Impact of Yeast Nutrient Supplementation Strategies on Hydrogen Sulfide Production during Cider Fermentation

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ACADEMIC ABSTRACT

Hydrogen Sulfide (H_2S), is a negative off aroma produced during yeast fermentation and is common in cider and leads to consumer rejection. H_2S has a very low odor detection threshold (ODT) and is often described as “rotten egg”. H_2S is produced when juice is deficient in yeast nutrients, such as amino acids and yeast assimilable nitrogen (YAN), which is a common problem in apples since they naturally low in nutrients. The purpose of this research was to investigate the effects of yeast nutrient addition to cider fermentation by adding four different nitrogen-rich supplements and evaluating the effects on H_2S production, fermentation kinetics, and aroma quality during cider. Three yeast strains (M2, EC1118 and ICV OKAY), four yeast nutrients (Fermaid K, Fermaid O, Experimental Nutrient, and DAP) and single addition versus split addition of nutrient were tested. For single addition, all nutrient was added pre-fermentation and for split additions, the first addition was pre-fermentation and the second at one-third total soluble solid (TTS) depletion as measured by °Brix. Sensory evaluation was conducted on selected treatments. The greatest H_2S was produced by M2 yeast strain (525 .63 ± 53.31 µg mL^{-1}) while the least H_2S on average was produced by EC1118 (118.26 ± 26.33 µg mL^{-1}) and ICV OKAY produced an intermediate amount of H_2S (209.26 ± 31.63 µg mL^{-1}). Significant differences were observed between treatments and total H_2S production within yeast strains. Yeast strain had the largest effect on H_2S production. The second largest effect was yeast nutrient type. Classical text analysis of descriptions of cider aroma were evaluated and 25 attributes were chosen to describe the ciders. Check- all-that-apply (CATA), a rapid sensory

technique that askes panelists, revealed that there was no clear pattern between variables tested. This work demonstrates that yeast nutrient type and yeast strain affect H_2S production during cider fermentation. These findings provide a basis for improving the effectiveness of strategies used to prevent H_2S production in cider fermentation.

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GENERAL AUDIENCE ABSTRACT

Cider, an alcoholic beverage made from fermenting apple juice, has grown in popularity and production in the United States in recent years. With increased in production and sales there is increase demand for high quality cider, but cider is prone to sensory faults. A common fault in cider aroma includes negative off aromas know as volatile sulfur compounds (VSCs). These aromas are often described as “rotten eggs”, or “cabbage” and lead to consumer rejection of the product. One of the most recognized VSCs is hydrogen sulfide (H_2S) which has a characteristic smell of “rotten eggs”. These negative off aromas are thought to be produced during yeast fermentation under nutrient lacking conditions. Apples, depending on cultivar, ripeness, and other factors, naturally lack yeast assimilable nitrogen, vitamins, amino acids, and other nutrients needed for a successful yeast fermentation leading to off aromas. Yeast nutrients can be added to apple juice to increase nutrient availability, but little research has been focused on nutrient addition and timing of additions to prevent H_2S production in cider. Most research focused on H_2S production has been studied in wine must or grape juice. This knowledge may be limited when applying practices to apple juice due to differences in juice chemistry. Providing cider makers with specific scientific strategies to prevent off aromas, such as H_2S, is important to the continued growth of the cider industry. This research is focused on exploring aroma quality and H_2S prevention strategies in cider by evaluating how yeast nutrient addition via four exogenous nitrogen rich yeast nutrient and timing of yeast nutrient addition affect H_2S production, fermentation kinetics, and consumer perception of aroma in cider fermentation.

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Chapter 1: Introduction

Cider, a fermented beverage made from apple juice, is a popular drink around the world. Cider is rapidly growing in popularity in the United States and is a well-established alcoholic beverage in Europe (A. Lea & Drilleau, 2003). In the United States in 2018, there were 877 operating cider producers located in 49 states (Lombardo, 2018). With the increased consumption of cider, there is also an increase in demand for high quality cider. In addition to the marked effect of Prohibition in the United States, past decreases in cider sales volume have been attributed to poor product quality, with the vast majority of ciders being considered "scrumpy" and a source of cheap alcohol for poor farmers (Bamforth, 2005; A. Lea & Drilleau, 2003). Poor quality ciders are often a result of off flavors and aromas. The most commonly known and widely recognized off aromas are hydrogen sulfide (H_2S) and other volatile sulfur compounds (VSCs) such as dimethyl sulfide, furfurylthiol, and methanethiol (Marchand, Gilles de, & Bertrand, 2000). These VSCs are typically described as "rotten eggs", "cabbage" or "reductive" aromas.

The process of cider making is closely related to white wine making, but there are key differences in fruit chemistry between apples and grapes, making it necessary to research apples and cider processing independently. Unlike grapes, apples lack key nutrients needed for yeast metabolism (A. Lea & Drilleau, 2003). Apple chemical composition differs from grapes in carbohydrates, organic acids, amino acids, minerals, polyphenols and yeast assimilable nitrogen (ammonium ions and primary amino nitrogen), all of which can effect fermentation outcomes (Downing, 1989; Guyot, Marnet, Sanoner, & Drilleau, 2003). The chemical composition of both apples and grapes are variable and depend on many factors including cultivar, ripeness, climate, and agricultural practices. Nonetheless, the differences between the category apple and grape are

more significant than differences observed within each category (Downing, 1989; Guyot et al., 2003). The primary organic acid found in apples is malic acid while the primary acid in grapes is tartaric acid (Lamikanra, Inyang, & Leong, 1995; Wu et al., 2007). They also differ in polyphenol composition. Apples have six predominant classes of polyphenols which include flavanols, procyanidins, hydroxycinnamic acids, dihydrochalcones, flavonols, and anthocyanins while in grapes the five main classes of polyphenols are anthocyanins, flavanols, flavonols, stilbenes, and phenolic acids (Verdu et al., 2014; Xia, Deng, Guo, & Li, 2010). Preliminary data also suggests that grapes have different amino acid compositions and yeast assimilable nitrogen (YAN) concentrations than apples (S. Ma et al., 2018; Spayd & Andersen-Bagge, 1996). All of these factors may influence fermentation outcomes (Lamikanra et al., 1995; A. Lea & Drilleau, 2003; Wu et al., 2007; Xia et al., 2010). Wine fermentation (i.e. fermentation of grape juice or must) has been the subject of decades of research, but limited research reports on fermentation of apple juice and cider quality exist. With the increase in cider popularity and production, it is important to have research focused on apple juice fermentation as opposed to extrapolating research knowledge from grape must, considering the differences in juice chemistry and the potential of these differences to affect fermentation outcomes.

1.1 Long Term Objective

The long term objective of this research project is to develop and test yeast nutrition strategies for cider fermentation, focusing on identifying nutrient source and timing protocols for specific combinations of juice or concentrate matrices and yeast strains.

1.2 Overall Objective and Hypothesis

The overall objective of this project is to examine and understand the relationship between yeast strain (high vs. medium vs. low hydrogen sulfide producing strains), nitrogen

supplementation via complex yeast nutrient addition, and timing of nitrogen supplementation additions on hydrogen sulfide production (H_2S) during cider fermentation in a juice-from-concentrate matrix.

1.3 Specific Objectives and Hypothesis

Specific Objective 1:

Determine how pre-fermentation addition of a yeast nutrient (Fermaid K, Fermaid C, Fermaid O, or Diammonium Phosphate) influence fermentation duration and total hydrogen sulfide production by three yeast strains (M2, EC1118, ICV OKAY)

Working Hypothesis 1:

Addition of a yeast nutrient (Fermaid K, Fermaid C, Fermaid O, or Diammonium Phosphate) pre-fermentation will decrease fermentation duration and decrease total hydrogen sulfide production, and this effect will vary depending on yeast strain (M2, EC1118 and ICV OKAY).

Specific Objective 2:

Determine whether adding a yeast nutrient (Fermaid K, Fermaid C, Fermaid O, or Diammonium Phosphate) in two steps (rather than adding all nutrients at one time, pre-fermentation) influences fermentation duration and total hydrogen sulfide production based on three different yeast strains (M2, EC1118 and ICV OKAY).

Working Hypothesis 2:

Two step nutrient addition for a specific yeast nutrient (Fermaid K, Fermaid C, Fermaid O, or Diammonium Phosphate) will decrease fermentation duration and decrease overall total H_2S produced depending on yeast strain (M2, EC1118, ICV OKAY).

Chapter 2: Literature Review

2.1 Cider Definition and Production in the United States

In the United States, the term "cider" may refer to several different beverages made from apple juice. In the United States, the term "cider" may be used to refer to unfiltered, unpasteurized non-alcoholic apple juice (i.e. "sweet cider") whereas the term "hard cider" refers to a fermented apple product. In Europe, the fermented product is simply referred to as "cider" (A. Lea & Drilleau, 2003). In this document, the term "cider" will be used to refer to the fermented, alcoholic beverage made from apples.

The popularity of cider in the United States has increased over the past decade. The number of cider makers drastically increased from 400 in 2014 to a total of 877 cider producers in 2018 (Lombardo, 2018). In the United States in 2018, cider profits reached $23.5 million with an annual growth rate of 19.8% between 2013 and 2018. The cider category in the United States is predicted to continue to grow at an Annual Growth Rate of 2.0% between years 2018 and 2023. There are now cider producers in almost every state in the U.S, with the largest numbers of producers found in New York followed by Michigan and California (Lombardo, 2018). With the continuing increase in popularity in the cider category, additional research focused on cider production methods and the chemical and sensory characteristics of cider is needed.

2.1.1 Cider Production

Cider, like wine, can be produced using a wide variety of techniques and practices chosen by the cider maker to produce the desired finished product. There is high variability between producers that depends on agriculture practices, apple cultivars, juice chemistry, fermentation practices, and multiple other factors, some of which will be discussed further in this review.

Several, processing steps are needed for cider production: harvesting apples, milling, pressing, and fermentation. Apples are often harvested at peak ripeness to ensure high sugar content in the fruit. Peak ripeness can vary between cultivar, region and producer but some have suggested that dessert or eating apples should be picked when 9.0%-12.0%_total soluble solids (TSS) (Blanpied & Kenneth, 1992; Mohebi, Babalar, Askari, & Talaei, 2017). Apples can be harvested in different manners depending on the size of the cider producer or apple orchard. Some large producers may use mechanical harvesters such as limb shakers and air blasters, while smaller producers hand pick apples (P. Li, Lee, & Hsu, 2011). A common harvesting practice for certain cultivars in Europe is to allow apples to fall off the tree and be harvested from the ground, instead of being picked of the branches to ensure that the fruit is ripe (A. Lea & Drilleau, 2003). However this practice in the United States may not be legal under the new Food Safety Modernization Act due to food safety concerns (Ewing & Rasco, 2019).

After harvest, fruit can be stored for several weeks in refrigeration to allow more unfermentable sugars (starch) to be converted to fermentable sugars. Postharvest storage can be used as method to potentially increase polyphenols for certain cultivars (Ewing, Peck, Ma, Neilson, & Stewart, 2019; A. Lea & Drilleau, 2003). Apples may also be pressed immediately after harvest with no storage period. A good practice after harvest is to sorted and eliminate rotten or moldy apples and then wash apples to reduce unwanted microbial hazards such as patulin, a mycotoxin

produced by mold, but this is not always done (Acar, Gökmen, & Taydas, 1998; A. Lea & Drilleau, 2003). Once the fruit is ready to be juiced, apples can be milled to reduce the size of the apple particles prior to pressing. Size reduction increases juice yield and ease of pressing. Milling produces a pulp which is then pressed to yield apple juice (A. Lea, 2015; A. Lea & Drilleau, 2003). Different kinds of presses may be used including basket press, bladder press, rack, and cloth press and among many other variations of presses. Pressing aids such as rice hulls or cotton hulls can be used to increase drainage of juice when pulp is too milled or naturally too soft which can be a problem when pressing dessert apples (A. Lea, 2015). Juice can then be clarified using the enzyme pectinase to remove solids and sometimes is clarified further through filtration to produce a clear juice. Juice is then fermented using *Saccharomyces cerevisiae* or allowed to ferment "naturally" with yeast and bacteria present from the fruit itself or the environment (this process can be called a wild, native or ambient fermentation) (A. Lea & Drilleau, 2003). Commercial yeast strains are readily available and commonly used to inoculate cider. Different commercial yeast strains are available due to their different nutrient needs, fermentation rates and have been known to have an effect on many cider attributes (Fairbairn, McKinnon, Musarurwa, Ferreira, & Bauer, 2017; Giudici & Kunkee, 1994; Manginot, Roustan, & Sablayrolles, 1998). After fermentation, cider is separated from dead yeast (lees) and the final cider can be stored until it is ready to be consumed.

2.2 Starting Material for Cider Fermentation

The starting raw material for cider fermentation is apple juice. This juice can be obtained from fresh pressed apples or from apple juice concentrate (AJC). It may be blended from a variety of sources and may have been treated physically or chemically prior to fermentation.

For certain cider styles, juice from cider apple cultivars is highly desired to achieve an optimal balance of sugar, tannins, and acids (Bamforth, 2005; A. Lea & Drilleau, 2003). Cider

apples are grouped in four different categories, as determined by their chemical composition: sharp, bittersharp, bittersweet, and sweet. Bittersharps and bittersweets are particularly important for cider due to their tannin compositions and acid content. Some cider apples cultivars, compared to dessert or culinary apples, may have a high fibrous structure that makes pressing apples easier (Guillermin et al., 2006; A. Lea & Drilleau, 2003). Table 1 illustrates the two major chemical components that determine these four traditional cider apple classifications: tannin content and acid content. Tannins are critical for overall mouthfeel and body of the cider by contributing to bitterness and astringency in the finished cider (Bamforth, 2005; A. Lea, 2015; A. Lea & Drilleau, 2003). Blending of different cultivars is common to achieve the desired balance of acids and tannins (A. Lea, 2015; A. Lea & Drilleau, 2003). It is also common to blend cider apple juice with non-cider apple cultivars such as dessert apples or with AJC to extend juice volume and reduce cost compared to cider apples alone, which are higher value products. Dessert apples, such as Granny Smith, Golden Delicious, and Honeycrisp, are more commonly grown and in higher supply than the traditional cider cultivars, especially in the US. AJC is relatively inexpensive ("Milne Fruit Apple Juice Concentrate," n.d.; "Tree Top Apple Juice Concentrate," n.d.), widely available year-round, and can be stored for long periods of time before use (Bamforth, 2005; A. Lea, 2015; A. Lea & Drilleau, 2003).

Table 3: Classification of Cider Apples Based on Tannin and Acid Content

Type of Apple	Tannin content (%)	Acid Content (%)	Example
Bittersharp	>0.2	>0.45	Backwell Red,
Bittersweet	>0.2	<0.45	Dabinett
Sharp	<0.2	>0.45	Brown's Apple
Sweet	<0.2	<0.45	Northwood

(A. Lea, 2015)

Apple juice concentration is the process of removing water from apple juice. This can be achieved in various ways, including by thermal evaporation, membrane separation (reverse osmosis, nanofiltration and other methods), freeze drying, filtration, distillation, vacuum evaporation, or drying by clathrate hydrates to decrease weight and volume and increase storage capabilities of juice (Adnan, Mushtaq, & Islam, 2017; Downing, 1989; Jiao, Cassano, & Drioli, 2004). Apple juice is usually concentrated five to seven times depending on the concentration method and desired final sugar concentration. Most apple juices are concentrated from 11°Brix to 70°Brix (Adnan et al., 2017). Juice must be clarified before concentration to avoid more difficult filtering post concentration (Adnan et al., 2017; A. G. H. Lea, 1995). During processing, especially thermal processing and during storage above 5°C, vitamins, minerals, antioxidants, flavors, and aromas can be easily degraded (Downing, 1989; A. G. H. Lea, 1995; Polydera, Stoforos, & Taoukis, 2003). These are clear disadvantages of using AJC for cider production, compared to fresh juice.

Amino acids are also consumed during Maillard browning which does not occur in fresh apple juice. Maillard browning occurs when food products are heated, especially during apple juice processing, concentration and storage. During thermal concentration methods, Maillard browning (non-enzymatic browning) occurs when reducing sugars and amino acids in juice are heated in the presence of oxygen. Essential amino acids are consumed during Maillard browning and as a result Maillard by-products such 5-hydroxymethyl furfural (HMF) and furfural are produced (Camire et al., 1990; Martins, Jongen, & Boekel, 2001). Furfural has been shown to inhibit yeast, such as *Saccharomyces cerevisiae*, and inhibits growth and alcohol production (Banerjee, Bhatnagar, & Viswanathan, 1981b, 1981a; Sanchez & Bautista, 1988). HMF is an indicator of nutrient loss

(amino acids and sugars) in AJC and may also have slight inhibitory effects on yeast fermentation (A. G. H. Lea, 1995; Taherzadeh, Gustafsson, Niklasson, & Lidén, 2000).

Monitoring levels of HMF and color indexes of AJC are commonly used as an indicator for quality in apple juice. HMF can represent the presence of other inhibitory Maillard products and amino acid consumption (Bengoechea et al., 1997; Z. Li et al., 2019). HMF continues to be formed during AJC storage if not properly stored below 5°C (A. Lea & Drilleau, 2003). Babsky (1986) observed a significant loss of amino acids during AJC storage at 35°C. Glutamic acid were decreased from 57.6 mg $100g^{-1}$ to 1.5 mg $100g^{-1}$, asparagine were depleted from 296.8 mg 100 ml^{-1} to 28.7mg 100 ml^{-1} and glutamine decreased from 296.8 mg 100 ml^{-1} to trace amounts. As a result of the amino acid consumption, a total of 44 mg 100 mL^{-1} of HMF was reported when AJC was stored for 110 days (Babsky, Toribio, & Lozano, 1986). Temperature of storage also effects how amino acids are degraded during storage. Beudo (2000) found in peach juice concentrate storage at 15°C, 30°C and 36°Cover 42 days, decreased total amino acid content by 8%, 35% and 60% respectively (Buedo, Elustondo, & Urbicain, 2000). Although no know studies have addressed degradation of B vitamins in AJC processing, it is reasonable to believe that B vitamins, that are essential to yeast fermentation, could be degraded during heat processing (Edwards & Bohlscheid, 2007). Further research needs to be conducted to elucidate amino acids and vitamin degradation in AJC so knowledge can be applied to cider making and the interactive effects of H_2S production.

Total HMF concentration in AJC has been investigate. Bengoechea (1997), observed a range of HMF from 0.38 to 2.48 mg L^{-1} in ten different store bought AJC along with other Maillard browning by-products (Bengoechea et al., 1997). In a newer study Kus (2005), found that a store bought AJC had 4.5mg^{-1}L of HMF which was significantly higher than other concentrates observed (peach, strawberry, cherry and orange) (Kus, Gogus, & Eren, 2005). Burdurlu (2003),

reported increased HMF concentration in AJC with increased storage temperatures and TSS measured as °Brixvalues after 4 months of storage, demonstrating that storage temperature and brix value of AJC can effect HMF concentration (Selen Burdurlu & Karadeniz, 2003). In AJC, the European Fruit Juice Association recommend a maximin level of 20 mg L^{-1} of HMF due to its cancerogenic effects in humans (Z. Li et al., 2019). All the above reported values are below this recommend limit for human health but there is no recommendation for cider making or yeast fermentation. It has been suggested that improvements in concentration technologies such as vacuum concentration can limit Maillard reactions to less than 20 ppm, while older concentration methods like thermal evaporation have HMF ranges from 100 ppm to 700 pm (A. Lea & Drilleau, 2003; A. G. H. Lea, 1995).

