nitrogen (YAN) is composed of two components: Free Amino Nitrogen (FAN), sometimes referred to as Primary Amino Nitrogen (PAN) or primary organic nitrogen; and ammonia or ammonium ion, sometimes referred to as inorganic nitrogen. Though grape and apple

juice may contain other nitrogen sources, such as proteins, peptides, amines, nitrates and etc., these are not assimilated or metabolized by yeast during normal fermentations [4].

FAN components include all primary amino acids and small peptides which are readily consumed by yeast. Though it is normally grouped with FAN components, proline is unable to be metabolized by *Saccharomyces cerevisiae* under normal anaerobic conditions [19-22]. Though proline can be metabolized once exposed to oxygen, yeast will preferentially select ammonia and other FAN components before metabolizing proline [19]. Therefore, it is important to measure FAN and not total amino nitrogen when determining YAN content, because proline can comprise a considerable portion of amino acid content in both grape and apple juice used for wine and cider making [7, 22-26]. Amongst the FAN components, arginine is typically the largest contributor and also the amino acid most readily consumed by yeast during fermentation [26-29]. Because of the dominance of arginine among YAN sources in grapes, for some cultivars the ratio of arginine to proline can provide an accurate means of quantifying the ratio of assimilable and non-assimilable nitrogen. [25, 27, 29]. In fact, it was found in Cabernet Sauvignon that the arginine to proline ratio of grape juice was demonstrated to be a relatively accurate method of measuring the ratio of YAN:YNAN (yeast non-assimilable nitrogen) [4, 25]. Most importantly, it should be noted that common methods for total nitrogen content determination in foods such as the Kjeldahl method are inappropriate for YAN determination, as many forms of nitrogen in juice or must are not assimilable to yeast.

There have been several studies conducted to determine which of the FAN components are preferentially assimilated, but with no clear consensus across the results of these studies. Arginine has been cited in several studies as the amino acid most preferentially consumed during fermentation, though results are not conclusive [4, 30]. This is because arginine may be consumed in

higher proportions due to its high concentration in solution compared to other amino acids [4, 26, 28]. In a publication by Ough *et al*, it was found that when glutamine and alanine are in excess, arginine is not consumed excessively, suggesting that arginine is not always preferentially consumed, depending on other components in the must [22]. Another confounding variable relating to arginine's consumption is that it is typically not consumed when ammonium is present, as ammonium is known to be a preferentially assimilated nitrogen source [4, 30]. Conversely, a study by Monteiro and Bisson found that an addition of arginine to nitrogen-deficient musts increased fermentation rate and biomass, but additions of diammonium phosphate (DAP) as an ammonium source did not decrease the total amount of arginine consumed [16]. Amino acids such as glutamine, asparagine and tryptophan have been noted as preferentially consumed during fermentations, but other amino acids such as alanine are also consumed in significant portions [9, 22, 25, 26, 30-33]. Despite this, it has been found that mixtures of amino acids can provide a greater rate of growth as compared to single amino nitrogen sources or ammonia alone [9, 17, 31, 34, 35]. These contradicting results are difficult to explain and are most likely due to the complex nature of yeast metabolism. Nitrogen-catabolite repression is a cellular mechanism that regulates uptake of nitrogen sources from the matrix surrounding the yeast[36]. When a preferred source of nitrogen is present in the extracellular fermentation medium, genes encoding the amino acid transporters needed for the yeast cell to take up less-preferred nitrogen sources are not expressed[36]. This mechanism is not yet well understood and results of studies of this phenomenon have varied across yeast strains and across conditions with different total YAN concentrations. There is little doubt that studies of the order of uptake of individual nitrogen sources within a complex medium have been confounded by nitrogen catabolite repression. Additionally, yeast strains vary in their demand for nitrogen[14] and therefore would likely also have variable responses to nitrogen catabolite repression. Other factors, such as

temperature and yeast micro- and macro-nutrients other than nitrogen also impact nitrogen uptake and metabolism by yeast. The interaction effect of these multiple factors with nitrogen concentration and composition have made setting an absolute target YAN concentration for cider fermentation a formidable challenge.

The total concentration of amino acids can vary greatly amongst cultivars, production practices harvest, growing season and fruit maturity at harvest in both wine grapes and cider apples. Most research on amino acid levels have focused on wine grapes, and the few studies investigating amino acid content of apples reflect notable similarities and differences between apple and grape chemistry. In grapes, amino acids make up 51-92% of the total juice YAN content upon harvest [4]. Reported average levels of amino nitrogen in grapes are 136 mg/L [37], 24.9-224.3 mg/L [28], and 92.0 mg/L [26], showing wide variation in average concentration across studies. Amino acid concentrations in grapes can be much higher than those found in apples, such as those reported by Butzke which found total amino acids in levels above 1000 mg/L [37]. Amino acid concentration in grapes is highly variable (Table 1). Factors influencing the concentration of amino acids in grapes are cultivar[4], growing season[4], and vineyard management practices such as fertilization, pruning, crop load management[4, 38, 39], among others. For cider apples, one study reported FAN content at 38.3 mg/L [7]. Other studies reported individual amino acid concentrations from 0.9-61.2 mg/L [23].

Table 1 – The identity and concentration of amino acids found in the whole grape and/or juice at harvest (reproduced without permission from Bell and Henschke, 2005)

Amino acid	Concentration range reported in the literature (mg/L)
Alanine	10 – 227
Arginine	20 – 2322
Asparagine	1 – 171
Aspartic acid	10 – 138
Citrulline	0.1 – 83
Cysteine	1 – 8.2
Glutamine	9 – 4499
Glutamic acid	27 – 454
Glycine	1 – 20
Histidine	5 – 197
Isoleucine	1 – 117
Leucine	2 – 160
Lysine	0.7 – 45
Methionine	1 – 33
Ornithine	0.1 – 27.2
Phenylalanine	2.8 – 138
Proline	9 – 2257
Serine	13 – 330
Threonine	9 – 284
Tryptophan	0.2 – 11
Tyrosine	2 – 33
Valine	7 – 116

The ammonium ion is the other major component of YAN. Yeast assimilate ammonium preferentially as compared to FAN components [9, 13, 17]. Jiranek *et al,* found that addition of ammonium to a model media inhibited the assimilation of amino acids, indicating that ammonium is considered an initially preferred source of YAN than FAN during fermentation by yeasts [30]. In fact, ter Schure *et al* has indicated that ammonium can be the sole nitrogen source in normal fermentative conditions[36]. Addition of diammonium phosphate (DAP) during normal wine and cider making practices provides ammonium as a supplemental nitrogen source to rectify YAN deficiency. Therefore, it can be implied that the addition of DAP inhibits the assimilation of FAN components naturally present in the must. However, Beltran *et al* reported that mixtures of amino acids and ammonium increased fermentation rate as compared to limiting to either only FAN or only ammonium sources [9]. And although ammonium is considered to be a sufficient nitrogen source to conduct a complete fermentation, several studies have been published indicating that either mixtures of FAN and ammonium or solely FAN sources produce a higher rate of fermentations or more desirable wine aromas[9, 12, 17, 31, 34, 35]. Addition of exogenous nitrogen sources which contain amino acids as well as DAP (such as the complex yeast nutrient Fermaid K) can hence be expected to produce more desirable fermentations and final wine or cider products

Ammonium can make up 2-52% of the total YAN content of fermentable juice in grapes [4]. However, data on the ammonia content alone of apple juice for hard cider fermentation is limited, and prior to this work there was no available survey data on the ammonia content of cider apples. This could potentially have a major influence on the production of H_2S during cider fermentation as it relates to yeast metabolism.

The formation of hydrogen sulfide has been examined in enological conditions since the early 1960s [5]. Though a common issue for centuries, its identification and prevention was only studied extensively within the last half-century.

Hydrogen sulfide is formed by *S. cerevisiae* through the sulfate reduction sequences (SRS) [8]. The SRS is activated when levels of sulfur-containing amino acids (such as methionine and cysteine) are deficient in the juice, as the products of the SRS are sulfur-containing amino compounds [8]. This system transports extracellular sulfate into the cell to be assimilated to produce sulfur-containing amino compounds, including cysteine and methionine [8] when there is demand for these amino acids. The sulfate is converted enzymatically to sulfite, and then sulfide, where specialized pathways convert the sulfide to amino acids and other amino compounds, as shown in Figure 1 [8]. Note that methionine and cysteine can also be extracellular sources of sulfur, but are usually found in insufficient concentrations to contribute significantly to the SRS (less than 10 mg/L), especially as compared to sulfate, which can be found in grape juice at concentrations as high as 400 mg/L [8, 40]. Sulfite can also be a source of extracellular sulfur, especially when added by wine and cider makers for antimicrobial and antioxidant purposes.
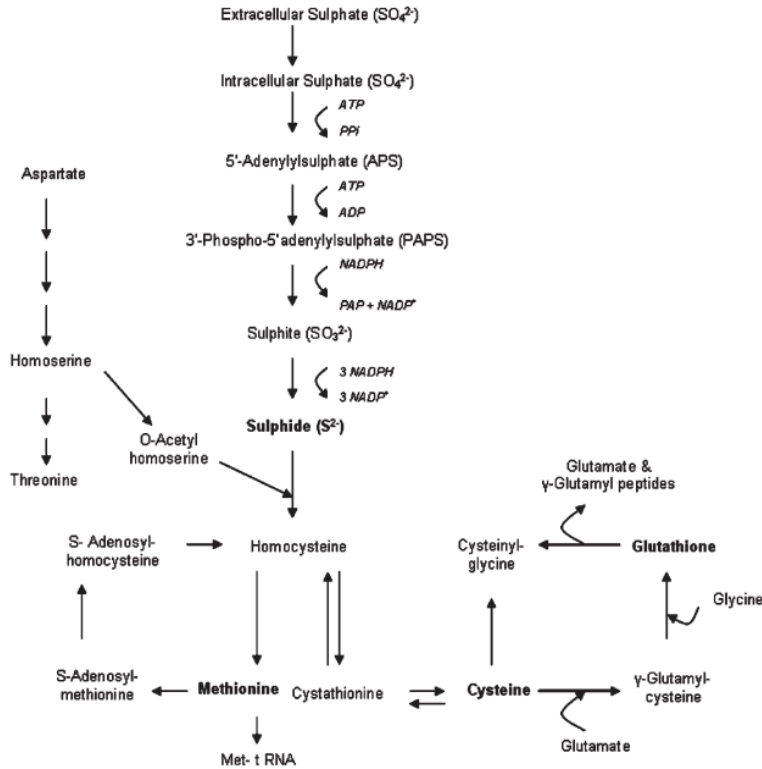
Figure 1 – Diagram of the sulfate reduction sequence pathway and sulfur-containing amino compound biosynthesis in S. cerevisiae (reproduced without permission from Rauhut 2009)

If the SRS pathway is active under conditions of adequate nitrogen supply, the formation of H_2S from sulfide will be inhibited, as the enzyme *O*-acetylhomoserine converts sulfide to methionine and cysteine [8, 41]. However, under nitrogen-deficient conditions, free sulfide can accumulate in the cell and eventually diffuse into the juice, because the enzymatic precursors necessary for the production of sulfur-containing amino compounds require nitrogen for formation [40, 41]. This indicates the importance of adequate levels of nitrogen in preventing the formation of H_2S.

Several factors can influence the amount of H_2S produced during wine and cider fermentations. Yeast strain is most often identified as the primary cause of high versus low

production of H_2S overall during alcoholic fermentation. Different yeast strains can produce levels of H_2S as high as 300 µg/L, and others produce no detectable H_2S [42]. Rankine first identified yeast strain as a major influence on H_2S production in 1963, noting that there was a seven-fold increase in H_2S production amongst some strains, while others produced no detectable H_2S [5]. Several papers have corroborated Rankine's results, noting that yeast strain is either the most important factor or a major influence when determining the quantity of H_2S produced during fermentation [43-48].

Several genes have been identified whose activity leads to a lower level of H_2S production and these genes seem to be present in those yeast strains that produce limited H_2S during fermentation. These genes encode specific enzymes in the SRS and can hence affect the production and control of sulfide (Figure 1). In a 2000 publication by Spiropoulos and Bisson, analysis of a low-H_2S producing strain of *S. cerevisiae* was found to be correlated with the overexpression of the *MET17* gene [49]. This gene leads to the overproduction of *O*-acytelhomoserine sulfhydrylase, which limits the amount of sulfide which can be accumulated in the cell, and hence would limit H_2S production [49]. However, in another strain it was found that H_2S production was not inhibited by expression of the *MET17* gene, implying that *O*-acytelhomoserine sulfhydrylase activity is not the sole determining factor for final production and accumulation of H_2S [49] by yeast during alcoholic fermentation.

Similarly, in a publication by Linderholm in 2010, the low-H_2S-producing strain of *S. cerevisiae* UCD932 was found to be controlled by a genetic variant in the SRS pathway [50]. It was found that the gene *MET10* was unique to this strain, and when this allele was transferred to a high-H_2S producing strain, it was found that H_2S formation was prevented, confirming that the *MET10* gene plays a major role in the prevention of the formation of H_2S [50]. Interestingly, this mutant *MET10* gene does not display a requirement for sulfur-containing amino acids to activate

the SRS, and therefore it was found that the SRS in this strain was perpetually active [50]. Further research is needed to determine if this mutant *MET10* gene is the root cause of all elevated H_2S production in strains of *S. cerevisiae*.
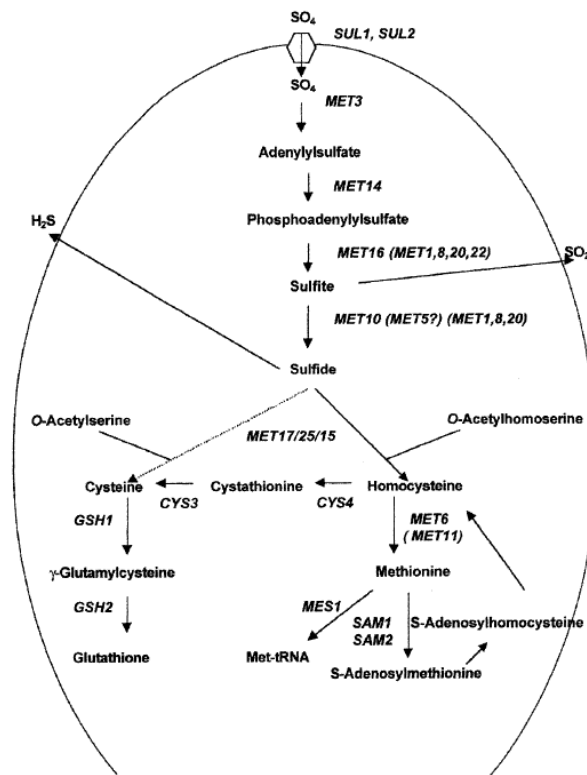

Figure 2 – The SRS pathway and the major genes which encode the enzymes found in each step of the pathway (reproduced without permission from Spiropoulos and Rose, 2000)

Other biochemical factors are major contributors to the production of H_2S during alcoholic fermentation. Pantothenate (pantothenic acid or Vitamin B_5) deficiency has been shown to lead to elevated levels of H_2S production. Wainwright found that pantothenate levels lower than 160 μg/L lead to elevated H_2S production, peaking at approximately 40 μg/L, and that H_2S production under

pantothenate deficiency is exacerbated by other nutrient deficiencies such as limited glucose, sulfate and nitrogen [51]. Several studies have confirmed that adequate levels of pantothenate are necessary for the prevention of H_2S production during fermentation [51, 52]. Notably, Bohlscheid found that interactions with various levels of pantothenate, YAN, biotin, and temperature significantly influenced the total requirement of each nutrient [52]. These results corroborated previous findings on the interactivity of YAN and pantothenate by Wang *et al* [53], and in part have motivated us to investigate not only the effect of fungicide residues, but also the interactive effect of fungicide residues and YAN on fermentation performance.

Sulfite is another known source of H_2S production during fermentation. Though formed under normal conditions in the SRS pathway, excess SO_3 can lead to elevated H_2S formation, usually under the same conditions found in typical H_2S production. This is because sulfites can be emitted extracellularly from the SRS, and in high concentrations reduce to form other volatile sulfur compounds including H_2S. Such elevated levels may come as a result of the addition of sulfite used to prevent oxidation and the growth of adverse microbes, usually in the form of potassium metabisulfite. Several publications have correlated concentrations as low as 30 mg/L of sulfite with increased H_2S production in winemaking conditions [8, 41, 54].

Yeast Assimilable Nitrogen and Hydrogen Sulfide

It has been widely reported and is now generally accepted by wine and cider makers that insufficient YAN can contribute to elevated levels of hydrogen sulfide production during alcoholic fermentation[4, 6, 48, 55]. Though most studies have focused on the impact of YAN on H_2S production in winemaking and brewing conditions, the findings and implications are generally extrapolated

to cidermaking conditions. Several relationships between YAN content and H_2S formation are examined below.

In general, previous research has shown that increased YAN concentrations in the fermentable must leads to a decrease in H_2S production. Several studies have corroborated this claim, finding that the addition of YAN or YAN components to nitrogen-deficient must decreases the amount of H_2S produced [6, 8, 44, 53, 56, 57] (Figure 3). Rankine first stated in 1963 that there was a correlation between fermentation vigor (defined as CO_2 evolution and sugar utilization) and H_2S evolution [5]. Because YAN is correlated with fermentation vigor, including CO_2 evolution, sugar utilization, and biomass production [13, 18, 58-60] (Figure 4), it can be inferred that H_2S production is correlated to YAN concentration.
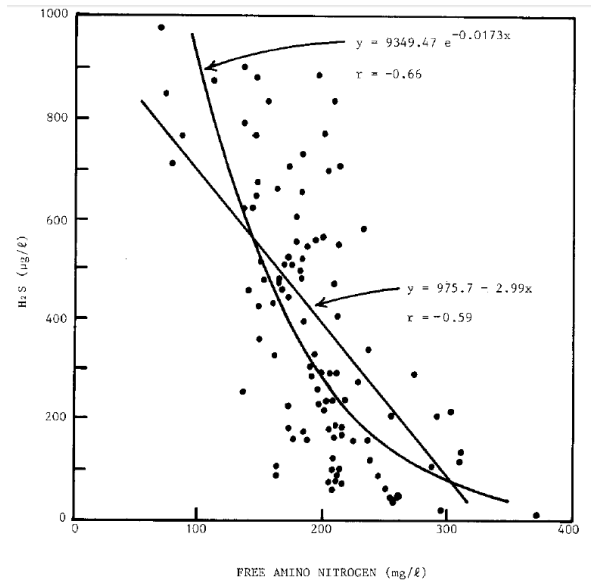

Figure 3 – Linear and curvilinear regressions of H2S development (μg/L) during fermentation on concentration of free amino nitrogen (mg/L) in musts (reproduced without permission from Vos and Gray, 1979)
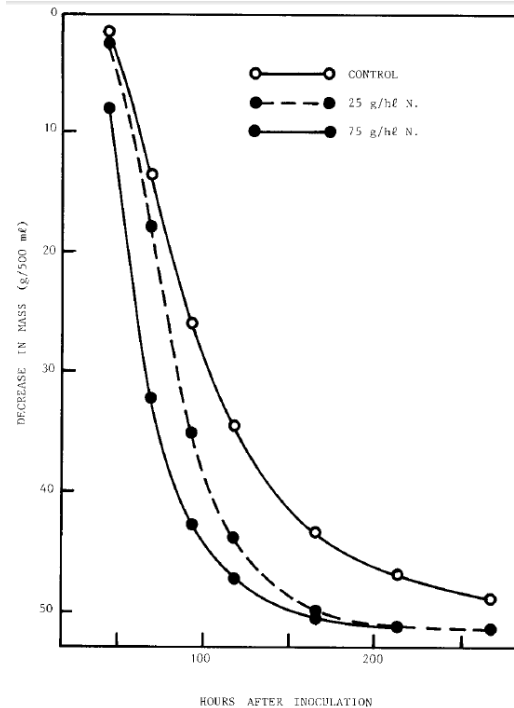

Figure 4 – Effect on fermentation rates by addition of diammonium phosphate (reproduced without permission from Vos and Gray, 1979)

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Vos & Gray hypothesized that that the major contributor to the formation of H_2S is the individual and unique nitrogen requirements of specific yeast strains rather than the activity of the sulfur reductase sequence [6]. In their study, there was no significant correlation (p>0.05) found between H_2S and soluble solids, titratable acidity, pH, turbidity, total nitrogen, protein nitrogen, and non-protein nitrogen [6]. There was a significant correlation (p=0.026) found between ammonia nitrogen and H_2S evolution [6]. However, the r-values for the correlation between H_2S evolution and FAN contain substantial deviations (linear r = -0.59, curvilinear r = -0.66), indicating that FAN cannot be the only factor influencing the formation of H_2S [6].

Jiranek *et al* found that the greatest amount of H_2S production occurred during the exponential phase of yeast growth in nitrogen-deficient musts [56]. When ammonium was added during the exponential phase, H_2S production was almost entirely inhibited [56]. This result was
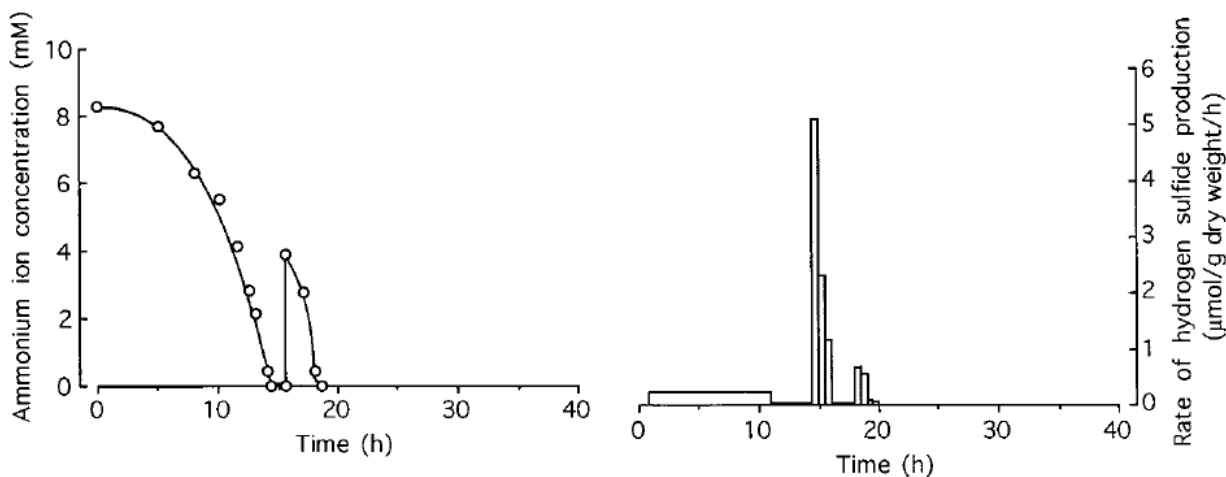
Figure 5 – The effect of the addition of ammonium on the production of hydrogen sulfide. Maxiumim H_2S evolution occurs upon ammonium depletion but is alleviated upon further addition of NH_4 (reprodcued without permission from Jiranek et al., 1995)

found to be true upon the addition of several primary amino acids as well [56]. Therefore, we can conclude that the production of H_2S is not only directly correlated with YAN concentration, but

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also that the addition of YAN or YAN components can act to decrease the formation of H_2S during fermentation (Figure 5).

Several studies have noted the importance of monitoring H_2S concentration post-fermentation and post-bottling. One study noted that H_2S evolution was prominent during fermentation, but that there were no off-aromas present in the finished wine [57]. It was hypothesized that this was due to carbon dioxide purging, in which the volatile H_2S was removed from solution by the CO_2 released during normal fermentative conditions [57]. Because H_2S is detected in most laboratory trap methods as it is carried out of the fermentation vessel via the evolution of CO_2, this seems to be a plausible explantation for the lack of H_2S off-aromas in the finished wine. Similar results were obtained in a study by Ugliano *et al* which found that H_2S generation during fermentation was not correlated with the H_2S concentration in the finished wine [55]. Indeed, the results of this study found that wines fermented with low and moderate initial nitrogen concentration produced wines with significantly higher H_2S compared to wines in high nitrogen fermentations ($p<0.01$) [55] (Figure 6). Ugliano made a similar interpretation of this finding as Park, in that CO_2 evolution effectively purged H_2S from the system[55]. However, because moderate-nitrogen fermentations had a residual H_2S concentration as high as the low nitrogen fermentation but had a notably higher fermentation rate, the fermentation vigor of the solution can only be partially correlated with final H_2S concentrations [55]. Another study found that the concentration of H_2S in the final wine was not related to or correlated with the addition of nitrogen during fermentation [61]. These discrepancies can be reasonably attributed to differences in removal of H_2S by CO_2 gas evolved during fermentation at different fermentation rates, since fermentation rate can be influenced by many factors incliding yeast strain and YAN concentration and composition.
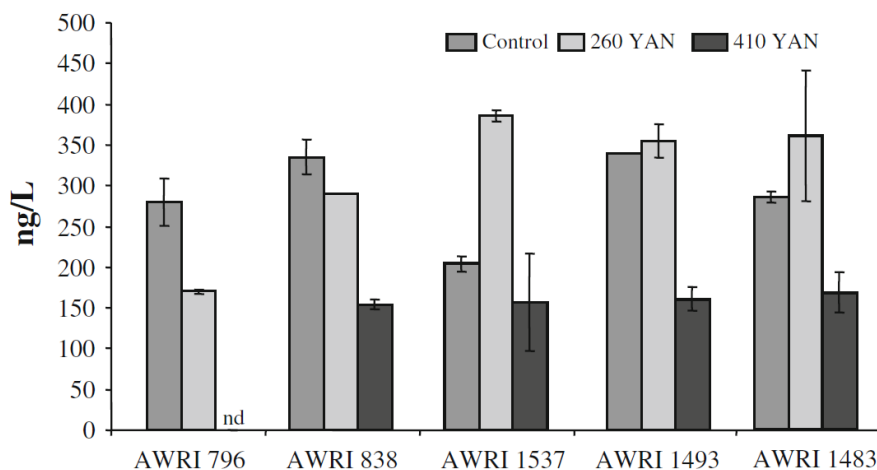
Figure 6 – Concentrations of H₂S in finished wines at different nitrogen levels. Control juice

contained 110 mg/L N (reproduced without permission from Ugliano et al., 2009)

As would be expected, the residual H₂S has more of an impact on the sensory quality of finished wines and ciders as compared to the amount of H₂S produced during fermentation. Levels as lows as 1.6 µg/L can be detected in white wines by sensory evaluation [62]. The presence of H₂S in finished wines and ciders is associated with a "rubbery" or "sulfury" aroma [63]. It was found that high concentrations of residual H₂S lead to "reduced," "earthy/dirty" and "vegetal" aromas and a repession of most fruity aromas [63]. In another study by Ugliano *et al*, it was concluded via sensory evaluation by a trained panel that high nitrogen fermented wines were the lowest in sulfide-related and rubbery off-aromas [63].

