

Effects of Hydroxycinnamates and Exogenous Yeast Assimilable Nitrogen on Cider
Aroma and Fermentation Performance

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ABSTRACT

Heritage apple cultivars for cider-making are often distinguished by a high concentration of tannins (phenolic compounds), and/or acid. The phenolic content of some cider apples far exceeds that of white wine, however most cider fermentation practices are directly taken from white winemaking, not accounting for effects of high concentrations of phenolic compounds on yeast fermentation. The objective of this study was to determine the impact of ferulic acid, *p*-coumaric acid, and chlorogenic acid—at concentrations reported in apples—and their interactions with yeast assimilable nitrogen (YAN) on fermentation kinetics and cider aroma. Our hypothesis was that the phenolic compounds present in high-tannin cider apples would negatively impact fermentation kinetics, but not alter the aroma, and that added YAN would reduce these effects. Ferulic acid negatively affected fermentation performance ($p < 0.05$), but *p*-coumaric acid and chlorogenic acid did not. *p*-Coumaric acid led to the greatest changes in cider aroma. Differences were also detected for different concentrations of ferulic acid. Chlorogenic acid did not affect aroma. Yeast strain influenced fermentation performance and cider aroma. Finally, addition of exogenous YAN improved fermentation performance for the low concentration ferulic acid condition, but not for the high concentration. Adding YAN also changed cider aroma in the presence of *p*-coumaric acid.

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GENERAL AUDIENCE ABSTRACT

Hard cider is increasingly popular in the United States. Heritage apple cultivars are traditional cider apples, often distinguished by a high concentration of tannins (phenolic compounds), and/or acid, unlike dessert apples that are typically higher in sugar than tannins. While the phenolic content of some cider apples far exceeds that of white wine, most cider fermentation practices are directly taken from white winemaking, and do not account for the effects of high concentrations of phenolic compounds on fermentation performance. The objective of this study was to determine whether three phenolic compounds—ferulic acid, *p*-coumaric acid, and chlorogenic acid—at concentrations reported in apples, would inhibit fermentation or alter the aroma of the cider, and if adding yeast assimilable nitrogen (YAN), a yeast nutrient, would reduce these effects. Our hypotheses were that the phenolic compounds present in high-tannin cider apples would negatively impact fermentation performance, but not alter the aroma, and that added YAN would reduce these effects. Ferulic acid negatively affected fermentation performance ($p < 0.05$), but *p*-coumaric acid and chlorogenic acid did not. Addition of nitrogen improved fermentation performance for the low concentration ferulic acid condition, but not for the high concentration. *p*-Coumaric acid led to the greatest changes in cider aroma, with differences in aroma also detected for ferulic acid ciders. Chlorogenic acid did not affect aroma or fermentation performance. Yeast strain and YAN addition also influenced fermentation performance and aroma.

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Attributions

Several people contributed to the work found in Chapter 3. Their contributions are described here.

Leah Hamilton, a current graduate student in the Department of Food Science and Technology at Virginia Tech, assisted in the text analysis of the sensory characteristics of cider aroma.

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Amanda C. Stewart, PhD, a current professor in the Department of Food Science and Technology at Virginia Tech, assisted with the study design, and compilation and submittal of the manuscript.

Chapter 1: Introduction

Craft cider consumption in the United States is rapidly increasing, with craft cider from small producers sold locally or regionally up 39% in 2016 from 2012¹. With this increasing local cider market, research is needed on cider chemistry and fermentation management strategies for cider production.

As of 2018, over 20% of all U.S. cider makers claim to be using heritage apple cultivars². Most cider makers do not specify what type of apples they are using but these can include culinary apples, dessert apples, or even apple-juice concentrate from local or imported sources. Heritage apple cultivars can be classified into four categories developed by Professor B.T.P. Barker of the Long Ashton Cider Institute³, listed in Table 1. Characteristic heritage cultivars for cider are the bittersharp and bittersweet varieties that possess tannin and/or acid content that is lacking in traditional dessert apples³.