Another by-product from Maillard browning, furfural, has been shown to inhibit yeast growth and alcohol production in *Saccharomyces cerevisiae* (Banerjee et al., 1981b, 1981a; Sanchez & Bautista, 1988). Banerjee (1981) found that furfural above 1mg mL^{-1} decreased CO_2 production in *S. cerevisiae* and Sanchez (1987) observed inhibition effects of furfural at 1.5mg mL^{-1} and complete inhibition above 2mg mL^{-1} in *S. cerevisiae* (Banerjee et al., 1981b; Sanchez & Bautista, 1988). Limited studies show amount of furfural in apple juice concentrate but one study suggested concentrations of 20 ppb in AJC (A. Lea & Drilleau, 2003). HMF has also been studied for inhibition effect on yeast growth. Sanchez (1988), reported a prolonged lag phase in yeast fermentation with 2 g L^{-1} of HMF but complete inhibition was not observed. Pfeife (1984), reported that concentrations of 5 mg mL^{-1} of HMF showed little change in glucose conversion in *Saccharomyces carlsbergensis* W34 (Pfeife, Bonn, & Bobletter, 1984). Although this paper studied a different yeast species, it demonstrates metabolic differences across yeast. More research

should be conducted on the inhibitory effects of Maillard products in AJC and levels of furfural in AJC in specific terms of cider fermentation.

Cider producers must consider storage time and temperature of storage when choosing AJC for these reasons. If the product is subjected to temperature abuse or very long periods of storage, HMF and furfural could be higher than originally thought. Even with observed advances in concentration technologies such as vacuum evaporation, that use less heat, the most common method of concentration currently used is thermal concentration evaporators (Adnan et al., 2017).

Maillard browning, consumption of amino acids and production of yeast inhibiters such as furfural, continue during storage, especially during incorrect storage and may result in an apple juice deficient in nutrients needed for yeast growth and an inhibitory medium for yeast growth. These compounding factors could all have negative consequences in cider production and cider aroma. These factors should be considered when using AJC as the base for cider making. Issues like production of furfural and HMF are not associated with fresh apple juice adding to problematic fermentations in AJC based fermentations (Banerjee et al., 1981b; A. Lea & Drilleau, 2003).

Despite these issues, AJC is extensively used in cider making for several reasons. AJC is sometimes chosen over fresh apple juice because it can be stored for long periods of time without apparent negative deterioration compared to fresh juice or fresh apples. AJC is convenient for Just-In-Time cider production businesses and year round operations when fresh apples are unavailable and there are limited apple producing trees (A. Lea & Drilleau, 2003). AJC is commonly made from a variety of apple cultivars that are not cider apples cultivar and some producers make cider solely from AJC. While most AJC are not made from 100% cider apples, it is more common for fresh juice made from dessert apples, culinary apples and cider apples to be reinforced with AJC to achieve ideal levels of tannins and acidity (A. Lea & Drilleau, 2003).

2.2.1 Pre-fermentation Additions and Treatments

Several different pre-fermentation juice treatments can be applied to alter the juice chemistry. These treatments can include altering the pH, acid concentration, sugar level, tannin level, and nutrient level. These pre-fermentation juice treatments are used to improve fermentation performance and/or create different sensory and taste outcomes.

Fermentable sugars in juice are converted into alcohols during yeast fermentation (Zoecklein, Fugelsang, Gump, & Nury, 1995). This is important to overall flavor and style of the beverage. Some producers may add sugar in the form of cane sugar or beet sugar to the apple juice or grape must prior to fermentation to get a higher percent alcohol, increase fermentation performance or achieve a specific style in the finished product (A. Lea, 2015). In grapes and apples, sugar accumulation occurs as the fruit maturation occurs (Jackson & Lambard, 1993). The total amount of sugar found in the fruit can be affected by growing temperatures, season length, agriculture practices, harvest time and many of other factors (Jackson & Lambard, 1993). Apples consist of three types of sugar: fructose, glucose and sucrose. Fructose is usually present in the highest concentrations followed by glucose, but sugar concentrations can vary greatly between cultivars (Fuleki, Pelayo, & Palabay, 1994; B. Ma et al., 2015; Wu et al., 2007). If the fruit does not have the desired level of sugar wine makers may add sugar to achieve desired final products.

Lowing the pH is another pre-fermentation treatment that can affect the fermentation. If the pH of a starting juice is above 3.6 it can become subject to unwanted microbial contamination and low quality taste (Boulton, Singleton, Bisson, & Kunkee, 1997; Jackson & Lambard, 1993; Kodur, 2011). To prevent this unwanted microbial growth, juice can be acidified to below 3.8 and sulfur dioxide (SO_2) can be added. SO_2 is added pre-fermentation to prevent unwanted microbial growth such as *Acetobactor aceti* or *Brettanomyces* species that are already present on the skins

of fruit (Zoecklein et al., 1995). Allowing SO_2 to stand overnight to 24 hours allows enough time to inactive any existing microbes and bacteria in the juice. Acidification is important for SO_2 addition because SO_2 is pH dependent and at a pH above 3.8, SO_2 may not be as effective against microbes (Deniz Bozoglu et al., 2015; Zoecklein et al., 1995).

Addition of tannins has become a widely accepted practice in wine making, especially with regards to red wine. A wide range of exogenous tannins are commercially available that are marketed to improve texture, perception of sweetness, color and ageing of wine or cider (García-Estévez, Alcalde-Eon, Puente, & Escribano-Bailón, 2017; "Scott Laboratories Cider Handbook," 2018). These tannins can be derived from a variety of sources including grapes, chestnuts and other wood or plant extracts (Sanz, Martínez-Castro, & Moreno-Arribas, 2008; "Scott Laboratories Cider Handbook," 2018). Some research has explored replacing the use of SO_2 with addition of tannins and lysozyme in grape must fermentations due to tannins antioxidant capacity and lysozymes ability to act on cell walls of unwanted microorganism that contribute to malolactic fermentation (Sonni, Chinnici, Natali, & Riponi, 2011). In this research, tannins are used to prevent oxidation of stored wine (Sonni, Cejudo Bastante, Chinnici, Natali, & Riponi, 2009). Other research has explored how tannins affect red wine color with some studies finding a positive increase in anthocyanin-derived colors and other seeing no effect on color (García-Estévez et al., 2017; Parker et al., 2007).(Parker et al., 2007)(Parker et al., 2007) This area remains a topic of current research, with conflicting results, notwithstanding both wine and cider producers may purchase and add commercially available exogenous tannins for these potential increases in positive attributes in the finished product.

Additional yeast nutrients, to increase total nitrogen available and other nutrients, are common pre-fermentation additions. A variety of yeast nutrients are commercially available,

including Diammonium Phosphate (DAP) which consists of only inorganic nitrogen, and inactive dry yeast nutrient (IDY) which consist of varying nutrients such as amino acids, thiamine and ammonia ions. Addition of IDY products and DAP have the potential to increase fermentation rate, increase completeness of sugar consumption and retention of aroma compounds but increasing total YAN concentrations and available nutrients (Bell & Henschke, 2005).

2.2.2 Negative Sensory Attributes of Hydrogen Sulfide

The transformation of fruit juice into an alcoholic beverage such as cider or wine is possible due to alcoholic fermentation by yeast strains that convert fermentable sugars into ethanol, gases, and acids. The most important yeast in beverage fermentation is *Saccharomyces cerevisiae* (Swiegers & Pretorius, 2005). During yeast metabolism, byproducts (other than ethanol and CO_2) are produced that provide aroma and flavor in the end product. The pathways by which these flavor-active compounds are produced are influenced by many factors, including but not limited to available nutrients in the juice, aroma precursors in the juice, and fermentation temperature and rate. Common negative byproducts of yeast metabolism during alcoholic fermentation are VSCs such as dimethyl sulfide, furfurylthiol, methanethiol, and H_2S. VSCs are commonly descried as rotten eggs, decaying seaweed, or reductive taste and have very low sensory thresholds that often lead to poor product quality and even consumer rejection of the product (Marchand et al., 2000; Swiegers & Pretorius, 2005). In general, these VSCs have varying limits of detection in different matrixes (Mestres, Busto, & Guasch, 2000). Table 2 lists common VSCs formed during yeast fermentation in wine and the observed sensory thresholds in wine (Davis & Qian, 2011; Manginot et al., 1998; Swiegers & Pretorius, 2005). Similar VSCs can be expected in yeast fermentation in apple juice since there are similar fermentation methods

and yeast strains used in cider and wine making. However, there is still a gap in knowledge of factors influencing production of VSCs specifically in the cider matrix. Grape juice or must and apple juice differ in juice chemistry which can have a large effect on sensory outcomes. Sensory impacts of VSCs in cider could have a larger effect in cider matrix due to cider having relatively fewer complex aromas compared to the complexity of aroma in wine.

H_2S is the most commonly recognized VSC, generally considered as a negative sensory attribute. It is possible for humans to detect H_2S in extremely low concentrations, with a wide range of sensory thresholds reported, from 1ng L^{-1} to 105µg L^{-1} (Mestres et al., 2000). H_2S can also affect the perception of other positive aroma compounds in wine. A study by Franco-Luesmam (2016), found that when H_2S was present in concentrations ranging from 0-40 µg L^{-1}, decreased positive aroma attributes such as cooked, candied, and floral aromas and increased negative attributes such as rotten eggs and reductions aromas were reported (Franco-Luesma et al., 2016). With H_2S being a very common negative off aroma produced in wine and cider it is important to understand the mechanisms that contribute to its production such as agricultural crop treatments, yeast strain, nutrient additions, nutrient limitations, beverage processing, and storage.

Table 4: Volatile Sulfur Compounds Found in Wine

Compound Name	Aroma descriptor	Aroma Threshold in Wine ($\mu g\ L^{-1}$)
Hydrogen Sulfide	Rotten Egg	0.001 -150
Methanethiol	Rotten egg, cabbage	0.3-1.82
Ethanethiol	Onion, rubber	0.19-1.1
S-methyl thioacetate	Cheesy, onion, burnt sulfury	300
S-ethyl thioacetate	Cheesy, onion, burnt sulfury	40
Dimethyl sulfide	Quince, truffle, asparagus, corn	10-160
Diethyl disulfide	Cooked vegetables, onions, garlic	4.3-40
Dimethyl disulfide	Garlic, burnt rubber	20-45
Benzothiazole	Rubber	24-50
Thiazole	Popcorn, peanut	38
4-methylthiazole	Green hazelnut	55
Acetythiazole	Roasted hazelnut	3
2-furanmethanethiol	Roasted coffee, burnt rubber	1 ng L^{-1}
Thiophene-2-thiol	Burnt rubber, roasted coffee	0.8

Adapted and compiled from Swiegers 2005, *Yeast Modulation of Wine Flavor* and Davis and Qian 2011, *Progress on Volatile Sulfur Compound Analysis in Wine*.

2.2.3 Hydrogen Sulfide Quantification

In order to evaluate strategies for prevention of H_2S production during beverage fermentation, the H_2S must be measured during fermentation. H_2S has been measured using selective gas traps by trapping targeted gas and preforming colorimetric analysis to quantify H_2S (C. S. Thomas, Boulton, Silacci, & Gubler, 1993; Vos & Gray, 1978). This method is not ideal as it requires a significant amount of time, expensive materials, and toxic chemicals (Acree,

Sonoff, & Splittstoesser, 1971; Ugliano & Henschke, 2010). Another method to measure H_2S used paper strips coated with lead acetate or silver nitrate that darken when H_2S comes in contact with the strip (Natusch, Sewell, & Tanner, 1974). This method is less time-consuming but can only test for the presence of H_2S and cannot give quantitative concentrations of H_2S (Ugliano & Henschke, 2010). Until more recently quantifying H_2S was inconvenient and expensive until Park (2008) developed an easy, rapid, and convenient way to quantify H_2S with minimal cost using coulometric H_2S pre-calibrated detector tubes packed with silver nitrate or lead acetate that darken upon exposure to H_2S (Park, 2008). The tubes are commercially available, require minimal training to use, and are most often used in environmental safety monitoring. They are pre-calibrated to a known concentration of H_2S. Park ran a standardization study on the tubes and found a good linear relationship (R2=0.9997) between a known injected amount of H_2S and the reading on calibrated scale on the tube (Park, 2008). Using the same method Ugliano and Henschke (2009) found 95-98% recovery of known concentrations of H_2S and no interference from SO_2 and mercaptans (Ugliano & Henschke, 2010). Since then, H_2S detector tubes have been used in many studies to detect and quantify H_2S concentration.

H_2S tubes are convenient for measuring H_2S but there are drawbacks to this method including the precision of the method. Depending on the pre-calibrated scale on the tube, the marked intervals indicating H_2S concentration are large. For example, 120SF tubes are calibrated with intervals of 100 $\mu g\ mL^{-1}$. When the darkened silica gel is in between the intervals, concentration readings must be estimated and small increased from day to day may not be visible on this scale. Another downfall to using H_2S tubes is the need to be replace the tubes when the pre-calibrated scale has reached its maximum. To replace the tubes, the tube must be removed from the stopper and replaced with a new one. This could be a source of error, as H_2S and CO_2

gas may be lost from the head space. Although these draw backs exist, the H_2S tubes still provide relative and quantitative information for testing different treatments.

2.3 Yeast Strain and Hydrogen Sulfide

Saccharomyces cerevisiae is one of the most widely used yeast species for winemaking and cider making. Yeast strain is a major factor affecting total H_2S produced during fermentation (Manginot et al., 1998; C. S. Thomas et al., 1993; Ugliano et al., 2009). H_2S is formed as a metabolic intermediate during yeast metabolism as part of the sulfate reduction sequence (SRS). The SRS pathway is responsible for the biosynthesis of sulfur-containing amino acids needed for yeast growth. In matrices that lack sulfur-containing amino acids (methionine and cystine) the SRS pathway is triggered to meet metabolic requirements of cystine, methionine, and glutathione (Bell & Henschke, 2005). Metabolic requirements of sulfur-containing amino acids and the SRS pathway may vary by yeast strain which can contribute to the varying ranges of H_2S produced by different yeast strains (Fairbairn et al., 2017; Giudici & Kunkee, 1994).

Apple juice and grape juice commonly lack sulfur-containing amino acid concentrations needed and have high sulfate concentrations, activating the SRS which reduces sulfate to sulfide that will bond with *o*-acetyldserine or *o*-acetylhomoserine to form cystine and methionine respectively (Swiegers & Pretorius, 2005; Ugliano & Henschke, 2009). Simply put, under nitrogen limiting conditions, amino acid precursors *o*-acetyldserine and *o*-acetylhomoserine will be limited, halting the pathway after sulfide is reduced. This reduced sulfide will be converted into H_2S that will then accumulate in the cell and diffuse into the media (Jiranek, Langridge, & Henschke, 1995b). H_2S binds with *o*-acetylhomoserine to form serine but with limited precursors H_2S will likely remain unbound or react with ethanol to produce mercaptans which can also be

oxidized to form polysulfide, both of which have negative sensory attributes (Swiegers & Pretorius, 2005).

Several genetic variations among S. *cerevisiae strains* have been demonstrated to affect total H_2S production by altering the SRS pathway. In the case where amino acid precursors are limited, over-expression of the MET17 gene, which encodes for *o*-acetylersine (OAS) and *o*-acetylhomoserine sulfuhyrylase (OAH SHLase), is thought to decrease H_2S formation in some strains by increasing production in OAS and OAH SHLase needed for sulfite binding (Spiropoulos & Bisson, 2000). Bisson (2000) modified yeast strains to overexpress the MET17 gene and saw no decrease in H_2S formation in UCD522 when OAS/OAH SHLase production was increased, but a significant reduction of H_2S formation in UCD713 was observed without an increase in OAS/OAH SHLase production (Spiropoulos & Bisson, 2000). In this study, a decrease in H_2S production was observed when MET17 was overexpressed in one yeast strain (UCD713) and not in the other yeast strain (UCD552). This study alludes to the complexity of yeast strains genetics, expression and regulation as relates to hydrogen sulfide production. A study by Mendes-Ferreira (2010) backed up the idea of complexity in yeast strain genetics, expression and regulation by showing that different MET genes (MET3, MET7, MET10, MET17 and SSU1) in two yeast strains (UCD522 and PYCC4072) were expressed differently under different nitrogen conditions (267 mg N L^{-1} and 402 mg N L^{-1})(Mendes-Ferreira, Barbosa, Jiménez-Martí, Del Olmo, & Mendes-Faia, 2010).

Other genes can also regulate the SRS pathway and lower H_2S production. Hansen (1994) was the first to observed that yeast strains with the MET10 gene grown on a BIGGY plate that detects H_2S, produced much lighter color plates indicating lower H_2S after six days of growth on the media compared to strains without the MET10 gene (Hansen, Cherest, & Kielland-Brandt,

1994). Linderholm's study (2010) backed up Hansen's theory when he added the MET10 gene to a high H_2S producing yeast strain and resulted in prevention of H_2S formation in BIGGY plates (Linderholm, Dietzel, Hirst, & Bisson, 2010). The MET10 gene, encodes for the alpha portion of sulfate reductase that reduces sulfate to sulfide, has been linked to UDC935 yeast that is known as a very low H_2S producing yeast strain (Linderholm et al., 2010). These are just a few examples of the vast research field studying yeast strain genetics and how genes expressed regulate SRS pathway and affect H_2S production. This body of work underscores the important influence of yeast strain on H_2S production during fermentation.

Table 3 provides reports of the addition of single amino acids, methionine and cystine, and the reported effects of additions on H_2S production and yeast strain differences. Methionine additions can lower H_2S production by acting as an inhibitor in the SRS pathway although interactive effects have been reported with yeast strain and concentration of total YAN (Boudreau, Peck, O'keefe, & Stewart, 2017; Wainwright, 1970) Additions of methionine and cysteine have been found to increase H_2S liberation when these amino acids are the only source of nitrogen. Jiranek (1994) found that when the sole source of nitrogen in media was either methionine or cystine, H_2S liberation increased in both AWRI 77 and AWRI 72 yeast strains compared to un-supplemented media (Jiranek et al., 1995b). When methionine is added in addition to other nitrogen sources it can decrease H_2S formation. Bourdeau (2017) found the addition of 5mg L^{-1} of methionine decreased H_2S production in EC1118 yeast when total YAN was 53mg L^{-1} of YAN but 50mg L-1 of methionine was needed to decrease H_2S formation when YAN was 153 mg L^{-1} while no amount of methionine added decreased H_2S in UCD522 yeast (Boudreau Iv et al., 2017). One potential explanation for this observation is that the increase in YAN increased the cell biomass. The increased cell biomass could lead to a higher methionine

concentration needed to inhibit the SRS pathway and in turn lower the H_2S production (Boudreau Iv et al., 2017). There are considerable complex interactions between total YAN concentration, amino acids, vitamins, yeast strain, and starting material that all play a role in H_2S production, but amino acids and the lack thereof can certainly affect H_2S production during cider fermentation.

Table 3: Amino Acids Additions and Reported Results

Amino Acid	Reported Results with Addition of Specific Amino Acid	Matrix Tested In	References
Methionine	Decreased H_2S dependent on YAN concentration and yeast strain Increase H_2S liberation	Cider Wine Cultured media	(Boudreau Iv et al., 2017) (Wainwright, 1970) (Jiranek et al., 1995b)
Cystine	Resulted in increased H_2S	Cultured media	(Jiranek et al., 1995b)

The total amount of VSCs and H_2S produced during beverage fermentation is affected by several different factors including yeast strain, availability of sulfur containing amino acids and gene expression. Even if VSC compounds do not seem to be present during or after fermentation based on sensory evaluation, VSCs may still be present in subthreshold concentrations that are not detectable by analytical or sensory means. This can, however, result in increased levels of VSCs or other negative aromas occurring via chemical reduction reactions when stored in very low oxygen and ambient conditions. This, in turn, can result in high H_2S and methyl mercaptan, another undesirable aroma, when uncorked (Ugliano et al., 2011). Although the mechanism behind post bottling evolution of H_2S is still poorly understood, it has been hypothesized that metal ions such as Cu^{+2} added as a fining agent to reduce reductive off aromas such as H_2S in finished wine can later act as a catalyst for H_2S formation and other VSC during storage (Chen, Jastrzembski, & Sacks, 2017; Ugliano & Henschke, 2010). Other additives to wine post-

fermentation but pre-bottling such as tripeptide glutathione used to reduce free SO_2 have also been shown to be associated with increases in H_2S during storage (Ugliano et al., 2011). SO_2, added post-fermentation as an antioxidant and antimicrobial has been linked to increased VSCs (Bekker, Smith, Smith, & Wilkes, 2016). Further research needs to be conducted to elucidate the mechanism behind post bottling H_2S formation.