Despite the common winemaking belief that low levels of YAN contribute to increased H₂S production during fermentation and the multiple studies which corroborate this idea, several other studies have found no significant correlation between YAN content and H₂S production [43,

$^{45,\ 48,\ 49,\ 55,\ 61,\ 63}$. In some cases, the correlation between H_2S and YAN was significant with some yeast strains, but there was no relationship in others [43, 45, 49, 55]. This is in accord with earlier findings which cited the dependence of H_2S formation on yeast strain [43-46, 48, 55, 58, 61, 63]. In a publication by Bohscheid *et al*, it was found that H_2S production was influenced by the interactions between different levels of YAN, biotin, pantothenic acid, and temperature, but not by YAN concentration alone [52]. However, the interactive effect of YAN with one or more of the following factors: biotin concentration, pantothenic acid concentration, and temperature, was significantly correlated with H_2S production ($p<0.001$) [52]. This corroborates the hypothesis that YAN is merely one of several components contributing to the production of H_2S during fermentation.

In fact, several studies found that addition of YAN to nitrogen-deficient musts lead to a direct *increase* in the production of H_2S [43, 48, 55, 61, 63, 64]. This seems to be partially yeast-strain dependent, as nitrogen addition can decrease H_2S production in some yeasts and increase H_2S production in others under the same fermentative conditions [55]. In a study in 2011 by Ugliano *et al*, addition of nitrogen to nitrogen-deficient must (110 mg/L N to 260 mg/L N) increased H_2S production across all five yeast strains tested [48] (Figure 7). Higher nitrogen levels (410 mg/L N) led to production of lower levels of H_2S as compared to medium nitrogen must across all five strains, and three strains produced higher levels of H_2S at high nitrogen levels as compared to low nitrogen levels [48] (Figure 8). However, as previously stated, this study found that lower nitrogen concentrations produced wines with significantly higher residual H_2S (Figure 6). Several points must be made when analyzing these findings. Nitrogen addition was done in the form of diammonium phosphate (DAP) addition, which does not account for the impact of FAN components on the production of H_2S, which is the main focus of our planned study [48]. Also, the impact of CO_2 purging may correlate strongly with the inverse relationship between H_2S produced

during fermentation and levels found in the finished wines [55]. Therefore, the distinction must be made when quantifying H_2S during fermentation and between residual in the finished wine in order to gain an accurate representation of the real impact of any H_2S produced during fermentation on cider flavor and quality.
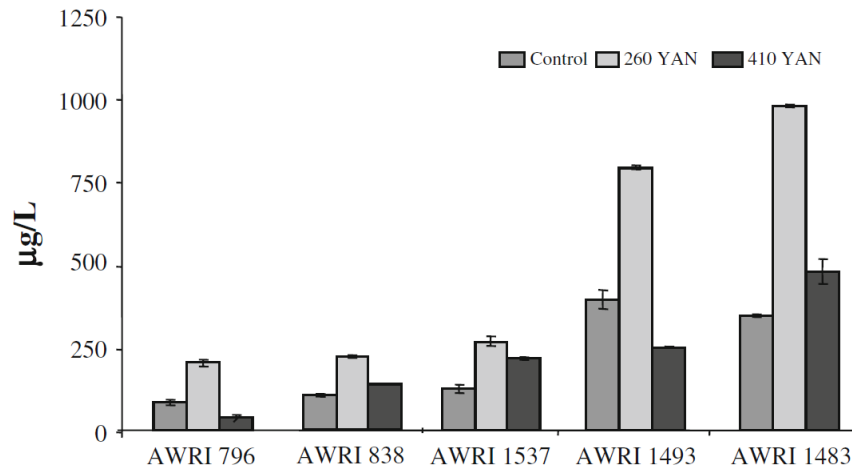
Figure 7 – Effect of yeast strain and nitrogen content on total H₂S produed during fermentation

(reproduced without permission from Ugliano et al., 2011)

In addition to total YAN concentration, YAN composition can have a major impact on the formation of H_2S. Specifically, amino acid concentration and composition can have major impacts on the production of H_2S. Several key findings are noted in the relationship of H_2S produced during fermentation to amino acid concentration and compostion.

In general, the addition of amino acids acts in a similar manner to increasing the total YAN or the ammonium component of YAN, including through the addition of ammonium ions added in the form of diammonium phosphate. Higher concentrations of amino acids leads to a decrease in the production of H_2S [44, 56, 57]. The addition of amino acids to nitrogen deficient juice reduces the total amount of H_2S formed during fermentation, including the amount of H_2S produced in high-sulfide producing yeast strains [57]. If amino acid levels are insufficient and nitrogen levels are deficient overall, H_2S formation will increase steadily even past the peak rate of fermentation where H_2S production is typically the highest [57]. Two studies cited the addition of non-sulfur containing assimilable amino acids (i.e. excluding methionine and cysteine) reduced the amount of H_2S produced during fermentation, supporting the findings produced by Park *et al* [44, 56].

Table 2 – The effect of the addition of different amino acids at varying concentrations on the production of H_2S (reproduced without permission from Giudici and Kunkee, 1994)

Amino acid	mM NH$_4$+ equivalent	H$_2$S
Arginine	0	-
	0.67	+
	3.35	+
	6.70	-
	13.40	-
Alanine	0	-
	0.67	+
	3.35	-
	6.70	-
	13.40	-
Asparagine	0	-
	0.67	+
	3.35	+
	6.70	+
	13.40	-
Methionine	0	-
	0.67	-
	3.35	-
	6.70	-
	13.40	-
Cysteine	0	-
	0.67	+
	3.35	+
	6.70	+
	13.40	+

Additions of sulfur-containing amino acids, specifically cysteine, have been found to increase the amount of H_2S produced during fermentation. Excess cysteine is known to cause H_2S production by yeast as a result of the SRS pathway, where yeasts directly decompose excess cysteine to pyruvate, ammonia and sulfide ions [64]. The addition of cysteine to fermenting juice has been shown to increase the total H_2S produced [44, 56]. It was found that additions of cysteine to nitrogen-deficient juice led to an increase in H_2S produced in all yeast strains selected and at all

concentrations of cysteine added to the must [44] (Table 2). Similarly, Jiranek *et al* found that the addition of cysteine increased the production of H_2S 10- to 35-fold when added as the YAN was depleted during fermentation [56]. A study by Jordan and Slaughter found that the addition of cysteine not only increased the production of H_2S, but also reduced cell growth in the fermentative media [65]. Therefore, excess cysteine present in the fermentative must can lead to a measurable increase in H_2S production. It has been surmised, however, that cysteine concentrations are too low in grape juice (<10 mg/L) to significantly impact the formation of H_2S [5, 6]. Cysteine concentrations in apple juice have been reported at 15 mg/L in one study [66] and preliminary findings observed in Virginia-grown cultivars analyzed in our lab have found concentrations even lower than this (Ma et al., unpublished). However, concentrations higher than approximately 100 mg/L cysteine have been shown to be the minimal amount necessary to increase H_2S production during fermentation[56, 65].

Conversely, insufficient levels of methionine in grape juice have been shown to lead to an increase in H_2S production during wine fermentation [44, 49, 51, 54, 56, 57, 64, 65]. When methionine is present in sufficient levels during fermentation, its production is inhibited in the SRS pathway, which will not be activated and will decrease the formation of the sulfide ion [8]. *O*-acytelhomoserine is the enzymatic precurser to methionine in the SRS, and acts as a result of sufficient methionine in inhibiting the formation of sulfide ions and decreasing the total amount of H_2S produced as a result of the SRS pathway [8, 51] (Figure 2).

In a multitude of studies, it was shown that there was a significant decrease in the formation of H_2S upon methionine addition [44, 49, 51, 54, 56, 57, 65]. In fact, in some cases the addition of methionine inhibited the formation of H_2S completely [44, 65]. The effectiveness of methionine addition seems to be increased when added with increasing levels of ammonium as a nitrogen source [44, 49]. This

finding is supported by previous publications which state that increased YAN reduces H_2S production and that methionine inhibits sulfide formation in the SRS pathway.

Minimum concentrations of methionine have been suggested, but a widely accepted minimum value has not been established. Observed concentrations of methionine in grape juice have ranged from 0mg/L to 52 mg/L (Table 2). Similarly, the observed methionine content of apples ranges from 0 mg/L to 43.3 mg/L (Table 2). Though the maximum and minimum levels of methionine initially appear similar in grape and apple juices, the average levels of methionine are typically lower in apple juices as compared to grape juices [4, 25, 28, 44, 66-68]. Eschenbruch suggests that levels lower than 20 mg/L can lead to the formation of H_2S [64]. Several studies have been conducted using methionine additions at one millimolar concentrations, which is equivalent to 149 mg/L methionine [51, 54], while another study used a 5 mM (745 mg/L) addition [65]. These concentrations are far outside the range naturally present in grape and apple juices, and would not be representative of pre-fermentation juice or must conditions.

*Table 3 – Average and Range of Concentrations of Methionine in Apple and Grape Juices. *np indicates no published data available.*

Methionine Content Range (mg/L)	Average Methionine Content (mg/L)	Fruit Type	Reference
0 - 4	1.2	Apples	Elbert 1959
0.59 - 4.3	2.0	Apples	Wu 2007
3.4	-	Apples	Ye 2014
1 - 33	np	Grapes	Bell 2005
0 - 52	23.4	Grapes	Park 2000
0 - 5	np	Grapes	Huang 1991
0 - 25	2.7	Grapes	Kluba 1978

Fungicide Residues and Hydrogen Sulfide

The use of late-season fungicide applications in grape and apple production is an industry-wide practice used to prevent and control the growth of a range of fungi and molds which cause plant disease and decreased fruit quality (plant pathogens). As yeast are fungi, the persistence of these residues on grapes and apples used for wine and cider production can be detrimental to yeast health and fermentation kinetics. Notably for this study, correlations have been observed between the persistence of fungicide residues on wine grapes and the occurrence of hydrogen sulfide in wine.

Of all the late-season fungicide residues used in wine and cidermaking, elemental sulfur (S^0) residues have been shown to be the most prominent cause of H_2S (Figure 9) [5, 47, 69-72]. Rankine first identified S^0 as a source of H_2S in 1963 [5]. It was found that colloidal sulfur (cosan) led to much higher proportional levels of H_2S production as compared to dusted S^0, implying that particle size of the sulfur particle plays a direct role in the amount of H_2S produced during fermentation [5, 70]. This result is correlated with particle size; spray particles are significantly larger (50 μm) than colloidal sulfur (1 μm) [5]. This result is consistent with the findings of Shuetz and Kunkee, who found that colloidal sulfur resulted in significantly higher H_2S production as compared to wettable and dusting sulfur [71] (Figure 8). S^0 is relatively insoluble in water, which may be related to the impact of colloidal sulfur on H_2S production as compared to dusted sulfur [71]. This may also be a factor of particle size as found in the Rankine paper, since it was found that S^0 must directly contact the yeast cell in order to form H_2S [71].
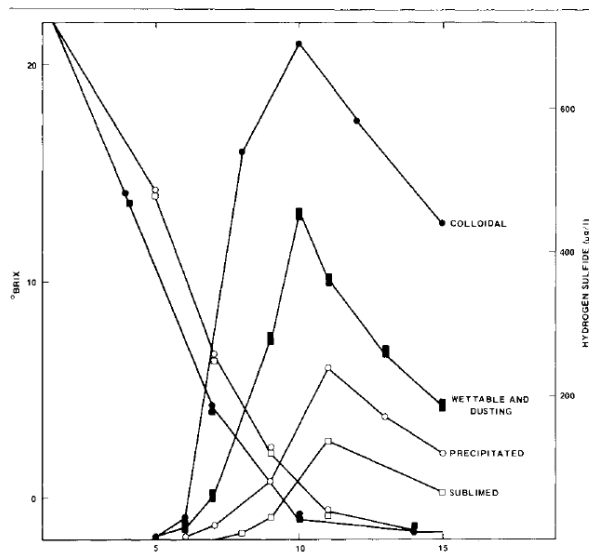
Figure 8 – The dependency of H₂S formation on the type of elemental sulfur in fermenting medium (reproduced from Schuetz and Kunkee, 1977)
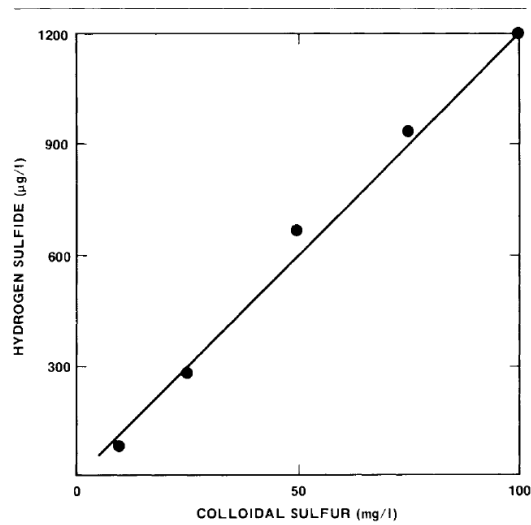
Figure 9 – The relationship of sulfur levels to the production of H₂S (reproduced from Schuetz and Kunkee, 1977)

In study by Acree *et al* in 1972, it was found that out of five fungicides tested only elemental sulfur led to a significant increase in H_2S production [69]. This result was consistent across all three yeast strains tested with residual S^0 in solution at 0.5-1.5 mg/L in a sulfate medium, which is a mock grape juice medium containing an addition 10 mg/L ammonium sulfate [69]. When the S^0-treated wines were subjected to sensory evalutation, they were rated as containing "sulfide-like" aromas at all levels (104-210 μg/L H_2S) [69].

Elemental sulfur concentrations decrease rapidly after application and after pre-fermentation settling of the pressed juice. In one study, S^0 concentration reached negligible levels in the juice (<2 μg/g berry weight) in 2-4 weeks post-application on the berry before harvest [47, 70]. A recent study with wine grapes demonstrated that S^0 residues in juice decrease rapidly after pressing, the process of removing the juice from the skins in white winemaking. Sulfur applied 12 days pre-harvest decreased from 13 μg/L in pressed juice to trace concentrations after 24 hours of settling [70] (Figure 10). H_2S production was significantly lower in musts that were macerated and

Figure 10 – S^0 residue persistence in relation to time after pressing (reproduced from Kwasniewski et al., 2014)
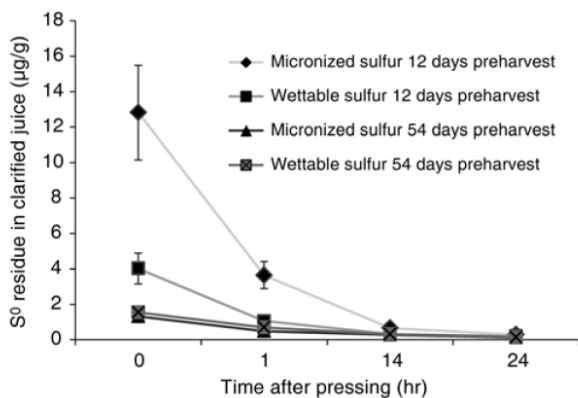
Table 4 – Processing techniques and their impact on S^0 levels and H_2S production during fermentation (reproduced without permission from Kwasniewski et al., 2014)

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

subjected to 24-hour skin contact prior to pressing as compared to those that were macerated for 1-2 weeks before pressing [70] (Table 4). White wines are typically settled prior to fermentation, where red wines are not [70]. Hard cider fermentation procedures vary in the practice of settling, maceration and/or washing, so the persistence of fungicide spray residues in the fermenting juice is possible and warrants further investigation.

High concentrations of S^0 present in the fermentation medium are not always consistently correlated with increased H_2S production. Thomas *et al* found that there was no significant correlation between H_2S production and amount of S^0 added to the fermentation [47]. In fact, it was found that the addition of low levels of S^0 actually decreased the amount of H_2S produced as

compared to the control juice [47]. It is unclear what lead to the difference observed in this study as compared to those in previous publications.

In practice, the observed S^0 content found in unclarified grape juice after pressing but before fermentation is variable. Kwasniewski found residual S^0 concentrations in unclarified juice as high as 100 mg/L when applied as colloidal sulfur 8 days prior to harvest [70]. As stated above, these residual concentrations decreased significantly as application time before harvest was increased [70]. Thomas *et al* found that over the course of the 1989 and 1990 experimental growing seasons that the average S^0 residues in the grape juice were 2-12 mg/L depending on the time of harvest in relation to the latest sulfur application [72]. It is therefore clear that the concentration of residual S^0 in the fermenting juice is dependent on a multitude of variables, such as time of harvest, rate of application, and post-harvest processing and winemaking practicies[70].

The effect of many other relevant fungicides on the formation of H_2S has not been studied in wine grapes or cider apples. One study showed that combined use of S^0 and Captan increased the amount of H_2S produced during fermentation [69]. There are no studies investigating the influence of Scholar® (fludioxonil) (Figure 12) or Indar® (fenbuconazole) (Figure 11) on the production of H_2S, with especially limited information as it pertains to hard cider fermentations. Scholar is used as a post-harvest storage fungicide in apples, and hence is applied outside of the orchard in conditions which are not common in vineyard management practices. Though fludioxonil is used in viticultural applications, the use of Scholar as a post-harvest fungicide may lead to increased risk of fludioxonil residues in cider fermentation, since fruit in post-harvest storage is not exposed to environmental conditions that would reduce residual fungicide concentrations after vineyard application such as wind and rain, among others). Indar fungicide is used much more frequently in orchard management as compared to vineyards, and hence

fenbuconazole is a relatively unique concern for cider fermentations. Hence, these two fungicides are of particular importance for investigation in relation to cider fermentation kinetics and H_2S formation.

Fenbuconazole can be used to prevent several plant diseases, including powdery mildew, leaf blotch, and numerous rot diseases. Fenbuconazole acts as a sterol inhibitor for the specific inhibition of demethylation. This prevents the synthesis of ergosterol in fungal plasma membranes[1]. Because of this, fenbuconazole may influence yeast of the strain *Saccharomyces cerevisiae* which is part of the fungi kingdom. Fludioxonil acts by interfering with signal transduction pathways of fungi[73]. Previous research has indicated that it inhibits spore germination in *Botrytis cenera*[74]. However, the impact of fludioxonil on wine yeast strains has not been studied.

One study found that fungicide residues, including residual concentrations of 1 and 5 mg L^{-1} fludioxonil, produce significant differences in the volatile acidity of wine, but also noted that final concentrations of these aroma and flavor compounds do not exceed typical sensory perception thresholds [75]. These studies did not monitor fermentation kinetics or H_2S production as a result of residual fludioxonil.

There are few reports of residual concentrations of late-season fungicides found in apple juice. One study found that residual concentrations of fludioxonil were as high as 1.25 mg/kg in red grape must when applied 7 days before harvest, and as high as 1.79 mg/kg when applied immediately prior to harvesting [76]. Though fludioxonil was investigated in a research vineyard, it is not currently used commercially in vineyards. No study to date has tested the late-season residue content of fenbuconazole in grape or apple juice. The USDA found that out of 379 juice samples, no residues of fludioxonil or fenbuconazole were present in commercial apple juice. This may be a result of pesticide application rate, pre-harvest intervals, as well as the processing methods used

on the juices, such as concentration and pasteurization as well as any filtration used before post-production. However, the FDA cites maximum residual limits on the following fungicides in apple juice: Indar® (fenzobuconazole) at 0.4 mg/L, and Scholar (fludioxonil) at 5.0 mg/L.
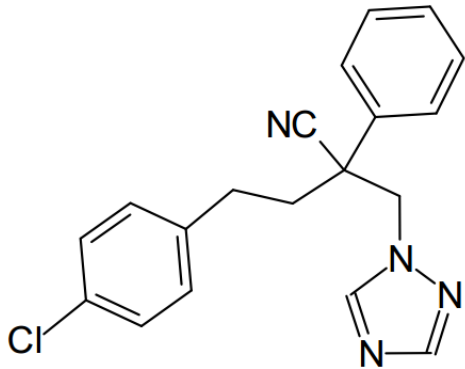
Figure 11 – Fenbuconazole, the active compound in Indar®. Kills fungi through DiMethylation Inhibitors (DMI) which prevents the biosynthesis of ergosterol, a major plasma membrane component) [1]
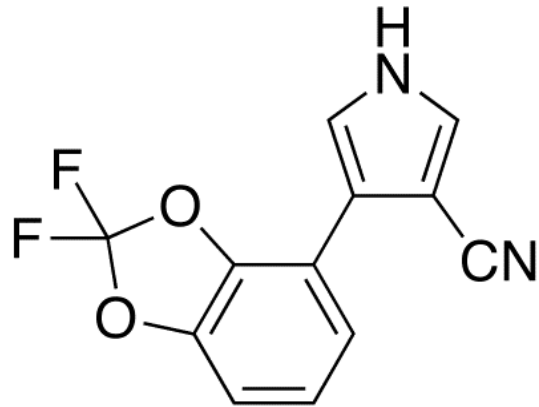
Figure 12 – Fludioxonil, the active compound in Scholar®. Kills fungi by disrupting several key transport mechanisms.

Chapter 3: Materials and Methods

Apple Samples

Traditional cider apple cultivars were grown at the Alson H. Smith, Jr. Agricultural Research and Extension Center prior to harvest. Fifeteen cultivars were collected in the 2014 harvest season, and 12 cultivars in the 2015 season. Apples were juiced and stored in 50 mL aliquots corresponding to field samples and analytical replicates. Samples were stored at -20°C and thawed to 22°C prior to analysis. Samples were centrifuged at 1096 xG for 5 minutes and the supernatant was sampled prior to analysis in order to remove sediment and to approximate the juice that would be used in cider making. YAN was determined using the following standard enzymatic and spectrophotometric methods: Primary Amino Nitrogen, (Megazyme PANOPA Enzymatic Kit, Megazyme International, Wicklow, Ireland); and Ammonium ion (Megazyme Ammonia (Rapid) Enzymatic Kit, Megazyme International, Wicklow, Ireland).

Apple Juice Composition

Commercially available, pre-packaged pasteurized apple juices without added sorbates or other preservatives were analyzed in order to determine an ideal juice for experimental fermentations. The primary goal was matching a YAN concentration similar to those found in previous research and preliminary research (below 100 mg/L YAN) while also retaining typical apple juice chemistry with Brix, pH, and TA not deviating from typically observed values. Using a commercially available juice was preferred to obtain a consistent product in sufficient quantity for all experimental treatments. The juice selected for this project was WhiteHouse Fresh Pressed 100% All Natural Apple Juice (National Fruit Product Co., Inc., Winchester, VA, USA). This juice

was selected because it contained no added ingredients or preservatives, which is indicative of what would typically be used for hard cider fermentations. The juice also had a yeast assimilable nitrogen concentration of 52 mg N/L, which is representative of reported apple cider YAN concentrations and is below the generally recommended YAN concentration of 140 mg N/L, which allowed us to use the control juice as a typical low nitrogen condition. Low initial juice YAN in the control juice was essential in order to allow targeted YAN supplementation in the experimental treatments.

Bottles of juice were combined in 5-gallon containers previously sanitized with a citric acid and sodium metabisulfate solution in order to homogenize the apple juice and ensure uniformity between fermentations. To mimic commercial cidermaking practices and inhibit the growth of undesirable microbes, the free sulfite concentration was raised to 50 ppm through addition of potassium metabisulfite. The potassium metabisulfite target concentration was selected in order to provide 0.8 mg L^{-1} molecular SO_2 at the juice pH of 3.7.

The following parameters were quantified in the control juice prior to application of experimental treatments: pH (probe, Thermo Scientific ROSS Ultra Triode Electrode Model 8107BNUMD, Thermo Fisher Scientific, Waltham, MA, USA); ^{o}Brix (Brix Refractometer Model RF10, Extech Instruments Corporation, Nashua, NH, USA); Residual Sugar (Megazyme Glucose/Fructose Enzymatic Kit, Megazyme International, Wicklow, Ireland); Titratable Acidity, standard method as reported by Amerine and Ough [77]; Total YAN, which includes; Primary Amino Nitrogen, (Megazyme PANOPA Enzymatic Kit, Megazyme International, Wicklow, Ireland); and Ammonium ion (Megazyme Ammonia (Rapid) Enzymatic Kit, Megazyme International, Wicklow, Ireland); and Free and Total SO_2, standard method as reported by Iland [78].

Yeast Strains

For this experiment, two commercially available yeast strains were used to evaluate differences in hydrogen sulfide production in response to the experimental treatments. This is because yeast strain is cited as the most prominent determining factor for the formation of H_2S during alcoholic fermentation [43-46]. The use of two yeast strains allows determination of the yeast strain effect as well as the interaction effect of yeast strain with the experimental treatments. Strain EC1118 (Prise de Mousse, *Saccharomyces bayanus*) (Lallemand, Montreal, Quebec, Canada) was chosen due to its resilience, robustness, wide temperature fermentation range, low nitrogen requirement, and its inhibition of malolactic fermentation due to its relatively high production of SO_2, as well as its common use in similar areas of research [72, 79]. The strain UCD522 (Montrachet, *Saccharomyces cerevisiae*) (Lallemand, Montreal, Quebec, Canada) was chosen due to its tendency to produce relatively high concentrations of H_2S [47, 79]. Juice was inoculated by first rehydrating the active dry yeast in 40° C water for 20 minutes. Yeast was then added at the prescribed dose recommended by the manufacturer, approximately 250-300 mg/L.