Table 1: Cider Apple Cultivar Classification

Classification of cider apples³		
	Acid (%)	Tannin (%)
Sharp	>0.45	<0.2
Bittersharp	>0.45	>0.2
Bittersweet	<0.45	>0.2
Sweet	<0.45	<0.2

While the tannin—or phenolic compound—content of traditional bittersharp and bittersweet cider apples can far exceed that found in grape juice, most fermentation-management strategies are taken directly from white winemaking, and do not account for the inhibitory effects that some phenolic compounds can have on yeast during fermentation.

Certain phenolic compounds have been shown to inhibit the growth of *Saccharomyces cerevisiae*⁴, but the impact of high tannin apple cultivars on fermentation rate has not yet been

investigated. These phenolic compounds have also been shown to influence the aroma development of red and white wines.^{9,115,6} Further investigation is needed to determine the impact of phenolic compounds and of YAN on cider aroma.

1.1 Long-Term Objective

The long-term objective of this research program is to develop fermentation management strategies specifically for the cider industry that take into account the chemical differences between cider apples and white wines.

The expected outcomes of our aims are the development of a fundamental understanding of how phenolic compounds and interactions of phenolic compounds and yeast assimilable nitrogen (YAN) affect fermentation rate and aroma development in apple juice. This will advance our understanding of fermentation in cider apples and help to develop robust and effective fermentation management strategies for high-tannin, low YAN cultivars.

1.2 Overall Objective and Hypothesis

The overall objective of this project was to determine the impact of phenolic compounds and the interaction of phenolic compounds and YAN on fermentation kinetics and the extent to which the interactive effects of tannin concentration and yeast nutrients contribute to the sensory perceptions of aromas generated during cider production.

Our central hypothesis was that the type and concentration of individual phenolic compounds present in a typical high tannin cider apple would negatively impact fermentation kinetics compared to low-tannin juice fermentation, but not alter the aroma, and that added YAN would reduce these effects.

1.3 Specific Objectives and Hypotheses

Specific Objective 1:

Determine whether the concentrations of three phenolic compounds (ferulic acid, *p*-coumaric acid, and chlorogenic acid) influence fermentation performance.

Hypothesis 1:

Higher concentrations of ferulic acid and *p*-coumaric acid will slow fermentation rate. Chlorogenic acid will not impact fermentation rate.

Specific Objective 2:

Determine whether there is an interactive effect of total YAN and phenolic compound concentration on fermentation kinetics.

Hypothesis 2:

Lower levels of YAN will result in slower fermentation rate in the presence of high phenolic compound concentrations, while added YAN can help overcome the phenolic inhibition on fermentation rate.

Specific Objective 3:

Determine the extent to which the interactive effects of tannin concentration and yeast nutrients contribute to the sensory perceptions of aromas generated during fermentation.

Hypothesis 3:

Phenolic compounds and yeast nutrients will not noticeably impact the aromas generated during cider production.

Specific Objective 4:

Determine whether there are measurable concentrations of inhibitory phenolic compounds (ferulic acid, *p*-coumaric acid, and chlorogenic acid) present in Virginia apples.

Hypothesis 4:

High tannin cultivars will have these compounds present at inhibitory levels.

Chapter 2: Literature Review

2.1 History and Definition of Cider

In the United States (US), cider was initially the most popular alcoholic beverage consumed. Cider consumption per capita of the population 15 years and older in 1790 was 34 gallons/yr. In colonial America, cider was the number one alcoholic beverage consumed, compared to spirits at 5.1 gallons/yr per person, wine at 0.6 gallons/yr per person, and beer at negligible consumption. However, cider rapidly declined in popularity with consumption negligible by 1845⁷. The term “cider” in the United States is often used to refer to cloudy unfiltered apple juice, unlike the traditional English definition for the term, which refers to the fermented alcoholic beverage known as “hard cider” in the U.S. Today the term “cider” is becoming more widely accepted in the U.S. to mean the fermented alcoholic beverage made from apples, which is the definition that will be used here⁸.