For now, prevention of production of H_2S seems to be the best strategy to reduce negative sensory aromas. If negative sensory compounds are never produced during fermentation, there is no need to find post fermentation strategies to deal with H_2S after the fact. The research cited in this literature review is almost all conducted in wine matrices. The cider fermentation matrix specifically, has seen very limited research in all of these areas, and the extrapolation of research from grape must fermentation should be done with caution due to differences in juice chemistry that has been mentioned in previous sections. Due to these factors, there is a need for research specifically focused on the cider matrix, and how cider matrix factors influence the production of H_2S and other aroma compounds during fermentation.

2.4 Yeast Assimilable Nitrogen, Yeast Nutrients and Hydrogen Sulfide

2.4.1 Yeast Assimilable Nitrogen and Nitrogen Utilization

Nitrogen found in amino acids and ammonium ions are essential yeast for yeast metabolism. A lack of nitrogen in juice can lead to a “stuck” or “sluggish” fermentation which can result in slow fermentation rates, incomplete fermentation of sugars, production of higher alcohols, low fatty acid production, and production off flavors and aromas (Alexandre & Charpentier, 1998; Bell & Henschke, 2005; Blateyron & Sablayrolles, 2001; Sablayrolles, Dubois, Manginot, Roustan, & Barre, 1996). Yeast assimilable nitrogen (YAN) refers to the

nitrogen in apple juice and grape juice that is present in a form that is available for yeast metabolism. YAN is generally defined as being composed of two parts: Free Amino Nitrogen (FAN) also known as Primary Amino Nitrogen (PAN) which is composed of organic nitrogen (amino acids, some definitions also include small peptides) and of inorganic nitrogen (ammonium ions).

Ammonium is one of the first nitrogen sources to be internalized into the yeast cell for metabolism in *S. cerevisiae* (Bell & Henschke, 2005). Amino acids contribute to the PAN but have varying rates of utilization dependent on the yeast strain and on the total amount of nitrogen present in the environment which is known as nitrogen catabolite repression (NCR) (Broach, 2012; Jiranek, Langridge, & Henschke, 1995a; Magasanik & Kaiser, 2002). Nitrogen sources in the literature have been loosely grouped into "preferred" and "non-preferred sources" depending on how well the individual nitrogen source can support yeast growth and how well it can repress non-preferred nitrogen utilization, and this is all yeast strain dependent (Ljungdahl & Daignan-Fornier, 2012). Dependent on the yeast strain, almost all amino acids can be used for yeast cell growth except for lysine, cystine and histidine (Gobbi et al., 2013; Ljungdahl & Daignan-Fornier, 2012) In has been reported that glutamine and asparagine are considered by some to be more "preferable" nitrogen sources after the depletion of ammonia ions occurs (Bell & Henschke, 2005). Some research has found that supposed "preferable" nitrogen sources such as arginine did not support growth of *S. cerevisiae* well which could be due to differences in yeast strains (Fairbairn et al., 2017). Proline has been reported to have limited viability as a nitrogen source for yeast due to anaerobic conditions of fermentation and is not typically included in PAN concentrations (Bell & Henschke, 2005).This demonstrates the importance of yeast strain and the need for more research on how nitrogen sources are utilized in fermentations. In general, it is

thought that a “balanced” mixture of amino acid and ammonia ions are ideal for fermentation by *S. cerevisiae* (Bell & Henschke, 2005), although the specific concentrations of each component that make up this “balance” are not well-defined and seem to vary depending on yeast strain and other factors. The suggested minimum amount of YAN in grape must for a complete fermentation is between 120-140 N mgL^{-1} in starting matrix, all though some have suggested even higher concentration of nitrogen of 267 mg N L^{-1} or 350 mg N L^{-1}(Alexandre & Charpentier, 1998; A. Mendes-Ferreira, Mendes-Faia, & Leão, 2004). Grape must can have a wide range of YAN concentration between 40 N mgL^{-1} to 559 N mgL^{-1}(Alexandre & Charpentier, 1998; Christian E. Butzke, 1998). Boudreau (2017) surveyed PAN and YAN concentrations in 12 apple cultivars grown in Virginia and found an average YAN of 59 mg N L^{-1} which some cultivars being as high as 249 mg N L^{-1} and 94% of the cultivars having concentrations lower than 140 mg N L^{-1} (Boudreau, Peck, O’Keefe, Stewart, & Stewart, 2018). This was a small survey, and further data is needed to better understand the global variation in YAN concentration and composition of cider apples. Apple juice and AJC is generally believed to have lower available nitrogen than grapes, however as very limited reports are available for ranges of YAN concentrations in apples, this is a subject that merits further study. Similarly, the YAN requirements for cider fermentation and factors that interact with these requirements, are also not well-established.

2.4.2 Addition of Yeast Nutrients and Timing of Nutrient Addition

Often total YAN is increased by winemakers or cidermakers through pre-fermentation supplementation with Diammonium Phosphate (DAP). DAP is added to apple juice or grape must to increase ammonium ion concentration, resulting in increased yeast population and

fermentation rate. Other forms of yeast nutrients such as inactive dry yeast products (IDY) can be used to increase available nitrogen and other nutrient sources in juice.

Many products are commercially available for yeast nutrient supplementation. IDY products are produced in a variety of ways depending on the manufacturer and application. These products often are made of either inactive yeast, yeast autolysates, yeast cell walls, or yeast extracts and can provide a variety of nutrients such as proteins, peptides, amino acids, sterols, fatty acids, vitamins, and minerals (Ángeles Pozo-Bayón et al., 2009). Some IDY products are also mixed with DAP or vitamins such as thiamine to provide both ammonium ions and nutrients for inactive yeast. The addition of IDY products and DAP have been linked to increased fermentation rates, flavor intensity, reduction of H_2S production and positive aroma compounds and are being further investigated for their positive impact on fermentation duration and effect on aroma (Ángeles Pozo-Bayón et al., 2009; Bell & Henschke, 2005; Gobbi et al., 2013; Pozo-Bayón, Andújar-Ortiz, & Moreno-Arribas, 2009). In a study by Soulbeyrand (2005) IDY added at high levels (100 mgL^{-1}) during active dry yeast rehydration showed an increase in fermentation rate. It was proposed that the IDY served to help repair active dry yeast that was damaged during freeze drying (Soubeyrand et al., 2005). IDY products can also be used to decrease fermentation duration during exponential growth phase by the addition of nitrogen sources (inorganic and organic) thought the soluble fraction (yeast cell walls, amino acids, and ammonia) and the insoluble fraction (cellulose) (Ángeles Pozo-Bayón et al., 2009). In this study the effectiveness of IDY depended on their composition and processing methods.

IDY, have also been used to increase positive aromas in wine and grape must. Suklje (2016) reported the addition of two separated IDY to Sauvignon blanc grapes during vine ripening and reported increased fruity aromas in grapes treated with IDY compared to control

wine with no IDY additions. Control wines were associated with greener sensory descriptors (Šuklje et al., 2016). Different aromas attributes were associated with each of the different IDY tested; one wine was described as gooseberry, apricot or banana while the other wine using the second IDY was described as peach, citrus, and apples aromas (Šuklje et al., 2016). Another study by Pozo-Bayón (2009), reported aroma compounds in wine were effected differently depending on the type of IDY, how the IDY was produced and the concentration of addition of IDY (Pozo-Bayón, Andujar-Ortiz, Alcaide-Hidalgo, Martín-Álvarez, & Moreno-Arribas, 2009). Yet another report suggested that addition of glutathione rich IDY to rosé, increased fruity aromas and decreased yeasty aromas when observed by a trained panel (Andújar-Ortiz, Chaya, Martín-Álvarez, Moreno-Arribas, & Pozo-Bayón, 2014).

Limited research on the addition of IDY effects on aroma have been reported and it is unknown the specific type of IDY being applied since product names and composition of IDY were not revealed. The mentioned above studies give a limited glimpse of how IDY products could be used in wine making and how it might affect wine aroma. These studies does not represent cider production or cider aroma with IDY additions and further research into cider aromas.

Not only is the total concentration of YAN important to yeast fermentations for metabolic growth and preventing stuck or slow fermentations, timing of nutrient additions has also been suggested to affect H_2S production and aroma. It is believed that H_2S is produced during the exponential growth phase after the yeast have consumed the most preferred nitrogen sources and start to consume lesser preferred sources of nitrogen (Ugliano, Kolouchova, & Henschke, 2010). Addition of nutrient during the exponential growth phase is thought to reduce nitrogen depletion and increase fermentation rate. Beltran (2005) found regardless of timing of addition (30, 72, 144

or 240 hours), nitrogen addition from DAP decreased fermentation duration. He also concluded that addition of DAP during the exponential growth phase decreased total fermentation duration even more compared to addition in lag phase growth. The consumption of ammonia ions also depended on timing, if ammonia was added towards the end of the fermentation (114 or 240 hours) it was hardly consumed and remained in the cider environment, but when added during the first half (30 or 72 hours) of the fermentation it was highly consumed (Beltran, Esteve-zarzoso, Rozes, Mas, & Guillamo, 2005). Typically, in wine making, nutrient additions are made at 1/3 brix depletion and no later due to the threat of unwanted microbial growth. This practice is done to prevent residual yeast nutrients that are not taken up by the yeast remaining in the wine post-fermentation, which would promote unwanted microbial growth.

The timing of yeast nutrient additions can influence the production of H_2S. Mendes-Ferreira (2009) evaluated timing of DAP additions in fermentation using the UCD522 yeast strain and found that adding 145 mg L^{-1} of DAP to fermentations pre-fermentation increased H_2S compared to grape juiced with no DAP addition pre-fermentation (824.2 µg L^{-1} compared to 403.8 µg L^{-1}) while addition of 145 mg L^{-1} of DAP at 72 hrs after inoculation resulted in less H_2S being produced (537.7 µg L^{-1}) (A Mendes-Ferreira, Barbosa, Inê, & Mendes-Faia, 2009). Another study by Mendes-Ferreira (2004) found that when DAP was added during the stationary phase, to a grape juice medium inoculated with PYCC 4072 yeast, maximum cell population and maximum fermentation rate increased (A. Mendes-Ferreira et al., 2004). Jimenez-Marti found similarly, that when adding amino acids, ammonia or a combination of both to sluggish and nitrogen-depleted synthetic grape must using Fermicru Primeur yeast, increasing the total YAN to 240 mg N L^{-1}, increased sugar consumption rate, ethanol production and shortened fermentation duration (Jiménez-Martí, Aranda, Mendes-Ferreira, Mendes-Faia, & del Olmo,

2007). Jiranek (1995) hypothesized when nitrogen depletion occurs later in the fermentations, there is less time for H_2S to accumulate and be released from the cell. Addition of nutrients, such as DAP, may delay nitrogen depletion until later stages in the fermentation and allowing less accumulation of H_2S (Jiranek et al., 1995b).

Further research focused on timing of nutrient additions is needed to elucidate the interaction between timing and H_2S production in cider as well as wine. Cider may differ from wine for a wide variety of reasons including natural chemical differences in grapes and apples. Cider may differ in pH, macro- and micro-nutrient content, sugar concentration, acid content, polyphenols and ethanol concentration among other traits. These matrices will also differ due to production practices including source of fruit, fruit cultivar or species, agriculture practices and many other factors. These differences make it difficult to extrapolate and directly apply wine research to cider. While insufficient YAN has been directly linked to H_2S production, recent studies suggest that meeting a recommended value for total YAN does not completely safeguard against H_2S formation (C .E. Butzke & Park, 2011; Giudici & Kunkee, 1994; Huang, Walker, Fedrizzi, Gardner, & Jiranek, 2017; Jiranek et al., 1995b; A Mendes-Ferreira et al., 2009; Vos & Gray, 1978; Wainwright, 1971). Some studies have shown that when nitrogen levels (total YAN) are increased using DAP additions (inorganic nitrogen), H_2S production by yeast during fermentation can increase. Mendes-Ferreira (2009) found that in two specific yeast strains (UCD522 and PYCC4072) the highest levels of H_2S were formed when YAN was 267mg N L^{-1} in the initial media and the lowest H_2S formation was in media containing 66mg N L^{-1}(Ana Mendes-Ferreira, Barbosa, Falco, Leão, & Mendes-Faia, 2009), demonstrating that increasing YAN may in some cases actually increase H_2S production. Ugliano (2010) found similar correlations with moderate initial YAN (260mg N L^{-1}) producing the highest concentration of

H_2S and interestingly the highest levels of initial nitrogen (410 mg N L^{-1}) producing similar or lower H_2S than the non-supplemented (110 mg N L^{-1}) (Ugliano et al., 2010). Yet another study by Ugliano (2009) supports increased YAN from DAP supplementation to final YAN concentration of 250 and 400 mg L^{-1} increased H_2S compared to non-supplement YAN of 100 mg L^{-1} (Ugliano et al., 2009). Again Boudreau (2017), found that fermentations using EC1118 yeast with a total YAN concentrations of 153 mg N L^{-1} had the highest total production of H_2S compared to fermentations with a total YAN of 53 mg N L^{-1} and 253 mg N L^{-1}(Boudreau Iv et al., 2017). While cell mass or maximum cell concentration during fermentation was not monitored in these studies, one potential explanation for these observations is that the high initial concentrations of nitrogen are depleted early, resulting in a higher population of yeast that are then nitrogen-deficient by mid-fermentation. This theory underlies the interest in strategies involving splitting the yeast nutrient additions into an early and mid-fermentation addition. With recent research calling into question the general notion that increasing YAN concentrations decreases H_2S, other factors that affect H_2S production by yeast have been explored. In recent years, H_2S production has been known to be affected by elemental sulfur addition in vineyard pesticide treatments, amount of pantothenic acid, and other vitamins and amino acids, yeast strain, bottling and storage conditions.

Almost all the above cited research has been conducted in wine or synthetic grape juice media. These studies provide a sound starting point for cider fermentation strategies; however, the findings may not translate wholly into cider matrices due to the differences in matrix constituents that may interact with YAN requirements. When extrapolating knowledge and methods developed for wine making to cider making, unexpected outcomes can result. The increasing evidence of factors that are not usually measured in fruit juice (e.g. vitamins,

minerals, fungicide residues, etc.) to influence fermentation further justifies the need for more research focused specifically on the cider matrix and how processing techniques, total YAN, pre-treatment additions and aroma compounds affect quality.

2.4.3 Vitamin Deficiencies and Amino Acid Deficiencies

Deficiencies of vitamins such as biotin and pantothenic acid have been shown to contribute to problematic fermentations as seen in Table 4. Pantothenic acid (pantothenate or vitamin B5) deficient fermentations may lead to H_2S production by depletion of metabolic precursors such as *O*- acetylserine and *O*-acetylhomoserine in the SRS pathways responsible for H_2S formation (Wainwright, 1971; Wang, Bohlscheid, & Edwards, 2003). Wainwright (1970) identified that using Guinness yeast strain 1164 fermentations with less than 160µg L^{-1} of pantothenic acid had no H_2S formation (Wainwright, 1970). This is in alignment with Edwards (2007) study that found when using EC1118 and UCD522 yeast strains in grape juice, addition of 200 µg L^{-1} of pantothenic acid pre-fermentation or early-fermentation and mid-fermentation (48hr or 96hrs) ceased all H_2S production for both yeast strains (Edwards & Bohlscheid, 2007). Interaction effects between assimilable nitrogen and pantothenic acid were also reported. Wang's (2003) study found that when using yeast strains EC1118 and UCD522 that H_2S production was higher in fermentations that contained high concentrations of nitrogen and lower concentrations of pantothenic acid compared to those that had high nitrogen and high pantothenic acid (Wang et al., 2003). Bohlscheid (2011) concluded that YAN, biotin, pantothenic acid, and fermentation temperature affect H_2S production by yeast strains EC1118 and UCD 522 and found that the YAN concentration alone did not significantly affect H_2S production ($P>0.05$) while fermentation temperature and vitamins (biotin and pantothenic acid) did have significant effects on H_2S production (Jeffri C. Bohlscheid, Osborne, Ross, & Edwards, 2011).

Table 4: Fermentation Problems Reported when Vitamin is Deficient in Matrix

Vitamin	Reported Problems when Deficient	Matrix Tested In	Refences
Pantothenic Acid Vitamin B5	Decrease cell growth Increase H_2S production	Wine Cider	(Hosono, Ko, & Uemura, 1972) (Edwards & Bohlscheid, 2007) (J. C. J. C. Bohlscheid, Osborne, Ross, & Edwards, 2011) (Wang et al., 2003) (Slaughter & McKernan, 1988)
Biotin Vitamin B7	Decrease in viable yeast cell count Decreased fermentation rate Remaining residual sugars Increase H_2S production	Wine	(Ough, Davenport, & Joseph, 1989) (J. C. Bohlscheid, Fellman, Wang, Ansen, & Edwards, 2007)

The research mentioned above demonstrated the importance of understanding the starting concentrations of vitamins in the apple juice. It is important to evaluate the components that contribute to the total YAN concentration. Different components that add to YAN concentration can influence the SRS pathway that leads differing H_2S concentrations. Low concentrations of vitamins can contribute to the problems found in both grapes must and apple juice dependent on yeast strain used for fermentation.

2.5 Cider Sensory Analysis

Aroma plays a large role in consumer acceptance of fermented beverages. Even with a quantitative method to measure total H_2S production during fermentation, it is still unknown how the measured H_2S values translate to consumer liking and descriptors associated with cider aroma. Due to the quantitative nature of the method used in this study to measure H_2S and the exclusivity of the method (only H_2S was monitored), final H_2S values measured is an indication of the total H_2S produced over the entire fermentation time. This may not, however, provide an accurate or useful representation of the overall aroma from the consumer's perception. VSCs that are considered highly volatile (boiling point <90°C) like H_2S, are released more easily, and may

decrease over time. Other VSCs that are less volatile (boiling point >90°C) such as methionol, may be present in fermented beverages and attribute to negative off aromas (Moreira et al., 2002). Other aromas (sulfur containing or not) produced during fermentation should also be taken into consideration during the evaluation of the cider aroma since H_2S is only one of many aromas that could be produced during fermentation (Rita, Zanda, Daina, & Dalija, 2011; Xu, Fan, & Qian, 2007), and the production of which has been linked to nitrogen concentration and composition in the juice. For this reason, sensory evaluation can be used to understand consumers' overall liking of the aroma and used to identify descriptors for the cider aroma.

Sensory evaluation is used to evoke, measure, analyze and interpret characteristics of products based on consumer perception using the five human senses (H. T. Lawless & Heymann, 2012). Conventional sensory evaluation techniques such as Descriptive Analysis (DA) require highly trained judges that produce a standardized list, known as a lexicon, of descriptors to describe a group of products. This can be very time consuming and expensive to conduct (H. T. Lawless & Heymann, 2012). In traditional lexicon generation, panelists need to be trained on the sample set that is considered the "frame of reference" which is a product category or group of products being evaluated (Drake & Civille, 2003). For this reason, rapid methods to achieve similar results with untrained consumers have been developed to evaluate products in a more time efficient manner. Rapid methods such as Projective Mapping, Check-All-That-Apply (CATA), Free Sorting Tasks and Flash Profiling can be used to evaluate product properties with untrained consumers (Dehlholm, Brockhoff, Meinert, Aaslyng, & Bredie, 2012; Dooley, Lee, & Meullenet, 2010).