Detection of Hydrogen Sulfide

Detection of H_2S was conducted using the lead acetate method as first described by Rankine [5]. The reaction produces a darkening of the silica upon contact with H_2S in a glass tube packed with lead acetate in an inert medium according to the following reaction:

$$H_2S + Pb(CH_3CO_2)_2 \rightarrow PbS + 2CH_3CO_2H$$
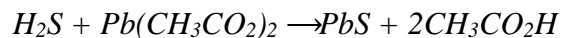
This method is accurate for measuring H_2S up to 150 µg per 100 cm^3 purged gas. Above this value, quantification is inaccurate. For quantification of higher amounts of H_2S during

fermentation, the reaction with silver nitrate produces a similar darkening of the silica upon contact. This is shown by the reaction below:

$$H_2S + 2AgNO_3 \longrightarrow Ag_2S + 2HNO_3$$
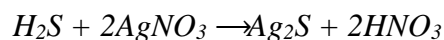
Observation of the degree of darkening through the formation of a band in the graduated glass tube allows quantification of the amount of H_2S produced. As the observation is the total amount of H_2S produced in the fermentation, the measurement can be made at any time during the process to provide a quantitative measure of the total amount of H_2S produced up to that point in the fermentation.

Lead acetate and silver nitrate tubes pre-prepared for the detection of H_2S were purchased ready-to-use from Komyo Kitagawa (Tokyo, Japan). The detection tubes are calibrated according to the following concentrations: Hydrogen Sulphide models 120SD (1-30 ppm), 120SB (3-150 ppm) and 120SF (50-1,000 ppm) (Kitagawa America, Pompton Lakes, NJ, USA). This method was found by others to have a very strong correlation of H_2S measured and actual amount produced (R^2 = 0.9971) and a 90-98% recovery [80]. It was also found that this detection method is not interrupted in the presence of other sulfur-containing compounds such as SO_2, dimethyl sulfide, or methyl mercaptan [80]. This method is accurate at concentrations typically found in laboratory-scale fermentations with an average H_2S production volume of 70 µg per 200 mL fermentation [80]. This has been demonstrated by others to be an effective method for continuous H_2S production during alcoholic fermentation [80].

The tubes are calibrated to detect concentration of hydrogen sulfide in parts per million per a 100 cubic centimeter sample volume (i.e. 100 cubic centimeters of gas passing through the tube). These values were converted to micrograms H_2S produced total mass by calculating the volume of CO_2 purged from the fermenter based on weight and adjusting to the calibration scale on the

tube. The final values for H_2S were given as total micrograms H_2S produced during each time period recorded during the fermentation.

In order to monitor H_2S production during fermentation, the lead acetate or silver nitrate tube was directly affixed to the rubber stopper used to cap the flask. It acts as an airlock while simultaneously measuring the H_2S content as it is purged by normal CO_2 evolution during fermentation.

Fermentations

Fermentations were carried out in 250 mL Erlenmeyer flasks containing a single magnetic stir bar fitted with a rubber single-hole stopper fitted with the H_2S detector tube. Flasks containing the stir bar were autoclaved prior to filling with juice. Stoppers and detector tubes were washed with a citric acid/potassium metabisulfite sanitizer solution. Flasks were filled with 200 mL juice for each fermentation run, experimental additions of fungicides and/or sources of exogenous nitrogen were made, and inoculated with the experimental yeast strain to initiate fermentation.

Fermentation rate was monitored by measuring the mass of each fermenter twice daily. During fermentation, carbon dioxide is produced through normal yeast metabolism and is purged out of the fermenter into the atmosphere, which translates to loss of mass from the fermentation flask system. Mass lost due to evolution of CO_2 is directly correlated with the fermentation rate of the fermenting medium [13, 18, 58-60]. Therefore, determination of relative rate of yeast metabolism, sugar consumption and fermentation rate can be approximated by regularly weighing the fermenter during fermentation.

In the present study, mass of the fermenter was recorded twice daily for a period of 15 days or approximately 340 total fermentation hours. Fermentations were stirred at 800 rotations per minute for 5 minutes at each monitoring time point. This prevented yeast settling to the bottom of the relatively small volume fermentation. Keeping the yeast in suspension was necessary to prevent incomplete or "stuck" fermentations. Stirring also ensures that any added insoluble treatments (such as elemental sulfur) were homogenized to prevent precipitation, hence inhibiting their effects. Upon completion of stirring, each fermentation flask was weighed to a precision of 10 ± 5 µg (Adventurer Pro AV412, Ohaus Corporation, Parsippany, NJ, USA). The H_2S production running total was recorded according to discoloration of the lead acetate or silver nitrate silica. The time of the weight and hydrogen sulfide measurement was recorded.

Fermentations were carried out at 18° C in a walk-in laboratory cooler. Temperature was monitored using a data logger (RHT20 Relative Humidity and Temperature Data Logger, Extech Instruments Corporation, Nashua, NH, USA). Fermentations were assumed to have fermented to completion when CO_2 evolution was below 0.2 mg/day, and residual sugar determination was performed as part of the fermentation kinetics assessment to confirm whether fermentations were in fact complete when CO_2 evolution had slowed to less than 0.2 mg/day.

Upon completion of the fermentation, 15 milliliter samples were taken from each fermentation for chemical analysis and stored at -20° C for further analysis. After sampling, the remaining volume of the triplicate fermentation runs for each treatment were pooled to obtain a sufficient volume for further sensory evaluation, and then stored in 500 mL screw cap Pyrex bottles. The headspace was purged with nitrogen gas for 30 seconds to limit oxidation during storage and mimic traditional cider bottling techniques. The bottles were then stored at 4° C for 78 days to approximate typical cider storage prior to sensory analysis.

Figure 1 outlines the overall experimental design and treatment plan. Detailed descriptions follow the diagram.
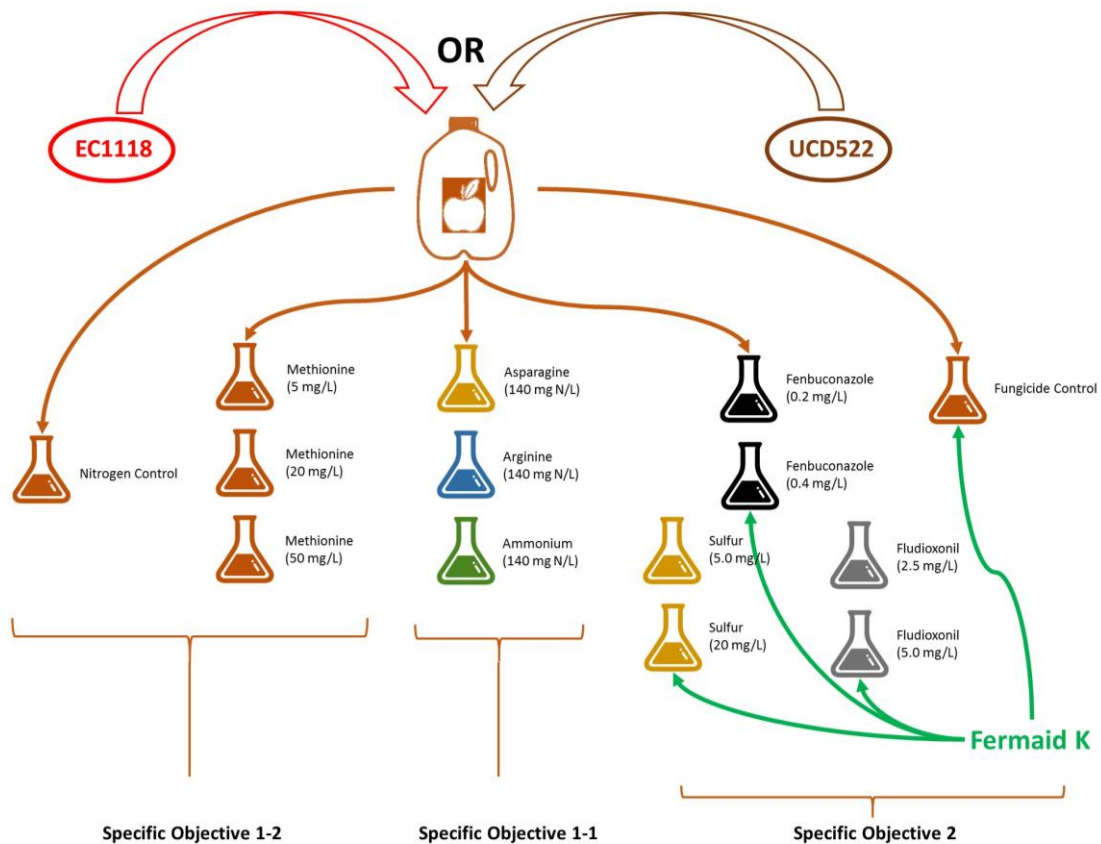
Figure 1 –Diagram of experimental design and treatment plan for specific objectives 1 and 2. Experimental fermentations were conducted in both yeast strains EC1118 and UCD522. Fermaid K yeast nutrient was added to all fungicide treatments and the fungicide control.

The first experiment of specific objective one involved addition of asparagine (L-Asparagine Monohydrate, Sigma-Aldrich, St. louis, MO, USA), arginine (L-Arginine, Sigma-Aldrich, St. louis, MO, USA), and ammonium in the form of DAP (Diammonium phosphate, Scott

Laboratories, Inc., Petaluma, CA, USA). These treatments were added to apple juice at concentrations of 140 mg/L nitrogen. Each treatment was run in triplicate, and two yeast strains representing tendencies for low H_2S production and high H_2S production (EC1118 and UCD522, respectively) were utilized in this experiment. Treatments were compared to determine if there was a significant difference in total H_2S production among different nitrogen sources at the same total YAN concentration. A total of 18 samples were fermented for this experiment.

The second experiment of specific objective 1 is the addition of methionine (L-Methionine, Thermo Fisher Scientific, Waltham, MA, USA) to apple juice at concentrations of 5, 20 and 50 mg/L. These intervals reflect concentrations of methionine which are low, medium and high concentrations which have been observed in grape juices. These concentrations have been shown to cause different levels of H_2S production [64]. The three methionine treatment levels were compared to a control apple juice fermentation with no added YAN. Each sample was run in triplicate with each yeast strain EC1118 and UCD522. A total of 18 samples were fermented in this experiment, compared to 6 control fermentations.

The first experiment of specific objective 2 is the addition of elemental sulfur (Wettable Sulfur Fungicide, Microthiol Disperss, Nufarm Americas Inc., Alsip, IL, USA) at concentrations of 5 and 20 mg/L. These levels were selected based on prior work which found residual S^0 on fruit after vineyard applications, 5 mg/L in one study [72] and 20 mg/L in another [71]. Each fermentation also contained 12.5 mg/L of Fermaid K yeast nutrient (Scott Laboratories Inc., Petaluma, CA, USA) which contained 25 mg/L N and other yeast micronutrients in order to alleviate the impact of insufficient YAN on the production of H_2S. Each sample was run in triplicate with each yeast strain EC1118 and UCD522. A total of 12 samples were fermented in this experiment.

The second component of specific objective 2 is the addition of the fungicide Indar (Fenbuconazole 23.5%) (Indar 2F Fungicide, Dow AgroSciences LLC Indianapolis, IN, USA) at concentrations of 0.2 and 0.4 mg/L. These concentrations reflect the intermediate and maximum acceptable residue levels of fenbuconazole in apples by USDA standards[81]. Each fermentation also contained 50 mg of Fermaid K yeast nutrient which contained 25 mg/L N and other yeast nutrients to reduce the impact of insufficient YAN on the production of H_2S. Each sample was run in triplicate with each yeast strain EC1118 and UCD522. A total of 12 samples were fermented in this component of specific objective 2.

The third component of specific objective 2 is the addition of the fungicide Scholar (Fludioxonil 20.4%) (Scholar Fungicide, Syngenta Crop Protection LLC Greensboro, NC, USA) at concentrations of 2.5 and 5.0 mg/L. These concentrations reflect the intermediate and maximum acceptable reside concentrations of fludioxonil in apples by USDA standards[81]. Each fermentation also contained 50 mg of Fermaid K yeast nutrient which contained 25 mg/L N and other yeast nutrients to reduce the impact of insufficient YAN on the production of H_2S. Each sample was run in triplicate with each yeast strains EC1118 and UCD522. A total of 12 samples were in this component of specific objective 2.

Each of the fungicide treatments conducted under specific objective two were compared to a control fermentation which contained no fungicide residue in order to determine if there was a significant difference in H_2S production when fungicides were present in the juice. The control fermentations were inoculated with each yeast strains EC1118 and UCD522. Each fermentation also contained 50 mg of Fermaid K yeast nutrient which contained 25 mg/L N and other yeast nutrients to reduce the impact of insufficient YAN on the production of H_2S. A total of 6 samples were fermented in the control component of specific objective 2.

A total of 84 fermentations were conducted. 69 were conducted from 23 July 2015 to 5 August 2015, and the final 15 completed from 13 August 2015 to 26 August 2015.

Interaction of YAN and Methionine and Fenbuconazole

Figure 2 below outlines the overall experimental design and treatment plan. Detailed descriptions follow the diagram.
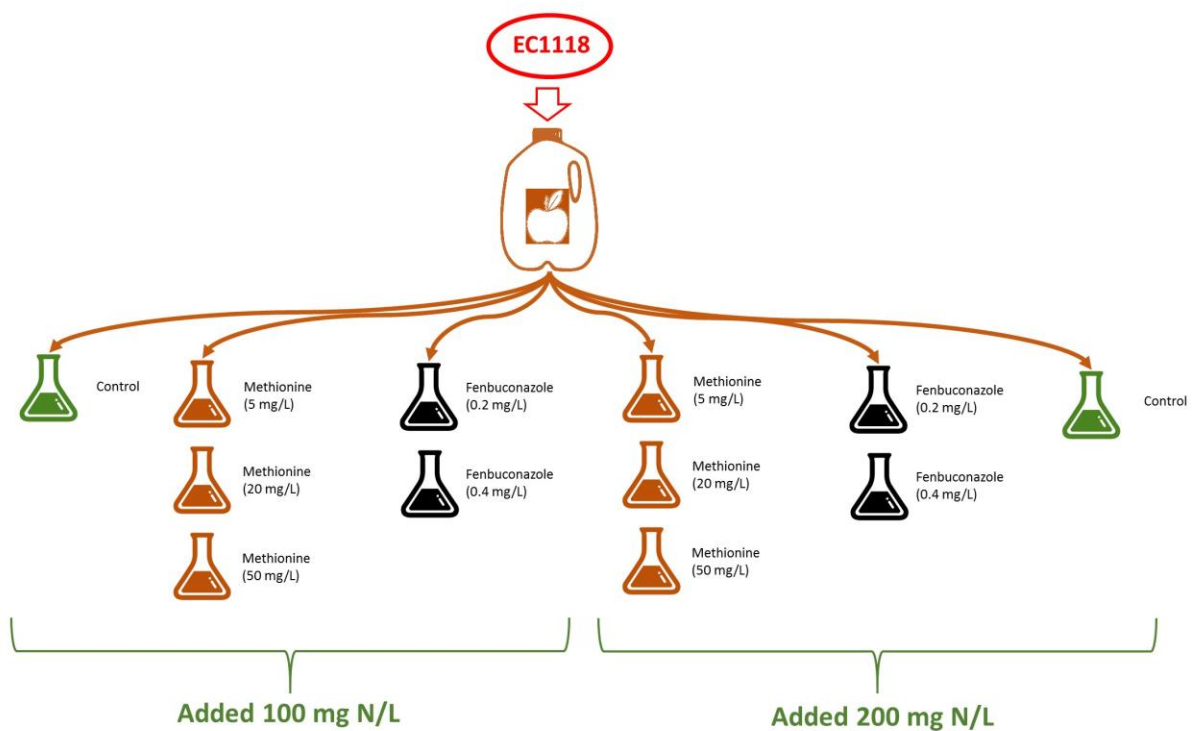

Figure 2 – Diagram of experimental design and treatment plan for specific objective 3. Experimental fermentations were conducted with yeast strain EC1118 only. Nitrogen was added in the form of DAP. Total YAN concentrations were 153 and 253 mg/L, respectively. Results of this experiment were compared to results in objective 2 with no added YAN, representing the "low" YAN treatment.

Specific objective 3 was to determine the interactive effect of total juice YAN and additions of specific YAN components or fungicide residues. Methionine and fenbuconazole were chosen to be added to YAN-supplemented juice based on their effect on fermentation kinetics and H_2S production observed in specific objective 2.

Additional fermentations were conducted to investigate the interaction effect of total juice YAN and methionine and fenbuconazole. Juice samples were supplemented with 100 and 200 mg N/L in the form of DAP to raise total juice YAN to 153 and 253 mg N/L, respectively, Within each YAN concentration, fermentations were individually supplemented with 5, 20 and 50 mg/L of methionine and 0.2 and 0.4 mg/L of fenbuconazole. Fermentations with no added methionine or fenbuconazole were used as a control. For this experiment fermentations were conducted in triplicate with the yeast strain EC1118. A total of 32 fermentations were conducted under specific objective 3.

Post-Fermentation Analyses

Once fermentations were completed, a 15 mL aliquot was collected from each fermentation flask and stored at -20º C until the time of analysis. For fermentations being retained for sensory analysis, the remaining volume of the triplicate fermentation runs for each treatment were pooled to obtain a sufficient volume for further sensory evaluation, and then stored in 500 milliliter screw cap bottles. The headspace was purged with nitrogen gas for 30 seconds to limit oxidation during storage and mimic traditional cider bottling techniques. Blended samples were stored at 4º C to stop fermentation. A 25 milliliter sample was taken from each blended treatment for further analysis. Analytical samples were stored frozen at -20º C to stop fermentation and preserve analytes.

Prior to analysis, samples were thawed at 22°C. The following analyses were performed: residual sugar (Megazyme Glucose/Fructose Enzymatic Kit, Megazyme International, Wicklow, Ireland); total YAN, which includes; free amino nitrogen, (Megazyme PANOPA Enzymatic Kit, Megazyme International, Wicklow, Ireland); and ammonium ion (Megazyme Ammonia (Rapid) Enzymatic Kit, Megazyme International, Wicklow, Ireland).

Pooled samples for sensory evaluation were thawed to approximately 22°C and analyzed for the following basic cider chemistry parameters to provide the representative cider chemistry of each experimental treatment subject to sensory evaluation: pH (probe, Thermo Scientific ROSS Ultra Triode Electrode Model 8107BNUMD, Thermo Fisher Scientific, Waltham, MA, USA);); Titratable Acidity, standard method [77].

Sensory Analyses

Sensory evaluation was conducted in order to determine whether the perceived aromas upon completion of fermentation and storage in bottle after 78 days were significantly different between control fermentations and fermentations containing added fungicides or YAN components. Four samples were chosen for sensory evaluation based on H_2S production during fermentation. These included treatments of methionine at 50 mg/L compared to the control in both yeast strains, as well as treatments of fenbuconazole at 0.4 mg/L compared to the control in both yeast strains. These were chosen due to detected decreases in H_2S formation in methionine treatments and increases in H_2S formation in fenbuconazole treatments. Prior to sensory evaluation, IRB approval was obtained to ensure consumer safety (see supplemental documents).

The triplicate fermented samples were blended for each fermentation to achieve uniformity and sufficient volume for sensory analysis. A triangle test was conducted to test for significant differences between control samples and experimental samples. In this experiment type as described by Lawless and Heymann [82], a panelist is presented with three coded samples in covered tasting glasses at 22°C in 5 mL aliquots under daylight-type lighting. Panelists compared the experimental samples to the control sample. For each triangle test, two of the samples presented to the panelist were the same, and the third was different; these samples were presented in randomized order and were different between panelists (i.e., the first panelist may receive control-control-sample, the next sample-control-sample, the third sample-control-control, etc.). The panelist was instructed to choose which sample is different from the other two based on the aroma of the finished ciders. The results of the triangle tests were analyzed to determine if untrained panelists are able to distinguish differences in aromas across the treatments imposed in these experiments. A total of 40 panelists conducted the sensory evaluation. This number was chosen based on the assumed perceivable differences between samples and to achieve a β value of 0.80 [82].

Statistical Analyses

Statistical testing was performed in order to determine if the addition of different components and concentrations of YAN, varying concentrations or types of late-season fungicide residues significantly affects the production of H_2S. Analyses were carried out using the total quantities of H_2S produced in each fermentation component in each specific objective. Analysis of variance (ANOVA) will be used to determine whether significant differences exist between each sample set using GraphPad Prism v.6 (GraphPad Software, Inc., La Jolla, CA, USA). Data

for each experiment will be compared using one-way ANOVA at ($p<0.05$) with the following hypotheses: $H_0 = \mu_1 = \mu_2 \cdots = \mu_n$ vs. H_a: at least one mean differs, where μ is the mean level of H_2S production in each fermentation set and n represents each experimental unit (experimental unit = component added, concentration, and yeast strain). If significant differences were found, determination of which treatment means are significantly different from one another were made using Tukey's Honest Significant Difference (HSD) test at $p<0.05$.

For experiments investigating the interaction of total YAN concentration and supplementation with methionine or fenbuconazole, two-way ANOVA at a significance of $p<0.05$ was applied. This analysis compares both a row and a column factor (in this case, total YAN concentration and methionine or fenbuconazole concentration) in order to determine whether significant differences exist across treatments. If at least one treatment mean was significantly different, determination of which samples are significantly different from one another were conducted using Tukey's Highly Significant Difference (HSD) test at $p<0.05$.

For the sensory analyses, statistically significant differences were determined using SIMS sensory software (Sensory Computer Systems, Berkeley Heights, NJ, USA). Results of a triangle test were analyzed at a confidence level of $p<0.05$, $\beta = 0.80$ and perceived sensory difference of 0.30. Differences were determined based on the number of panelists who were successfully able to determine significant differences between samples.

Chapter 4: Survey of YAN in 12 Apple Cultivars

Abstract

Yeast assimilable nitrogen (YAN), is an essential yeast nutrient known to impact cider fermentation kinetics and the formation of both desirable and undesirable aroma compounds by yeast during fermentation. The YAN concentration and composition of 12 apple cultivars grown in Virginia over the course of two seasons was determined through enzymatic assay of the two components of YAN: free amino nitrogen (FAN) and ammonium. YAN concentration ranged from 9 to 249 mg N L^{-1}, with an average of 59 mg N L^{-1}. Of the YAN values observed in this survey, FAN was the predominant component, with an average of 51 mg N L^{-1} as compared to an average concentration of 8 mg N L^{-1} of ammonium. 94% of samples analyzed contained YAN concentration below 140 mg N L^{-1}, a concentration generally considered sufficient to complete fermentation. In the 2014 harvest, only one of the 12 cultivars analyzed and no cultivar in the 2015 harvest contained an average YAN concentration exceeding 140 mg N L^{-1}. There was a correlation between PAN and YAN for each sample, but no correlation between ammonium and YAN or ammonium and PAN. This indicates that unlike YAN in grape juice or must, YAN in apple juice could be predicted based on PAN measurement.

Key words: Primary amino nitrogen, ammonium, stuck fermentations, apple juice.

Introduction

Yeast assimilable nitrogen (YAN) is a yeast nutrient essential for completion of fermentation in wine and cider production. Aside from fermentable sugars, YAN is the most

important nutrient required by yeast during alcoholic fermentation. In winemaking conditions, deficiencies in YAN lead to sluggish or stuck fermentations [12, 13, 60]. This is a result of insufficient YAN concentration in fermenting juice leading to lower yeast biomass [83, 84]. Furthermore, low YAN concentration is known to contribute to increased hydrogen sulfide production, a volatile aroma compound widely associated with negative perception of wine and cider aroma and flavor [6, 48, 56]. Similarly, low YAN has been shown to lead to wines with lower sensory quality as compared to nitrogen-sufficient fermentations [63]. This is often attributed to the production of undesirable compounds, such as higher alcohols, as well as the lack of positive flavor compounds formed, such as esters and volatile fatty acids, under conditions of YAN starvation [4].

There is no universal consensus on the concentration of YAN required to successfully complete fermentation. Though the most commonly accepted minimum is 140 mg N L^{-1} [11], minimum YAN concentrations as high as 267 mg N L^{-1} [85] or as much as 350 mg N L^{-1} in juices containing Brix concentrations higher than 20° [59] have been recently suggested. YAN requirements for proper yeast metabolism during fermentations are dependent on many distinct factors, including yeast strain [14] and nitrogen source [86].

One YAN component, ammonium, is preferentially assimilated by yeasts [30] and can be the sole YAN source used to complete fermentation [36]. However, free amino nitrogen (FAN) is also an essential part of healthy fermentations and can lead to higher fermentation rates when used in combination with ammonium [9]. Similarly, fermentations with high FAN concentrations lead to desirable flavor production in wines as compared to ammonium alone [86].

Although extensive research has been conducted on the YAN concentration and composition of grape juice and wines, there is limited data concerning the YAN concentration and composition of apple juice. One study found that the average FAN concentration of 51 apple

cultivars was 38.3 mg N L^{-1}, but YAN was not quantified [7]. The objective of this study was to determine the concentration and composition of YAN in 12 apple cultivars grown in Virginia during the the 2014 and 2015 growing seasons.

Materials and Methods

Juice Samples. Apple juice samples were collected from the Alson H. Smith, Jr. Agricultural Research and Extension Center experimental orchard in Winchester, VA. Fruit samples from each of the twelve apple cultivars were harvested from one to three trees and then separated into three lots for downstream analyses. Fruit from each lot was juiced, placed into 15 mL aliquots, and frozen at -20° C until analyzed. Fruit samples were collected from the same trees in both the 2014 and 2015 harvests.

Analytical Methods. YAN was quantified using enzymatic assays for free amino nitrogen (K-PANOPA kit, Megazyme, Wicklow, Ireland) and ammonium ion (Ammonia-Rapid kit, Megazyme, Wicklow, Ireland). Samples were thawed to 22°C and centrifuged at 1096 xG for 5 min prior to analysis.

Statistical Analysis. Descriptive statistical analyses were conducted using GraphPad Prism v.6 (La Jolla, CA, USA). One-way analysis of variance (ANOVA) was conducted to test for differences in total YAN between cultivars within a growing seasons. Two-way analysis of variance was conducted to test for differences in total YAN among cultivar and growing season. ANOVAs were conducted using significance of $p<0.05$ followed by parametric mean testing using Tukey's Honest Significant Difference (HSD) using GraphPad Prim v.6 (La Jolla, CA, USA).

Significant correlation coefficients were calculated by conducting a regression analysis at significance of $p < 0.05$.