2.2 Cider Industry Growth

Cideries have been rapidly opening in the U.S. over the last ten years, with 90% of all active commercial cider producers having begun production since 2008² (Figure 1). Cider has grown in market acceptance and is reemerging as an individual category of alcoholic beverage. With consumers migrating to locally made artisan products, craft cider from small producers sold locally or regionally is experiencing a large (39%) increase in sales between 2012 and 2016. Growth in craft cider continued with 16.7% of all off-premise cider sales in 2016 represented by

small craft cider, up from 8% in 2014¹. With the growing number of cideries, over 20% of which claim to use heritage apple cultivars², the demand for these apples is growing. This growth illustrates the need for cider research, as little research has been conducted on cider, and heritage apples in particular, compared to that of wine and beer.

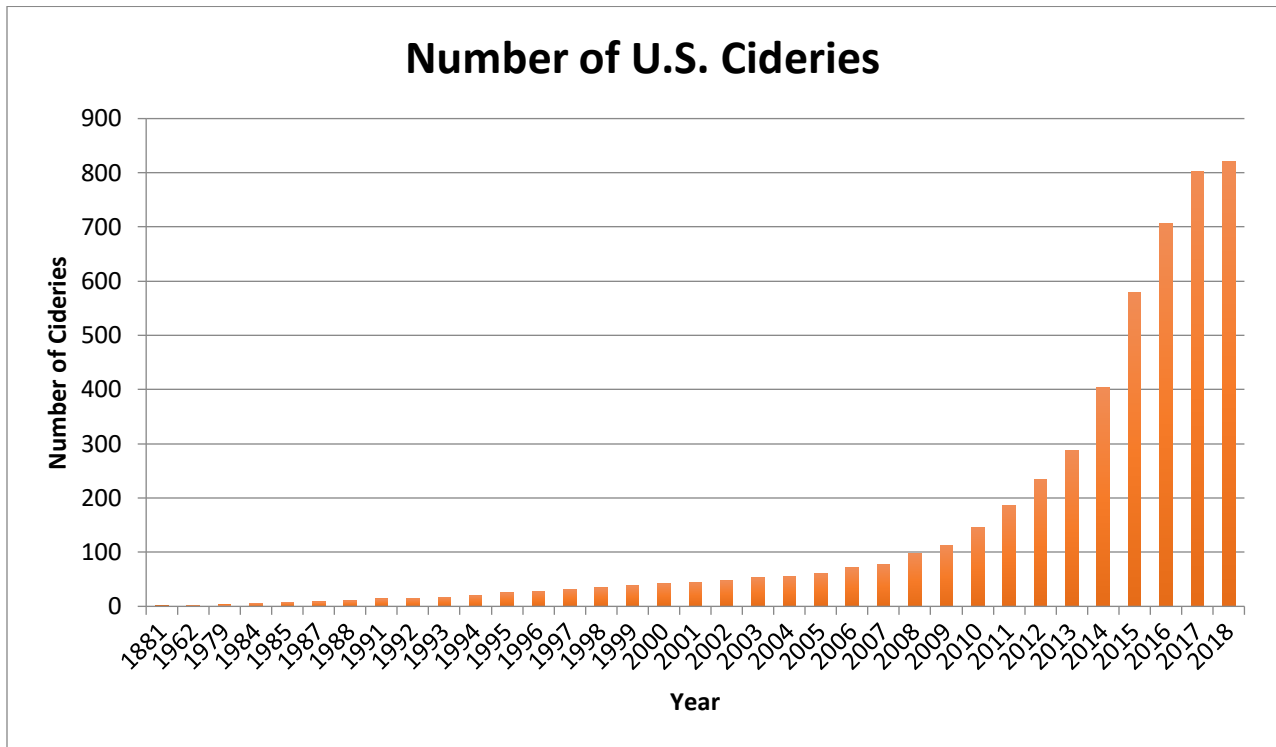


Figure 1: Number of U.S. cideries currently active by year they started production (adapted from Cyder Market LLC, 2018³)