In a CATA task, untrained consumers are provided with a list of predetermined attributes and asked to choose which attributes describe each product. CATA has been used to describe

many products and in some cases has provided results similar to traditional DA (Gastón Ares, Varela, Rado, & Giménez, 2011; Bruzzone, Ares, & Giménez, 2012). One of the main challenges in CATA method is the development of the attribute list (Varela & Ares, 2012). Some CATA studies develop a list of attributes using a trained panel of judges, conducting a DA and developing a lexicon. A lexicon is defined as a standardized vocabulary that helps in the communication of perceived sensory attribute including flavor, aromas, taste and mouthfeel (Drake & Civille, 2003; L. Lawless & Civille, 2013; Phetxumphou, Cox, & Lahne, 2020). CATA lists can be developed from a pre-existing lexicon, or untrained consumers can create a lexicon using free choice profiling (FCP) (Alencar et al., 2019; Gastón Ares et al., 2011; Dooley et al., 2010; Phetxumphou et al., 2020). Using a pre-existing lexicon already developed by trained panelists can be time saving but these lexicons may not exist for the desired product (G. Ares & Jaeger, 2015; L. Lawless & Civille, 2013). For example, the aroma wheel developed for wine by Dr. Ann C. Noble could be used to develop a list of attributes to describe wine (Noble et al., 1987). In the case of hard cider there is no such lexicon of standardized words. It has been observed that there is confusion among cider consumers when using terms to describe cider leading indicating the need for more standardized vocabulary (Jamir, Stelick, & Dando, 2020). Using a frame of reference in conjunction with rapid lexicon development that uses untrained consumers the development of attributes used for a CATA task becomes less expensive and less time consuming.

When a pre-existing lexicon is not an option, the development of a lexicon using untrained panelists and open ended response questions can be used to generate terms. Open ended responses allows consumers to freely describe the product in their own words free of restrictions from researchers in the form of providing a list of attributes (Varela & Ares, 2012).

The disadvantage of this method is that many different descriptors that may be highly unique to a given individual and hard to interpret by others may be used to describe the same perceived attribute making the data more subjective and timing consuming to review after the test (ten Kleij & Musters, 2003).

In order to create a standard list of attributes, responses from open ended comments can be analyzed using text analysis (ten Kleij & Musters, 2003). The three phases of text analysis include 1) breaking up phrases into single words, 2) lemmatization of works and 3) groups word into synonyms (Lahne, Trubek, & Pelchat, 2013). The third step takes several assessors to evaluate the lemmatized words separately and group them by similarity. Assessors then meet and agree on groupings to words to condense the list of attributes to a reasonable number of terms (Jaeger et al., 2015; Lahne et al., 2013) This method of analyzing free text from open-ended coments has been used to evaluate free text on products such cheese, milk dessert and apples (Gastón Ares & Deliza, 2010; Lahne et al., 2013; Symoneaux, Galmarini, & Mehinagic, 2012). Using this method creates a standardized list that can then be used in a CATA task.

There are several ways to interpret attribute data from CATA tasks. CATA data is usually transformed into numerical values (0 or 1) to represent checked or uncheck attributes, where 1 represents a checked attribute and 0 represents an unchecked attribute. A common statistical test used on CATA data is Cochran's Q test. Cochran's Q test investigates differences between treatments applied to the samples using binary outcomes to determine how well each attribute was discriminated between samples (Varela & Ares, 2008). In other words, Cochran's Q can be used to determine whether consumers detected differences between samples for each term presented in the CATA task (G. Ares & Jaeger, 2015; Meyners, Castura, & Carr, 2013; Varela & Ares, 2008).

A common multivariate analysis applied to CATA data is Correspondence Analysis (CA), which can be used to evaluate a relationship between checked attributes and products (Greenacre, 2013). CA uses categorical data collected from CATA tasks to create a contingency table and displays the data on a two dimensional biplot (Greenacre, 2013). The contingency table formed from the CATA responses can be organized by rows and columns where rows represent consumers response to a product and columns represent each attribute (Lahne et al., 2013; ten Kleij & Musters, 2003). The cell count is the number of times the specific attribute was checked for that sample (Greenacre, 2013; Lahne et al., 2013).Relationships between the rows and columns of the contingency table can be evaluated by the chi-square distance (X^2-distance). X^2-distance can be computed by subtracting the expected value from the original value for each attribute (Greenacre, 2013). Then a simple mathematical transformation of X^2-distance can be performed so that X^2-distance can be accurately represented in plain ordinary space on the biplot (Greenacre, 2013; Varela & Ares, 2008). The total inertia, which is a measure of how much variance is in the contingency table, is also used in the visualization the biplot to determines the spread of the points in the represented two dimensions. The higher the total inertia the greater the association between the rows and columns (Greenacre, 2013). The resulting graph of the X^2-distance and the total inertia of the contingency table gives a visual representation of the differences of the two variables applied to the samples(Greenacre, 2013; Varela & Ares, 2008). From the CA biplot, product descriptions can be suggested based off the most commonly selected attribute associated with the product.

Hedonic liking is often used in conjunction with a CATA task There are several ways to test liking, but the most commonly used method is using a 9-point hedonic scales with 9 equally spaced points anchored go from “Like Extremely” to “Dislike Extremely” with “Neither Dislike

nor Like" as the middle point. The 9-point scale has been widely used to evaluate product liking(H. T. Lawless & Heymann, 2012). For analysis of hedonic liking data, each of the 9-points on the scale are assigned a value of one through nine with nine being "like extremely".

Sensory evaluation can be used to give insight on which sensory attributes are associated with a products and connect those attrbiures to overall liking for that product. In this project sensory evaluation can be used to understand how yeast nutrients, H_2S and aroma effect consumer perception of cider aroma as well as giving insight on liking of the product. The generation of a lexicon by untrained consumers rather than trained judges for a specific group of ciders could achieve a more accurate representation of the descriptors to be used in a CATA task. Lexicon generation using untrained consumers followed by CATA task was used in the experiment to generate a list of descriptors that represents the specific group of cider samples.

2.6 Conclusion

Pre-fermentation additions such as sugar, SO_2, tannins, and yeast nutrients to juice are often chosen by the cider or wine maker depending on the style of the finished product and chemistry of the starting juice or must. All pre-fermentation additions can influence fermentation outcomes and a wide diversity of different style products result from the differences. Cider makers should carefully consider what pre-fermentation additions are used when producing cider due to these effects on cider outcomes. Cider markers should also consider what type of starting juice to ferment there are pros and cons to both fresh juice and AJC. Fresh juice must be fermented quickly and cannot be stored for long periods of time like AJC, but AJC can contain Maillard browning products that could inhibit yeast fermentation and lack amino acids which could lead to off aromas like H_2S.

The presence of H_2S can have a large effect on consumer acceptance of fermented beverages, including cider. Many factors affect how much H_2S is formed. Yeast strain selection, nutrient availability in juice and total YAN, vitamin availability and type of juice being used during fermentation are all known to contribute to H_2S formation in wine fermentation but the vast majority of research investigating H_2S formation is in grape juice or must, which limits the knowledge when applied to cider matrix. Differences in in polyphenols, tannins, sugar content, nutrient availability, pH, amino acids among many other matrix differences needs to be taken into consideration while considering H_2S formation in cider. The need to study H_2S in cider matrix is of importance because of the growth in cider production and popularity. Research in a wine must is a good starting point for cider research but cannot be applied directly without the possibility of differences due to the nature of the two starting matrixes. The purpose of this research is to explore how nitrogen source, timing of nitrogen source and yeast strain affect total production of H_2S, fermentation duration, cider chemistry and quality of cider aroma.

2.7 References

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Chapter 3: Complex Yeast Nutrient Addition and Timing of Additions Influence on Hydrogen Sulfide Production and Aroma in Cider Fermentations

3.1 Introduction

Cider, an alcoholic beverage made from apples, is rapidly growing in popularity in the United States. In 2018, the US had a total of 877 cider producers with cider sales reaching $520.5 million in 2018 (Lombardo, 2018). With the increased consumption of cider, there is also an increase in demand for high quality cider. Past decreases in cider sales volume have been attributed to poor product quality caused by suboptimal processing practices, including poor nutrient availability for yeast during fermentation (Bamforth, 2005). Cider processing is closely related to white wine processing, but there are key differences in fruit chemistry between apples and grapes that may result in poor quality cider when winemaking practices are directly applied (A. Lea & Drilleau, 2003). One of these key differences is nutrient availability for yeast fermentation in apple juice.

Saccharomyces cerevisiae is the most used yeast strain for wine and cider fermentation. Yeast used for alcoholic fermentation, need specific nutrients such as sulfur containing amino acids, B vitamins, and ammonium ions among other nutrients to ferment juice into ethanol(Boudreau Iv et al., 2017; Edwards & Bohlscheid, 2007; Jiranek et al., 1995a; Magasanik & Kaiser, 2002; D. Thomas & Surdin-Kerjan, 1997). Lack of nutrients, including yeast assimilable nitrogen (YAN), can lead to “stuck” or “sluggish” fermentations which can result in slow fermentation rates, incomplete fermentation of sugars, production of higher alcohols, low fatty acid production, and production off flavors or aromas (Abdi & Williams, 2008; Alexandre & Charpentier, 1998; Bell & Henschke, 2005; Blateyron & Sablayrolles, 2001; J. C. Bohlscheid et al., 2007; Sablayrolles et al., 1996). One of the most common aroma compounds that causes of off aromas in cider is hydrogen sulfide (H_2S) which is described as “rotten eggs”, “cabbage” or

"vegetal" and leads to consumer rejection (Marchand et al., 2000; Waterhouse, Sacks, & Jeffery, 2016). H_2S has a very odor detection threshold; the literature suggests it to be as low as 1 µg L^{-1} and may negatively interact with positive fermentation aromas (Davis & Qian, 2011; Franco-Luesma et al., 2016; Mestres et al., 2000; Siebert, Solomon, Pollnitz, & Jeffery, 2010). Apples are naturally low in YAN needed for yeast fermentation and processed apple juices may have even fewer nutrients available due to amino acids consumption during heat processing (Banerjee et al., 1981b; Boudreau et al., 2018; Martins et al., 2001).

Many factors contribute to H_2S production in wine fermentation. Yeast strain has a large effect on the potential for H_2S production during wine fermentation, due to differences in metabolic pathways and nutrient demands (Manginot et al., 1998; C. S. Thomas et al., 1993; Ugliano et al., 2009) among yeast strains. While this phenomenon has been researched for decades in wine systems, less is known about factors affecting H_2S production during cider fermentation. Yeast nutrient availability been linked to H_2S production. In grape juice, the generally recommend minimum YAN concentration is between 120-140 mg N L^{-1} and if juice falls below the range, nitrogen additions are typically recommended. Diammonium phosphate (DAP), which only consists of ammonium ions, is commonly added to increase YAN and prevent stuck and slow fermentation, although increasing YAN does not completely safeguard against H_2S formation (C .E. Butzke & Park, 2011; Giudici & Kunkee, 1994; Huang et al., 2017; Jiranek et al., 1995b; A Mendes-Ferreira et al., 2009; Vos & Gray, 1978; Wainwright, 1971). Some studies have shown that increasing YAN levels with DAP additions actually increased H_2S production, contrary to the general assumption that this addition would decrease H_2S production (Boudreau Iv et al., 2017; Ana Mendes-Ferreira et al., 2009; Ugliano et al., 2010). With increasing recent findings challenging the general notion that increasing YAN concentrations

decrease H_2S, other factors that effect H_2S production in fermentation have been explored, including addition of micronutrients and lipids, and nutrient addition timing strategies, wherein nutrients are added at different stages during the fermentation, rather than all being added pre-fermentation(Andújar-Ortiz et al., 2014; Ángeles Pozo-Bayón et al., 2009; "Scott Laboratories Cider Handbook," 2018).

Yeast nutrient formulations other than DAP, such as inactive yeast products, are increasingly used to improve nutrient availability and subsequent wine aroma. These products contain more than just ammonia ions such as such as proteins, peptides, amino acids, sterols, fatty acids, vitamins, and minerals (Ángeles Pozo-Bayón et al., 2009). Inactive yeast products can be made of either inactive yeast, yeast autolysates, yeast cell walls, or yeast extracts and are suggested to increase fermentation rate and produce positive aroma compounds, but these products have not been investigated on their ability to lower H_2S production.(Ángeles Pozo-Bayón et al., 2009). These nitrogen rich products could provide more complex nutrients compared to only ammonia additions in the hope to prevent H_2S production but no know research is available on these nutrient types.

Nutrient addition timing has also been suggested to affect H_2S production. It is believed that H_2S is produced during the exponential growth phase after the yeast have consumed the most preferred nitrogen sources and start to consume lesser preferred sources of nitrogen (Ugliano et al., 2010). Addition of nutrient during the exponential growth phase is thought to reduce nitrogen depletion and increase fermentation rate. It has been reported that addition of DAP during exponential growth phase, decreased fermentation duration and addition of DAP at 72 hours into fermentation produced less H_2S than when adding DAP pre-fermentation (Beltran et al., 2005; A Mendes-Ferreira et al., 2009). Research is limited in this area and further research

focused on timing of nutrient additions are needed to elucidate the interaction between timing and H_2S production in cider as well as wine.

Almost all the above cited research has been conducted in wine or synthetic grape juice mediums. With the increase in cider popularity and production, it is important to have research focused on apple juice fermentation as opposed to extrapolating research knowledge from grape must, considering the differences in juice chemistry and the potential effects of these differences on fermentation outcomes. The complexity of H_2S production related to cider nutrient availability is limited and so is the information on timing. The objective of this study was to evaluate the effects of four different exogenous nitrogen-rich supplements on H_2S production, fermentation kinetics, and aroma quality for cider fermentation.

3.2 Experimental

3.2.1 Apple Juice

Kroger Brand Frozen 100% Apple Juice Concentrate with vitamin C (Kroger CO., Cincinnati, Ohio 45202) was chosen for this study because there are no added sorbates or other preservatives that would be expected to inhibit yeast growth during fermentation. Ascorbic acid, or Vitamin C, is not known to affect yeast growth. A total of 150 (354 mL each) cans of frozen apple juice concentrate (AJC) were mixed together for homogeneity. The proportion of apples from each country and cultivar are unknown but 54 AJC cans where labeled as “Juice form USA, China and Poland” and 96 cans were labeled “Juice from USA, China and Ukraine”. Once mixed, concentrate was poured into pre-sanitized 3.5 gallon plastic buckets and frozen at -20°C and saved for further use. Upon use, AJC was thawed at 18°C until it could be poured with ease. AJC was rehydrated with water by adding one part AJC and three parts MilliQ water. Rehydrated AJC yielded apple juice (AJ) used for fermentation.

The following parameters were used to measure unfermented apple juice: pH (probe, Thermo Scientific ROSS Ultra Triode Electrode Model 107BNUMD, Thermo Fisher Scientific, Waltham, MA,USA) total soluble solids (Brix Refractometer Model RF10, Extech Instruments Corporation, Nasgua, NH, USA) titratable acidity (AOAC procedure 962.12 and 936.16), total YAN consisting of Primary Amino Nitrogen (PAN)(Megazyme PANOPA Enzyme Kit, Megazyme International, Wicklow, Ireland) and ammonia (rapid) (Megazyme Ammonia ion (rapid) Enzyme Kit, Megazyme International, Wicklow, Ireland), UPLC amino acid analysis (ACQUITY UPLC H-Class Bio Amino Analysis, Waters Corporations, Milford, USA) (Calton, Gillingwater, Hammond, & Cooper, 2007), and total sugars (HPLC method sugar analysis).

3.2.2 Microscale Fermentations

All fermentation treatments were conducted in triplicate. AJC was rehydrated to apple juice (AJ) as described above and AJ was treated with potassium metabisulfite at 0.8mg L^{-1} of molecular SO_2 to prevent microbial spoilage (Boulton et al., 1997).A sanitizer solution was used to sanitize equipment, stoppers, and H_2S tubes. Sanitizer was made up each day by mixing 11.36 L of water, 8.00 g of potassium metabisulfite and 12 g of citric acid. Sanitized equipment, stoppers, and H_2S tubes were airdried before use. 200 mL of apple juice was aliquoted into previously autoclaved 250 mL Erlenmeyer flasks with autoclaved stir bars.

Four yeast nutrients used for this experiment were provided by Lallemand Inc. (Lallemand Inc. Petaluma, CA). Fermaid K is described by the producers as a blend of inactive yeast hulls, amino acids from the inactive yeast hulls, sterols, unsaturated fatty acids, DAP, niacin, calcium pantothenate, folic acid, and thiamine(“Scott Laboratories Cider Handbook,” 2018). Fermaid O is listed as containing inactive yeast hulls rich in organic nitrogen (“Scott Laboratories Cider Handbook,” 2018). DAP contains inorganic nitrogen in the form of

ammonium slats(“Scott Laboratories Cider Handbook,” 2018). Experimental yeast nutrient, not commercially available, is 35% organic nitrogen and contains 30% of the same nitrogen source as Fermaid O.(“Scott Laboratories Cider Handbook,” 2018) Reported concentration of total YAN added by a 25g hL^{-1} nutrient addition is as follows: Feramid K provide 25 mg N L^{-1}, Fermaid O provides 10 mg N L^{-1}, and DAP provides 50 mg N L^{-1}.

Each yeast nutrient formulation, four total (Experimental yeast nutrient, Fermaid K, Fermaid O and DAP), were dissolved in 35°C milliQ water and mixed for 20 minutes. Each yeast nutrient solution was added either in one single addition or in two split additions. For single addition treatments, 25 g hL^{-1} of yeast nutrient was pipetted in one dose pre-fermentation. For split yeas nutrient addition, half the amount of yeast nutrient (12.5 g hL^{-1}nutrient) was added pre-fermentation and remaining half (12.5 g hL^{-1}) of yeast nutrient was added during the fermentation at 1/3 °Brix depletion. Control fermentations received the same amount of water to substitute for yeast nutrient in other treatments. Each nutrient treatment was fermented with one of three different yeast strains (EC1118, ICV OKAY and M2). Each yeast strain was rehydrated for 20 minute and inoculated at 25g hL^{-1}. Treatments were applied in a full factorial design.

To measure H_2S over fermentation time (hrs), hydrogen sulfide sampling tubes supplied by Kitagawa America (Pompton Lakes, New Jersey) were utilized as airlocks to measure total H_2S production as described by Ugliano and Henschke (Ugliano & Henschke, 2010) and by Park (Park, 2008). Tubes Total H_2S production was calculated by taking the sum of all H_2S tube reading over the entire fermentation duration. Two different detector tubes were used based on preliminary test fermentations: 120SF tubes (50-1,000 ppm) for M2 yeast strain fermentations and 120SB (6-150 ppm) for EC1118 and ICV OKAY yeast strain fermentations. Via fermentation-derived CO_2 entrainment, H_2S reacted with lead acetate or silver nitrate packed

silica gel in tubes, resulting in a color change. Tubes were pre-calibrated and color change readings on tube were recorded in µg L^{-1} of H_2S gas.

After inoculation, both ends of the H_2S tubes were broken and placed into the rubber stopper acting as an airlock. Fermentation flasks were wrapped in parafilm around the stopper to minimize gas leakage around stopper. Fermentations were carried out at 18°C. Flasks were stirred at 600 RPM for 5 minutes twice per day. After being stirred, the flasks were weighed to track CO_2 loss and evaluate fermentation progression. When the second addition of yeast nutrient was added, H_2S tubes were removed from stopper, nutrient was added through stopper hole and H_2S tubes was replaced back into stopper. This may have allowed some gas to escape and could be a source of error for the method. H_2S tube concentrations were recorded twice per day and replaced when approaching maximin range.