Results

Over both the 2014 and 2015 seasons, YAN ranged from 9 to 249 mg N L^{-1} across all cultivars analyzed, with an average and standard error of 59 ± 3 mg N L^{-1} (Table 1). FAN ranged from 6 to 249 mg N L^{-1} with an average of 51 ± 3 mg N L^{-1} and ammonium ranged from 0 to 79 mg L^{-1} with an average of 8 ± 1 mg N L^{-1} (Table 3). Of the 12 cultivars analyzed over two seasons, 94% contained YAN concentrations below 140 mg N L^{-1}, the generally recognized minimum YAN concentration required to complete fermentation in grapes. In fact, 81% of samples contained YAN concentrations below 100 mg N L^{-1}, indicating severe nitrogen deficiency.

There were many differences between YAN concentrations by cultivar (Table 2). In the 2014 harvest, Golden Delicious had the lowest YAN concentration (25 ± 2 mg N L^{-1}) and Enterprise had the highest YAN concentration (172 ± 12 mg N L^{-1}) (Table 1). In the 2015 harvest, Blacktwig had the lowest YAN concentration (19 ± 2 mg N L^{-1}) and Enterprise again had the highest YAN concentration (119 ± 13 mg N L^{-1}) (Table 1). In the 2014 harvest, 11 out of 12 cultivars analyzed had an average YAN concentration less than 140 mg N L^{-1} with the exception of Enterprise. In the 2015 season, no cultivar had a YAN concentration which exceeded 140 mg N L^{-1}. With the exception of Enterprise in both seasons, all cultivars had an average YAN concentration below 100 mg N L^{-1}.

There was an interactive effect ($p < 0.0001$) between cultivar and harvest season. Of the 12 cultivars analyzed, five had differing concentrations of YAN from the 2014 to 2015 harvest. Golden Delicious and Northern Spy increased from 2014 to 2015, whereas Enterprise, Granny

Smith and Empire decreased. There was a correlation between YAN concentration and harvest season (p=0.049) despite the interactive effect observed between cultivars (Figure 2).

In all samples and in both seasons, FAN comprised the largest proportion of YAN found in the juice. Ammonium only accounted for 15% of the total juice YAN on average. There was a correlation between FAN concentration and total YAN in both harvest seasons (Figure 3A, 3B). However, there was no correlation between ammonium concentration and total YAN (Figure 3A, 3B) or ammonium and FAN concentration (Figure 4) in either harvest season.

Discussion

There are very few studies on the YAN composition and concentration of apples used for cider production. In one study of three apple cultivars, the FAN component measured averaged 38.3 mg N L^{-1}, but YAN was not quantified [7]. The same study quantified total nitrogen by the Kjeldahl method which was found to be 155.8 mg N L^{-1} [7], but this total includes proline as a nitrogen source which is not able to be assimilated by yeast under typical fermentation conditions [4]. The present study provides new information on the YAN concentration and composition of several apple cultivars used for cider making in the United States.

The majority of the cultivars examined in this study contained less than 140 mg N L^{-1} and can be considered nitrogen-deficient for fermentation. Concentrations of YAN below 140 mg N L^{-1} contribute to sluggish or stuck fermentations in wine [12, 13, 60]. The minimum YAN required to complete cider fermentations has not been established, but previous research on wine has been used as a baseline for cider fermentations. Based on model media fermentations which may better reflect cider nitrogen requirements, a minimum of 267 mg N L^{-1} is needed to complete

fermentation [85], well above the average YAN concentrations found in apple cultivars in this study. Adverse fermentation conditions such as low fermentation rate due to limited YAN may be exacerbated by the limited ammonium concentrations found in this study. Research on yeast metabolism has indicated that ammonium is consumed preferentially by yeast during fermentation [30, 36], and that ammonium consumption inhibits the assimilation of FAN component when both are present in the fermenting medium [30]. Therefore, the very low concentrations of ammonium present in apple juice as compared to grape juice may further influence observed differences in yeast metabolism and growth in low YAN conditions in apple as compared to grape.

In the two harvest seasons observed in this study, year-to-year differences in YAN concentration were observed within five out of the twelve cultivars analyzed. There was no trend within a cultivar of an increase or decrease in YAN from one harvest to the other (Table 1). Similarly, there was only a slight correlation ($R^2 = 0.3323$) between harvest season in the YAN concentration of each cultivar. In fact, YAN concentration was shown to increase by as much as 280% or decrease by 70% within cultivar from the 2014 to 2015 harvests (Table 1). This indicates that orchard managers and cider makers should be aware of the irregularity of YAN concentration from season to season, regardless of cultivar or location. The fact that YAN concentrations which have been sufficient in past harvests may decrease significantly in subsequent harvests emphasizes the need for cidermakers to quantify YAN seasonally to enable targeted correction of YAN deficiencies prior to fermentation.

In both harvest seasons, there was a strong correlation between FAN concentration and YAN concentration (Figure 3). This observed effect was due to the consistently low concentrations of ammonium regardless of FAN concentrations observed in this study, making FAN by far the largest portion of the total YAN. Orchard managers and cider makers may be able to accurately

estimate total YAN by measuring FAN alone, lowering costs and time associated with laboratory analyses. Further research is necessary to determine if this finding can or should be extrapolated beyond the set of cultivars or the growing region evaluated in the present work.

There was no correlation between ammonium and FAN concentration in either harvest season. This is similar to previous research on surveys of YAN concentration and composition in grape juice [37].

Conclusion

Analysis of 12 apple cultivars grown in Virginia over the 2014 and 2105 harvest season revealed that the vast majority of juices were deficient in YAN. Of the 12 cultivars analyzed, only one had a YAN concentration above 140 mg L^{-1} on average. There was a large variation in YAN concentration across cultivars. Furthermore, YAN concentration varied significantly from harvest to harvest, indicating that there is a significant risk of YAN deficiency regardless of season, location or cultivar. Cider makers in the mid-Atlantic should be aware of the prominence of YAN deficiencies within the region with regard to measuring and correcting for juice YAN prior to fermentation.

A correlation between FAN and total juice YAN was found in both seasons evaluated in this study. For cider makers using the cultivars evaluated in this study grown in the mid-Atlantic region of the United States, our findings indicate that total YAN can reasonably be predicted by FAN measurement.

Tables

Table 1 – Average YAN concentration of apple cultivars [a]			
	YAN 2014	**YAN 2015**	**Average YAN**
Albemarle Pippin	77 ± 4	85 ± 11	81 ± 6
Golden Delicious	25 ± 2	70 ± 4	48 ± 6
Enterprise	172 ± 12	119 ± 13	145 ± 11
Virginia Gold	37 ± 1	44 ± 3	40 ± 2
Northern Spy	37 ± 2	61 ± 5	49 ± 4
Field Red	31 ± 2	23 ± 3	27 ± 2
Arkansas Black	64 ± 4	46 ± 4	55 ± 3
Blacktwig	42 ± 3	19 ± 2	30 ± 3
Winesap	50 ± 4	42 ± 5	46 ± 3
Old York	65 ± 4	49 ± 8	56 ± 5
Granny Smith	97 ± 5	29 ± 5	63 ± 9
Empire	95 ± 7	34 ± 5	64 ± 8

[a] values expressed as average ± standard error

Table 2 – Multiple comparison of average YAN concentration of apple cultivars [a]

|	Albemarle Pippin	Golden Delicious	Enterprise	Virginia Gold	Northern Spy	Field Red	Arkansas Black	Blacktwig	Winesap	Old York	Granny Smith
Empire	ns	ns	****	*	ns	****	ns	****	ns	ns	ns
Granny Smith	ns	ns	****	*	ns	****	ns	****	ns	ns	
Old York	**	ns	****	ns	ns	***	ns	**	ns		
Winesap	****	ns	****	ns	ns	ns	ns	ns			
Blacktwig	****	ns	****	ns	ns	ns	**				
Arkansas Black	**	ns	****	ns	ns	**					
Field Red	****	ns	****	ns	*						
Northern Spy	***	ns	****	ns							
Virginia Gold	****	ns	****								
Enterprise	****	****									
Golden Delicious	****										

[a] Significant differences determined through one-way ANOVA followed by Tukey's HSD. Significance is as follows: ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

Table 3 – Average FAN and ammonia concentrations of apple cultivars in 2014 and 2015 harvests

	FAN (mg N L^{-1}) [a]		Ammonia (mg N L^{-1}) [a]	
	2014	**2015**	**2014**	**2015**
Albemarle Pippin	74 ± 3	64 ± 6	3 ± 1	22 ± 10
Golden Delicious	19 ± 1	65 ± 3	7 ± 1	5 ± 2
Enterprise	165 ± 13	114 ± 12	6 ± 2	5 ± 3
Virginia Gold	32 ± 2	41 ± 2	5 ± 2	3 ± 1
Northern Spy	33 ± 2	48 ± 5	4 ± 2	13 ± 3
Field Red	30 ± 2	15 ± 2	1 ± 1	8 ± 2
Arkansas Black	59 ± 3	38 ± 4	5 ± 2	8 ± 2
Blacktwig	35 ± 3	14 ± 2	7 ± 2	5 ± 1
Winesap	43 ± 4	30 ± 3	7 ± 1	12 ± 5
Old York	59 ± 3	39 ± 5	7 ± 1	11 ± 5
Granny Smith	87 ± 5	20 ± 3	9 ± 2	9 ± 3
Empire	91 ± 7	17 ± 3	4 ± 1	17 ± 3

[a] values expressed as average ± standard error
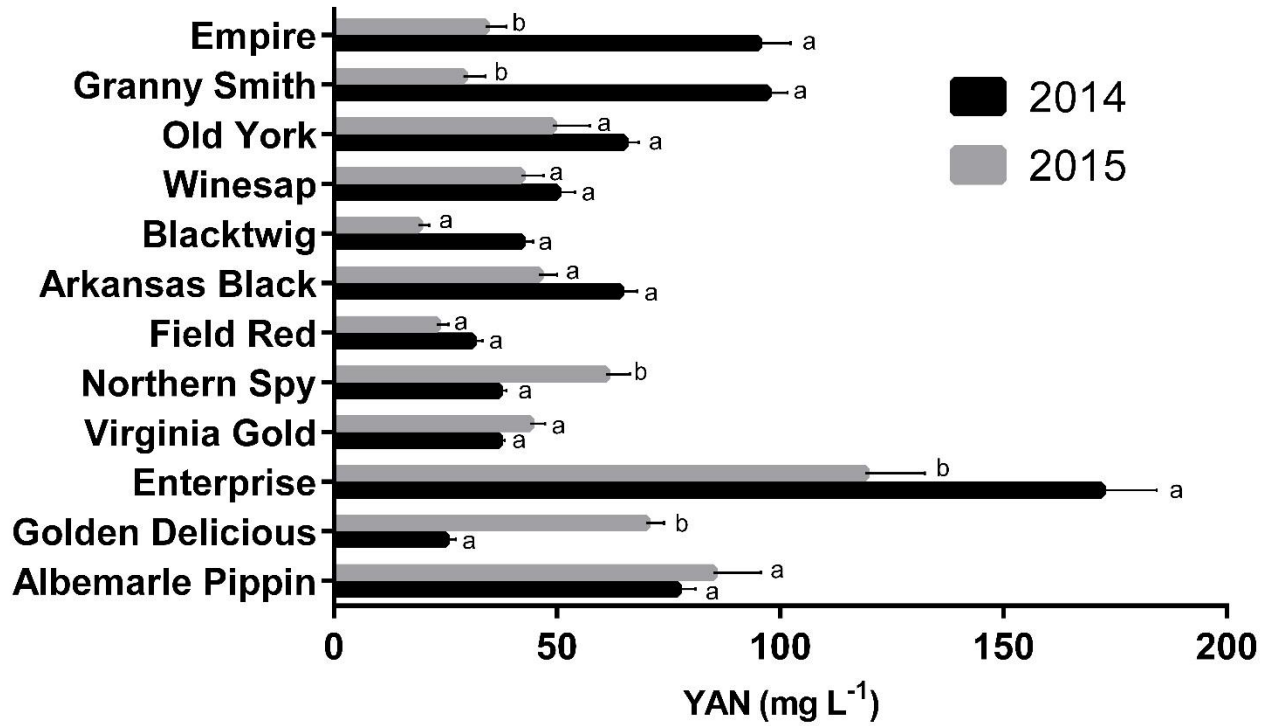
Figure 1. Concentration of YAN quantified in 12 Virginia cider apple cultivars in the 2014 and 2015 harvests. Bars labeled with different letters have significantly different YAN concentrations between harvests. Analyzed using two-way ANOVA followed by Tukey's HSD (P<0.05).
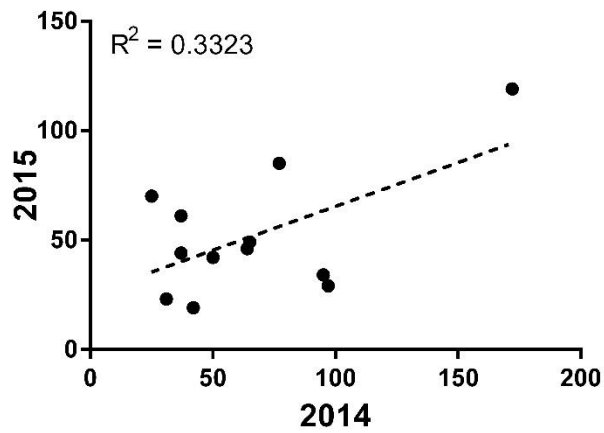
Figure 2. Linear regression and correlation coefficient for apple cultivars in 2014 and 2015 harvest seasons. Units are expressed as mg L^{-1} YAN. The R^2 value of 0.3323 is statistically significant (p=0.049).
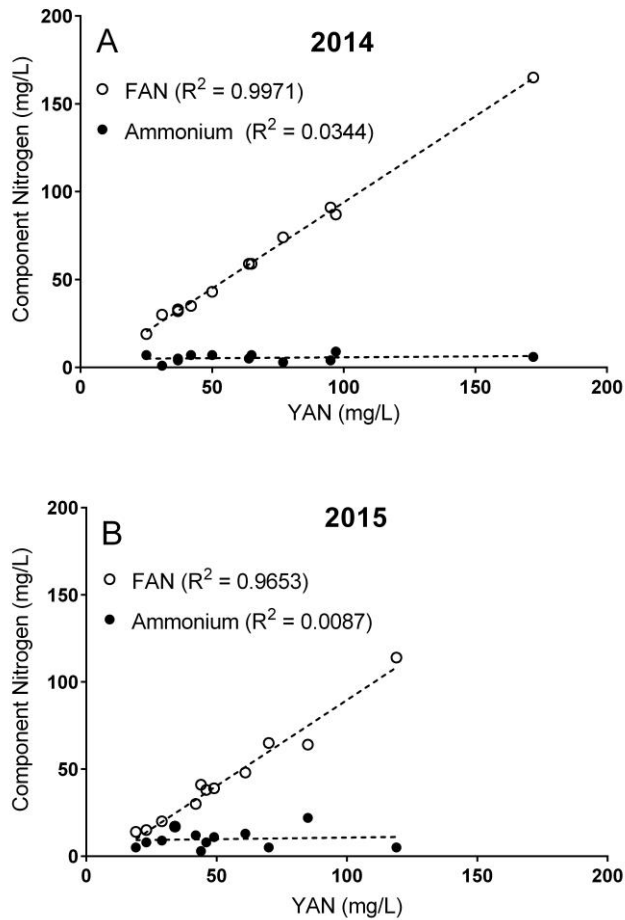
Figure 3. Linear regressions and correlation coefficients of YAN and FAN/ammonia in the 2014 and 2015 harvests. FAN and YAN were correlated in both years (p<0.0001, y=0.9805x-4.127 for 2014 and y=0.9853x-8.905 for 2015). FAN and ammonia were not correlated in either 2014 (p=0.5637) nor 2015 (p=0.7736).
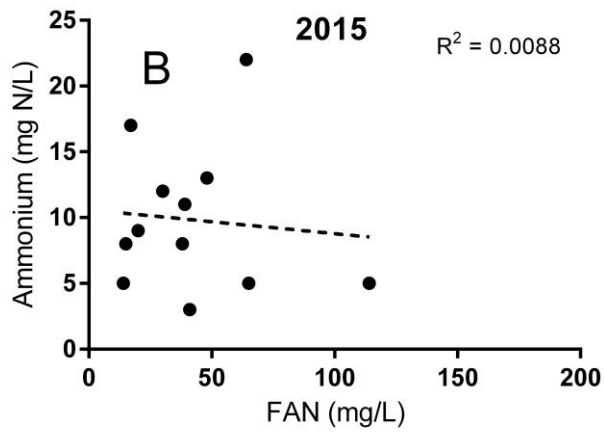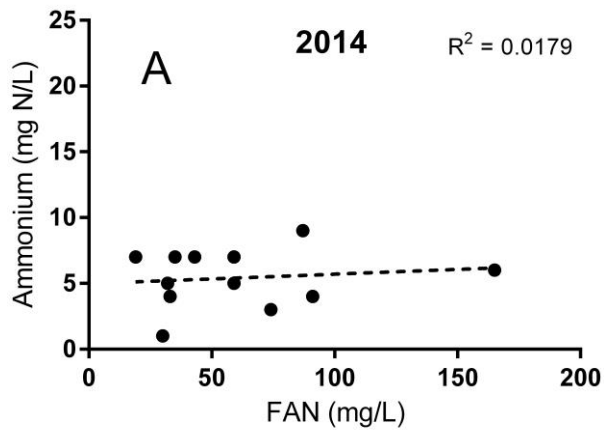
Figure 4. Linear regressions and correlation coefficients of FAN and ammonia in the 2014 and 2015 harvests. There was no correlation between FAN and ammonia in either 2014 (p=0.6789) nor 2015 (p=0.7724).

Chapter 5: Impact of YAN on H$_2$S Production and Fermentation Kinetics

Abstract

Yeast assimilable nitrogen (YAN) concentration and composition impacts hydrogen sulfide (H$_2$S) production and fermentation kinetics during wine fermentation but has not been extensively studied in cider fermentations. Due to differences in amino acid concentration and composition between apples and grapes, it is reasonable to expect differences in yeast metabolism between the two distinct systems. Nitrogen-deficient apple juice was supplemented with asparagine, arginine, methionine and ammonium and fermented with two yeast strains. There was no difference in H$_2$S production among fermentations with nitrogen added as asparagine, arginine, and ammonium. Additions of methionine at concentrations as low as 5 mg L^{-1} decreased H$_2$S production in juice in one yeast strain at 53 mg L^{-1} YAN. At 153 mg L^{-1} YAN, only methionine at concentrations of 50 mg L^{-1} decreased H$_2$S production, and no methionine treatment decrease H$_2$S production at 253 mg L^{-1} YAN. When juice was supplemented to 153 mg L^{-1} YAN there was an increase in H$_2$S production regardless of methionine treatment. H$_2$S production decreased when juice was supplemented to 253 mg L^{-1} YAN. Following sensory evaluation, panelists were able to discern differences in the aroma of samples supplemented with ammonium and methionine, and the differences were correlated with observed differences in H$_2$S production during fermentation.

Key words: Arginine, asparagine, ammonium, methionine, fermentations, apple cider, *Saccharomyces cerevisiae*

Introduction

In winemaking conditions, yeast assimilable nitrogen (YAN) deficiencies are widely known to contribute to increases in hydrogen sulfide (H_2S) production during alcoholic fermentation [6, 44, 53, 56]. However, the formation of H_2S can be influenced by many other biochemical and environmental factors. Yeast strain [45, 48], temperature and deficiencies in yeast nutrients such as biotin and pantothenic acid [52] also contribute to H_2S formation during wine fermentation. Although the knowledge gained through decades of wine research in this area is often applied to cider fermentations, differences between cider and wine fermentation have not been investigated.

YAN [primary amino nitrogen (PAN) + ammonium] concentration affects fermentation rate, where lower concentrations of YAN lead to slow or stuck fermentations [12, 18]. Ammonium is preferentially assimilated by yeasts during alcoholic fermentation [30]. Despite this, sources of nitrogen as PAN lead to increased yeast growth rates [31] as compared to sources of nitrogen as ammonium alone. Mixtures of ammonium and PAN have been shown to increase growth rates as well [9]. Similar results indicating the benefits of amino nitrogen on yeast growth rate have been confirmed in apple fermentations [7]. Therefore, the source of yeast assimilable nitrogen has been demonstrated to impact yeast growth rates and in turn fermentation kinetics in cider fermentation.

Similarly, a link between yeast assimilable nitrogen, yeast growth rate and total H_2S production has been demonstrated [43]. Addition of ammonium and most amino acids leads to a decrease in H_2S formation during fermentation [44, 56]. In apples, asparagine is often cited as the PAN component found in the highest or near-highest concentration [66, 68]. However, arginine is usually cited as the most prominent PAN component found in wine grapes [4]. Prior research has indicated that varying amino acid sources can differentially affect volatile aroma compounds[87] and

H_2S production during fermentation, and that asparagine may proportionally increase H_2S production as compared to arginine [44].

When methionine is present in juice during fermentation, there is a notable decrease in H_2S production [44, 64]. This is a result of methionine acting as an inhibitor in the sulfur reduction sequence (SRS) which would otherwise produce free sulfur ions during normal yeast metabolism, causing H_2S to be produced [8]. The suggested minimum concentration of methionine to inhibit H_2S production has not been established, but has been suggested to be at least 20 mg L^{-1} [64]. While concentrations of methionine exceeding 20 mg L^{-1} are normally found in grapes, methionine concentrations are typically far lower than 20 mg L^{-1} in apples [66, 68]. In general, grapes contain a higher concentration of total PAN than apples, although relatively little data is available on yeast assimilable nitrogen in apples [4, 57].

The objective of this study was to determine the impact of composition and concentration of yeast assimilable nitrogen on the production of H_2S and on fermentation kinetics during cider fermentation. One experiment in this study evaluated the impact of the amino acids asparagine, arginine and ammonium on H_2S production and fermentation kinetics. A second experiment investigated the effect of increasing concentrations of methionine on H_2S production and fermentation kinetics during cider fermentation.

Materials and Methods

Apple Juice. Commercially available pasteurized apple juice, WhiteHouse Fresh Pressed Natural Apple Juice (National Fruit Product Co., Winchester, VA, USA), was used to ensure consistency of juice across samples and to best represent large-scale cidermaking practices.. Multiple bottles of the juice were combined into one homogenous lot then stored in 1 L aliquots

at -20°C until use, and then thawed to 22°C prior to inoculation. The primary juice chemistry for the starting material was as follows: 12.9° Brix by refractometer, pH 3.7, titratable acidity (TA) 3.4 g L^{-1} malic acid equivalent using standard methods [88] and 53 mg L^{-1} YAN. YAN was quantified using commercially available Megazyme (Wicklow, Ireland) kits for PAN (K-PANOPA) and ammonium (Ammonia-Rapid).

Nitrogen Additions. Amino acids were obtained from Sigma-Aldrich (St. Louis, MO, USA). Diammonium phosphate (DAP) was obtained from Scott Laboratories, Inc. (Petaluma, CA, USA). L-arginine, L-asparagine and DAP were added at concentrations of 140 mg L^{-1} nitrogen to respective fermentation treatments. L-methionine was added at concentrations of 5, 20 and 50 mg L^{-1} to respective fermentation treatments. Juice with no nitrogen addition served as a control. Interactive effects of YAN and methionine were examined by supplementing YAN in the apple juice to 153 and 253 mg N L^{-1} using DAP added at concentrations of 100 and 200 mg L^{-1} nitrogen Each YAN treatment level was then further supplemented with 0 (control), 5, 20 and 50 mg L^{-1} L-methionine.

Fermentations. Experimental fermentations were carried out using two yeast strains. Prise de Mousse *Saccharomyces bayanus* EC1118 (Lallemand, Montreal, Canada) was selected to represent a low-H$_2$S producing strain and Montrachet *Saccharomyces cerevisiae* UCD522 (Lallemand, Montreal, Canada) was selected to represent a high-H$_2$S producing strain. Fermentations comparing the interactive effect of total YAN and methionine were only conducted with the strain EC1118. Juice was inoculated with 0.05 g of active dry yeast rehydrated in 35°C water for 20 min. Fermentations were carried out according to the method as described by Ugliano and Henschke for rapid determination of H$_2$S formation during alcoholic fermentation [80]. These were conducted in 250 mL Erlenmeyer flasks fitted with a one-hole rubber stopper to which a H$_2$S

detector tube was affixed (described below). Fermentations were not aerated, but were stirred twice per day at 800 rpm for 5 min to prevent yeast settling. Fermentations were carried out at 18°C in triplicate. Fermentation rate was monitored by measuring the mass of the fermentation vessel as a proxy for CO_2 evolution. Fermentations were deemed to be complete when CO_2 production did not exceed 0.2 g per 24-hour period. Upon completion, triplicate fermentations were combined and transferred to 500 mL screw-cap bottles. Headspace was purged with N_2 gas for 30 seconds prior to bottling to prevent oxidation. Bottles were cooled to 4°C to inhibit further fermentation. Finished cider was analyzed to determine pH, TA, residual YAN and residual sugar. Residual sugar was analyzed using the D-Fructose/D-Glucose (K-FRUGL) enzymatic kit (Megazyme, Wicklow, Ireland).

Hydrogen Sulfide Detector Tubes. The H_2S detection and quantification method was described by Ugliano and Henschke [80]. Detector tubes were obtained from Komyo Kitagawa (Tokyo, Japan). CO_2 produced during fermentation carried H_2S through the detector tube. H_2S reacts with the lead acetate (Tube 120SB, 120SD) or silver nitrate (Tube 120SF) contained in the tube, creating a discolored band. The different tubes have different capacities for total H_2S quantification, and were selected based on total H_2S production in a given treatment as determined through preliminary experimentation. H_2S reacts with the chemical contained in the tube (lead acetate or silver nitrate) which creates a discolored band. The length of the band is proportional to the amount of purged H_2S. If at any reading point the tube appeared to be near saturation, the tube was replaced with a new tube. This method may have allowed a small amount of H_2S gas to escape, but was employed consistently across treatments in this study.

Quantification of Amino Acids. Amino acids were quantified using chromatographic separation. Analysis was conducted on an ACQUITY UPLC (Waters Corporation, Milford, MA,

USA) using the AccQ•Tag Ultra Derivitization Kit method adapted for cell culture analysis to quantify free amino acids. Standards for L-Glutamine, γ-Aminobutyric acid (GAGB), and L-Asparagine (Sigma Aldrich, St. Louis, MO) were added to the standard mix to adapt the free amino acids method for apple and grape juice analysis (Waters Corporation, Milford, MA, USA) quantified via UV detection using a photodiode array detector. Norvaline (Sigma-Aldrich, St. Louis, MO) was used as an internal standard.