Cider is being produced commercially in 48 states and the District of Columbia, excepting only Nevada and Wyoming. However, nearly 70% of all cider producers are in the more traditional apple growing regions of the northeast, upper Midwest, western, and northwestern United States. Virginia is tenth on the list of number of cider producers, with 23 cider makers². However, Virginia is the sixth largest apple producer in the country with over 50 orchards around the state from the southwest to north of the Shenandoah Valley⁹. This demonstrates the potential for continued growth of cider production in Virginia.

2.3 Cider Production

Cider can be produced in a variety of ways, with quality and characteristics differing greatly depending on fruit selection and preparation methods⁸. Fruit selection, processing, fermentation, and storage conditions are all key aspects of cider making.

2.3.1 Cider Fruit

“True” cider apples can be defined as “those cultivars raised and grown for no other purpose and indeed often regarded as inedible”(page 17).³ The Bittersharp and Bittersweet cultivars (Table 1) are those most desired for cider making due to their high tannin content. Since these true cider cultivars have been bred for cidermaking and are selected specifically for this purpose, they have many advantages to the cider maker including potentially high sugar levels (up to 15%), a range of acidities (0.1-1.0%), a fibrous structure that makes pressing easier, long term storage potential, and a high tannin (phenolic compound) level¹⁰. These “true” cider apple cultivars are also referred to as heritage cultivars. Broadly speaking, these are historic fruits that have maintained their genetic identity much longer than their normal lifespan due to human cultivation and breeding¹¹.

Heritage fruit generally yields a more complex and interesting cider, but some heritage cultivars produce lower yields and are harder to grow than typical dessert apples¹⁰. Despite this, heritage apple usage in cider production in the U.S. is increasing, with cider makers paying a premium for these particular apples. Cider makers can end up paying over four times as much for heritage cultivars as they would for dessert apples. In a 2016 survey the mean price per bushel of dessert apples was \$4.30, while the mean price per bushel for specialty cider apples was \$19.00¹². Between December 2015 and January 2018, the total number of cideries producing ciders with heritage apples increased from 133 cideries to 171².

Due to the sugar, acid, and tannin balance needed to achieve a successful cider, it is rare to only use one apple cultivar to make a cider. Blends are typically created to achieve the desired characteristics of the cidemaker¹⁰. In cidemaking, it seems that blends are more frequently made of the apples before pressing or of the pressed juices before fermentation, in contrast to winemaking, in which blends are typically made after fermentation^{3,13,14}. Little research has been done on blending in cider, which could be a possible method for moderating potential inhibitory effects of high polyphenol concentration in certain apple cultivars.

2.3.2 Apple Processing

After harvest, apples must be milled and pressed into juice before cider can be made. This is different from winemaking, where grapes are soft and just need a light pressing to release their juices. Since apples are a hard fruit they must be ground up (milled) into a pulp before the juices can be extracted. Apples should be sorted and washed before milling to remove damaged fruits that could potentially impact the microbiology of the fermentation and the quality of the cider^{3,10}.

To extract the juice many different types of presses are used across the industry. A traditional method involves stacking layers of apple pulp wrapped in cloth between wood or plastic boards and applying pressure to extract the juice. Today industrial methods for juice extraction can include a hydraulic (for example a bladder press) or enzymatic juice extraction coupled with a chopping or milling step.¹⁵ Good juice yields are around 75% by weight and above, but vary dramatically depending on press type and apple cultivar and age^{3,10}.

2.3.3 Juice Preparation

After pressing, the juice must be prepared for fermentation. This can include blending juices, and adding acids, yeast nutrients, exogenous tannin sources, enzymes, and possibly other fermentable sugar sources to reach the desired sugar, tannin, and acidity levels. These adjustments to juice chemistry are made to impact both the flavor of the cider and the microbial ecology during fermentation. Antimicrobials and exogenous nitrogen sources (yeast nutrients) may also be added to promote a complete, rapid fermentation to dryness¹⁰.