Fermentations were considered complete when weight measurements were consistent for four data points (2 total days). Fermentation performance was also tested by residual sugars using the D-fructose and D-glucose enzyme kit. Fermentations were considered complete when there was less than 2 g L^{-1} of total sugar remaining in the fermentation. Samples were collected from each individual fermentation in a 50 mL centrifuge tube and frozen at -80°C until further use.

3.2.3 Cider Chemistry and Nutrient Evaluation

Cider chemistry metrics were measured using analysis described above for apple juice materials section. In addition to the previously listed chemical analysis, residual sugars (D-Fructose/ D-Glucose Liquid Ready Reagents (K-FRGLQR) (Assay Kit, Megazyme International, Wicklow, Ireland) , ethanol content (AOC 984.14 using Gas Chromatography), total H_2S (µg L^{-1}) and total fermentation duration (hours) tracked through CO_2 loss.

Each yeast nutrient was evaluated for chemical composition including total YAN consisting of Primary Amino Nitrogen and ammonia ions and amino acid using the same methods listed above in apple juice materials section. methods

3.2.4 Statistical analysis

All cider chemistry data is reported as mean ± standard error of the mean (SEM) for n=3 analytical replicates. A 3-Way-ANOVA was performed on the influence of three variables (yeast strain, yeast nutrient type and timing) for pH, TA, residual sugars, total H_2S production, total fermentation duration, and ethanol data. For each data set, there were three yeast strain levels (M2, EC1118, ICV OKAY), five nutrient type levels (control, K, experimental, O, and DAP) and two timing of nutrient addition levels (single and split). Significance was defined as 0.05. Interaction plots were used to evaluate three way interactions effects between yeast strain type, yeast nutrient type and yeast nutrient timing. Tukey's HSD was performed as a post hoc test to evaluate the differences between average means of all chemistry data.

3.2.5 Pilot Scale Fermentation for Sensory Evaluation

Results from previous cider chemical analysis, preliminary sensory evaluation and the limitation of sensory panelists lead to the selection of specific cider treatments. Two yeast strain levels (M2 and EC1118), three nutrients (Fermaid O, experimental yeast nutrient and DAP) and two timing of nutrient additions (single addition and split). Yeast strains were chosen based on largest differences between total H_2S produced in microscale fermentations and the thought that higher H_2S concentration would lead to more off aromas. Yeast nutrients were chosen based on differences in composition. Experimental yeast nutrient consists of both ammonia ions and amino acids fractions. Fermaid O only consists of organic nitrogen and DAP is only ammonium ions. The two timing additions were kept for sensory evaluation because this is the recommended use of the products. -A total of twelve treatments were chosen for evaluation in order to prevent

panelist fatigue. Selected treatments were scale up and fermented in triplicate in 1 gallon glass jugs. Fermentations were conducted in the same manner using a scaled up method based off the microscale fermentations. Nutrient additions and yeast strains remained the same at 25 g hL^{-1}. All scale up cider fermentations treatments were conducted in triplicate.

Fermentations were carried out at 18°C and gently hand mixed twice per day. Fermentation performance was monitored by testing residual sugars using the D-fructose and D-glucose enzyme kit. Fermentations were considered complete when there was less than 2 g L^{-1} of sugar remaining in the fermentation. H_2S tube could not be used as airlock for scale up fermentations due to the pre-calibrated scale of the tubes and the size of the scale up fermentations. Maximum readings would be reached too quickly before researchers could change the tubes. To measure H_2S produced in scaled up fermentation, H_2S of the head space for each fermentation vessel was measured using a hand pump and a H_2S tube (Kitagawa America).Post-fermentation, the triplicate treatments were combined into a homogeneous lot. The homogenate was then aliquoted into 375 mL bottles that had previously been purged with nitrogen and capped. Bottles was stored at 0°C prior to sensory evaluation.

3.2.6 Sensory Evaluation

Sensory analysis was conducted on selected cider formulations to determine differences in aroma between treatments. The goal was to understand differences in consumer liking and descriptions of the cider aromas among the treatments. Sensory evaluation was conducted in two separate parts: lexicon generation and Check-All-That-Apply (CATA) task.

3.2.7 Samples

For both portions of the study, cider samples were evaluated at room temperature and about 22mLof cider was poured into a black wine glass to conceal any color variation. Sample

glasses were covered with a watch glass to concentrate aromas. Samples were labeled with 3 digit blinded code. Each panelist received 6 of the 12 cider samples to prevent sensory fatigue. The samples were presented in randomized design. Samples for lexicon generation were presented as a modification of Williams Latin Square designed using R statistical software and samples for CATA were presented using Series 36 Raghavarao predetermined by Compusense sensory evaluation software.

3.2.8 Panelists

All panelists were 21 years of age or older and recruited from the Virginia Tech and Blacksburg area. Each panelist could take part in each part of the test only once. Panelist that took part in the first portion of the study could participate in the second part of the study. Panelist were asked to only smell the cider. No consumption of cider was allowed.

3.2.9 Lexicon Generation

For lexicon generation, panelists (N_1=45) were presented with a single cider at a time and asked to remove the watch glass and smell the sample. First for each cider, they were asked to rate the aroma on based on liking. The 9-point hedonic scale was used with 1 being “dislike extremely” and 9 being “like extremely”. They were then instructed to smell the cider again and describe the aroma in their own words posed as an open ended response.

3.2.10 Check-all-that-apply

Data collected from the lexicon generation was used to generate a list of 25 descriptors presented in the CATA task. For lexicon generation, panelists (N_1=63) were presented with a single cider at a time and asked to remove the watch glass and smell the sample. First for each cider, they were asked to rate the aroma on based on liking using the same 9-point hedonic scale. They were then instructed to smell the cider again and asked to select at least one but as many descriptors from the list of 25 attributes that describe the aroma of the sample. Panelists also had

the option to enter attributes in the “other “options when an attribute could not be found on the list.

3.2.11 Sensory Statistical Analysis

Responses from lexicon generation were compiled and used to generate the list of attributes for the CATA portion. This was done by the use of classical text analysis (Lahne et al., 2013; Phetxumphou et al., 2020; Symoneaux et al., 2012). Words that were duplicates were combined, non-descriptive words were deleted, and synonyms were combine. Words with similarities were grouped into one word that described the attribute. Analysis was performed independently by two researchers to minimize bias. Researches met after grouping words individually and then discussed differences to merge descriptors into one list.

Liking scores collected for CATA task were averaged for each of the induvial ciders and presented as average liking score ± STD and graphed as a box and whisker plot and Tukey’s HSD test was used to determine differences between average mean liking scores with significance set at 0.05. Responses from the CATA study were compiled and analysis using R statistical software. CATA attribute data was analyzed with correspondence analysis (CA) to visually represent attributes and ciders(Greenacre, 2013; Meyners et al., 2013).

3.3 Results and Discussion

3.3.1 Starting Juice Chemistry and Yeast Nutrient Evaluation

Primary juice chemistry of initial starting apple juice (AJ) is shown in Table 5. All parameters for juice chemistry (pH, TA, total YAN, and total sugars) are within the recommend ranges for cider fermentation (A. Lea & Drilleau, 2003). This specific AJ was chosen for the low total YAN concentration of 53.16 ± 2.94 mg N L^{-1} because it is a general representation of YAN concentrations found in apples and the goal of this research was to evaluate how addition of yeast nutrient and timing of nutrients, affects H_2S production (Boudreau et al., 2018). Ammonia

concentration in initial AJ was below the enzymatic kits limit of detection of 0.07 mg N L^{-1}. Amino acids were measured and is reported in Table 5. AJ was highest in asparagine followed by aspartate (33.83 ± 0.00 mg L^{-1} and 10.19 ± 0.00 mg L^{-1} respectively) with other trace amino acids present. This amino acids profile is typical of amino acids found in reconstituted apple juice (Wu et al., 2007; Ye, Yue, & Yuan, 2014).

Table 5. Primary Juice Chemistry of Reconstituted Apple Juice used for all microscale fermentations and scale up fermentations

Primary Juice Chemistry		
	Average	SEM
pH	3.68	0.02
Titratable Acidity (g L^{-1} as malic acid equivalents)	4.62	0.03
Ammonia Concentration (mg N L^{-1})	0.00	0.00
PAN Concentration (mg N L^{-1})	53.16	2.94
Fructose (g L^{-1})	57.80	0.70
Glucose (g L^{-1})	29.56	0.36
Sucrose (g L^{-1})	9.55	0.12
Total Sugars (g/L)	96.91	
Amino Acids (mg L^{-1})	Average	SEM
Histidine	0.24	0.01
Asparagine	33.83	0.00
Serine	1.25	0.36
Glutamine	1.18	0.01
Arginine	0.30	0.00
Glycine	0.22	0.00
Aspartate	10.19	0.00
Glutamate	1.72	0.03
Threonine	0.42	0.00
Alanine	1.46	0.02
Gamma Aminobutyric acid	0.63	0.01
Proline	0.32	0.00
Cysteine	0.00	0.00
Lysine	0.00	0.00
Tyrosine	0.22	0.00

Methionine	0.11	0.00
Valine	0.31	0.00
Isoleucine	0.42	0.00
Leucine	0.12	0.00
Phenyalanine	0.14	0.00

Yeast nutrient chemistry of all four yeast nutrients are shown in Table 6. Reported total YAN concentrations in the manufacturer's handbook are similar to total YAN concentrations measured via enzymatic kit during nutrient evaluations. Most notably, each yeast nutrient had different concentrations of PAN and ammonia. Experimental yeast nutrient and Fermaid K consisted of both ammonia ions and PAN, Fermaid O consisted only of PAN, and DAP is comprised of ammonia ions. The faction of PAN and ammonia ions each yeast nutrient agrees with the reported compositions in the manufacturer's handbook. Amino acids in each yeast nutrient varied in concentration and presence. Experimental yeast nutrient contained 19 amino acids and Fermaid O contained 17 amino acids out of 20 amino acids measured. In Fermaid K only 7 of the 20 amino acids measured amino acids were detected. Each yeast nutrient provided different levels of amino acids and total YAN concentrations to the AJ. No specific amino acid information was provided by the manufacturers for the use of comparisons. The Waters ACQUITY UPLC H-Class Bio Amino Analysis uses a derivatization agent that must not be fully consumed in the reaction and remain in excess in order to measure amino acids. With the high levels of ammonia ions, reagent concentration was doubled and may be a source of error for this method. Additional method development should be conducted to measure amino acids in samples with high ammonia ions but is beyond the scope of this experiment and paper.

Table 6. Yeast Nutrient Composition of all Four Yeast Nutrient in Water

Yeast Nutrient Composition								
25g h L^{-1} total yeast nutrient in water	**Fermaid K**		**Experimental Yeast Nutrient**		**Fermaid O**		**DAP**	
	Average	SEM	Average	SEM	Average	SEM	Average	SEM
pH	3.63	0.00	3.63	0.00	3.63	0.00	3.67	0.00
Titratable Acidity (g L^{-1} as malic acid equivalents)	4.59	0.07	4.65	0.05	4.65	0.05	4.59	0.02
Ammonia Concentration (mg N L^{-1})	21.33	0.12	38.25	0.26	0.00	0.00	46.74	6.288
PAN Concentration (mg N L^{-1})	3.59	0.71	4.79	0.16	10.02	0.04	0.00	0.00
Amnio Acids								
25g h L^{-1} total yeast nutrient in water	**Fermaid K**		**Experimental Yeast Nutrient**		**Fermaid O**			
mg L^{-1}	Average	SEM	Average	SEM	Average	SEM		
Histidine	0.00	0.00	0.10	0.01	0.31	0.00		
Asparagine	0.00	0.00	0.24	0.01	0.00	0.00		
Serine	0.00	0.00	0.32	0.00	0.58	0.04		
Glutamine	0.02	0.06	0.08	0.00	0.28	0.01		
Arginine	0.00	0.00	0.26	0.00	0.17	0.03		
Glycine	0.00	0.00	0.18	0.00	0.41	0.01		
Aspartate	0.00	0.00	0.39	0.00	0.91	0.02		
Glutamate	0.00	0.00	0.94	0.01	0.28	0.01		
Threonine	0.07	0.01	0.25	0.00	0.56	0.01		
Alanine	0.01	0.02	0.52	0.01	0.15	0.01		
Gamma Aminobutyric acid	0.01	0.02	0.14	0.00	0.19	0.00		
Proline	0.01	0.02	0.18	0.00	0.52	0.01		
Cysteine	2.49	2.13	0.00	0.00	0.00	0.00		
Lysine	0.00	0.00	0.37	0.00	0.37	0.00		
Tyrosine	0.00	0.00	0.27	0.00	0.32	0.14		
Methionine	0.00	0.00	0.27	0.13	2.13	0.85		
Valine	0.00	0.00	0.31	0.10	0.39	0.00		
Isoleucine	1.91	3.53	0.34	0.00	0.64	0.01		
Leucine	0.00	0.01	0.58	0.00	0.33	0.00		
Phenyalanine	0.00	0.00	0.61	0.03	0.00	0.00		

3.3.2 Microscale Cider Chemistry

Cider chemistry parameters (pH, TA, total residual sugars, and ethanol; see supplementary Table 8) were all measured as indicators of fermentation health and progression. Ciders were considered complete if residual sugars remaining after fermentation were less than 2 g L^{-1}. All fermentations were considered complete because sugars were below the detectible limit of the enzymatic kit at 133 mg N L^{-1}. Ethanol content ranged from 6.06% to 6.30% confirming that alcoholic cider was produced. pH of ciders ranged from 3.53 to 3.67 and titratable acidity presented in malic acid equivalent ranged from 5.07% to 6.37% malic acid equivalent. Three way ANOVA results indicated interaction effects (plotted in interaction plot supplementary Figure 10) between treatment factors. The ranges of percent ethanol, pH, and TA were significantly different from one another when performing Tukey's HSD test ($p < 0.05$) to test for significant differences between averages (supplemental data Table 8).In the grander scheme of cider aroma and cider taste, there is likely little discernable differences between ciders based off cider chemistry alone. The differences among pH, TA and so on would likely not be of concern in practical cider maker.

3.3.3 Total H_2S Production

A 3-Way-ANOVA was performed on the influence of three variables (Yeast strain, yeast nutrient type, and timing) on the total H_2S production. For total H_2S production there was a significant main effect for both yeast strain and nutrient timing. There were significant interaction effects between yeast strain and nutrient. All other interactions were not significant. Interactions plots for total H_2S production and total duration (supplemental Figure 11, panel A).

Total H_2S concentration (µg L^{-1}) over the entire fermentation duration for all treatments were grouped by the three treatment variables (yeast strain, yeast nutrient type and timing of yeast nutrient addition) in order to easily visual treatment effects. Total H_2S concentrations

across all treatments can be found in supplementary figures (Figure 9). Total H_2S concentrations are presented in bar graphs (mean of triplicate fermentations ± SEM) in Figure 1, Figure 2, and Figure 3. Ciders in this figure are coded with three letter codes. The first letter represent the yeast strain (M =M2, E =EC1118 and I= ICV OKAY). The second letter represents the yeast nutrient (CON = control, K = Fermaid K, E = Experimental, O = Fermaid O and D = DAP). The final letter will represent the timing of yeast nutrient, (S = single addition and D = split level addition).

In Figure 1, total H_2S concentration grouped by yeast strain. It is clear in Figure 1 that that fermentations with M2 yeast produced the most H_2S over all with an average of 525.63 ± 53.31 µg L^{-1} across all M2 treatments. EC1118 fermentations produced the least H_2S on average 118.26 ± 26.33 µg L^{-1}. Surprisingly, ICV OKAY produced more H_2S than EC1118 yeast on average over all treatments (209.26 ± 31.63 µg L^{-1} vs. 118.26 ± 23.33 µg L^{-1} respectively). ICV OKAY is considered "no/low H_2S producing yeast strain" when used in wine. This yeast strain may have specific nutrient requirements that were absent in the AJ matrix such as B vitamins, higher concentration of total YAN or other juice chemistry differences compared to grape juice (Jeffri C. Bohlscheid et al., 2011; Lamikanra et al., 1995; Wu et al., 2007; Xia et al., 2010). When observing total H_2S production, Figure 1 shows that yeast nutrient type did not affect H_2S production of ciders fermented with ICV OKAY but had a significant effect on H_2S production in cider fermented with M2 and EC1118 yeast strain. Within M2 yeast strain MCD, MES, and MCS produced the least H_2S. Within EC1118 yeast strain EED, EED, and EDS produced the least H_2S. It has been demonstrated by many others that yeast strain has are large effect on determining amount of H_2S produced (Manginot et al., 1998; A Mendes-Ferreira, Mendes-Faia, & Leao, 2002; Ana Mendes-Ferreira et al., 2009; Ugliano et al., 2009). Different yeast also require different nutrients which can explain why nutrient type influenced H_2S production on M2

and EC1118 but not ICV OKAY. Fermentation with ICV OKAY yeast strain may not have received the required nutrients from the tested yeast nutrients (Fairbairn et al., 2017; Gobbi et al., 2013; Ljungdahl & Daignan-Fornier, 2012). No yeast nutrient reduced H_2S production consistently across all yeast strains, suggesting that yeast nutrient should be selected based off of the yeast strain used rather than a one-size-fits-all yeast nutrient.

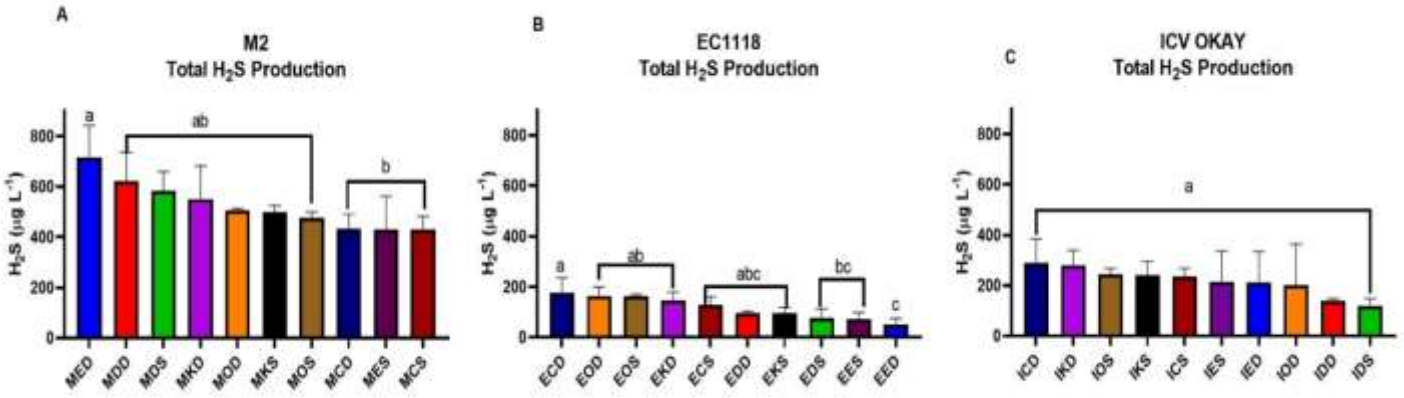

Figure 1: Total H_2S Production Grouped by Different Yeast Strains used for Fermentation. Tukey's HSD test was preformed to product letter groupings. If any one letter is shared between a bar there is no significant difference between the means. No common letter identifies significant difference at $\alpha = 0.05$.

In Figure 2, total H_2S concentrations are grouped by yeast nutrient type to clearly visually nutrient effect on H_2S production across all yeast trains. M2 yeast strain produced the highest levels of concentration regardless of the nutrient type added followed by ICY OKAY treatments. Yeast strain effect could not be overcome by nutrient type addition in this experiment. The highest concentration of H_2S produced and the worst treatment combinate of yeast trains and nutrient type was MED (716.66 ± 72.6 µg L^{-1}). The lowest H_2S production and best treatment combination was EED (50.66 ± 13.96 µg L^{-1}).