Determination of Fermentation Rate, Duration, and H₂S Production Rate. Maximum fermentation rate was determined by taking the slope of the fermentation curve during the exponential phase of yeast growth corresponding to the highest constant rate of CO_2 production, as reported by others [89]. Steeper slopes correspond to faster fermentation rate. Fermentations were determined to be complete when the CO_2 production rate decreased to less than 0.2 g day^{-1}. Fermentation duration is expressed in total hours starting from inoculation. Total H₂S production was determined by calculating the sum of H₂S production over the time course of fermentation. To determine the relative rate of H₂S production over the time course of fermentation, the fermentation duration was divided into four quartiles of equal time. The percent of H₂S produced in each quartile out of the total H₂S produced for a given fermentation was compared to determine if there was a significant difference in the H₂S production over the time course of fermentation across treatments.

Sensory Evaluation. Sensory analyses were conducted at the Virginia Tech Sensory Laboratory with approval from the Institutional Review Board (VT IRB #15-559). Cider samples were stored at 4° C for 78 days prior to evaluation. Samples were compared for sensory differences in cider aroma using a triangle test. Cider samples were served in 5 mL aliquots at 22° C in wine glasses covered with petri dishes to prevent the loss of volatiles. Each panelist performed 4

consecutive triangle tests examining only the aroma of each cider sample. A total of 40 untrained panelists conducted the sensory evaluation. No demographic information was collected for the panelists.

Statistical Analyses. Values were compared using a one-way analysis of variance (ANOVA) at a significance of $p<0.05$ followed by parametric mean testing using Tukey's Honest Significant Difference (HSD) using GraphPad Prism v.6 (La Jolla, CA). Analyses comparing the interaction between yeast strain/YAN concentration and fungicide residues were analyzed using a two-way ANOVA at a significance of $p<0.05$ and post-hoc testing by Tukey's HSD. Statistical analyses for sensory tests were conducted using SIMS Sensory Software (Sensory Computer Systems, Berkeley Heights, NJ, USA).

Results

Total Hydrogen Sulfide Production. There was no difference in H_2S production between fermentations with asparagine, arginine and ammonium added to nitrogen-deficient juice (Figure 1). Total H_2S production was higher in the yeast strain UCD522 as compared to EC1118. In juices fermented by strain EC1118, asparagine supplementation led to the lowest total volume of H_2S (8.9±3.3 μg) on average, in the strain UCD522 asparagine produced the highest total volume of H_2S of all samples, (80.0±4.8 μg) on average. Additions of methionine at all concentrations tested decreased H_2S production as compared to unsupplemented juice in the yeast strain EC1118 ($p<0.01$) (Figure 1). In fact, there was a nearly 40-fold decrease in H_2S production on average when methionine was added at concentrations of 20 mg L^{-1} in the strain EC1118. This effect was not observed in yeast strain UCD522, where no difference in H_2S production was observed between ferments with methionine added at any concentration and the unsupplemented juice.

Though there was no decrease in H_2S production in the strain UCD522, there was a linear correlation of decreasing H_2S production with increasing methionine in the strain UCD522 (Figure 2).

Although no difference in H_2S production was observed among additions of asparagine, arginine and ammonium, additions of these nitrogen sources significantly increased total H_2S production as compared to nitrogen-deficient juice in the strain UCD522 ($p<0.05$) (Figure 1). H_2S production in fermentations containing added sources of nitrogen were not significantly different from nitrogen-deficient juice in the strain EC1118, but H_2S production when asparagine (8.9 ± 3.3 µg), arginine (13.2 ± 8.9 µg) and ammonium (11.3 ± 4.5 µg) were added was higher on average as compared to nitrogen-deficient juice (3.9 ± 0.8 µg).

Hydrogen Sulfide Production Rate. There was no difference in amount of H_2S produced in a given quartile of the fermentation between different sources of added amino nitrogen for yeast strain EC1118 (Figure 3A). The majority of H_2S was formed in the second quartile, which also correlates with the highest rate of CO_2 production and the maximum fermentation rate. Minimal H_2S was produced during the first quartile for the experimental fermentations with yeast strain EC1118, which corresponds to the lag phase of yeast growth and limited CO_2 production. H_2S production was negligible in the fourth quartile, within which H_2S production did not exceed 9% of the total production for any fermentation. Similarly, there was no difference in H_2S production during a given quartile between any treatment of methionine and unsupplemented juice (Figures 3C and D). Additions of sources of nitrogen at high concentrations affected the rate of H_2S production as compared to nitrogen-deficient juice. When the juice was not supplemented with nitrogen, H_2S was produced steadily during the first three quartiles of the fermentation and a notable decrease in H_2S production was observed in the fourth quartile. All sources of nitrogen

produced less H_2S in the first quartile as compared to unsupplemented juice in both yeast strains, and almost all nitrogen sources produced less H_2S in the third quartile with the exception of asparagine in the strain EC1118 ($p<0.01$) (Figures 3A and 3B).

Fermentation Kinetics. In the strain EC1118, fermentations with added arginine had a lower maximum fermentation rate as compared to those with additions of ammonium ($p<0.05$), but maximum fermentation rate with added asparagine fell between the two and was not different than either ammonium or arginine (Table 2). There was no difference in fermentation rate between additions of nitrogen sources in the strain UCD522. However, arginine had the lowest fermentation rate when nitrogen sources were added in the strain UCD522. Methionine additions at 50 mg L^{-1} had a lower maximum fermentation rate compared to unsupplemented juice for strain EC1118 ($p<0.05$) (Table 3). Maximum fermentation rates did not differ at any concentration in the strain UCD522. In fact, maximum fermentation rate was highest on average when methionine was supplemented at concentrations of 50 mg L^{-1} in the strain UCD522 but was lowest when supplemented at 50 mg L^{-1} in the strain EC1118. Additions of 140 mg L^{-1} YAN increased the maximum fermentation rate as compared to nitrogen-deficient juice in both yeast strains ($p<0.001$) (Table 2, Figure 4). In fact, for both yeast strains, maximum fermentation rate nearly doubled on average for all sources of nitrogen added at 140 mg L^{-1}.

There was no difference in fermentation duration among different sources of added nitrogen in either yeast strain (Table 2). Additions of ammonium had the shortest fermentation duration on average for both yeast strains. Additions of methionine resulted in longer fermentation duration when added at concentrations of 50 mg L^{-1} in the strain EC1118 as compared to unsupplemented juice ($p<0.01$) (Table 3). For UCD522, addition of methionine at 20 mg L^{-1} resulted in the longest fermentation duration. These results correspond with the findings for

fermentation rate. Additions of nitrogen sources all resulted in shorter total fermentation duration in both yeast strains (p<0.0001) (Table 2). All nitrogen treatments in both yeast strains finished fermenting over 99 hours sooner on average than nitrogen-deficient juice. This corresponds to the higher fermentation rate observed with additions of nitrogen sources.

In treatments with different sources of added nitrogen, ammonium additions led to a greater mass of CO_2 lost, as compared to addition of arginine in the strain EC1118, but neither differed from CO_2 production by asparagine (Table 2). There was no difference in CO_2 production in the strain UCD522 across nitrogen addition treatments. In the strain EC1118, methionine additions at 20 mg L^{-1} increased CO_2 production (Table 3). Interestingly, in the strain EC1118 CO_2 production increased by 0.75 g on average when methionine was added at 5 and 20 mg L^{-1}, but only increased by 0.09 g on average when added at 50 mg L^{-1}. There was no difference observed in CO_2 production when methionine was added at any concentration in the strain UCD522. Additions of methionine decreased CO_2 production on average when added at all concentrations. CO_2 production increased when juice was supplemented with all nitrogen sources at 140 mg L^{-1} in the strain EC1118, but was not different in the strain UCD522 (Table 2).

There was no difference in RS between any sources of nitrogen in either yeast strain (Table 2). Similarly, there was no difference in RS between methionine additions and the control in either yeast strain (Table 3). However, additions of all sources of nitrogen at 140 mg L^{-1} decreased the amount of RS in both yeast strains (Table 2). In fact, compared to the control RS was 6% of the total on average in the strain EC1118 and was 2% of the total on average in the strain UCD522.

After fermentation was completed, there was a greater amount of residual DAP as compared to both arginine and asparagine in fermentations with yeast strain EC1118. There was

no difference in residual YAN sources in yeast strain UCD522, though DAP concentrations were nearly 5-times higher on average as compared to either asparagine or arginine.

Interactive Effect of Methionine and Total YAN. As previously stated, additions of methionine resulted in decreased H_2S production in the strain EC1118 at all concentrations of added methionine in nitrogen-deficient juice. When fermentations were supplemented to 153 mg L^{-1} YAN, only additions of methionine at 50 mg L^{-1} resulted in decreased H_2S production ($p<0.05$) (Figure 6). Additions of methionine did not decrease H_2S production at any concentration when fermentations were supplemented to 253 mg L^{-1} YAN. However, fermentations containing 253 mg L^{-1} produced less than 1 µg total H_2S across all treatments, and some treatments did not produce any detectable H_2S. When juice was supplemented to 153 mg L^{-1} YAN there was an increase in H_2S production across all treatments and in the control as compared to juice at 53 mg L^{-1} YAN ($p<0.05$) (Figure 6). Conversely, there was no difference in H_2S production between juice containing 53 mg L^{-1} YAN and juice containing 253 mg L^{-1} YAN in any methionine treatment. In fact, H_2S production was lower when supplemented to 253 mg L^{-1} YAN as compared to unsupplemented and 153 mg L^{-1} YAN in the control juice. Across all treatments, the highest volume of H_2S produced occurred when total juice nitrogen was 153 mg L^{-1}.

Additions of methionine did not affect fermentation rate at YAN concentrations of 253 mg L^{-1} (Table 4). In fermentations with YAN concentrations of 153 mg L^{-1}, methionine added at concentrations of 5 mg L^{-1} lead to lower fermentation rates, but had no effect when methionine was added to nitrogen-deficient juice (Table 4, Table 3). However, fermentation rate increased with increasing YAN, regardless of methionine treatment (Figure 7). Similarly, higher YAN concentrations decreased fermentation duration in both 153 mg L^{-1} and 253 mg L^{-1} as compared to juice not supplemented with YAN (Table 4, Table 3). Fermentation duration decreased by 117

hours or more on average across all treatments when supplemented from 53 mg L^{-1} to 153 mg L^{-1} nitrogen, and decreased by an additional 19 hours or more on average when further supplemented to 253 mg L^{-1} nitrogen by addition of DAP. There was no significant difference in fermentation duration between treatments with methionine. CO_2 production was not affected by treatments of methionine nor additions of YAN. Residual sugar decreased when juice YAN increased from 53 to 253 mg L^{-1}.

Sensory Evaluation. Sensory analyses were conducted to determine whether panelists could detect differences in cider aroma between control fermentations and fermentations containing added methionine at 20 mg L^{-1} and added DAP at 140 mg L^{-1} in products fermented with both strains EC1118 and UCD522. Aroma of ciders produced using DAP additions in both strains were different from the control ($p < 0.01$) and methionine additions in the strain EC1118 were different from the control ($p < 0.05$). No difference from the control was detected in the UCD522 fermentation with methionine added.

Discussion

The typical endogenous amino acid composition of apple juice is different from that of grape juice [4, 68]. The most prominent primary amino nitrogen (PAN) source in grapes is typically arginine [4], whereas asparagine is typically the most prominent PAN component in apples [66, 68, 90]. As previously stated, yeast are generally believed to assimilate ammonium preferentially to any other nitrogen source [30]. However, additions of ammonium do not decrease the amount of arginine assimilated during fermentation [17], and PAN sources may lead to higher fermentation rates as compared to ammonium alone [31]. Similarly, another study found that histidine, lysine and methionine are consumed preferentially to ammonium by percent consumed, and that asparagine,

aspartic acid and glutamic acid were consumed preferentially to ammonium by total volume consumed [31]. These inconsistent findings across studies demonstrate the complexity of the interaction of yeast metabolism and overall fermentation health. The results of this study indicate that, when treated with sources of YAN, there was higher residual ammonium remaining in cider fermented by strain EC1118, but not in cider fermented by strain UCD522 (Figure 5). For both yeast strains, the source of nitrogen which resulted in the highest residual concentration post-fermentation was ammonium. There was no difference in residual arginine or asparagine in either yeast strain. The higher residual ammonium may indicate that amino nitrogen is preferentially assimilated by yeast, or that the excess amino acids were taken up and stored by the yeast in intracellular pools, and removed with the yeast upon racking at the end of the fermentation. Despite this, fermentations treated with ammonium had the fastest fermentation rate with strain EC1118, and fermented to completion in the shortest amount of time in both strains. Fermentations with added DAP, asparagine and arginine produced similar quantities of H_2S in both yeast strains. Therefore, it does not appear that any one particular source of YAN contributes more to faster fermentations or decreased production of H_2S. This finding may be compounded by other factors which are known to influence fermentations kinetics, such as yeast nutrients such as biotin and pantothenic acid in the fermenting medium which were not investigated in this study [52]. Further research is required to determine the influence of YAN sources on the fermentation kinetics and H_2S formation during cider fermentation.

Fermentations conducted in nitrogen-deficient juice produced significantly less H_2S when containing additions of methionine in the strain EC1118. Additions of methionine at concentration of 5 mg L^{-1} decreased total H_2S produced, which is notably lower than 20 mg L^{-1} concentration previously prescribed as necessary to inhibit H_2S production in grape fermentations [64]. It is

possible that concentrations of methionine below 5 mg L^{-1} may also lead to decreased H$_2$S production, although lower concentrations were not investigated in this study. Additions of methionine did not decrease H$_2$S production in the strain UCD522, but as stated earlier H$_2$S production is highly dependent on yeast strain. The strain UCD522 is particularly prone to greater H$_2$S production due to its inefficiency in the basal expression of the sulfur reductase sequence (SRS) or the inability to effectively incorporate reduced sulfur in the SRS [49]. Previous research indicated that methionine additions can be effective in decreasing H$_2$S production in the strain UCD522 in artificial media [49], but this effect may be confounded when fermented in actual juice by other yeast nutrients and micronutrients present in the complex apple juice matrix. Within the range of concentrations examined in this study, there was a significant correlation between increasing methionine concentration and decreasing H$_2$S production in the strain UCD522 even if total H$_2$S production was not significantly decreased (Figure 2). This finding is particularly important for cidermakers, since apple juice often contains less than 5 mg L^{-1} methionine, whereas grape juice typically contains greater than 5 mg L^{-1} methionine [4]. Therefore, cidermakers may be at increased risk of H$_2$S production due to low methionine concentration as compared to winemakers.

Since concentrations of YAN above 140 mg L^{-1} have been reported to decrease H$_2$S production, the interactive effect of total YAN and additions of methionine was investigated. It was found that additions of methionine and ammonium could both decrease H$_2$S production in an interactive manner when fermented in artificial media [49], but this effect has not been investigated in cider fermentations. Only methionine at concentrations of 50 mg L^{-1} decreased H$_2$S production when juice was supplemented to 153 mg L^{-1} total YAN, as compared to juice at 53 mg L^{-1} YAN where any additions of methionine at 5, 20, and 50 mg L^{-1} resulted in decreased H$_2$S production.

This may be due to the highly complex interactions of yeast strain, yeast nutrient deficiencies, amino acid concentration and composition, and temperature, which all are known to affect H_2S production [52]. Fermentation rate increased when supplemented with YAN, which is known to be correlated with overall yeast biomass [84]. Therefore, it is possible that increased yeast biomass may require a higher overall concentration of methionine to effectively inhibit the SRS and prevent H_2S production. This is consistent with results obtained when juice was supplemented to 253 mg L^{-1} YAN, where methionine additions at any concentration did not affect total H_2S production. However, H_2S production was too low to accurately quantify in fermentations containing 253 mg L^{-1} YAN, hence some effects may not be observable due to the limit of quantification of our method exceeding the actual minimum H_2S production levels present in this study. H_2S production was almost completely inhibited in juice supplemented to 253 mg L^{-1} YAN. Therefore, under the conditions evaluated in this study, additions of high concentrations of YAN are more effective at decreasing H_2S production than additions of methionine, further evidence of the complex relationships of YAN concentration, composition and H_2S production in cider fermentations.

Contrary to previous findings, YAN supplementation to 153 mg L^{-1} resulted in a significant increase in H_2S production as compared to nitrogen-deficient juice. This was true when the primary nitrogen source was ammonium, arginine and asparagine, which indicates that H_2S production was not a factor of the efficacy of a specific YAN component in preventing H_2S production. The minimum YAN requirement typically recommended to avoid a stuck or sluggish fermentation in wine is 140 mg L^{-1} [11] but has recently been recommended to be no less than 267 mg L^{-1} [85]. However, H_2S may still be produced even when fermentations contain enough YAN to avoid stuck or sluggish fermentations. Additions of YAN have been shown to simultaneously increase fermentation rate and H_2S production in wine fermentations [48] which is in agreement with the

findings of this study. This is most likely due to the regulation of the SRS sequence in response to overall nitrogen limitation. Low nitrogen concentrations (66 mg L^{-1} YAN) down-regulated the SRS sequence and lead to decreased H_2S formation, where high nitrogen concentrations (267 mg L^{-1} YAN) upregulated the SRS and contributed to maximum H_2S production [91]. In the current study, YAN concentrations of 253 mg L^{-1} resulted in decreased H_2S production as compared to lower concentrations of 153 and 53 mg L^{-1}. However, fermentation rate increased and fermentation duration decreased with increasing YAN. This suggests that nitrogen additions increased yeast biomass and fermentation rate incrementally. At concentrations of 53 mg L^{-1} YAN, yeast growth is apparently inhibited, most likely due to nitrogen starvation. When supplemented to 153 mg L^{-1} YAN, it is possible that YAN is depleted during the first stage of fermentation, leading to nitrogen starvation during the logarithmic growth phase. This nitrogen starvation can lead to increased H_2S production as compared to low-nitrogen fermentation due to the higher yeast biomass [48]. If YAN is supplemented to 253 mg L^{-1}, starvation can be avoided altogether, thereby inhibiting H_2S production. For these reasons, research has suggested a two-step addition of nitrogen supplements, with half the total supplementation added prior to fermentation and the other half added at one-third Brix depletion [60]. Further research is needed to determine the mechanisms underlying the complex relationship between YAN consumption, yeast biomass and H_2S production.

Sensory evaluation revealed that additions of DAP resulted in differences in aroma between supplemented ciders when compared to unsupplemented ciders. Though the nature of these differences in cider aroma cannot be determined through difference testing alone, analytical results showed higher H_2S production in DAP supplemented ferments, suggesting that the difference may be correlated to the observed increase in H_2S production. High H_2S concentrations would lead to a prominent increase in "reduced" and "sulfur-like" aromas in the finished cider.

This is contradictory to the belief that additions of YAN in the form of DAP would necessarily protect against the occurrence of "reduced" aromas in finished ciders and wines. This also refutes the assumption by some cidermakers that H_2S is purged by CO_2 during the fermentation and the corresponding aroma is not present in the finished ciders and wines. Further research is necessary to determine the relationship between residual H_2S persisting in finished ciders based on total H_2S production and fermentation kinetics during cider fermentation. Similarly, ciders containing added methionine had differences in cider aroma compared to unsupplemented ciders, but only in ciders fermented using yeast strain EC1118. As indicated by our analytical results, methionine additions significantly decreased H_2S production by yeast strain EC1118 but did not affect H_2S production by strain UCD522. It is possible that the perceived difference in aroma could be attributable to a decrease in the "reduced" and "sulfur-like" aromas in finished ciders containing added methionine as compared to control fermentations, although further sensory evaluation such as descriptive analysis would be required to test this hypothesis.

Conclusions

Yeast assimilable nitrogen is known to affect hydrogen sulfide production in grape and wine fermentations, but the effect of YAN on cider fermentations has not been well examined. Differences in sources of YAN which simulate a more apple juice like matrix (asparagine rich) have no significant impact on the formation of H_2S in the fermenting juice when compared to arginine-rich juices more like the grape juice matrix. Supplementation of apple juice with methionine to concentrations normally observed in grape juice decreased H_2S production by yeast strain EC1118. This corresponds to a difference in cider aroma which may be due to decreased sulfur off-aromas. This finding suggests that relatively small additions of methionine pre-

fermentation could contribute to a substantial increase in cider quality. This information can be used to identify methods to decrease instances of H_2S formation for cidermakers. This research also indicates that additions of YAN do not necessarily decrease H_2S formation, but could in fact increase H_2S production when supplemented to the commonly recommended YAN concentration of 140 mg L^{-1}. This finding suggests that cidermakers and winemakers may encounter prominent H_2S production even at YAN concentrations generally considered sufficient for the prevention of sulfur off aromas. The complexity of this phenomenon points to the need for continued research on optimal pre-fermentation juice chemistry for cider fermentation without H_2S production.

Tables

Table 1 Amino acid composition of raw apple juice [a] [b]

Amino Acid	Concentration (mg L^{-1})
Histidine	3.7
Asparagine	38.5
Serine	3.7
Glutamine	nd
Arginine	2.8
Glycine	0.4
.	16.7
Glutamate	6.1
Threonine	1.0
Alanine	2.1
Proline	0.6
Cysteine	0.0
Lysine	0.0
Tyrosine	0.0
Methionine	nd
Valine	0.9
Isoleucine	1.6
Leucine	1.0
Phenylalanine	2.5

[a] Quantified using the method described in Materials and Methods section

[b] The designation "nd" indicates amino acids were not detectable

Table 2 Post-fermentation Analyses of Fermentations Treated with Different Sources of Amino Nitrogen [a][f]

Yeast Strain	Experimental Treatment	Fermentation rate (g CO_2/hr)	Fermentation Duration (hr)	Total CO_2 Production (g)	Residual Sugar[b] (mg/L)
EC1118	Control [c]	0.059 ± 0.000 a	282 ± 0 a	9.45 ± 0.11 a	95 ± 19 a
	Asparagine [d]	0.121 ± 0.005 bc	183 ± 3 b	10.3 ± 0.17 bc	6 ± 9 b
	Arginine [d]	0.114 ± 0.002 b	179 ± 10 b	10.21 ± 0.55 b	*nd* b
	Ammonium [de]	0.124 ± 0.003 c	172 ± 6 b	10.34 ± 0.05 c	1 ± 1 b
UCD522	Control [c]	0.057 ± 0.002 a	304 ± 5 a	10.03 ± 0.11 a	416 ± 262 a
	Asparagine [d]	0.122 ± 0.016 b	192 ± 5 b	10.24 ± 0.55 a	3 ± 3 b
	Arginine [d]	0.113 ± 0.009 b	195 ± 0 b	10.26 ± 0.44 a	9 ± 15 b
	Ammonium [de]	0.114 ± 0.001 b	184 ± 12 b	9.89 ± 0.32 a	2 ± 2 b

[a] Values expressed as mean ± standard deviation

[b] Quantified using the method described in the Materials and Methods section

[c] Control juice contained 52 mg L^{-1} YAN with no added nitrogen

[d] Added at concentrations of 140 mg nitrogen L^{-1}

[e] Added in the form of diammonium phosphate

[f] The designation "*nd*" indicates not detectable

Table 3 Post-fermentation Analyses of Fermentations Treated with Methionine [a]

Yeast Strain	Methionine [b] (mg/L)	Fermentation rate (g CO_2/hr)	Fermentation Duration (hr)	Total CO_2 Production (g)	Residual Sugar[c] (mg/L)
EC1118	0 [d]	0.059 ± 0.000 a	282 ± 0 a	9.45 ± 0.11 ab	95 ± 19 a
	5	0.054 ± 0.007 ab	293 ± 10 ab	10.20 ± 0.04 bc	135 ± 25 a
	20	0.059 ± 0.002 a	288 ± 10 a	10.22 ± 0.07 c	311 ± 295 a
	50	0.049 ± 0.002 b	307 ± 0 b	9.54 ± 0.12 b	165 ± 87 a
UCD522	0 [d]	0.057 ± 0.002 a	304 ± 5 a	10.03 ± 0.11 a	416 ± 262 a
	5	0.057 ± 0.001 a	318 ± 9 ab	9.97 ± 0.08 a	343 ± 103 a
	20	0.055 ± 0.002 a	323 ± 0 b	9.84 ± 0.10 a	606 ± 89 a
	50	0.058 ± 0.001 a	307 ± 0 a	9.94 ± 0.04 a	498 ± 134 a

[a] Values expressed as mean ± standard deviation
[b] Denotes concentration of nitrogen added
[c] Quantified using the method described in the Materials and Methods section
[d] Control juice contained 52 mg L^{-1} YAN with no added nitrogen

Table 4 Post-fermentation Analyses of Fermentations Investigating Interactive Effects of Methionine and Total YAN in fermentation by yeast strain EC1118 [a][b]

| YAN (mg/L)	Methionine (mg/L)	Fermentation rate (g CO_2/hr)	Fermentation Duration (hr)	Total CO_2 Production (g)	Residual Sugar [c] (mg/L)
153	0	0.141 ± 0.000 a	160 ± 4 a	9.69 ± 0.12 a	70 ± 32 a
	5	0.132 ± 0.003 b	165 ± 0 a	9.64 ± 0.14 a	105 ± 82 a
	20	0.135 ± 0.003 ab	160 ± 4 a	9.68 ± 0.12 a	82 ± 36 a
	50	0.137 ± 0.002 ab	165 ± 0 a	9.72 ± 0.12 a	83 ± 16 a
253	0	0.162 ± 0.001 a	141 ± 0 a	9.90 ± 0.12 a	13 ± 3 a
	5	0.162 ± 0.003 a	138 ± 5 a	9.71 ± 0.05 a	18 ± 9 a
	20	0.164 ± 0.002 a	135 ± 5 a	9.78 ± 0.13 a	22 ± 8 a
	50	0.164 ± 0.007 a	138 ± 5 a	9.69 ± 0.03 a	24 ± 12 a