2.3.3.1 Clarification

2.3.3.1.1 Pre-Fermentation Clarification

Immediately after pressing, apple juice is cloudy due to the large amounts of suspended solids in the juice. Depending on the cider maker, clarification may or not be performed before fermentation. Frequently, pectinase enzyme is used for clarification before fermentation, and sometimes as a processing aid to get a higher yield from pressing. If pectinase is not used at this stage it can lead to persistent pectin hazes at the end of fermentation, which can result in poor post-fermentation clarification³. Depending on the cider maker, this pectin haze could be desirable, or undesirable if a clear cider is sought.

In white wine, clarification is regarded as an important step contributing to final wine quality, although pre-fermentation filtration has been shown to negatively impact fermentation rate due to nutrient loss through filtration¹⁶. In cider, YAN losses of 50% have been shown to result from pre-fermentation juice clarification using pectinase.¹⁷ Different clarification treatments can have different effects on polyphenol and YAN composition. Ultrafiltration of apple juice results in reduced losses of polyphenols compared with traditional clarification

methods using a combination of gelatin and bentonite with 7% loss for ultrafiltration, compared to 28% loss using gelatin and bentonite¹⁸.

2.3.3.1.2 Post-Fermentation Clarification

There are many ways in which post-fermentation clarification can be achieved including ultrafiltration and centrifugation, although these processes require significant capital investment and are thus mainly restricted to very large-scale operations. Static settling is more often relied upon in smaller cideries wherein the solids are allowed to separate from the cider by gravity. Pectinase enzymes or gelatin fining can also be combined with static settling to aid in clarification, however pectinase enzymes are more often used in pre-fermentation clarification methods.

2.3.3.2 *SO₂ Addition*

Sulfur dioxide (SO₂) is an important additive in both wine and cider making. The potential for microbiological contamination exists at all stages of processing, and SO₂ is added to improve microbial stability¹⁹. The effectiveness of SO₂ is pH dependent, because only the undissociated form has antimicrobial activity. The addition rates outlined in Table 2 provide guidelines for adjustment of SO₂ additions according to pH. Juices with pH above 3.8 should be acidified until they are below 3.8 before SO₂ is added^{3,10,19}. It is typically best to wait overnight before yeast inoculation after SO₂ addition to allow sufficient time to act against any wild organisms in the juice, and to decrease the risk of inhibition of the yeast inoculum³. Since the effectiveness of SO₂ as an antimicrobial agent depends on a number of factors including the pH and the presence of microorganisms in the medium it is impossible to give a formulaic time for when yeast should be inoculated after SO₂ addition.

Table 2: Sulfur dioxide addition requirements for apple juice (table reproduced from Lea and Piggot, 2012)¹⁰

Sulfur Dioxide Addition Requirements	
pH	Addition Required (mg/L)
3.0-3.3	75
3.3-3.5	100
3.5-3.8	150

Juices above 3.8 should be acidified to this value before sulfur dioxide addition.

2.3.4 Fermentation

Once the juice is prepared it is either inoculated with a pre-selected yeast culture or allowed to ferment un-inoculated, using environmental microbiota. In a “natural” or un-inoculated fermentation, microorganisms present in the environment are allowed to grow in the juice, whereas in inoculated fermentations a large inoculum of a selected yeast strain is added to the juice, intended to proliferate quickly in the juice and outcompete the environmental microorganisms²⁰. In un-inoculated fermentations, microorganisms come from the environment, although the precise origin is generally unknown and may vary. They could be present on the fruit, come from the orchard, be present on the processing equipment, or come from any other sources that have contact with the cider and its components throughout the cider making process, including other tanks, workers, and equipment.