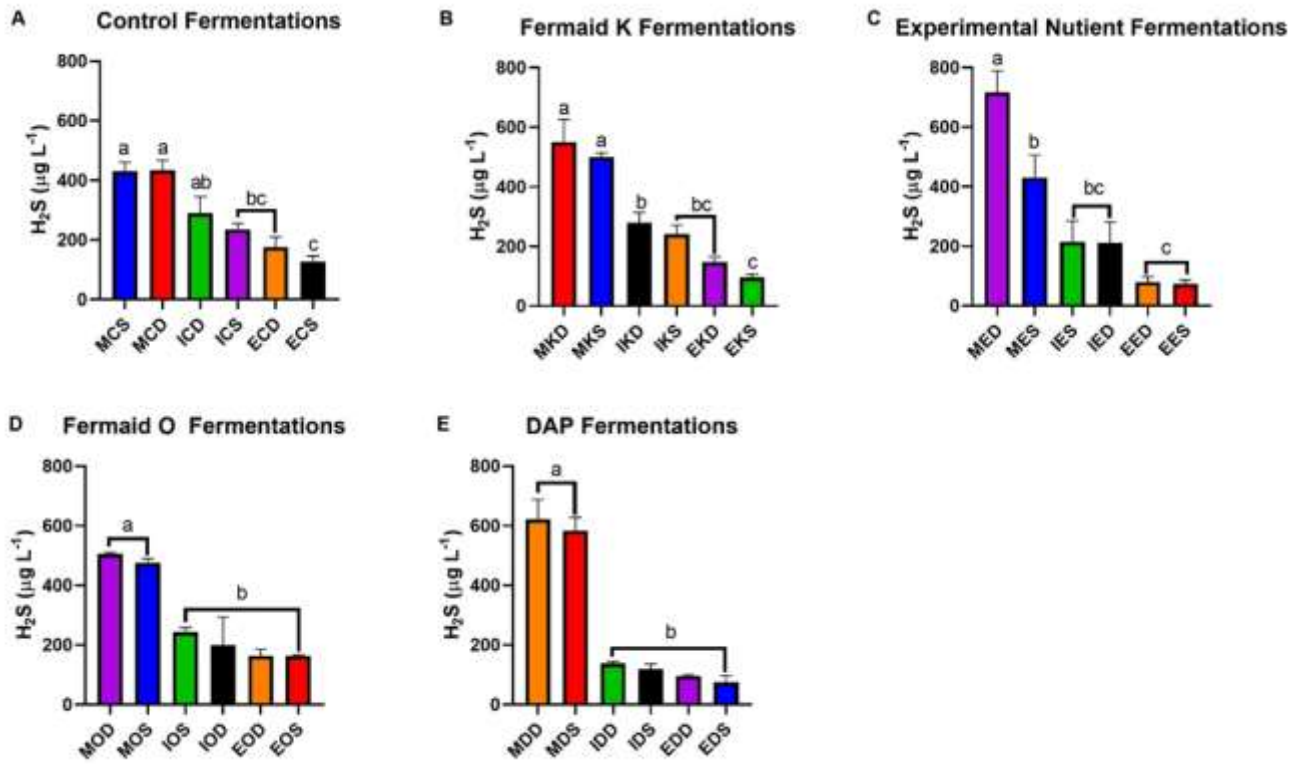

Figure 2:Total H_2S Produced (µg L^{-1}) Grouped by Type of Yeast Nutrient Used. Tukey's HSD test was preformed to product letter groupings. If any one letter is shared between a bar there is no significant difference between the means. No common letter identifies significant difference at $\alpha = 0.05$.

In Figure 3 total H_2S concentrations are grouped by yeast nutrient timing. Figure 3 suggests that timing of yeast nutrient did not significantly lower H_2S production between all treatments and in one case split nutrient addition increased total H_2S between MES and MED (Figure2 panel A). This could be due to the concentration of yeast nutrient used and the total amount of YAN in each cider treatment. The suggested minimum amount of YAN in grape must for a complete fermentation is between 120-140 N mgL^{-1} in starting matrix, although some have suggested even higher concentration of nitrogen of 267 mg N L^{-1} or 350 mg N L^{-1} (Alexandre & Charpentier, 1998; A. Mendes-Ferreira et al., 2004). The nutrient treatment with the highest level

of YAN in this experiment was DAP, which raised the total YAN to about 103 mg N L^{-1}, far below the minimum amount recommended.

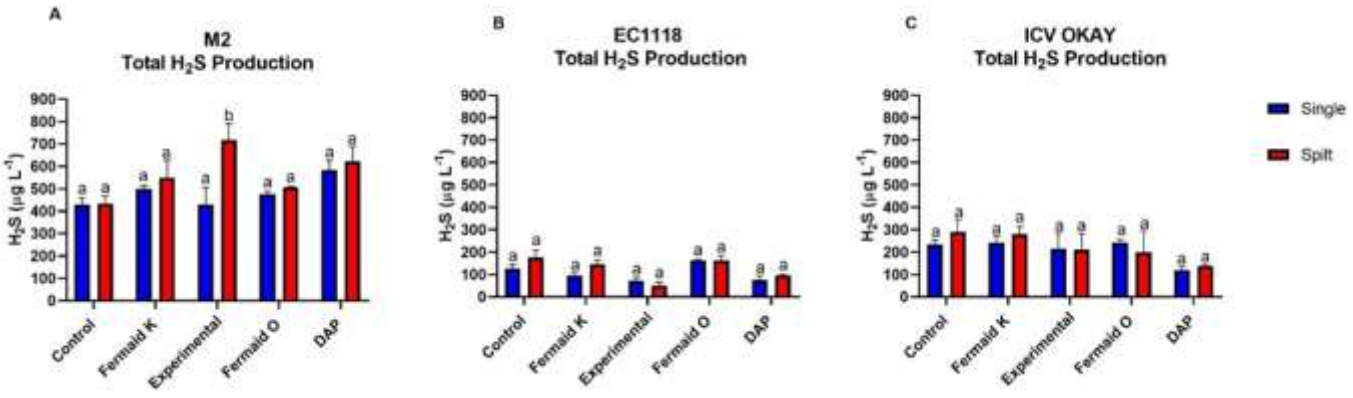

Figure 3: Total H_2S Produced (µg L^{-1}) Grouped by Timing of Yeast Nutrient. Tukey's HSD test was preformed to product letter groupings only within single and split nutrient timing of specific treatments. If any one letter is shared between a bar there is no significant difference between the means. No common letter identifies significant difference at $\alpha = 0.05$.

With multiple interacting factors on total H_2S production determination the most influential predictors factors to provide better understanding of factors was desired. Partition trees were made to identify the most important predictor factors for H_2S production (Figures 4). Figure 4, the partition tree for total H_2S, indicates the most important factor for total H_2S production was yeast strain, as indicated by the first split in the tree followed by nutrient type indicated in the split in the tree within yeast strain nodes. This figure demonstrates that yeast strain was the most important deciding factor for total H_2S production. This is not surprising as it has been reported that yeast strain plays a large role in H_2S formation (Manginot et al., 1998; C. S. Thomas et al., 1993; Ugliano et al., 2009). This also suggests that yeast strains could potentially be paired with specific yeast nutrients to provide optimal nutrient availability depending on the yeast strain. For example, if a cider producer wishes to use ICV OKAY yeast,

DAP or experimental yeast nutrient be used in rather than Fermaid K or Fermaid O. These yeast strain and nutrient type combination would likely yield lower overall total H_2S production. Different nutrient and yeast strain combinations can be further explored in cider research to provide optimization of nutrient additions.

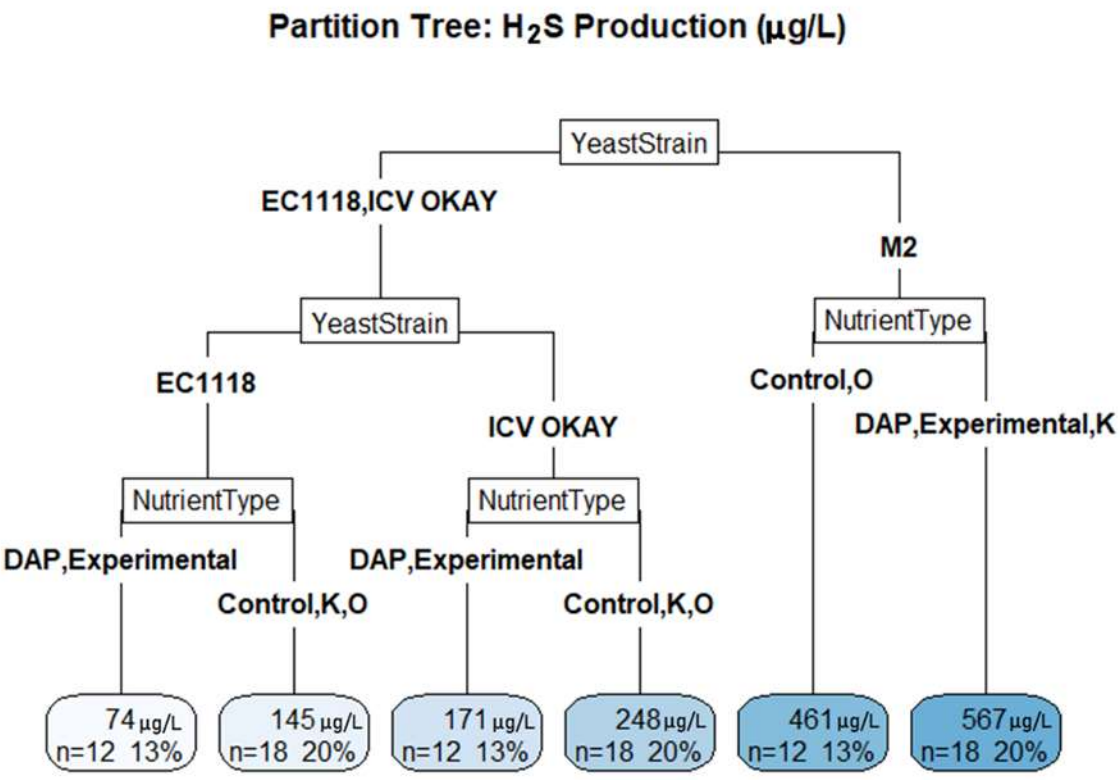

Figure 4: Partition tree for total H_2S (µg L^{-1}) production. Each split line represents the split value of the tree that shows where the predictor is less (splits left) or higher than the predictor value (split right side) ultimately leading to the final leaves (bottom blue boxes). Predictor values not shown. The percentage presented in each leaf, represents the total percent of observations included in that leaf. The numerical value presented is the average H_2S (µg L^{-1}) production of observations included in the leaf.

3.3.4 Results for Total Fermentation Duration

For total fermentations duration (hrs) was also evaluated in microscale fermentations. A 3-Way-ANOVA was performed on the influence of three variables (Yeast strain, yeast nutrient type, and timing) on total fermentation duration. The main effect of yeast nutrient type and yeast nutrient timing was significant. The interaction effect between yeast strain and nutrient type was significant. The remaining interactions were not significant. Interactions plots for both total H_2S production and total duration (supplemental data Figure 11, panel B). Interaction effects are not surprising due to the complex nature of yeast fermentation and metabolic pathways for H_2S production.

Figure 5 shows the total fermentation duration of all cider treatment. The longest fermentation, EDS, reached completion in and average of 312 hours while the shortest, MDD, completed in an average of 240 hours. The difference between these two fermentations is about 72 hours (3 days). To determine which factors were most relevant for producing fermentation duration, a partition tree was made. Figure 6, the partition tree for total fermentation duration, shows that the most important factor for duration of fermentation was yeast nutrient type, followed by timing of addition. When considering how to ferment cider, producers should consider the tradeoffs between H_2S production and total duration of fermentation. If shortening the fermentation time is most concerning, then producers should consider what type of yeast nutrients they would like to use, although in practical cider making a 3 day difference between cider fermentation times may be insignificant. If aroma and reduction of H_2S are the main priority for the producer then yeast strain should be the highest consideration.

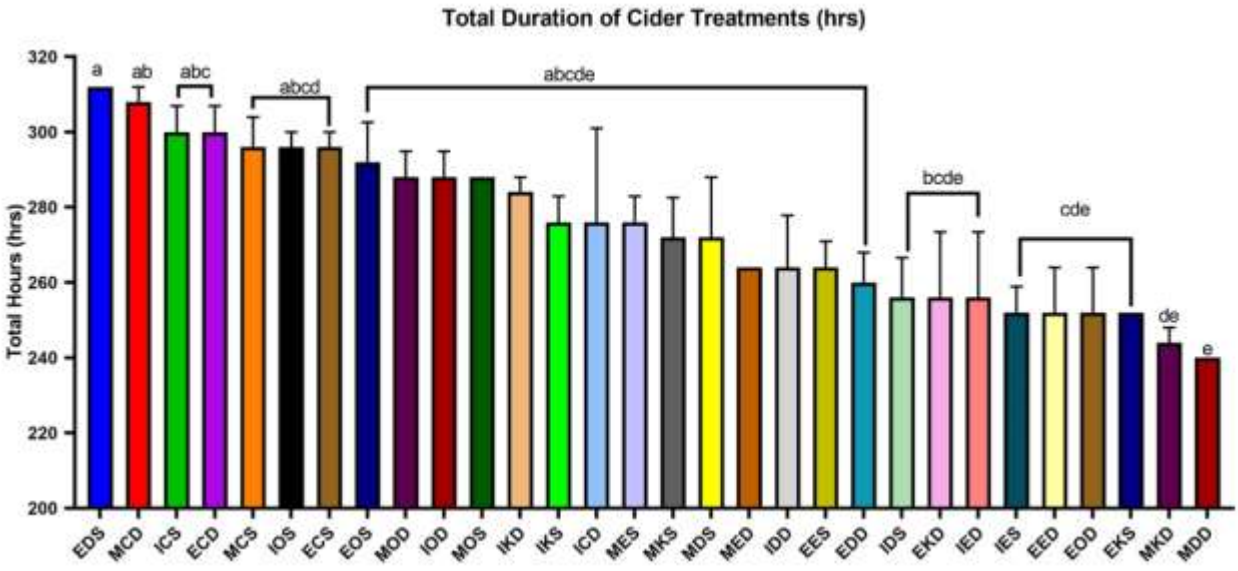

Figure 5: Total Fermentation Duration (hrs). Three way ANOVA with Tukey's HSD test was preformed to product letter groupings. If any one letter is shared between a bar there is no significant difference between the means. No common letter identifies significant difference at $\alpha = 0.05$.

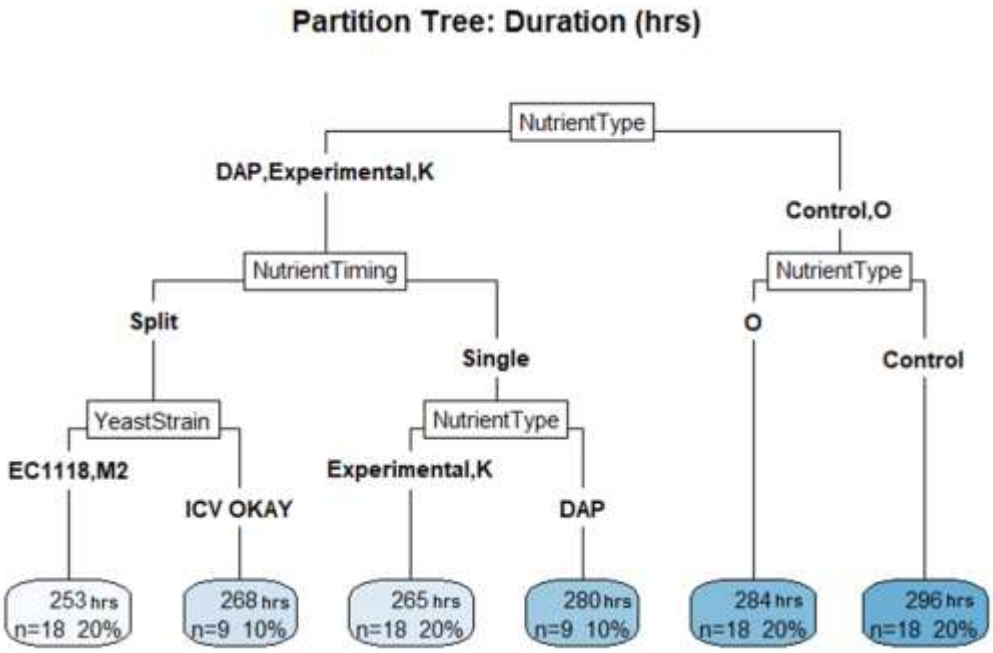

Figure 6: Partition tree for total fermentation duration (hrs) production. Each split line represents the split value of the tree that shows where the predictor is less (splits left) or higher than the predictor value (split right side) ultimately leading to the final leaves (bottom blue boxes). Predictor values not shown. The percentage presented in each leaf, represents the total

percent of observations included in that leaf. The numerical value presented is the average duration of fermentation (hrs) production of observations included in the leaf.

3.3.5 Aroma Results

A total of 375 descriptors were obtained from lexicon generation. After classical text analysis, a final list of 25 descriptors were derived and used in the CATA study as seen in the CATA contingency table in Table 7. The attributes represent the scaled cider samples from this study rather than all ciders on the market. The attributes are helpful to understand what aromas are produced the treatment variables chosen. The most frequently used aroma term in the CATA study were apple and alcohol followed by fruit, sweet and wine-like as seen in Table 7. The aroma descriptors used the least to describe this sample set were vegetal and meaty. Sulfur was checked a total of 37 times with it being selected the most for MED.

Table 7: Contingency Table from Check-All-That-Apply (CATA) Task of Selected Cider Treatments with Variations of Yeast Strain, Yeast Nutrient and Timing of Yeast Nutrient.

	Cider Treatments Evaluated												
Aroma	MES	MOS	MDS	MED	MOD	MDD	EES	EOS	EDS	EED	EOD	EDD	Total
Apple	8	17	17	9	15	11	12	11	18	7	17	8	150
Sweet	6	14	16	10	8	6	9	8	15	9	11	9	121
Sour	7	7	6	7	4	9	10	5	7	8	5	8	83
Alcohol	11	11	17	14	17	13	10	11	13	12	12	9	150
Fruit	12	13	13	10	8	13	9	9	13	9	15	8	132
Rotten	2	1	3	4	4	0	3	3	4	1	3	7	35
Sulfur	2	2	2	6	3	3	2	3	4	3	4	3	37
Wine Like	12	9	15	10	11	8	8	8	11	10	8	11	121
Floral	2	10	7	1	4	6	5	2	4	4	6	5	56
Musty	11	5	6	11	6	7	10	8	7	8	6	8	93
Earthy	6	4	5	3	5	8	9	8	4	4	2	4	62
Chemical	6	3	3	4	2	7	5	1	2	2	1	4	40
Candy	2	5	6	2	1	1	3	2	5	2	3	3	35
Vinegar	3	5	4	5	4	3	11	5	3	7	4	4	58
Green Apple	4	5	8	8	4	5	6	3	7	4	6	5	65
Grape	5	7	5	4	2	5	3	2	6	5	4	5	53
Vegetal	0	2	2	3	3	2	2	3	1	3	3	1	25
Meaty	4	0	1	1	3	3	4	2	0	2	3	3	26
Bready	4	0	3	7	5	5	4	5	0	1	5	2	41
Ripe	4	5	4	1	5	5	4	6	5	1	4	4	48
Band-Aid	1	3	0	4	1	2	3	6	3	2	1	0	26
Yeasty	5	4	8	8	8	5	4	5	6	7	6	7	73
Astringent	5	2	2	3	3	4	5	3	5	2	3	1	38
Juicy	1	6	6	2	2	6	5	6	7	2	1	5	49
Metallic	2	4	3	1	3	2	2	3	2	2	1	4	29
Other	2	2	0	1	2	5	1	2	1	5	1	1	23

Average liking of each cider aroma was calculated and tested for Tukey's HSD ($p < 0.05$) and graphically presented in Figure 7. MOS average liking score was significantly different from MED, EOD, MES, and EES average liking score as seen in Figure 7 represented by treatment not sharing grouping letters. The remaining ciders were not significantly different from any of the other cider average liking scores. The highest average liking was 6.42 ± 1.65 SD

for MOS which is in the range of "like slightly" and the lowest average liking of cider aromas was 5.00 ± 1.84 SD for EES which corresponds to "neither like nor dislike". Average liking scores suggest that panelists were indifferent about the cider aromas over the whole sample set.