[a] Values expressed as mean ± standard deviation
[b] Data for EC1118 fermentation with 53 mg N L^{-1} juice with methionine supplementation are presented in Table 3
[c] Quantified using the method described in the Materials and Methods section
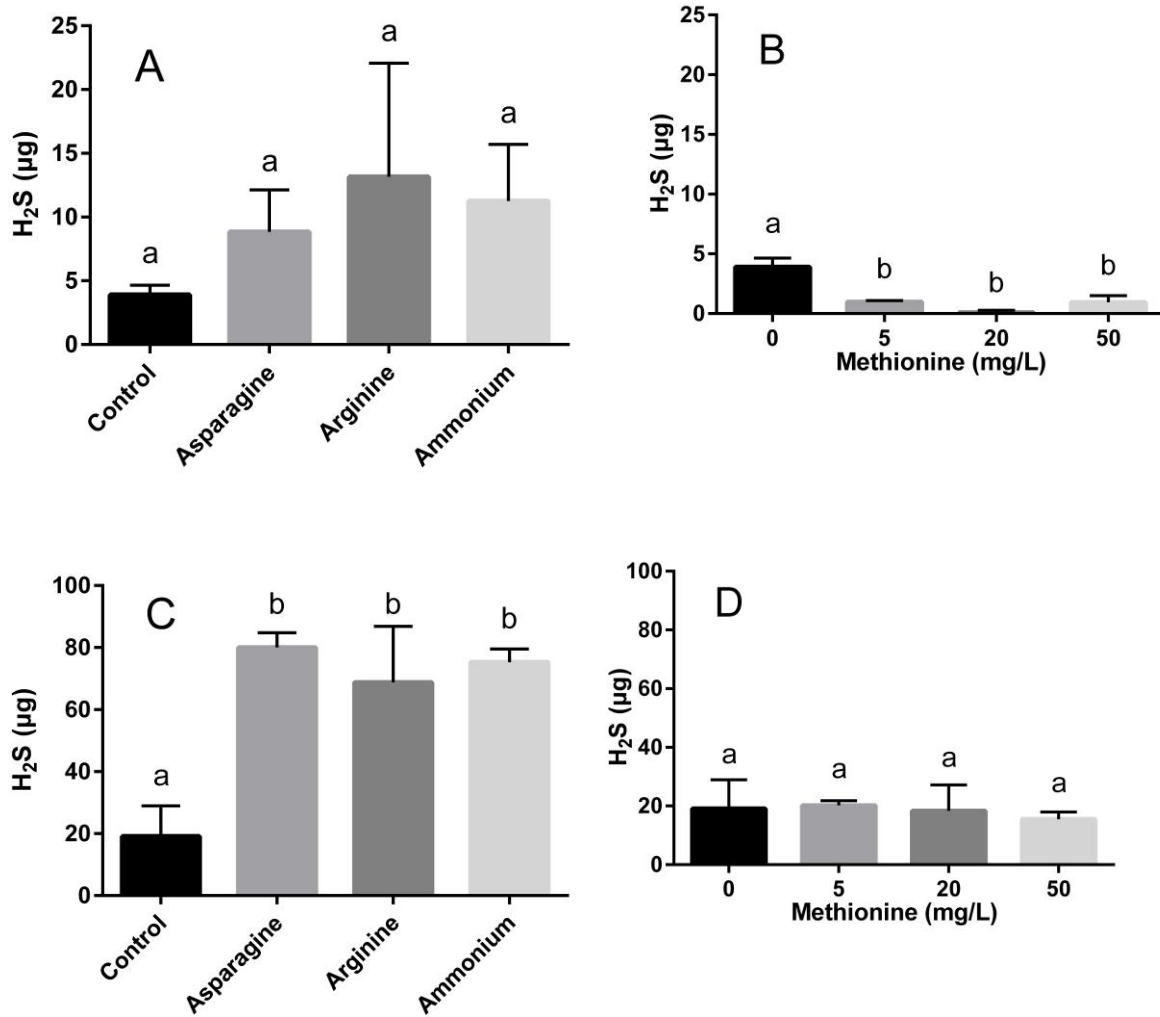
Figure 1 Total H₂S produced during fermentations with different sources of nitrogen and with added methionine. Values expressed as mean with error bars expressing standard deviation. Asparagine, arginine and ammonium were added at concentrations of 140 mg L^{-1} N. Ammonium was added in the form of diammonium phosphate. Control juice contained 53 mg L^{-1} YAN with no added nitrogen. Figures A and B represent fermentations with yeast strain EC1118, while figures C and D represent fermentations with yeast strain UCD522. Means not labeled with a common letter are significantly different (One-way ANOVA with Tukey's HSD, $p < 0.05$).
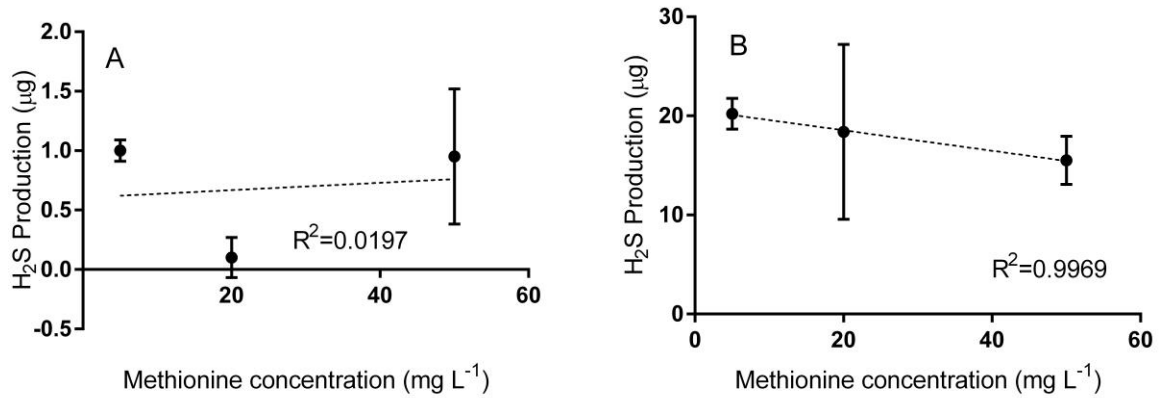
Figure 2 Linear regression H_2S production and methionine concentration. Graph A corresponds to fermentations using strain EC1118 and graph B corresponds to strain UCD522. There was no correlation of H_2S production and methionine concentration for yeast strain EC1118 but there was a correlation for yeast strain UCD522 (regression analysis, $p<0.05$).
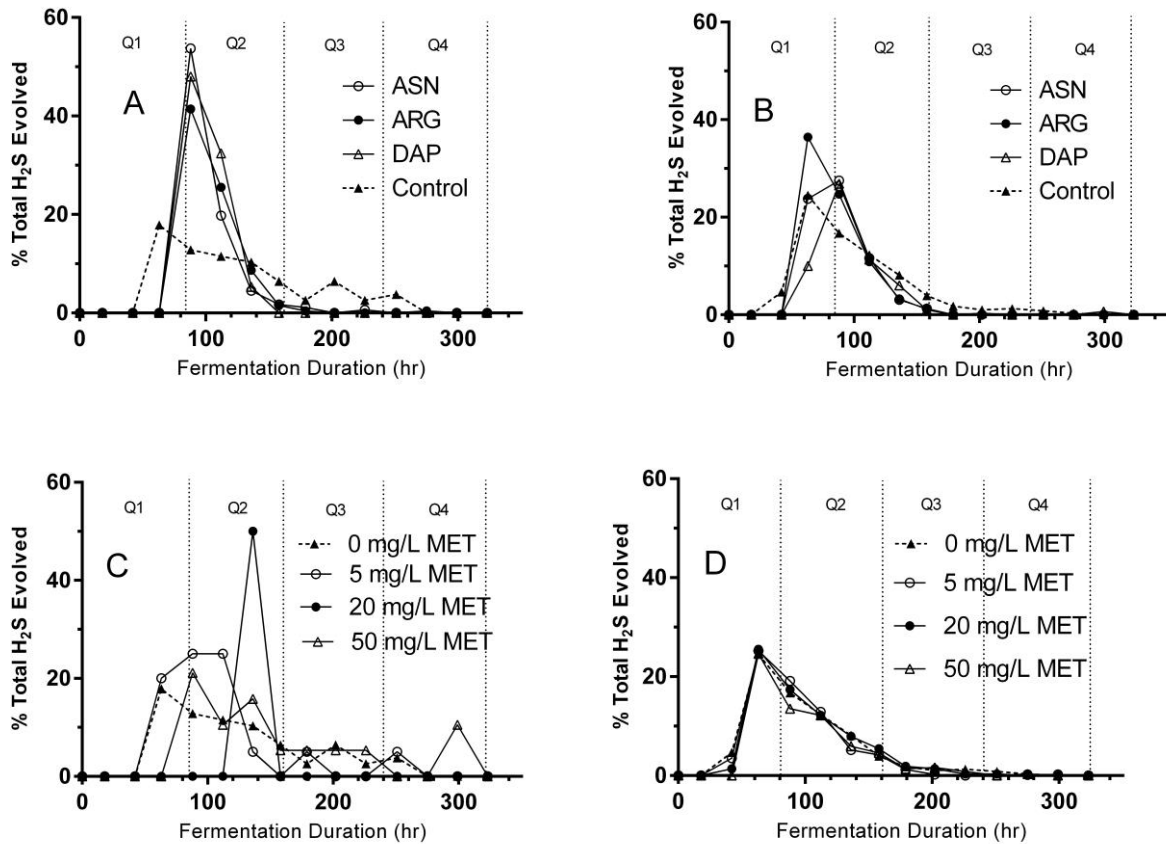
Figure 3 Time course of H_2S production during experimental fermentations. Figures A and C represent fermentations conducted with yeast strain EC1118, and Figures B and D represent fermentations conducted with yeast strain UCD522. Descriptors "Q1-Q4" represent each quartile of fermentation used to compare the rate of H_2S evolution over the time course of fermentation. There was no significant difference in H_2S production over the time course of fermentation between sources of nitrogen or between methionine treatments and control.
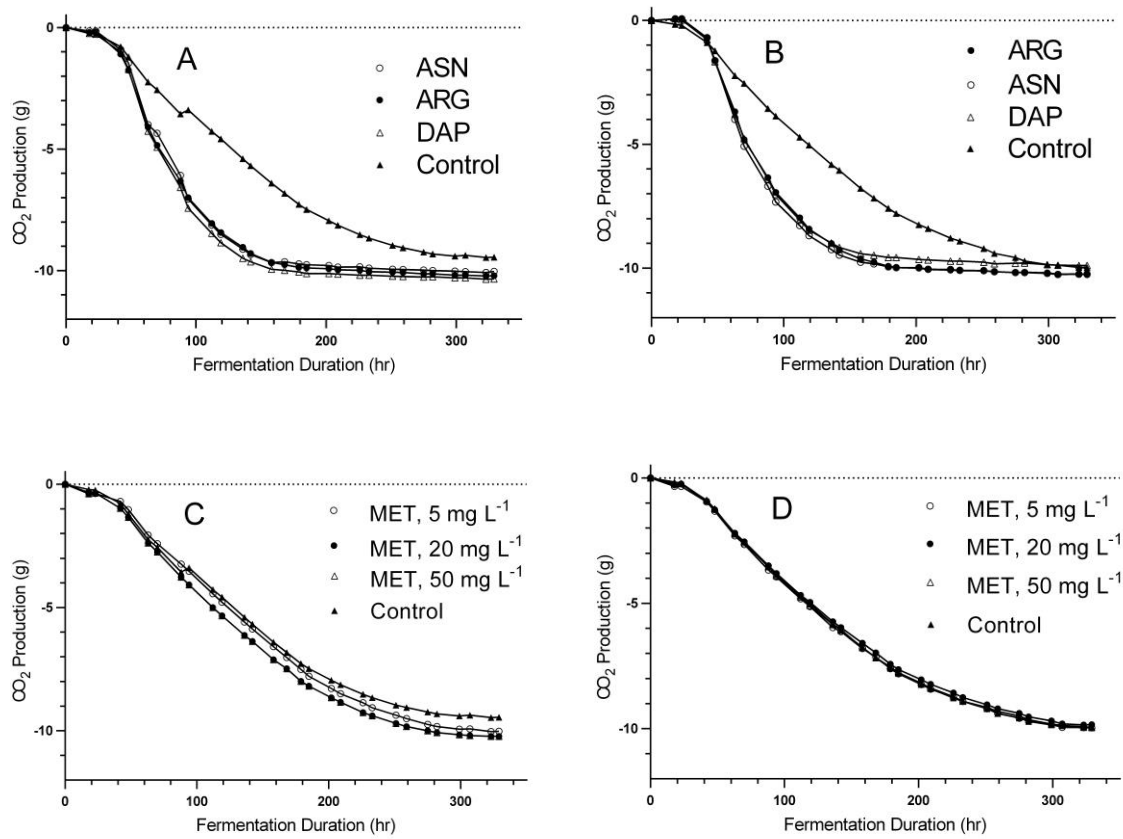
Figure 4 Fermentation curves for treatments containing added methionine and different sources of nitrogen plotted as the mass difference of the fermenter due to the loss of CO_2, which correlates to fermentation rate. The slope of the curve during the logarithmic phase of growth was used to calculate and compare maximum fermentation rates. Graphs A and C are fermentations using strain EC1118, graphs B and D are fermentations using strain UCD522. Treatments containing higher concentrations of added nitrogen fermented significantly faster as compared to low-nitrogen treatments. There was no significant difference in fermentation rate between treatments with methionine.
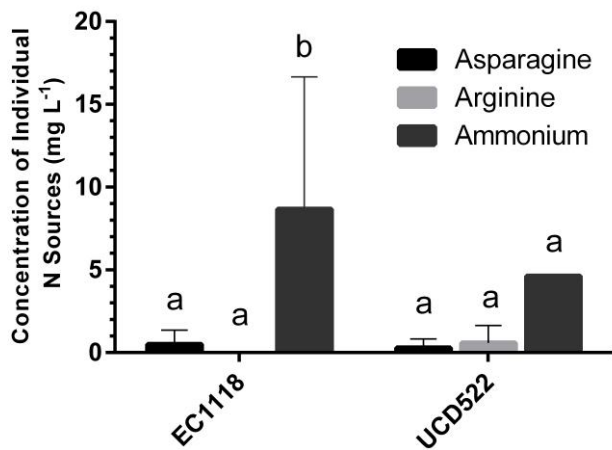
Figure 5 Residual nitrogen concentration for fermentations treated with different sources of YAN. Concentrations represent the residual concentration of the respective YAN sources post-fermentation after supplementation at 140 mg N L^{-1} each within each treatment. Means not labeled with a common letter are significantly different from one another within the specified yeast strain (One-way ANOVA with Tukey's HSD, $p < 0.05$).
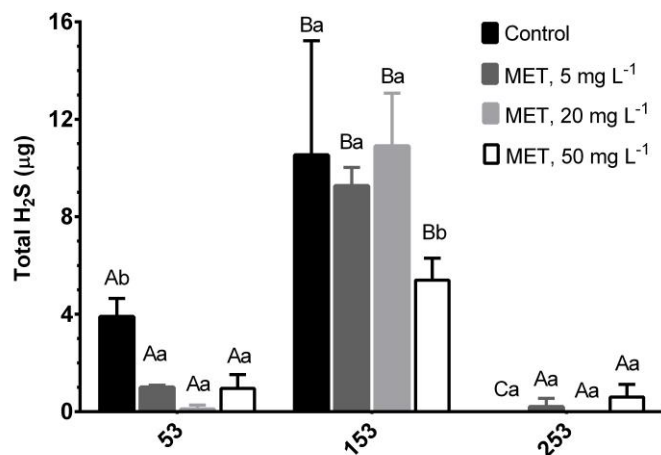
Figure 6 Total H₂S produced during fermentations comparing the interactive effects of total nitrogen and methionine. Columns are grouped corresponding to total juice YAN listed on the X axis. Values expressed as mean with error bars expressing standard deviation. Control juice contained no added methionine. Means not labeled with a common lowercase letter are significantly different within the specified YAN concentration. Means not labeled with a common uppercase letter are significantly different across YAN concentrations (Two-way ANOVA with Tukey's HSD, $p < 0.05$).
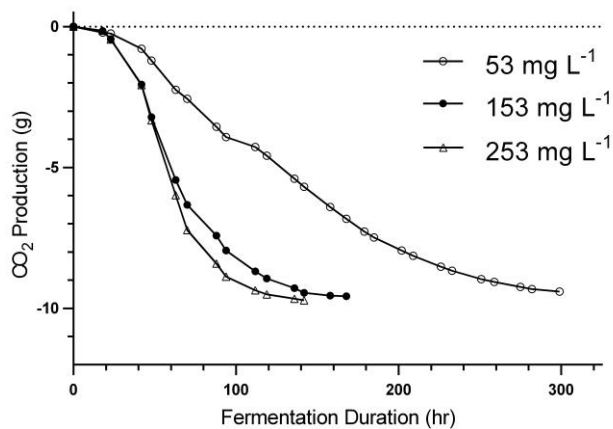
Figure 7 Fermentation curve for juices containing different concentrations of YAN in the strain EC1118 plotted as the mass lost due to CO_2 purged from the fermenter correlating to fermentation duration. The slope of the curve during the logarithmic phase of growth was used to calculate maximum fermentation rates. Fermentation rate increased significantly and fermentation duration decreased significantly with increasing YAN concentration (One-way ANOVA with Tukey's HSD, $p < 0.05$).

Chapter 6: Impact of Fungicides and YAN on Fermentation Kinetics and H$_2$S Production

Abstract

In cider production, residual fungicides on fruit may adversely affect yeast viability or metabolism during fermentation, leading to sluggish or stuck fermentation or the formation of undesirable aroma compounds such as hydrogen sulfide (H$_2$S). This study explored the effects of the fungicides elemental sulfur (S^0), fludioxonil and fenbuconazole added to apple juice at or below maximum residual concentrations allowable by the United States Department of Agriculture, on fermentation kinetics and H$_2$S formation during cider fermentation in two yeast strains. S^0, fludioxonil, and fenbuconazole all affected fermentation kinetics, but only S^0 increased H$_2$S production during fermentation. An interactive effect of yeast assimilable nitrogen (YAN) concentration and fenbuconazole was also demonstrated, wherein increasing YAN to 253 mg L^{-1} alleviated the negative effects of fenbuconazole on fermentation kinetics and H$_2$S production.

Introduction

Fungicide applications in orchards are essential for controlling diseases and thus ensuring sufficient fruit quality for cider production. However, fungicide residues can negatively impact yeast biology and therefore fermentation performance, and subsequently cider quality. In the US, unfermented apple juice is commonly referred to as "cider", however, in this work, as in the global cider industry, the term "cider" refers to the alcoholic beverage resulting from the fermentation of apple juice. The cidermaking process may be especially prone to increased fungicide residues due to the use of fungicides as post-harvest storage protectants, as well as the

fact that unlike grapes used in winemaking, apples used in cidermaking may be grown in the same orchard with multiple market destinations. These factors, combined with wide variation in practices for washing fruit prior to juicing, could contribute to the presence of residual fungicides in the fermenting juice. Such residues may contribute to adverse fermentation conditions and the occurrence of objectionable off-aromas including hydrogen sulfide (H_2S). H_2S is a common off-aroma occurring during alcoholic fermentation that negatively affects the quality of finished ciders. The causes of H_2S production during fermentation are numerous and have complex interactive effects that remain a topic of current research. Factors including yeast strain[45, 48], yeast assimilable nitrogen (YAN)[6, 44, 56] concentration and composition, temperature and deficiencies of other yeast nutrients such as biotin and pantothenic acid[52] have all been shown to contribute to overall H_2S production in wine fermentation. Certain fungicides are also known to increase H_2S production in wine fermentation and significantly impact wine flavor, but the impact of fungicide residues has not been thoroughly examined in cider fermentation. Similarly, insufficient YAN in the fermenting juice contributes to sluggish and/or stuck fermentations[4, 12, 18] and reduced wine quality through the production of higher alcohols and thiols combined with decreased production of desirable esters and long-chain fatty acids[4]. The interactive effects of YAN concentration and fungicide residue could reasonably be expected to significantly impact cider quality in much the same way as in grape-based wine production.

Elemental sulfur (S^0) can be used in orchards, and is commonly used in those that are organically-managed to limit powdery mildew incidence. The mode of action as a fungicide is not fully understood, but it is known that S^0 acts as an electron receptor in fungal respiratory chains and stimulates respiratory activities[92]. Sulfur is also likely related to oxidation of sulphydryl groups in important mitochondrial respiratory enzymes[93]. S^0 residues can be toxic to

native yeast found on grapes[94], however S^0 is not toxic to strains of *Saccharomyces cerevisiae* even at concentrations of 200 mg L^{-1}–a level which is far greater than reported residual S^0 concentrations in grapes or apples[95]. While non-toxic to yeast at practical residual concentrations, extensive research has indicated that S^0 residue can lead to substantial increases in H_2S production during wine fermentation[5, 47, 71]. In grape or apple juice fermentations, S^0 can non-enzymatically react with reducing compounds in the fermenting juice to form H_2S[96]. S^0 residues in concentrations as low as 1 mg L^{-1} have been shown to increase H_2S production during wine fermentation[97]. It is common for residues on fruit and in juice to greatly exceed these concentrations. One study found residue on Cabernet Sauvignon berries was 1.5 µg/g S^0 (approximately 1.7 mg L^{-1} S^0 in must) at harvest, but exceeded 12 µg/g S^0 (approximately 13.5 mg L^{-1} S^0 in must) a month after application on Pinot Noir berries[72]. The lack of data concerning persistence of residual S^0 in orchard systems leads to increased risk of residual S^0 in apple juice prior to fermentation, and subsequent formation of H_2S during yeast metabolism, which can negatively impact cider quality.

The impact of other fungicide residues on yeast growth and metabolism and resulting cider quality is not well understood. Fenbuconazole is a common fungicide used to prevent several plant diseases, including powdery mildew, leaf blotch and various rot diseases in both apple and grape production. Fenbuconazole is a sterol inhibitor, specifically a demethylation inhibitor (DMI) which prevents the biosynthesis of ergosterol in fungal plasma membranes[1]. Currently, there is no data on residual fenbuconazole in apple or grape juice, nor in finished wines or ciders. Fludioxonil is a broad-spectrum fungicide used frequently in post-harvest storage of apples, and operates by interfering with signal transduction pathways of fungi[73]. It has been found to inhibit spore germination in *Botrytis cenera*[74]. Residues of fludioxonil have been

cited as high as 0.15 mg L^{-1} in finished wines[98], but residues have not been quantified in grape juice, apple juice or ciders. Similarly, the impact of these fungicides on cider fermentation or finished cider or wine quality have not been extensively studied. García *et al* found that residual fludioxonil in Airen grape must at concentrations as low as 1 mg L^{-1} significantly alters the volatile fraction of wine aromas[75] but other flavor impacts are unknown, including the impact on the production of H_2S by yeast during fermentation.

The objective of this study was to determine the impact of fungicide residues on fermentation kinetics and the production of H_2S during apple cider fermentation. Laboratory scale fermentations were used to compare the effect of three fungicides [elemental sulfur (S^0), fludioxonil and fenbuconazole] on fermentation kinetics and H_2S production by two yeast strains commonly used in cider production.

Materials and Methods

Apple Juice. Commercially produced pasteurized apple juice was used to ensure consistency of juice across samples and to best represent large-scale cidermaking practices. The juice used was White House Fresh Pressed Natural Apple Juice (National Fruit Product Co., Winchester, VA, USA). Multiple bottles of the juice were combined into one homogenous lot then stored in 1L aliquots at -20°C until use, and then thawed to 22°C prior to yeast inoculation. The juice was measured to have 12.9° Brix by refractometer, pH 3.7 and titratable acidity (TA) 3.4 g L^{-1} malic acid equivalent using standard methods[88], and 53 mg L^{-1} YAN. YAN was quantified using commercially available Megazyme (Wicklow, Ireland) kits for primary amino nitrogen (K-PANOPA) and ammonium ion concentration (Ammonia-Rapid).

Fungicide Additions. S^0 was obtained in the form of Microthiol Disperss fungicide (80% sulfur, Nufarm Australia Ltd., Victoria, Australia) and added at concentrations of 5 and 20 mg L^{-1} S^0. Fludioxonil was obtained in the form of Scholar SC fungicide (20.4% fludioxonil, Syngenta Crop Protection LLC, Greensboro, NC, USA) and added at concentrations of 0.2 and 0.4 mg L^{-1} fludioxonil. Fenbuconazole was obtained in the form of Indar 2F fungicide (23.5% fenbuconazole, Dow Agrosciences LLC, Indianapolis, IN, USA) and added at concentrations of 2.5 and 5.0 mg L^{-1} fenbuconazole. Juice with no fungicides added was used as a control. Interactive effects of YAN and fenbuconazole residues were examined by adding YAN to the apple juice at concentrations of 25 mg N L^{-1} in the form of Fermaid K (Scott Laboratories, Inc., Petaluma, CA, USA) for the control, and further supplementing to 153 and 253 mg N L^{-1} using diammonium phosphate (DAP) for the medium and high YAN concentration treatments (Scott Laboratories, Inc., Petaluma, CA, USA). Each treatment was then supplemented with fenbuconazole at concentrations of 0 (control), 0.2 and 0.4 mg L^{-1} each.

Fermentations. Fermentations were carried out with two yeast strains for each treatment. Prise de Mousse *Saccharomyces bayanus* EC1118 (Lallemand, Montreal, Canada) was selected to represent a low-H_2S producing strain and Montrachet *Saccharomyces cerevisiae* UCD522 (Lallemand, Montreal, Canada) was selected to represent a high-H_2S producing strain. Juice was inoculated with 0.05 g of active dry yeast rehydrated in 35°C water for 20 min. Yeast nutrient was added in the form of Fermaid K (Scott Laboratories, Petaluma, CA, USA) to all fermentations at concentrations of 250 mg L^{-1}. This added an additional 25 mg L^{-1} nitrogen to the juice, bringing total juice YAN to 78 mg L^{-1} for the control, as the endogenous 53 mg L^{-1} N in the apple juice was not sufficient to complete fermentation. Fermentations were carried out according to the method as described by Ugliano and Henschke for rapid determination of H_2S

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formation during alcoholic fermentation[80]. These were conducted in 250 mL Erlenmeyer flasks

fitted with a one-hole rubber stopper to which a H_2S detector tube was affixed (described below).

Fermentations were not aerated, but were stirred twice per day at 800 rpm for 5 min to prevent

yeast settling. Fermentations were carried out at 18°C in triplicate. Fermentation rate was

monitored by measuring the mass of the fermentation vessel as a proxy for CO_2 evolution.

Finished cider was analyzed to determine pH, TA, residual YAN, and residual sugar. Residual

sugar was analyzed using a D-Fructose/D-Glucose (K-FRUGLU) enzymatic kit (Megazyme,

Wicklow, Ireland).