Fermentation kinetics are dependent on a number of factors including available nutrients, initial yeast inoculum, temperature, pH, shape and size of vessel, mixing, pesticide or fungicide residues in the juice, apple cultivar, polyphenol content, amount of available oxygen, and yeast strain^{16,19,21-27}. Kinetics of fermentation, as seen in Figure 2 are typically modeled by an initial lag phase, where the yeast inoculum is multiplying and consuming sugar to reach an ultimate concentration of at least 1×10^7 cells/mL juice³. This is followed by a “log” or exponential growth phase during which the juice becomes saturated with carbon dioxide (CO₂) that the yeast produces, creating an anaerobic environment in which the rest of the fermentation occurs. This is

followed by a stationary phase in which many of the sugars have been metabolized and the yeasts are running out of substrate on which to continue fermentation. Finally, the stationary phase is followed by a death phase²⁸.

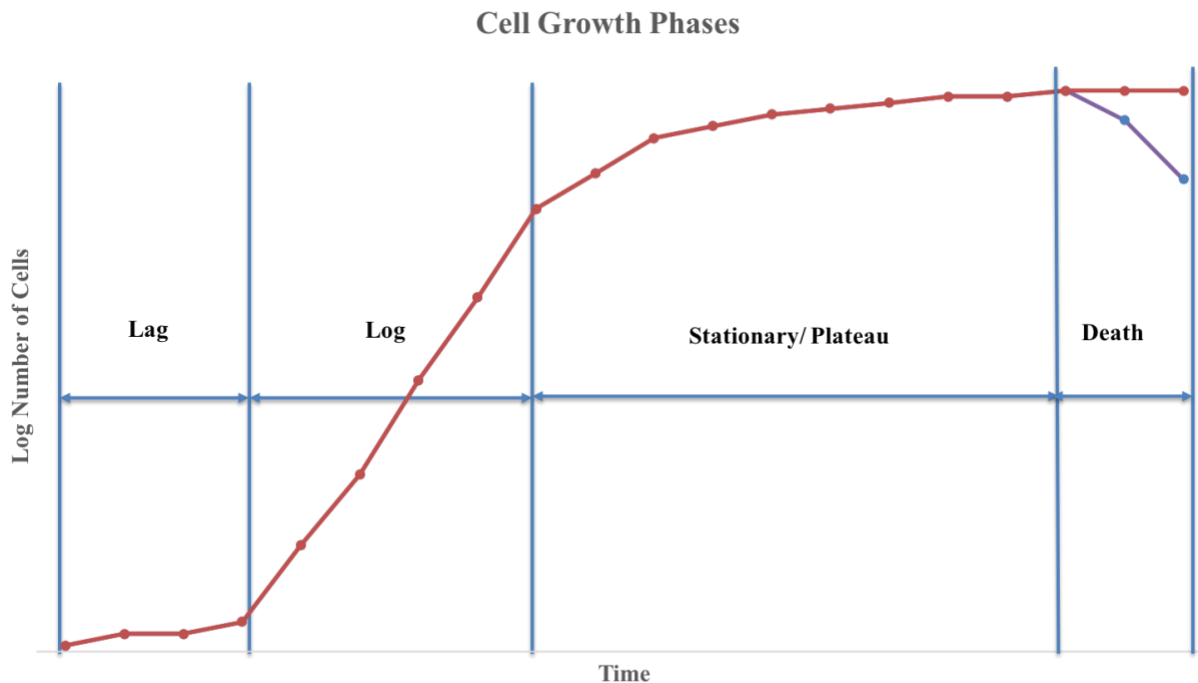


Figure 2: Cell Growth Phases During Fermentation. The red line indicated total number of cells and the purple line shown in the death phase indicates the amount of live cells.²⁸

The maximum fermentation rate (r_{\max}) is calculated during the log phase illustrated in Figure 2. Maximum fermentation rate can be influenced by a number of factors including yeast strain, fermentation temperature, juice composition, and YAN level, and is of interest to cidemakers because it can drastically change the production capacity or throughput of the cidery, which are utilized over a much greater portion of the year compared to wineries due to the storability of apples. A fairly easy method for monitoring the fermentation rate is by weight, measuring the amount of CO_2 released throughout the fermentation.^{16,29} The r_{\max} can be calculated by taking the slope of the greatest tangent line to the log phase of the cell growth curve. The shape of the cell growth curve is that of a first order reaction, so a first order reaction

could also be fitted to the curve and the rate constant, k , could be taken as the overall fermentation rate.^{22,24,29} This method however takes into account the total duration of the fermentation (including the death phase) and thus is generally a lower value than the maximum fermentation rate.