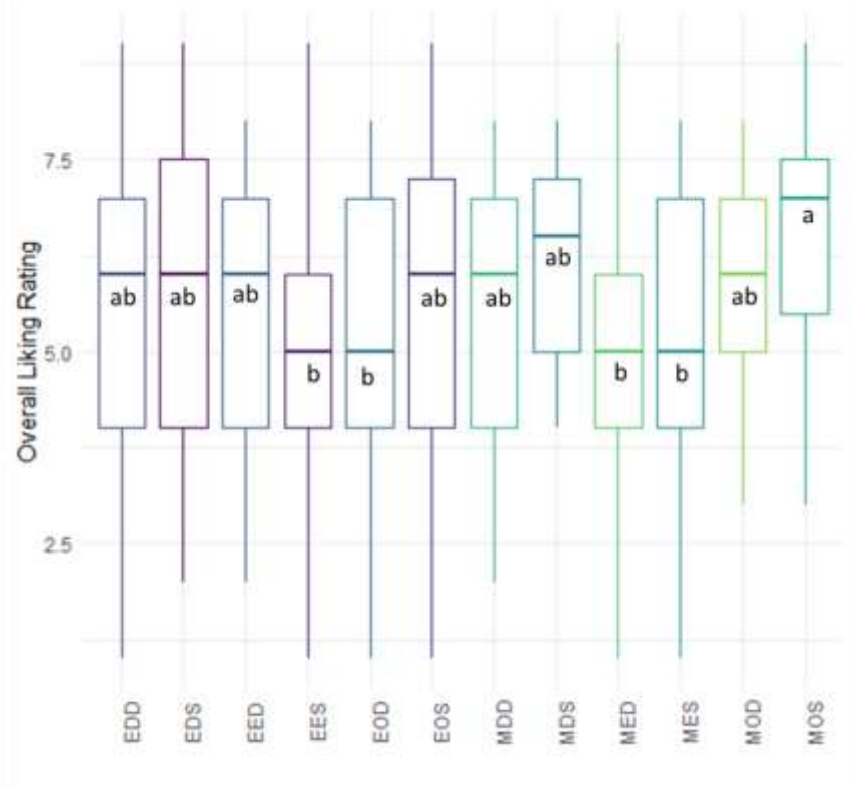

Figure 7: Selected sensory ciders were evaluated for aroma overall liking. Box and whisker plots represent the average liking score for specific ciders. Interquartile ranges, median and minimum and maximum ranges are included for each cider. Ciders were presented to panelist with a an incomplete block design and as a results each cider was evaluated different total number of times an replicates values (n) are as followed: EED(), EDS (n = 32), EES (n = 30), EOS (n = 32), EDD (n=31), EED (n=31), EOD (n = 32), MDS (n = 32), MES (n = 32), MOS= 31, MDD (n = 32), MED (n = 32), and MOD (n= 32).

Figure 8 depicts attributes checked for each cider in a correspondence analysis (CA) symmetrical biplot. A total of 51.92% of variance was explained by the first two dimensions in the CA. Dimension 1 separates meaty from candy and chemical from floral attributes while dimension 2 separates rotten from floral/chemical aroma attribute. Descriptors in the same

direction as a plotted cider signify aromas associated with that cider. For example, MOS is in the same direction as descriptors “floral” and “candy”. Figure 8 shows little indication of cider aromas being groups by any treatment used in this study (yeast strain, yeast nutrient type or timing). Regardless of a lack of a clear pattern by treatment, the biplot in Figure 8 indicates that two ciders, MCD and MOD, were more associated that sulfur and rotten attributes that other ciders like MOS as seen in the contingency table in Table 3. This could suggest that the combination of M2 yeast and the split addition of Fermaid O nutrients produced more noticeable sulfur aromas than other yeast strain and nutrient combinations. Single addition of Fermaid O, MOS, was more associated with floral, candy and grape attribute which indicated that a single addition of Fermaid O while using M2 yeast resulted in less sulfur aroma and more commonly used attributes to wine. Yeast nutrients, yeast type and yeast timing did contribute to different aromas attributes for each cider but there was not overall most liked cider aroma or clear grouping of ciders based off attributes for tested treatments.

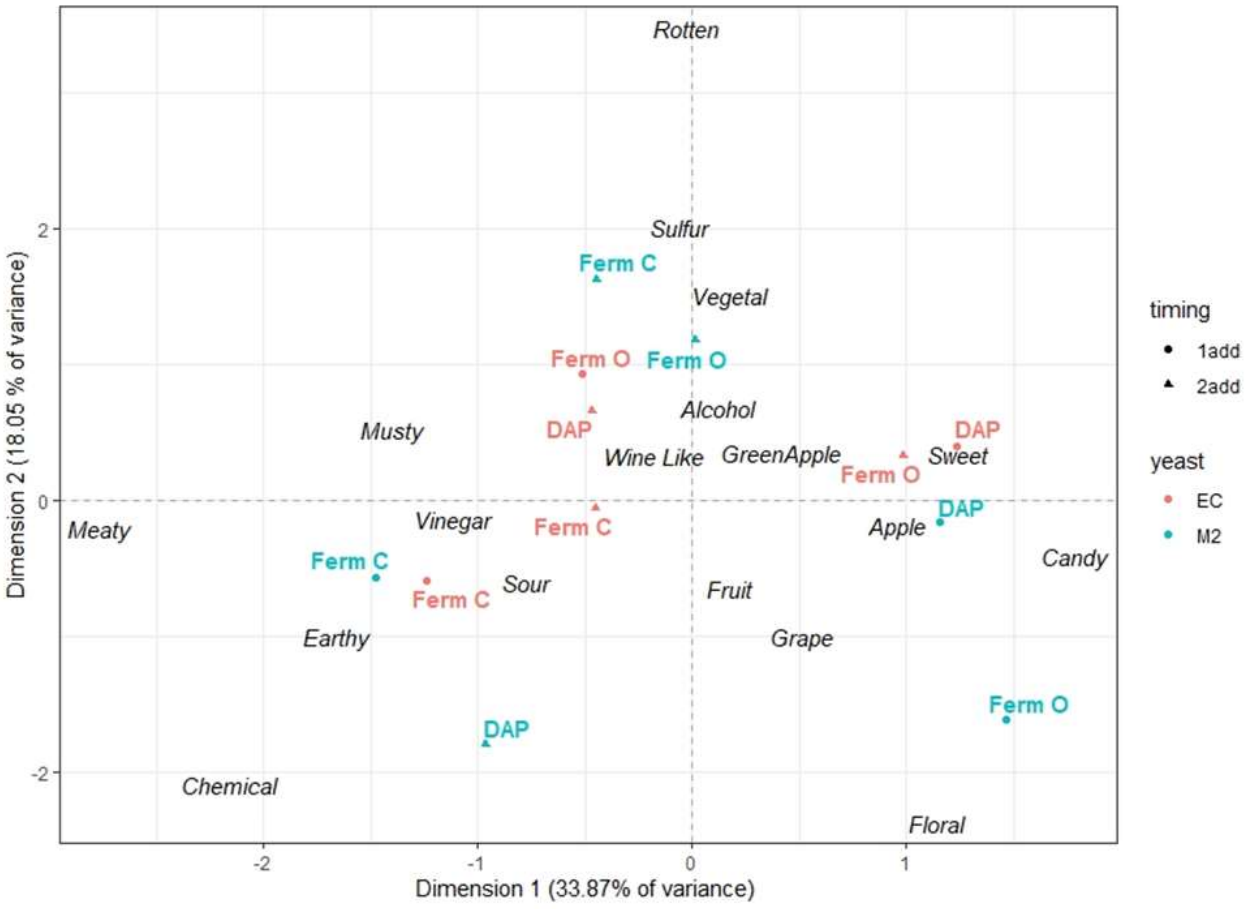

Figure 8: CA Factor Map for Ciders Evaluated by CATA Task.

3.4 Conclusion

This study evaluated the effects of four yeast nutrient supplements three of which were inactive yeast nutrients and the timing of addition of the yeast nutrients on H_2S production, fermentation kinetics and aroma quality in cider fermentation. Yeast strain was the most influential factor in H_2S production. M2 yeast strain produced the highest level of H_2S while EC1118 produced the least. Yeast nutrients only affected H_2S production in M2 yeast strain and ICV OKAY. The most important factor for total fermentation duration was yeast nutrient type followed by yeast nutrient timing. When evaluating cider aroma, selected ciders used in CATA sensory study showed no correlation by any variables tested in this study. This study demonstrates the need for more specific research focused of H_2S prevention strategies in cider

because wine making practices that are commonly used to prevent H_2S production does not reliably or consistently work in the cider matrix. For practical cider processing, cider makers should consider selection of yeast strain a prevention strategy for H_2S as well as continuing to consider the historical recommendations for YAN concentration.

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Declaration of Interest Statement

There are no potential conflicts of interest reported by authors.

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3.6 Supplemental Tables and Figures

Table 8: Cider Chemistry Parameters

Fermentation Treatment	pH			Titratable Acidity			Ethanol			Total Residual Sugars		
	pH	Standard Error	pH Groups	% Malic Acid	Standard Error	TA groups	% Ethanol	Ethanol Groups	Ethanol Groups	D-Glucose + D-Fructose (mg/L)	Standard Error	Residual Sugars Groups
MCONS	3.63	0.02	abcde	5.64	0.06	defgh	6.21	0.012	a	0.00	0.00	a
MKS	3.63	0.01	abcde	5.68	0.06	defgh	6.18	0.020	a	0.00	0.00	a
MES	3.65	0.01	abc	5.91	0.04	bcdef	6.21	0.032	a	0.00	0.00	a
MOS	3.61	0.01	bcdefg	5.56	0.21	efghi	6.22	0.009	a	0.00	0.00	a
MDS	3.58	0.01	fghi	5.87	0.11	cdef	6.20	0.043	a	0.00	0.00	a
ECONS	3.57	0.02	ghi	6.37	0.42	ab	6.23	0.030	a	0.00	0.00	a
EKS	3.59	0.01	defgh	6.37	0.25	ab	6.23	0.015	a	0.00	0.00	a
EES	3.55	0.02	hi	6.18	0.11	abc	6.16	0.027	a	0.00	0.00	a
EOS	3.53	0.01	i	6.19	0.14	abc	6.21	0.017	a	0.00	0.00	a
EDS	3.53	0.03	i	6.52	0.10	a	6.14	0.023	a	0.00	0.00	a
ICONS	3.63	0.01	abcdef	5.91	0.05	bcdef	6.17	0.031	a	0.00	0.00	a
IKS	3.64	0.01	abcd	6.06	0.04	abcd	6.30	0.009	a	0.00	0.00	a
IES	3.63	0.01	abcde	6.06	0.07	abcd	6.21	0.009	a	0.00	0.00	a
IOS	3.65	0.02	abc	5.74	0.12	cdefg	6.21	0.054	a	0.00	0.00	a
IDS	3.64	0.01	abcde	6.00	0.12	bcde	6.27	0.043	a	0.00	0.00	a
MCOND	3.62	0.01	abcdefg	6.10	0.09	abcd	6.16	0.038	a	0.00	0.00	a
MKD	3.61	0.01	bcdefg	5.96	0.07	bcde	6.18	0.013	a	0.00	0.00	a
MED	3.63	0.01	abdcef	5.74	0.09	cdefg	6.06	0.098	a	0.00	0.00	a
MOD	3.65	0.01	abc	5.56	0.08	efghi	6.21	0.032	a	0.00	0.00	a
MDD	3.59	0.01	defgh	5.70	0.04	defg	6.16	0.032	a	0.00	0.00	a
ECOND	3.60	0.01	cdefgh	6.03	0.04	bcde	6.14	0.038	a	0.00	0.00	a
EKD	3.62	0.01	abcdef	5.79	0.01	cdefg	6.15	0.067	a	0.00	0.00	a
EED	3.65	0.01	abc	5.64	0.14	defgh	6.27	0.052	a	0.00	0.00	a
EOD	3.64	0.01	abcde	5.37	0.03	ghij	6.21	0.021	a	0.00	0.00	a
EDD	3.58	0.01	efghi	5.46	0.02	fghij	6.20	0.040	a	0.00	0.00	a
ICOND	3.66	0.01	ab	5.84	0.06	cdefg	6.26	0.018	a	0.00	0.00	a
IKD	3.66	0.01	ab	5.11	0.05	ij	6.33	0.013	a	0.00	0.00	a
IED	3.65	0.01	abc	5.07	0.03	j	6.35	0.009	a	0.00	0.00	a
IOD	3.67	0.01	a	5.09	0.05	j	6.24	0.042	a	0.19	0.11	b
IDD	3.65	0.01	ab	5.19	0.02	hij	6.30	0.015	a	0.00	0.00	a

Table 9: Completed Cider Total H_2S Production and Total Duration of Fermentation

Fermentation Treatment	Total H_2S Production			Total Hours Fermentation		
	Total H_2S ($\mu g\ L^{-1}$)	SEM	Total H_2S Groups	Total Hours (hrs)	SEM	Total Hours Groups
MCS	430.00	21.23	bcde	296.00	4.62	abcd
MKS	487.50	10.21	abcd	272.00	10.58	abcde
MES	453.75	44.19	bcd	276.00	6.93	abcde
MOS	475.00	14.43	bcd	288.00	0.00	abcde
MDS	583.33	44.09	ab	272.00	16.00	abcde
ECS	127.00	19.09	fg	296.00	4.00	abcd
EKS	95.67	11.92	fg	252.00	0.00	cde
EES	73.33	14.53	fg	264.00	6.93	abcde
EOS	162.66	4.33	fg	292.00	10.58	abcde
EDS	75.00	21.52	fg	312.00	0.00	a
ICS	235.33	19.38	defg	300.00	5.93	abc
IKS	241.00	30.64	defg	276.00	6.93	abcde
IES	215.00	70.06	efg	252.00	6.93	cde
IOS	243.33	13.95	defg	296.00	4.00	abcd
IDS	119.00	17.15	fg	256.00	10.58	bcde
MCD	433.33	33.33	bcde	308.00	4.00	ab
MKD	550.00	76.37	ab	244.00	4.00	de
MED	716.66	72.65	a	264.00	0.00	abcde
MOD	505.00	5.00	abc	288.00	6.93	abcde
MDD	621.66	66.23	ab	240.00	0.00	e
ECD	175.66	34.65	fg	300.00	6.93	abc
EKD	146.60	18.55	fg	256.00	17.44	bcde
EED	50.66	13.96	g	252.00	12.00	cde
EOD	163.00	21.73	fg	252.00	12.00	cde
EDD	95.66	4.70	fg	260.00	8.00	abcde
ICD	290.00	55.08	cdef	276.00	24.98	abcde
IKD	280.33	33.99	cdefg	284.00	4.00	abcde
IED	211.00	71.63	efg	256.00	17.44	bcde
IOD	200.00	94.65	efg	288.00	6.93	abcde
IDD	138.33	6.01	fg	264.00	13.86	abcde

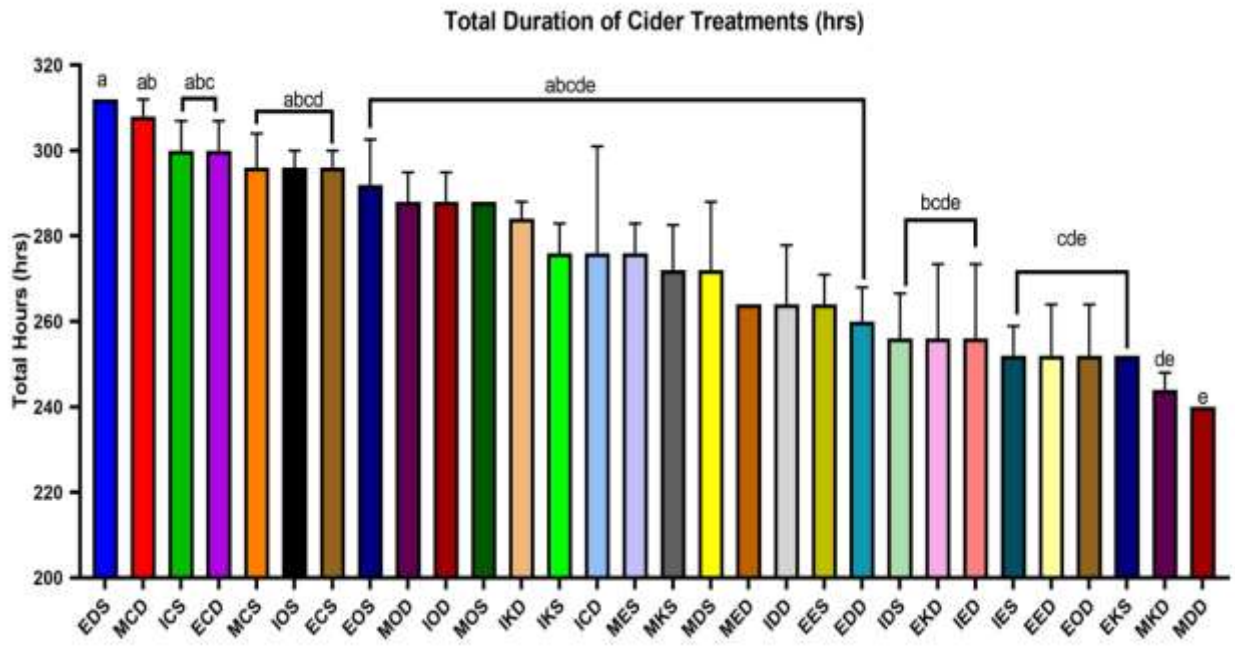

Figure 9: Total H_2S Production across all Cider Treatments. Ciders in this figure are coded with three letter codes. The first letter represent the yeast strain (M =M2, E =EC1118 and I= ICV OKAY). The second letter represents the yeast nutrient (CON = control, K = Fermaid K, E = Experimental, O = Fermaid O and D = DAP). The final letter will represent the timing of yeast nutrient, (S = single addition and D = split level addition). Each bar represents the mean (n=3) and standard error of the mean (SEM). Tukey's HSD test was preformed to product letter groupings. If any one letter is shared between a bar there is no significant difference between the means. No common letter identifies significant difference at $\alpha = 0.05$.