Hydrogen Sulfide Detector Tubes. The H_2S detection and quantification method was as

described by Ugliano and Henschke[80]. Detector tubes were obtained from Komyo Kitagawa

(Tokyo, Japan). Tubes were inserted into a one-hole rubber stopper to obtain a gas-tight seal.

CO_2 produced during fermentation carried H_2S through the detector tube. H_2S reacts with the

lead acetate (Tube 120SB, 120SD) or silver nitrate (Tube 120SF) contained in the tube, creating

a discolored band. The different tubes have different capacities for total H_2S quantification, and

were selected based on total H_2S production in a given treatment as determined through

preliminary experimentation. The length of the discolored band is proportional to the amount of

purged H_2S. Readings were taken twice daily to determine the H_2S production rate. If at any

reading point the tube appeared to be near saturation, the tube was replaced with a new tube.

This method may have allowed a small amount of H_2S gas to escape, but was employed

consistently across treatments in this study.

Determination of Fermentation Rate, Duration, and H_2S Production Rate. Maximum

fermentation rate was determined by taking the slope of the fermentation curve during the

exponential phase of yeast growth corresponding to the highest constant rate of CO_2 production,

as reported by others[89]. Steeper slopes correspond to faster fermentation rate. Fermentations were determined to be complete when the CO_2 production rate decreased to less than 0.2 g day^{-1}. Fermentation duration is expressed in total hours starting from inoculation. Total H_2S production was determined by calculating the sum of H_2S production over the time course of fermentation. To determine the relative rate of H_2S production over the time course of fermentation, the fermentation duration was divided into four quartiles of equal time. The percent of H_2S produced in each quartile out of the total H_2S produced for a given fermentation was compared to determine if there was a significant difference in the H_2S production over the time course of fermentation across treatments.

Statistical Analyses. Values were compared using a one-way analysis of variance (ANOVA) at a significance of $p < 0.05$ followed by parametric mean testing using Tukey's Honest Significant Difference (HSD) using GraphPad Prism v.6 (La Jolla, CA). Analyses comparing the interaction between yeast strain/YAN concentration and fungicide residues were analyzed using a two-way ANOVA at a significance of $p < 0.05$ and post-hoc testing by Tukey's HSD.

Results and Discussion

Fermentation Kinetics.

Fermentation duration, fermentation rate, total CO_2 production, and residual sugar for fermentations conducted using yeast strains EC1118 and UCD522 are reported in Tables 1 and 2, respectively, and fermentation curves are shown in Figure 1. These results are presented and discussed in the following paragraphs.

Elemental Sulfur.

Regardless of the concentration added, S^0 did not affect the fermentation duration, rate, total CO_2 production, or residual sugar with yeast strain EC1118 (Table 1). At the concentration of 5 mg L^{-1}, S^0 had no effect on fermentation duration, fermentation rate, or residual sugar with either yeast strain (Tables 1 and 2). The 5 mg L^{-1} S^0 treatment resulted in decreased total CO_2 production by UCD 522, but there was no effect in the fermentations with EC1118. The 20 mg L^{-1} S^0 treatment using strain UCD 522 containing added S^0 at 20 mg L^{-1} were longer in duration (p<0.05) by 25 hours on average, had a lower maximum fermentation rate (p<0.05), and lower total CO_2 production than the control, but this effect was not observed for yeast strain EC1118 at the same S^0 concentration. The interaction effect of yeast strain and S^0 was significant with regards to fermentation duration (p=0.0002, data not shown). Fermentation duration is known to differ among yeast strains[99] and the results of this study show that effect of S^0 on fermentation rate also differs across yeast strains. The significance of this interaction effect supports the need to employ at least two yeast strains in studies investigating the impact of juice chemistry on fermentation performance. This also emphasizes the importance of considering the yeast strain when applying research findings to cider production. Residual sugar concentration was not different for any S^0 treatment or yeast strain. CO_2 production is directly correlated to fermentation rate, since CO_2 evolution occurs concomitantly with sugar consumption by yeast. The lack of a significant difference in RS while significant differences in CO_2 production were observed in the added S^0 conditions for fermentations conducted by UCD522 can likely be explained due to the higher level of precision in measurements for the CO_2 production by mass as compared to the RS measurement by enzymatic assay. Taken together, these results suggest that cidermakers should be aware that residual S^0 above 20 mg L^{-1} in juice can result in

fermentation duration of up to one day longer at 18°C, but should not impact residual sugar concentration in the finished cider. The fermentation curves showing CO_2 production over the time course of fermentation in Figure 1 A and B further illustrate this conclusion. The observed impact of S^0 on fermentation kinetics as a whole is consistent with previous research which has indicated that sulfur residue does not significantly impact fermentation kinetics of *Saccharomyces cerevisiae* during fermentation[95].

Fludioxonil.

Additions of fludioxonil did not significantly impact fermentation rate or duration in either yeast strain with one exception. The 5 mg L^{-1} addition of fludioxonil slightly but significantly increased fermentation rate in the strain UCD522 (Table 2). No difference in CO_2 production or RS was observed for either yeast strain in fermentations containing added fludioxonil (Table 2). Fludioxonil did not impact the time course of CO_2 production in either yeast strain (Figure 1 C and D). Taken together from a practical perspective, these results suggest that fludioxonil does not significantly impact fermentation kinetics for EC1118 or UCD522.

Fenbuconazole.

Additions of fenbuconazole increased fermentation duration ($p<0.05$) and decreased fermentation rate ($p<0.01$) in both yeast strains (Tables 1 and 2, Figures 1E and 1F). Additions of fenbuconazole at concentrations of 0.2 mg L^{-1} decreased fermentation rate by approximately 10% on average in both strains. Fermentation rate decreased by 15% on average when fenbuconazole was added at concentrations of 0.4 mg L^{-1}. Consequently, this increased fermentation duration in both strains at all concentrations ($p<0.05$). On average, additions of fenbuconazole increased fermentation duration by approximately 55 hours regardless of yeast strain or concentration.

Additions of fenbuconazole decreased the amount of CO_2 produced during fermentation for both yeast strains, with the exception of additions of 0.2 mg L^{-1} in the strain UCD522 ($p<0.05$). Similarly, additions of fenbuconazole increased RS concentration in all fermentations with the exception of additions of 0.2 mg L^{-1} in the strain EC1118 ($p<0.05$). In fact, RS concentration were 34- to 185-fold higher on average in all fermentations containing added fenbuconazole (Tables 1 and 2). Fermentations supplemented with fenbuconazole at a concentration of 0.4 mg L^{-1} averaged 8.6 g L^{-1} RS. This concentration is too high for the finished cider to be considered "dry," therefore these fermentations are considered incomplete or "stuck," a term commonly used to describe incomplete fermentation in wine production. Even with cider styles where residual sugar is acceptable, stuck fermentation is not desirable because the outcome is unpredictable. Stopping the fermentation by chilling to stop yeast metabolism or centrifuging to remove yeast provide more reliable means of producing cider that contains residual sugar. Additions of fenbuconazole at increasing concentration have linear correlations with fermentation rate, fermentation duration, CO_2 evolution and residual sugar (Figure 2). Increasing fenbuconazole concentration resulted in decreased fermentation rate, increased fermentation duration, decreased total CO_2 evolved and increased residual sugar ($p<0.001$). As such, the results of this study indicate that residual fenbuconazole at 0.2 and 0.4 mg L^{-1} can adversely affect cider fermentation and the resulting cider quality.

The mechanism by which fenbuconazole impacts yeast growth and metabolism was not determined in this study. However, the decreasing fermentation rate with increasing concentration of fenbuconazole suggests that fenbuconazole may either lower cell viability, yeast cell biomass, or yeast growth rate. The fungicide mode of action of fenbuconazole is through the inhibition of ergosterol production. Ergosterol is an essential cell membrane constituent in fungi

and cellular death may result if the enzymes that generate ergosterol are disrupted[100]. In practice, residual fenbuconazole in apple juice merits consideration as a possible cause of sluggish cider fermentation.

Total Hydrogen Sulfide Production.

Elemental Sulfur.

Additions of S^0 to juice led to an increase in H_2S production (Figure 3). For yeast strain EC1118, H_2S production increased 9-fold on average when added at 5 mg L^{-1} S^0 and 20-fold on average when added at 20 mg L^{-1} S^0. Similarly, for UCD 522 H_2S production increased nearly 5-fold on average when added at 5 mg L^{-1} and increased nearly 30-fold on average when added at 20 mg L^{-1}. These results are in agreement with previous findings which have identified residual S^0 as a significant contributor to H_2S production[5, 47, 71]. In white wine production, the practice of settling the juice prior to beginning fermentation has been shown to minimize the negative impacts associated with high concentrations of S^0 in the fermenting medium[70]. Pre-fermentation juice settling is not a standard practice in cider production and cidermakers could potentially encounter higher residual S^0 in juice that can increase H_2S production. The concentration of residual S^0 present on apples prior to processing and fermentations has not been assessed in this study or by previous research. It is possible that orchards using S^0 as a fungicide may encounter higher residual concentrations of S^0 in juice as compared to residual concentrations found in grape juice or must prior to fermentation. All of these factors indicate that assessment of residual S^0 on apples and implementation of practices to prevent or limit residual S^0 concentrations in apple juice pre-fermentation is important to cider production.

Fludioxonil.

Additions of fludioxonil did not result in increased H_2S production for either yeast strain (Figure 3). This finding is complementary to the findings on fermentation rate and duration, indicating that residual fludioxonil does not adversely affect yeast growth or metabolism in cider fermentation.

Fenbuconazole.

Additions of fenbuconazole did not lead to increased H_2S production (Figure 3). Fenbuconazole at 0.4 mg L^{-1} in EC1118 fermentations resulted in a 254% increase of H_2S production as compared to the control, but this result was not significantly different from the control. At this concentration, fenbuconazole had no significant impact on H_2S production, although it did significantly impact fermentation kinetics. Yeast produce H_2S through the sulfur reduction sequence (SRS)[8]. In this process, sulfate ions are converted to free sulfur (S^{2-}) that then are used in yeast metabolism to produce sulfur-containing amino acids such as methionine and cysteine[8]. It is likely that fenbuconazole, at the concentrations tested in this study, did not interfere with SRS signal transduction, and hence did not affect H_2S production by yeast, even if it did impact cell growth and/or viability.

Relative Rate of Hydrogen Sulfide Production

The time course of H_2S production during fermentation may vary since H_2S can be effectively stripped from the fermenting media by CO_2 gas evolved during fermentation[43], especially during the period of maximum fermentation rate when CO_2 evolution is also at its maximum rate. It is possible that a cider in which less total H_2S had been produced but was produced later in the time course of fermentation could retain more of the unwanted H_2S character in the final product. This could impart a greater sensory impact on the finished product. Additions of S^0, especially at high concentrations, tended to alter the rate of H_2S production

(Figure 4 A and D). The percentage of the total production of H_2S for a given treatment was greater in the third and fourth quartiles at all concentrations of S^0 in the strain UCD522 ($p<0.05$), as compared to the control. At concentrations of 20 mg L^{-1}, H_2S production was greater in the third quartile in the strain EC1118 ($p<0.01$). The greatest proportion of the total H_2S was produced in the second quartile for the majority of fermentations. This correlates to the logarithmic phase of fermentation, which also corresponds to the highest rate of SRS activity in the yeast, leading to the observable spike in H_2S production during the highest rate of fermentation[57]. When S^0 is present in the fermenting juice, however, H_2S is produced throughout the duration of the fermentation, since SRS activity is not the primary cause of H_2S production[69]. Additions of fludioxonil did not affect the rate of H_2S production (Figure 4 B and E). H_2S production decreased in the second quartile when fenbuconazole was added at 0.2 mg L^{-1} in both yeast strains ($p<0.05$)(Figures 4C and 4F). However, higher concentrations of fenbuconazole did not significantly impact total H_2S production. It is possible that yeast cell density is correlated with a change in the relative rate of H_2S production and that fenbuconazole may decrease total cell biomass achieved during fermentation. The mechanism by which fenbuconazole affects H_2S production may be determined in future studies by monitoring cell density throughout the time course of fermentation and corresponding H_2S production.

Interactive Effect of Fenbuconazole and Yeast Assimilable Nitrogen.

The fermentation kinetics results of this study clearly illustrate that residual fenbuconazole can lead to sluggish and stuck cider fermentations. Insufficient YAN is also widely known to cause stuck and sluggish fermentations[12]. The generally accepted minimum recommended concentration of YAN to successfully complete fermentation is 140 mg L^{-1} for wine[11]. However, this recommendation is not universally accepted. The optimal concentration of

pre-fermentation YAN required for a given fermentation is difficult to determine due to interactions with numerous factors including, but not limited to, yeast strain[45, 48], micronutrient concentrations[52], biotin concentration[52], pantothenic acid concentration[52], and temperature[52]. The apple juice used in this study contained an initial YAN concentration of 53 mg L^{-1}, and was supplemented to 78 mg L^{-1} in all of the treatments. This relatively low YAN concentration is typical of apple juice[7]. However this concentration may still be insufficient to prevent sluggish or stuck fermentations and may have influenced the initial findings of this study on the impact of fenbuconazole on fermentation rate and duration.

Fermentation rate increased and fermentation duration decreased with increasing YAN concentration ($p<0.05$) (Figure 5, Figures 6A and 6B). This is in agreement with prior findings which indicate that increasing YAN is correlated with increasing fermentation rate and decreasing fermentation duration[4]. RS decreases with increasing YAN when fenbuconazole was present in concentrations of 0.4 mg L^{-1} (Figure 6D), but RS was not significantly affected by increasing YAN for the control (no fenbuconazole) or at 0.2 mg L^{-1} fenbuconazole. This corroborates previous research which indicated that increasing YAN minimizes sluggish or stuck fermentations[4, 12, 18], although the findings of this study emphasizes the influence of interactive effect of YAN with other parameters.

There was an interactive effect between additions of fenbuconazole and YAN on H$_2$S production ($p<0.0001$)(Figure 6E). Interestingly, H$_2$S production did not decrease when YAN was increased from 78 mg L^{-1} to 153 mg L^{-1}, but increased in the control (no fenbuconazole) and 0.2 mg L^{-1} fenbuconazole fermentations (Figure 7A). Increasing YAN from 153 mg L^{-1} to 253 mg L^{-1} decreased H$_2$S production in all fermentations ($p<0.001$) (Figure 7A). In fact, there was no detectable H$_2$S when YAN concentration was 253 mg L^{-1}. H$_2$S production decreases with

increasing YAN concentration at 0.4 mg L^{-1} fenbuconazole (Figure 7B), with both factors

significantly correlating (p=0.0012) (Figure 6E). This corroborates previous findings which

indicate that insufficient YAN supplementation can lead to increased H$_2$S production[48]. The

results of this study show that a minimum YAN concentration of 140 mg L^{-1} does not always

prevent the occurrence of stuck or sluggish fermentations. Furthermore, this study shows that a

minimum YAN concentration of 140 mg L^{-1} does not always prevent the formation of H$_2$S

during fermentation, even with the relatively low starting sugar concentration of apple juice (as

compared to grape juice, the media in which most YAN investigations have been conducted).

Additions of both 0.2 mg L^{-1} and 0.4 mg L^{-1} fenbuconazole lowered fermentation rate in

juice with both 78 mg L^{-1} and 153 mg L^{-1} YAN (p<0.05) (Table 3). However, there was no

difference in fermentation rate at any concentration of fenbuconazole at 253 mg L^{-1} YAN. At

YAN concentrations of 153 mg L^{-1} fermentations with added fenbuconazole at both

concentrations had a slower fermentation rate than the control with no fenbuconazole (p<0.01).

However, fermentation duration was not different among fenbuconazole concentrations when

YAN concentration was 253 mg L^{-1}. The interaction of YAN and fenbuconazole concentration

was not significant in terms of fermentation rate or duration (Figure 6A or 6B). With all other

variables held constant, increasing YAN concentrations correlate with an increase in yeast

biomass[84]. It is possible that residual fenbuconazole inhibited yeast growth and promoted yeast

mortality. One possible explanation for the results of this study is that increasing YAN

concentration may overcome this effect by allowing yeast biomass to increase to the point where

the impact of residual fenbuconazole on yeast cell populations becomes negligible. Interestingly,

fenbuconazole still adversely impacted fermentation kinetics when YAN concentration was 153

mg L^{-1}, a greater concentration than the minimum 140 mg L^{-1} typically considered sufficient to

complete fermentations in wine and cider. Therefore, the minimum YAN concentration required for fermentation is greater than the standard recommendation when fenbuconazole is present in the juice.

RS concentration increased with increasing concentrations of fenbuconazole at YAN concentrations of 78 mg L^{-1} 153 mg L^{-1} (Table 3). However, with 153 mg L^{-1} YAN, the RS concentration was 10-20 fold lower compared to juices with the same fenbuconazole concentration fermented at 78 mg L^{-1} YAN. When YAN concentration was 153 mg L^{-1}, all treatments fermented completely. Finally, there was no difference in RS concentration at any concentration of fenbuconazole when YAN concentration was 253 mg L^{-1} (Table 3). There was an interaction effect of YAN and fenbuconazole on RS (p=0.0006) (Figure 6D).The effect of fenbuconazole on RS at low YAN concentration becomes negligible at high YAN concentrations (Figure 6D). This further corroborates the finding that increasing YAN lessens the impact of fenbuconazole on yeast growth and metabolism. There was no difference in CO_2 when fenbuconazole was added to juice with153 or 253 mg L^{-1} YAN (Table 3). Even though CO_2 production did not decrease with increasing YAN from 153 mg L^{-1} to 253 mg L^{-1}, there was a significant correlation between increasing YAN and decreasing CO_2 when fenbuconazole is added at either concentration (p<0.01) (Figure 6C). This supports the hypothesis of this study that increasing YAN concentration contributes to increased fermentation rate, even when fenbuconazole is present. Additions of fenbuconazole did not affect H_2S production for any of the YAN treatments.

Due to the complex chemical nature of the apple juice/cider matrix and interactive effects of many factors on fermentation performance, it is difficult to determine the complete impact of fungicide residues on cider fermentation and product quality. Notwithstanding, this study

showed that fungicide residues can impact fermentation rate, particularly under conditions of low YAN concentration. The results of this study also demonstrate that fermentation kinetics and H_2S production was specific to the particular fungicide chemistry, and likely its mode of action. As fungicide applications differ widely across fruit production systems and regions, this observation is of special concern to emerging apple and grape growing regions that contend with relatively high fungal disease pressure, such as the Eastern United States. The results of this study indicate that the presence of fungicide residues should be added to the growing list of factors beyond starting Brix which should be taken into consideration when determining the minimum YAN concentration for a given cider fermentation. Determining regionally-specific guidelines for minimum YAN concentration in cider will require further research into the interactions between YAN and fungicide residues as well as other juice matrix factors on fermentation rate and H_2S production.

Figure 1. Fermentation curves for treatments containing additions of fungicides plotted as change in mass of the fermentation vessel due to CO_2 purged from the fermenter over the time course of fermentation. The slope of the curve during the logarithmic phase of growth was used to calculate and compare maximum fermentation rates. Graphs A, C, and E represent fermentations conducted by yeast strain EC1118. Graphs B, D and F represent fermentation conducted by yeast strain UCD522. Units ascribed to added fungicide concentrations are in mg L^{-1}. Control fermentations contained no added fungicides.

Figure 2. Linear regression of the maximum fermentation rate, fermentation duration, CO_2 evolved and residual sugar for treatments with increasing fenbuconazole concentrations. Concentrations of fenbuconazole are expressed in units of mg L^{-1}. All linear correlations were statistically significant ($p<0.01$).

Figure 3. Total H_2S produced during fermentations comparing treatments with fungicides at increasing concentrations. Values expressed as mean with error bars representing standard deviation. Concentrations are expressed in units of μg H_2S. Control fermentations contain no added fungicides. Figures A, B and C reflect fermentations using yeast strain EC1118. Figures D, E and F reflect fermentations using yeast strain UCD522. Y-axis scales are not consistent across this figure because they reflect maximum range of H_2S produced within each yeast strain. Means not labeled with a common letter within specified yeast strain are significantly different (One-way ANOVA with Tukey's HSD, $p<0.05$).

Figure 4. The percent of the total H_2S produced over the time course of fermentation. Graphs A, B and C represent ciders fermented using yeast strain EC1118. Graphs D, E and F represent ciders fermented using yeast strain UCD522. Markers "Q1 - Q4" represent quartiles of the

fermentation duration used to compare the rate of H_2S evolution over the time course of fermentation.

Figure 5. Fermentation curve for yeast strain EC1118 in apple juice containing different initial concentrations of YAN plotted as the mass of CO_2 purged from the fermenter over the time course of fermentation. The slope of the curve during the logarithmic phase of growth was used to calculate and compare maximum fermentation rates. Fermentation rate increased significantly and fermentation duration decreased significantly with increasing YAN concentration (One-way ANOVA with Tukey's HSD, $p<0.05$).

Figure 6. Linear regression of fermentation rate, fermentation duration, CO_2 evolved, residual sugar and H_2S production in fermentations treated with fenbuconazole and different concentrations of YAN. Concentrations of fenbuconazole are expressed in units of mg L^{-1}. Linearity is significant for fermentation rate at all concentrations ($p<0.05$), fermentation duration at all concentrations ($p<0.01$), CO_2 evolution for concentrations of 0.2 and 0.4 mg L^{-1} ($p<0.01$), residual sugar for concentrations of 0.4 mg L^{-1} ($p<0.01$), and H_2S production for concentrations of 0.4 mg L^{-1} ($p<0.01$). There was a significant interaction between fenbuconazole additions and YAN for residual sugar ($p=0.0006$) and H_2S production ($p=0.0109$).

Figure 7. Total H_2S produced during fermentations using yeast strain EC1118 comparing the interactive effects of total yeast assimilable nitrogen and concentration of added fenbuconazole. Columns for figure 7A are grouped corresponding to total juice YAN mg L^{-1}. Columns for figure 7B are grouped corresponding to fenbuconazole concentration in mg L^{-1}. Values expressed as mean with error bars representing standard deviation. Control juice contained no added fenbuconazole. Means not labeled with a common lowercase letter are significantly different

within the specified YAN concentration for figure 7A or within the specified fenbuconazole

concentration for figure 7B (Two-way ANOVA with Tukey's HSD, $p<0.05$).

Tables

Table 1 – Fermentation rate, fermentation duration, total CO_2 production and residual sugar for fermentations using yeast strain EC1118 [a][b]

Experimental Treatment [c]	Fermentation rate (g CO_2/hr)	Fermentation duration (hr)	Total CO_2 production (g)	Residual sugar (g/L)
Control	0.073 ± 0.002 a	259 ± 0 a	9.88 ± 0.13 a	0.05 ± 0.02 a
Sulfur, 5	0.071 ± 0.000 a	259 ± 0 a	9.63 ± 0.16 a	0.02 ± 0.01 a
Sulfur, 20	0.072 ± 0.001 a	256 ± 0 a	9.74 ± 0.23 a	0.03 ± 0.00 a
Control	0.073 ± 0.002 a	259 ± 0 a	9.88 ± 0.13 a	0.05 ± 0.02 a
Fludioxonil, 2.5	0.071 ± 0.003 a	251 ± 0 a	9.85 ± 0.26 a	0.04 ± 0.02 a
Fludioxonil, 5.0	0.068 ± 0.006 a	259 ± 0 a	10.08 ± 0.34 a	0.05 ± 0.03 a
Control	0.073 ± 0.002 a	259 ± 0 a	9.88 ± 0.13 a	0.05 ± 0.02 a
Fenbuconazole 0.2	0.063 ± 0.003 b	306 ± 24 b	9.36 ± 0.05 b	3.32 ± 2.92 a
Fenbuconazole, 0.4	0.059 ± 0.000 b	314 ± 13 b	9.12 ± 0.21 b	9.26 ± 0.98 b

[a] Values expressed as mean ± standard deviation
[b] Values marked with different letters are significantly different from the control within a specified fungicide treatment (ANOVA w/ Tukey's HSD)
[c] Fungicide concentrations are expressed in mg L^{-1}

Table 2 – Fermentation rate, fermentation duration, total CO_2 production and residual sugar for fermentations using yeast strain UCD522 [a][b]

Experimental Treatment [c]	Fermentation Rate (g CO_2/hr)	Fermentation duration (hr)	Total CO_2 production(g)	Residual Sugar (g/L)
Control	0.071 ± 0.001 a	259 ± 0 a	9.90 ± 0.09 a	0.07 ± 0.01 a
Sulfur, 5	0.070 ± 0.001 a	258 ± 10 a	9.63 ± 0.03 b	0.40 ± 0.07 a
Sulfur, 20	0.067 ± 0.002 b	284 ± 0 b	9.60 ± 0.14 b	0.06 ± 0.00 a
Control	0.071 ± 0.001 a	259 ± 0 a	9.90 ± 0.09 a	0.07 ± 0.01 a
Fludioxonil, 2.5	0.074 ± 0.002 ab	259 ± 0 a	9.76 ± 0.15 a	0.10 ± 0.02 a
Fludioxonil, 5.0	0.075 ± 0.002 b	259 ± 0 a	9.87 ± 0.14 a	0.07 ± 0.02 a
Control	0.071 ± 0.001 a	259 ± 0 a	9.90 ± 0.09 a	0.07 ± 0.01 a
Fenbuconazole, 0.2	0.065 ± 0.001 b	318 ± 9 b	9.94 ± 0.13 a	2.40 ± 1.19 b
Fenbuconazole, 0.4	0.061 ± 0.001 c	322 ± 13 b	9.41 ± 0.06 b	7.72 ± 0.99 c

[a] Values expressed as mean ± standard deviation when applicable
[b] Values marked with different letters are significantly different from the control within a specified fungicide treatment (ANOVA w/ Tukey's HSD)
[c] Fungicide concentrations are expressed in mg L^{-1}

Table 3 – Fermentation rate, fermentation duration, total CO_2 production and residual sugar for interactive fermentations of fenbuconazole and yeast assimilable nitrogen [a] [b]

| YAN [c]	Fenbuconazole concentration [c]	Fermentation rate (g CO_2/hr)	Fermentation duration (hr)	Total CO_2 production (g)	Residual sugar (g/L)
	0.0	0.071 ± 0.001 a	259 ± 0 a	9.90 ± 0.09 a	0.07 ± 0.01 a
78	0.2	0.065 ± 0.001 b	318 ± 9 b	9.94 ± 0.13 a	2.40 ± 1.19 b
	0.4	0.061 ± 0.001 c	322 ± 13 b	9.41 ± 0.06 b	7.72 ± 0.99 c
	0.0	0.141 ± 0.000 a	160 ± 4 a	9.69 ± 0.12 a	0.07 ± 0.03 a
153	0.2	0.131 ± 0.003 ab	181 ± 0 b	9.64 ± 0.10 a	0.53 ± 0.15 b
	0.4	0.128 ± 0.003 b	189 ± 0 b	9.63 ± 0.13 a	0.97 ± 0.27 c
	0.0	0.163 ± 0.001 a	141 ± 0 a	9.90 ± 0.12 a	0.01 ± 0.00 a
253	0.2	0.146 ± 0.004 a	152 ± 10 a	9.74 ± 0.09 a	0.05 ± 0.02 a
	0.4	0.144 ± 0.005 a	152 ± 10 a	9.82 ± 0.05 a	0.13 ± 0.01 a

[a] Values expressed as mean ± standard deviation

[b] Values marked with different letters are significantly different from the control within a specified fungicide treatment (ANOVA w/ Tukey's HSD)

[c] Concentrations are expressed in mg L^{-1}
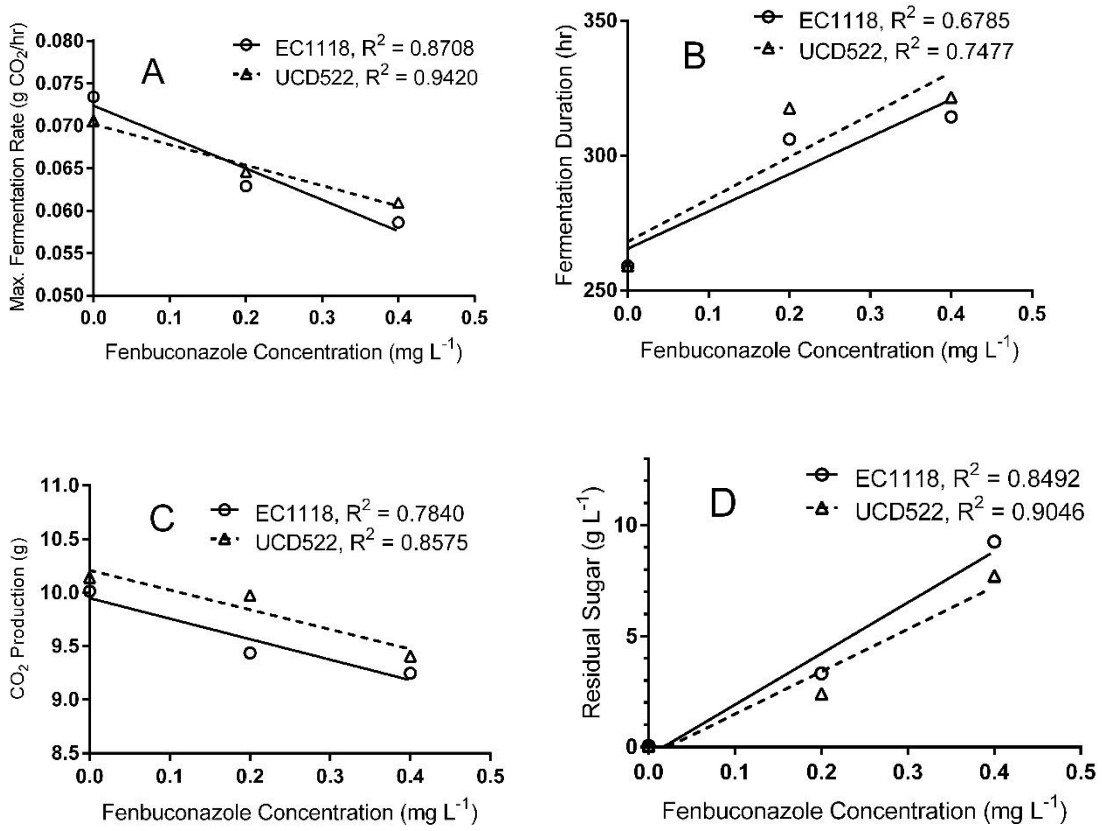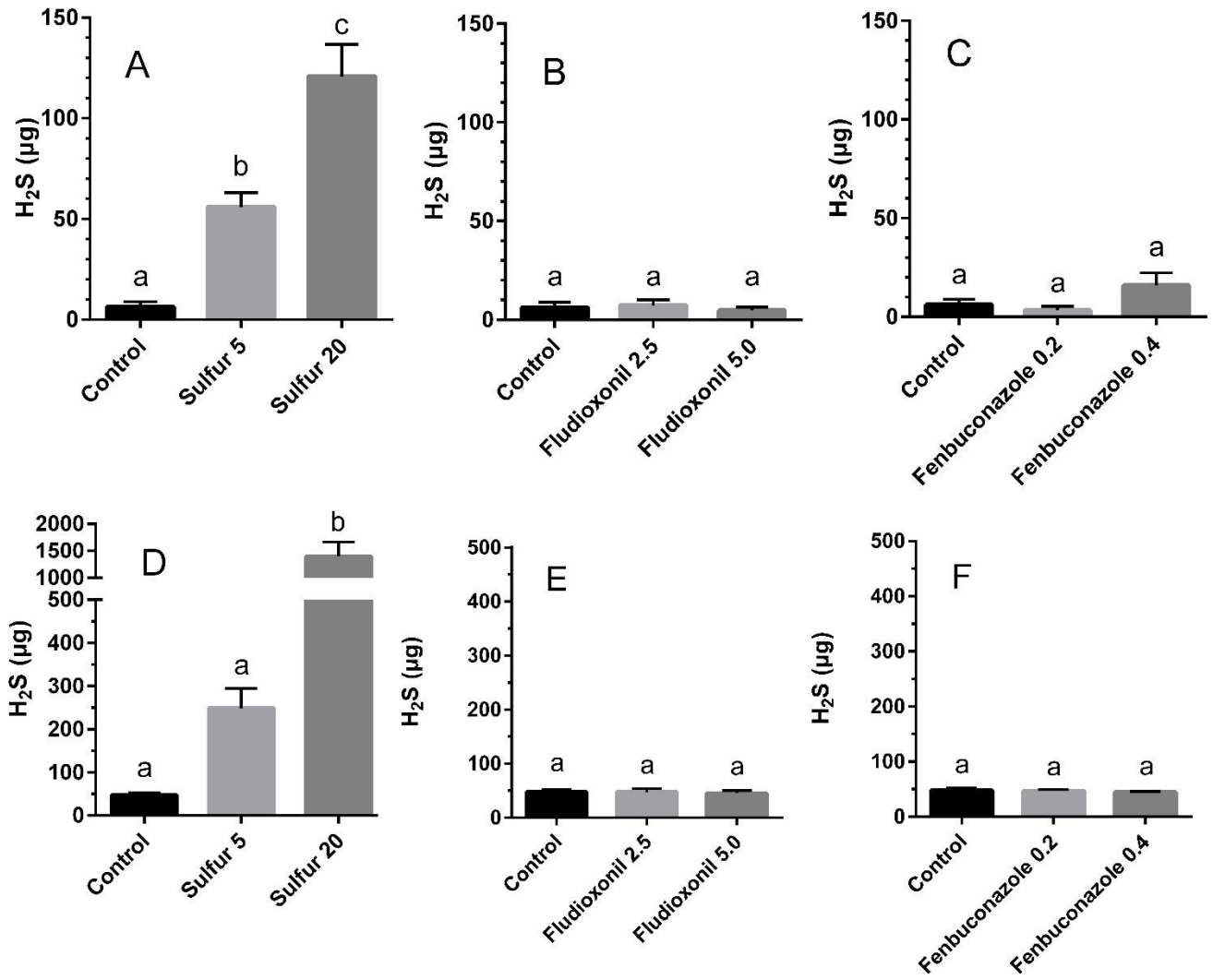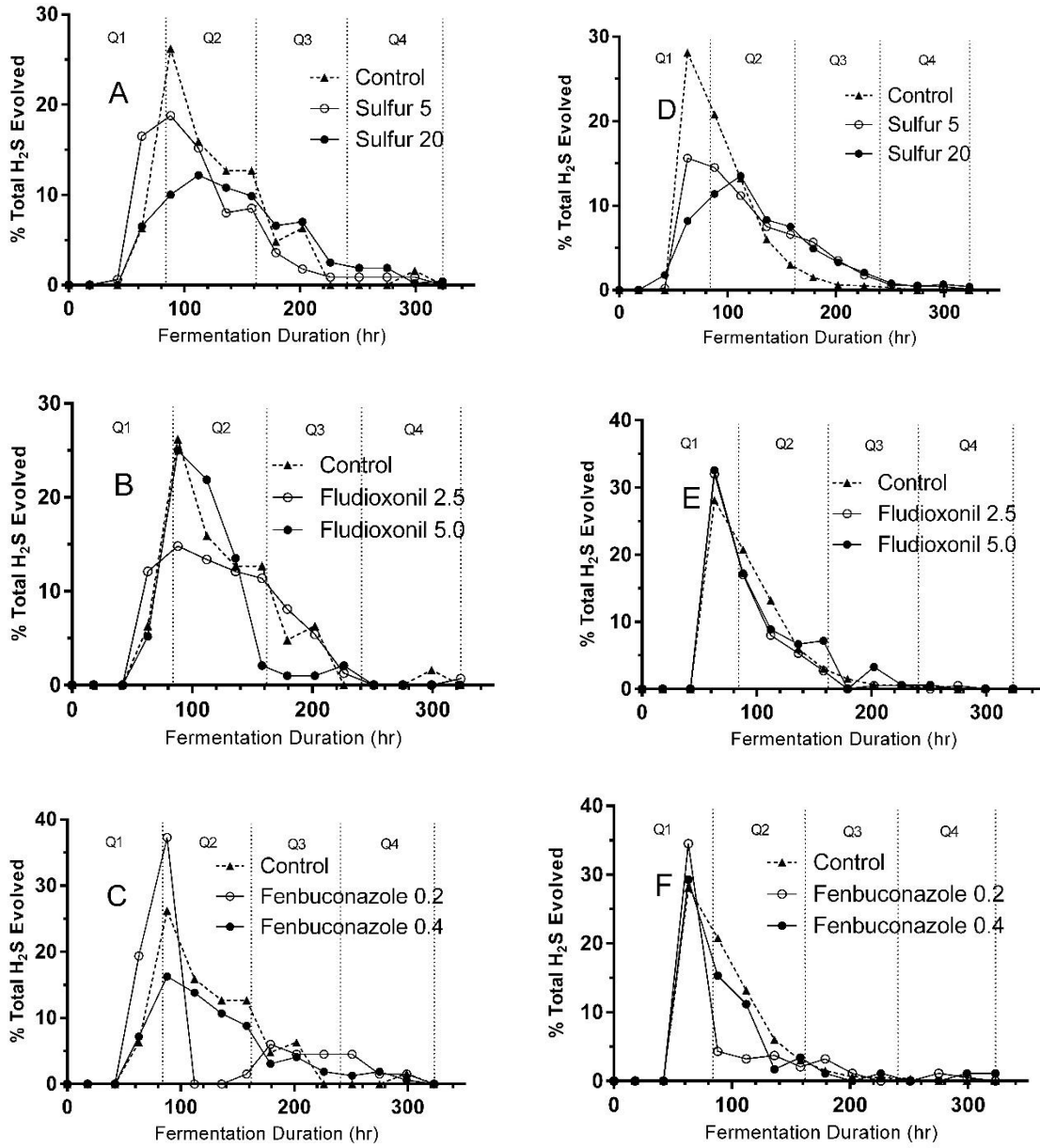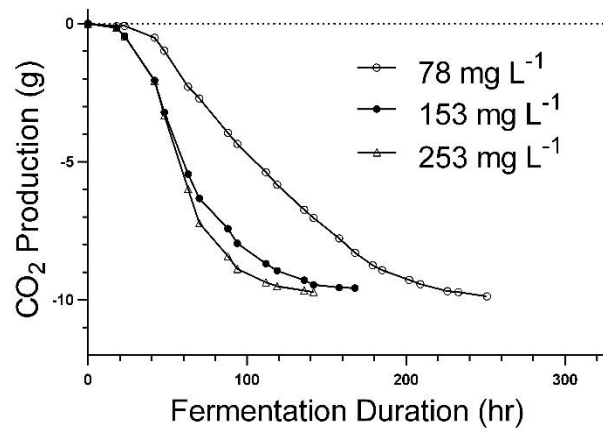
Figures

Figure 1.
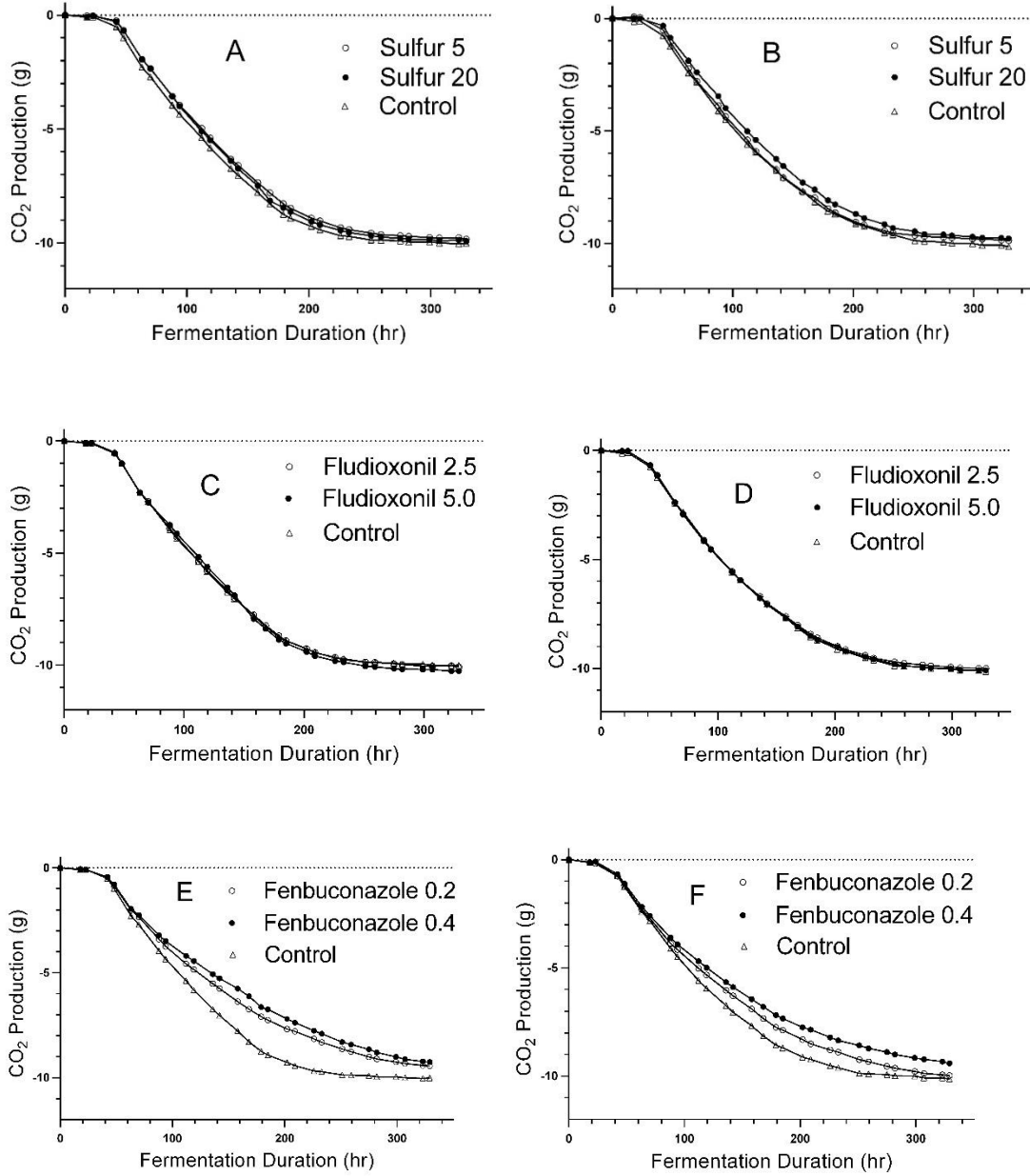
Figure 2

Figure 3

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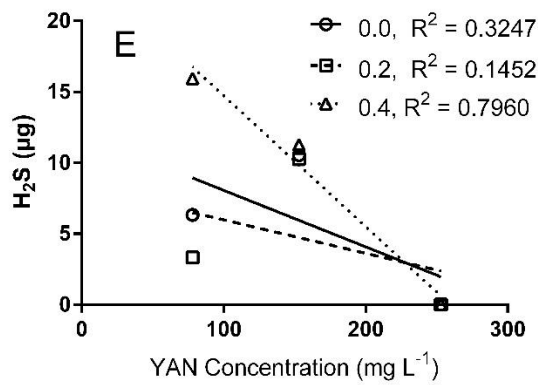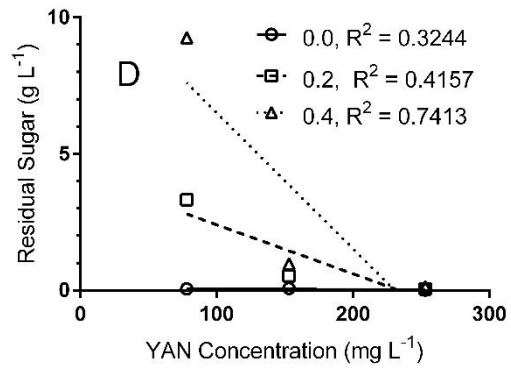
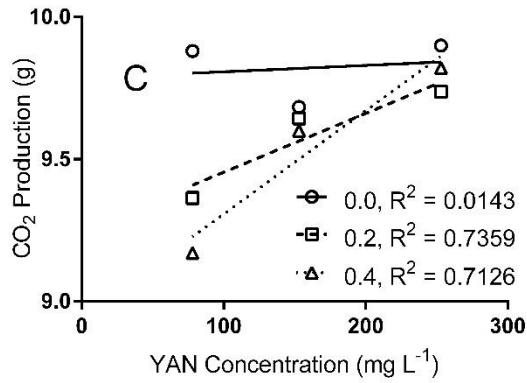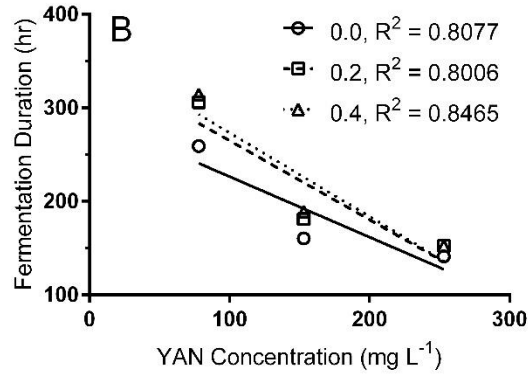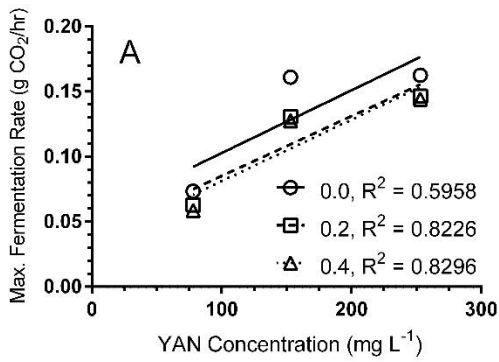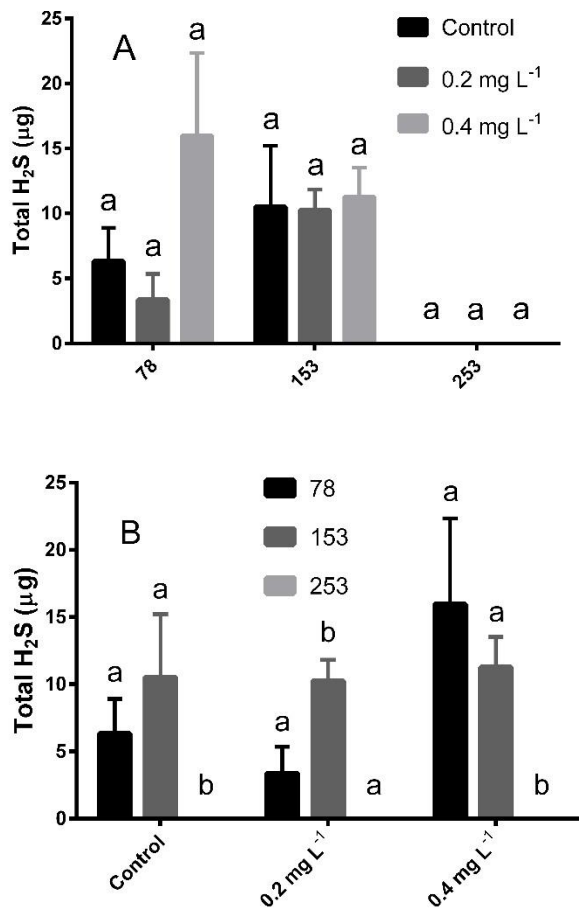**Figure 7**

Chapter 7: Summary and Conclusions

The results of this study provide valuable information regarding the complex role of yeast assimilable nitrogen (YAN) concentration and composition and interactions of YAN and fungicide residues in cider fermentations. Our findings will contribute to the development of targeted cider fermentation strategies based on fruit chemistry, orchard management practices and fermentation conditions.

The survey of 12 Virginia cider apple cultivars identified that the vast majority of apples surveyed were considered deficient in YAN. The generally low YAN concentrations, regardless of cultivar analyzed or harvest season, indicate that cider makers in the mid-Atlantic region should be aware of the high potential for low YAN status in their fruit, and the possible adverse fermentation conditions which may arise due to low YAN. The finding that the majority of apple juice YAN is composed of FAN with ammonium making up a very minimal proportion of total YAN provides useful insights. The correlation we observed between FAN and YAN suggests that cider makers may be able to quantify FAN alone to accurately quantify total juice YAN, saving laboratory time and expenses involved in measuring ammonia. The exceedingly low ammonium concentrations found in apple samples may also affect fermentation dynamics, as may be suggested by previous research in wine. Further research is needed to determine the proportionate concentration of ammonium in YAN in a wider range of cultivars and geographic regions and the impact of low ammonium concentrations on fermentation dynamics and cider flavor.

The study on interaction of amino acid composition and total YAN elicited several significant findings. Additions of methionine to supplement apple juice to methionine concentrations more typical of grape juice resulted in decreased H_2S production, even at very low methionine concentrations. However, this effect was diluted and eliminated at higher

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concentrations of total YAN. Nevertheless, the impact of methionine on fermentation health and cider aroma presents an interesting perspective on how differences between apple and grape juice chemistry, namely methionine concentration, may contribute to higher prevalence of H_2S production during cider fermentation as compared to white wine fermentation. These insights can help identify ideal juice composition and guide the design of targeted yeast nutrient supplements for cider fermentations. There was no difference in H_2S production or fermentation kinetics between fermentations where total YAN was rich in arginine (approximating grape juice), asparagine (apple juice with high endogenous YAN), and ammonium (apple juice supplemented with YAN). This dispels the notion that natural differences in primary YAN sources may differentially affect fermentation kinetics and H_2S production, especially in cider as opposed to wine fermentations. Further research is necessary to identify how juice YAN composition may impact flavor formation during fermentation and cider sensory characteristics.

Finally, fungicide residues play an important role in cider fermentations. Fludioxonil residues did not affect fermentation kinetics or H_2S production. As predicted, residual elemental sulfur (the positive control in this experiment) led to greatly increased H_2S formation during cider fermentation, which is in agreement with the results of previous research on elemental sulfur and H_2S production in wine fermentations. Fludioxonil is a fungicide used in post-harvest storage applications of apples prior to maceration and pressing for fermentation. These results indicate that fludioxonil residues do not adversely affect fermentations and hence are not of great concern to cider makers. However, fenbuconazole residues are detrimental to fermentation kinetics, leading to slower fermentation rates and higher residual sugar concentrations. This effect was minimized with increasing total YAN concentration. Therefore, cider makers and orchard managers should be aware of possible fungicide residues which may contribute to adverse fermentation conditions,

especially at low YAN concentrations which are typical to cider apple chemistry. Further research is needed to determine the prevalence of fungicide residues on apples used for cidermaking, as well as investigate the effects of other residual fungicides typically found on cider apples to determine the risk of negative fermentation performance.

Taken together, the results of this study provide valuable information relating juice chemistry to cider quality. Understanding the impact of total YAN, amino acid composition, fungicide residues and the interactions thereof on fermentation performance and cider quality is essential for developing research-based fermentation management strategies for production of consistent high-quality cider. Furthermore, our finding that supplementation with relatively small concentrations of methionine can decrease H_2S production can inform the design of targeted yeast nutrient supplements for cidermaking. In summary, white winemaking practices are an excellent starting point for development of cidermaking practices, however seemingly subtle differences in juice chemistry indeed necessitate the continued development of targeted practices designed specifically for cider fermentation.

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Supplemental Material

VirginiaTech

MEMORANDUM

DATE: June 5, 2015

TO: Amanda C Stewart, Thomas Francis Boudreau IV

FROM: Virginia Tech Institutional Review Board (FWA00000572, expires April 25, 2018)

PROTOCOL TITLE: Determination of the impact of nitrogen and fungucide residues on the formation of hydrogen sulfide in hard cider fermentations

IRB NUMBER: 15-559

Effective June 5, 2015, the Virginia Tech Institution Review Board (IRB) Chair, David M Moore, approved the New Application request for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report within 5 business days to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

All investigators (listed above) are required to comply with the researcher requirements outlined at:

http://www.irb.vt.edu/pages/responsibilities.htm

(Please review responsibilities before the commencement of your research.)

PROTOCOL INFORMATION:

Approved As: **Exempt, under 45 CFR 46.110 category(ies) 2,6**
Protocol Approval Date: **June 5, 2015**
Protocol Expiration Date: **N/A**
Continuing Review Due Date*:   **N/A**

*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

FEDERALLY FUNDED RESEARCH REQUIREMENTS:

Per federal regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals/work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee.

The table on the following page indicates whether grant proposals are related to this IRB protocol, and which of the listed proposals, if any, have been compared to this IRB protocol, if required.

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