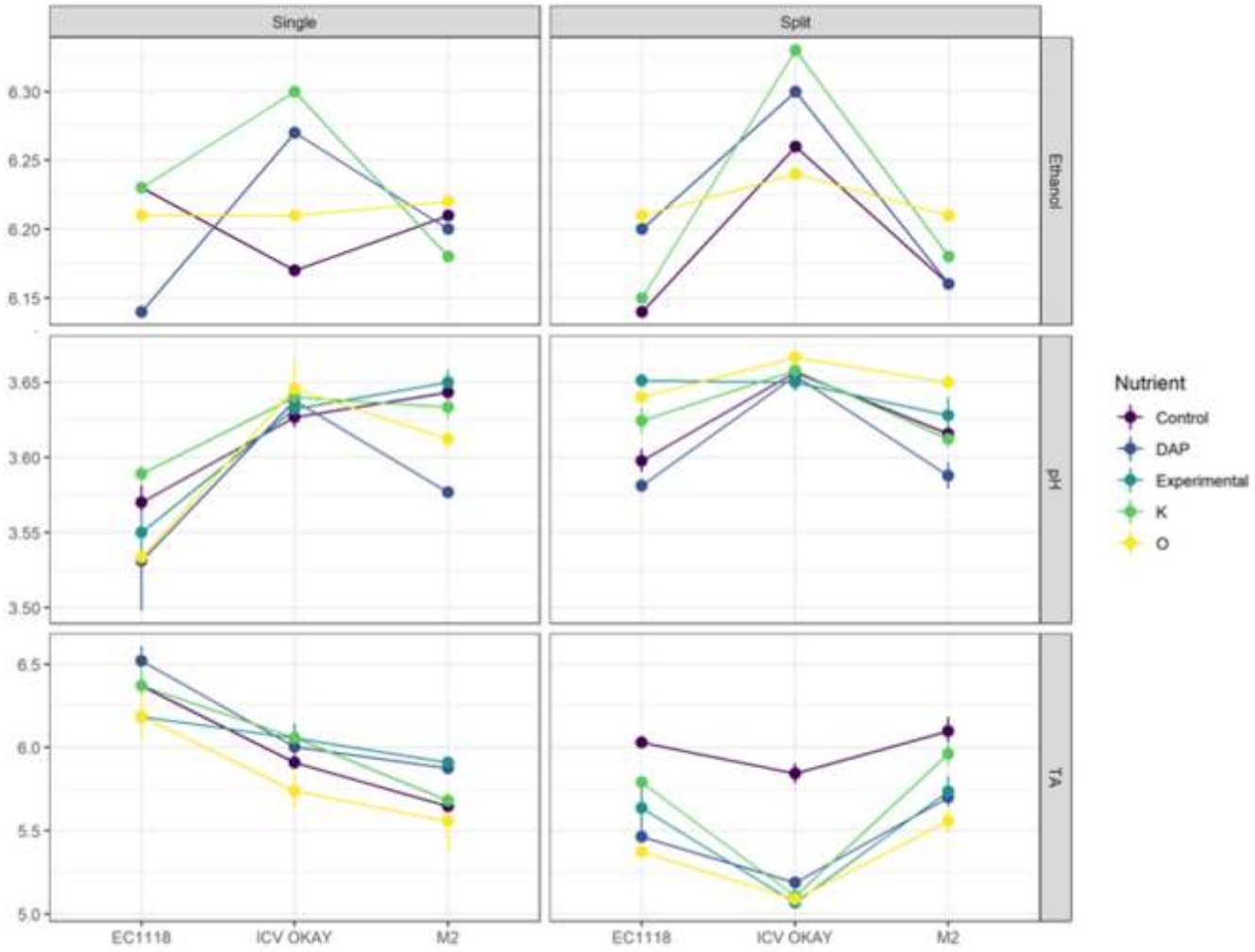

Figure 10: Interaction Plot for Cider Chemical Analysis. Parallel lines indicated no interactions effects. Non-parallel lines indicate interaction effects between the treatments.

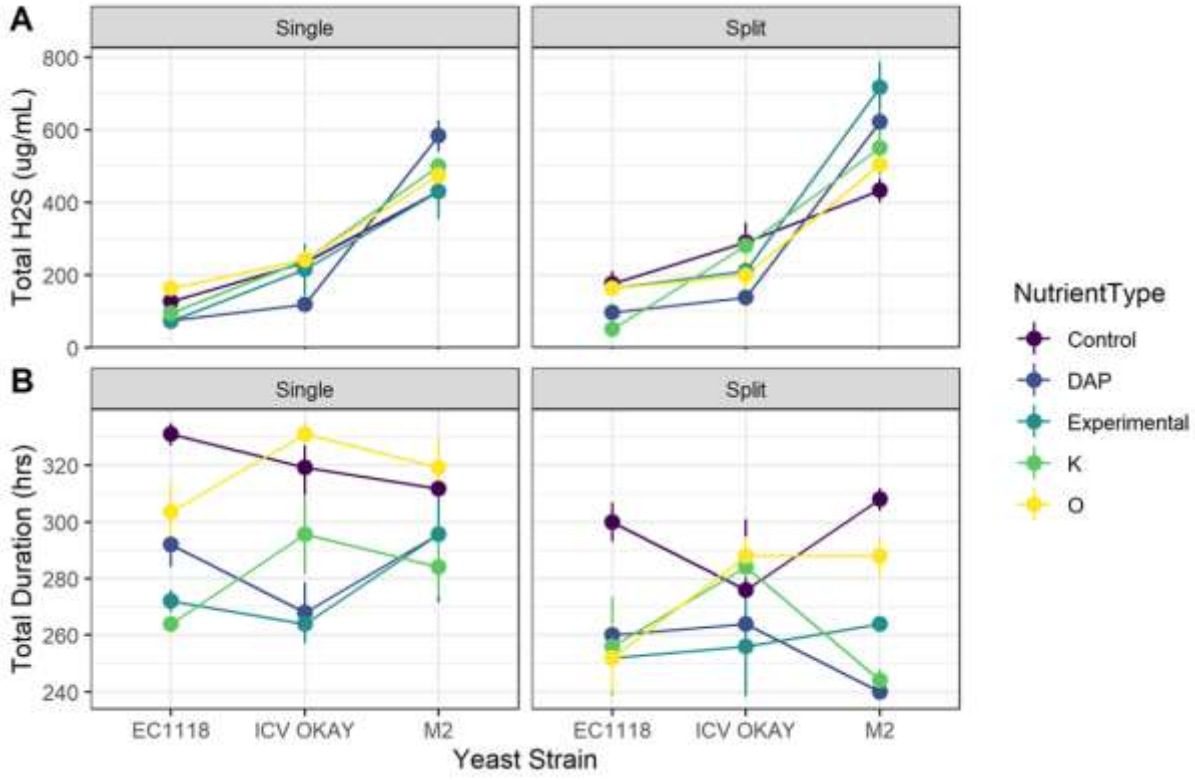

Figure 41: Interaction plots for total H_2S production (µg L^{-1} H_2S) (Graph A) and total fermentation duration (hrs) (Graph B). Interactions are represented by non-parallel lines. Parallel lines indicate not interaction.

Chapter 4: Conclusions and Future Work

4.1 H_2S Production and Fermentation Kinetics in Microscale Fermentations

4.1.1 Key Findings

H_2S was produced in different concentrations across many of the treatments. The most important factor for H_2S production was yeast strain followed by yeast nutrients. M2 and EC1118 yeast strain were significantly affected by yeast nutrient additions while ciders fermented with ICV OKAY were not affected by yeast nutrient addition. Yeast nutrient timing did not make a difference in total H_2S production except for one case in which yeast nutrient addition increased H_2S production when added in two dosages (MES vs. MED). There were many interactive effects between tested variables which was to be expected. M2 yeast strain produced the highest levels of H_2S while EC1118 produced the lowest levels of H_2S.

For total fermentation duration, yeast nutrient type as the most important deciding factor for followed by nutrient timing. The fastest fermentation was complete at 240 hours while the slowest was 319.3 hours which is a difference of 79.3 three hours (about 3.3 days). There were several significant interactive effects between the variables tested and total duration of fermentation. There did not seem to be a clear pattern linking treatment variables to shortening or increasing total duration of fermentation.

4.1.2 Limitations

Detector tubes used in this experiment were used to quantify H_2S based on their ease of use and advantage compared to other method of H_2S detection as discussed in the literature review, but tubes did have limitations. Tubes had to be removed from the stopper several times; when pre-calibrated scale had reached maximum detection and when second yeast nutrient

additions occurred. Tubes had to be removed from the stopper and replaced with a new tube. This could have caused loss of quantification H_2S in the head space. H_2S tubes also had to be removed during second nutrient addition. The second nutrient addition occurred during the exponential growth phase and H_2S gas could have been released when removing the tube from the stopper. H_2S tubes pre-calibrated scales also have large intervals which varied (100 mg μL^{-1} to 25 mg μL^{-1}) by the type of tube used. This resulted in a wide range of error between triplicate showing that this method of quantification of H_2S concentrations is not as accurate as other methods could be like HPLC. More difference may have been seen if there was lower SEM values.

In terms of nutrient addition, yeast nutrient does not completely dissolve in water. After adding yeast nutrients to water, an insoluble fraction remained and quickly settling out of solution. This could contribute to lower levels of available yeast nutrients than desired in the cider fermentation since yeast may not be able to utilize the insoluble fraction of the nutrient.

AJC could have also been a source of limitation in this experiment. Apple juice concentrate was purchased from Kroger and apples cultivars used in juice was unknown. Processing methods were also unknown which could have added to factors that would limit yeast fermentation in apple juice concentrate. Storage temperatures and duration was also unknown prior to purchasing the AJC.

4.1.3 Future Work

Future work would include increasing the amount of yeast nutrient added from 25 g hL^{-1}. The highest level of total YAN in this experiment was 103 mg N L^{-1} which is far below the recommend level of total YAN concentration. Increasing all nutrient additions so each treatment

is above 140 mg N L^{-1} of total YAN may yield different results and show more significant difference between H_2S production and fermentation kinetics.

More future research could be conducted on how frozen AJC effect H_2S production. Investigating deeper into nutrients availability and how processing methods affect nutrients in AJC could elucidate which nutrients are lacking. Many cider producers use apple juice concentrate for cider fermentation but there is little know about the effects of concentration methods on amino acids or nutrient available. Surveying different kinds of apple juice concentrates for nutrients such as pantothenic acid and amino acids that are very important to H_2S production may elucidate more information on how apple juice concentrate may play a role in H_2S production.

4.2 Sensory Evaluation of H_2S and Off aromas

4.2.1 Key Findings

Through classical text analysis 25 attributes were chosen to represent the selected cider samples. These attributes ranged from both positive and negative attribute. Through CA of the CATA data, ciders with different treatments were described by different attributes. Two treatments, MCD and MOD, were most often described as sulfur and vegetal which are likely attributes describing VSCs and H_2S. There were no clear patterns of groupings within CATA data suggesting that treatment variables did not predictably affect aroma. Through average liking scores of cider treatments, there was no clear best treatments as all average liking scores ranged from “neither dislike nor like” and “slightly like”.

4.2.2 Limitations

A limitation for sensory evaluation, was the total number of ciders tested. Only 12 out of the 30 samples were tested due to time limitations and the ability of panelist to evaluate cider before sensory fatigue. Another limitation was that there was no control in the sensory study. If a control cider was presented, differences between the control cider with no yeast nutrients and ciders with nutrient additions could have been evaluated for aroma attributes. Since a control cider was not presented, this was not possible.

Total subject sample size was also a limitation for this study. The ideal number of participants for the CATA study would have been closer to 100 people but only 63 total panelists evaluated the ciders. An increased number of panelists might have given more separation of treatments by descriptors.

4.2.3 Future Work

Future sensory work could include testing ciders fermented with different yeast nutrients against control cider. This could help identify different aromas due to treatment variables. Ciders with higher addition levels of yeast nutrient additions could also be investigated. Higher level of yeast nutrients could increase the total YAN, vitamins and increase metabolic pathways in yeast strains in the hopes to produce more positive aromas. Finally, more research could be conducted on the taste of the cider to investigate whether there are discernable differences between treatments since taste of ciders which were not investigated in this study.

4.3 Overall Conclusion

In this experiment, yeast strain played the largest role in reducing H_2S production which has been heavily reported on in previous wine research. The effect of yeast nutrient on H_2S was

yeast strain dependent, but not significantly affected by addition timing. Total duration of fermentation was most significantly affected by nutrient type, followed by nutrient timing, while yeast strain showed little impact on the duration of fermentation. CATA data revealed that treatment variables made no distinguishable patterns when observing the aroma attributes evaluated by untrained panelists. Continued research is needed to elucidate the full effect of yeast nutrient addition timing on H_2S production as well as the effects of nutrient composition on yeast metabolism and aroma production.

Production of H_2S in cider fermentation is extremely complex and dependent upon many interacting factors but cider is an import value added product that has gained national attention. In order to continue and encourage growth of cider production and consumption, research is needed to help provide scientific evidence on how to improve cider quality and aroma. Without targeted research, cider popularity could decline as it has in the past due to low quality. By continuing the investigation into the complex nature of H_2S production in cider better recommendations can be developed for cider producers to prevent H_2S. Overall, it is important to try to provide help and knowledge to the cider category to continue growth and enthusiasm.

Appendices

Appendix A

Microscale Fermentation Fitted with Hydrogen Sulfide Tubes Protocol

1) Autoclave Pyrex flasks (250mL) and stir bars by placing a small amount of water inside the flask and cover opening with foil. Add autoclave tape to ensure proper temperature is reach. Autoclave on Gravity 15 setting (~30min).
2) Make sanitizer by add 3 gallons of water (11.36L), 2 teaspoon of potassium metabisulfite (8g), 1 tablespoon Citric Acid (12g) to a large container. Place stoppers and H_2S tubes in sanitizer. Allow sanitizer to air dry completely before use.
3) Dissolve frozen apple juice concentrate by adding 1,062 mL of DI water for every one can of AJC (345 ml). Add all cans and water into a large container and mix thoroughly. Measure and pour 200 mL of juice into each 250 mL flask.
4) It is generally recommended 100mg L^{-1} potassium metabisulfite which yields 58.8 mg L^{-1} free SO^2 but due to large surface area, targeted free SO^2 is higher. Create a 0.02g mL^{-1} solution by dissolving 2.60 g potassium metabisulfite in 130mL distilled water. Add 1 mL of 0.02g mL^{-1} to each flask. Allow to stand for about 24 hours before yeast addition.
5) Add stoppers & tubes to flask to close off fermentation vessel to the environment and let stand overnight.
6) After letting juice stand overnight, rehydrate yeast. Add yeast at the recommended 25 g HL^{-1} by adding 3.25 g yeast to 65 mL of water that is 35°C. Stir gently to remove clumps. Let stand for 20 min and stir again. Take temperature of yeast to make sure difference between yeast solution and juice is NO more than 10°C difference. If more than 10°C yeast will go into cold shock. Add 1 mL of yeast solution to each flask.

7) Add yeast nutrients at recommend 25 g hl^{-1} by dissolving nutrient in water (30 - 40°C). Add 10 g of yeast nutrient in 200 mL water (.05 g ml^{-1}). Mixed for 20 minutes and allow to cool to room temperature. For fermentation receiving only one addition of yeast nutrients add **1mL** of solution to each flask except for control. Add 1mL of water to the control flask. For fermentations receiving split additions add **500 µL** of yeast nutrient solution into each treatment except for control. Add 500 **µ**L of water to the control flask.
8) Break H_2S tubes on BOTH ends, place into stopper and add to each flask and ensure that stopper is firmly in place.
9) Tightly wrap stopper and opening of flask with parafilm to ensure an airtight deal. This is to ensure that all gas from the fermentation is pushed through the H_2S tube.
10) Stir each flask at 600 rpm for 5 minutes twice a day.
11) Weight each flask and record its weight after being stirred each time.
12) For fermentations that receive as second nutrient addition, addition will be added at 1/3 brix depletion (in this case start of day 3) by dissolving 10 g of nutrients in 200 mL water (30 - 40°C) and mixed for 20 minutes. Allow to cool to room temperature.
13) Record weight of flasks before addition to obtain original weight and remove H_2S tube and insert thermo pipet with nutrient in tip into stopper hole. Add 500 µL of nutrient through stopper hole and quickly replace H_2S tube. Stir for 5 minutes at 600 rpm and reweigh flask.
14) Continue to stir flasks at 600 rpm for 5 minutes twice per day and weighing flasks until weight of flask has not changed for four data points (2 days).

Appendix B

Scale Up Fermentation Protocol

Materials

36 carboys 1 gallon carboy, Yeast (EC1118 and M2), 36 stoppers, 36 air locks, Kroger AJC 25 liters, DI Water, Sanitizer, Nitrogen Gas, 75 (375mL) wine bottles , 75 caps for wine bottles, SO_2 (Potassium metabisulfite), Yeast Nutrient (Fermaid C, Fermaid O and DAP), Large 5 gallon Nalgene container with spout & lid , tubing for racking , H_2S tubes for head space & pump

Day 1:

Juice Rehydration

1. Thaw AJC at 0°C overnight so it can be measured in its liquid form. Stir buckets well and make sure they are not settled from freezing. Then measure out 625 mL AJC and 1875 mL water. Pour directly into 1 gallon jug using a funnel and mix by shacking carboy. Sample apple juice for chemical analysis.
2. Add SO_2 by weighing 10 g of KMS and adding to 40 mL of cold water and stir until dissolved. Add 1 mL of solution per juice of juice. Once SO_2 is added, add stoppers and airlock to jug and allow to sit overnight at 18°C.
3. Yeast Nutrient rehydration: Dissolve 100 g of yeast nutrient in 320 mL water to make .3125 g L^{-1} solution and add 2 mL of nutrient to each jug for single addition treatments and add 1 mL for split level. Yeast nutrient was added at recommend 25g hL^{-1}.

Day 2: Addition of yeast

4. Rehydrate yeast by mixing yeast with water. Yeast was added at 25g hL^{-1}. Once yeast was added allow cider to ferment at 18°C. Check cider twice per day to make sure yeast

hasn’t settle. If yeast have settled, then gently stir jug by hand. Add water when needed to air locks.

Day 4: Second Nutrient Addition

5. Add second yeast nutrient addition at end of 3rd day. Remake nutrient fresh and add 1 mL of nutrient solution to cider by following directions in step 3.

End fermentations

6. Test cider towards the end of fermentation for total residual sugar using D-fructose and D-glucose enzymatic kit from Megazyme. Fermentation were considered complete when less than 2 g per liter of sugar was remaining in the cider

Bottling

7. Using H_2S tubes and H_2S pump, measure the H_2S remaining the measurements of headspace of each cider jug by removing airlock and sampling headspace as quickly as possible.
8. Once fermentation was completed, cider was rack off lees and combined replicates to ensure homogeneity. All tubing and materials were sanitized and allowed to air dry before use. Dispense cider into 375 mL bottles that have been purged with nitrogen. Ensure that fill line on each bottle is constant. Purge headspace for 30 seconds with nitrogen. Store in refrigeration at 0°C until sensory test.

Appendix C

Cider Lexicon and CATA Sensory Study Protocol

There are a total of 12 different cider samples. Each cider will be labeled with a different three digit randomized code and have a labeled designated area sectioned off on the lab bench with tape to help keep cider samples separated. Each section will have a coded cider bottle, a jigger and labels designated for each cider.

Set up:

1. Place 5-6 wine glasses in each of the 12 sections and label stem of glasses
1. Pour 10 oz (small side of the jigger) of cider into each wine glass at least 30min before first panelist arrive
2. Cover each wine glass with a watch glass

Through-out the day on a as need basis:

2. Place new wine glasses out and label each glass stem with correct code
3. Pour 10 oz (small side of the jigger) of cider into each wine glass
    a. Make sure code on wine glasses match the code on cider bottle
    b. Cider should be at room temperature
    c. Ensure to use the designated jigger for each cider sample
    d. Do not use the same jigger to pour all 12 samples
    e. Clean jiggers throughout the day as needed
4. Cover each wine glass with a clean watch glass
5. Provide each panelist with the correct coded sample when it appears on the Compusense screen by passing it though the booth and closing the booth hatch.
6. When sample is passed back through the hack discard the sample in a bucket or sink.

7. Remove coded sticker from the stem of the glass and place dirty wine glass in dishwasher rack.
8. Clean wine glasses when rack is full.
9. Pour new samples throughout the day as needed
    a. Each panelist will receive a new cider sample
    b. Discard of each sample after being presented to a panelist
    c. DO NOT reuse cider samples after it has been evaluated by a panelist