Once cider has reached the stationary phase, the fermentation is typically considered finished because there is very little sugar left to metabolize. Cider is typically considered “dry”, with little perceptible sweetness, when the residual sugar content is at 1.0-2.0 g/L³⁰. Depending on the style of cider desired, the cider maker may stop the fermentation before this point to retain a higher residual sugar concentration, or the cider may be sweetened to the desired level after fermentation to dryness. At this point the cider maker will rack (decant) the cider off of the yeast cells and other detritus that has flocculated to the bottom of the fermentation vessel and deposit it in another vessel for subsequent operations including clarification, aging, carbonation, and/or bottling.

2.4 Primary Chemistry of Apple Juice

There are many chemical constituents of apple juice that influence the overall fermentation process as well as the quality of the finished cider. Concentrations of all of these chemical constituents in juice or cider vary significantly depending on the cidemaker’s stylistic goals, which will determine the desired characteristics of the finished cider. In general, sufficient sugar content in juice is required to obtain the desired alcohol concentration in any cider. Acid and tannin contribute tartness, bitterness and astringency which can be essential contributors to the intended sensory characteristics or style of many ciders³. A suggested composition of the ideal cider apple juice according to Lea is presented in Table 3, but a quality cider can certainly be made with juice deviating from these recommended levels. While this table is a published

resource, it originates from the UK where cider is frequently made in the “Old World” style.³¹ Further research is needed to identify ideal juice compositions for current global cider styles. The true contributions of many of these categories to cider production and finished cider quality, especially with regards to YAN—made up of ammonium ions and amino acids and tannins, or phenolic compounds—is still unknown³².

Table 3: Representation of an ideal cider apple juice composition for UK cider styles (reproduced from Lea & Piggot, year 2012) ¹⁰

Composition of “Ideal” Cider Apple Juice	
Fructose (g/100mL)	7-11
Glucose (g/100mL)	1.5-3
Sucrose (g/100mL)	2-4.5
Sorbitol (g/100mL)	0.2-1.0
Starch	0 (but up to 2% may be present in unmaturred fruit)
Pectin (g/100mL)	0.1-1.0
Potassium (mg/L)	1200
pH	3.3-3.8
TA to pH 8.1 as malic acid (g/100mL)	0.3-0.5
Chlorogenic acid (mg/L)	300-700
Phloridizin (mg/L)	100-200
Epicatechin and Procyanidins (mg/L)	1000-1500

2.5 Yeast Assimilable Nitrogen (YAN)

Besides fermentable sugars, YAN is one of the main nutrients for yeast metabolism during fermentation of fruit juice. It is one of the major factors influencing yeast growth and fermentation rate, as well as a factor in sulfurous off-flavor production^{16,22,30,33,34}. The main source of nitrogen in cider production comes from the apples themselves in the form of proteins, peptides, alpha amino acids, and ammonium ions, but these compounds are present in apples at much lower concentrations than in grapes^{22,27}. YAN is generally considered to be composed of

the alpha amino acids (assimilable organic nitrogen) and ammonium ions (inorganic nitrogen), both of which are recommended by yeast nutrient manufacturers for a healthy fermentation²⁷. Yeast nutrient manufacturers typically recommend YAN levels of 100-200 mg N/L for cider making, but these guidelines are adapted from white wine research, as very little research has been conducted specifically on the optimum YAN levels for cider production^{22,27}. The range of YAN found in apples is vast, from 40 to 330 mg N/L^{22,30,34}. Thus, supplementation with exogenous YAN sources is typically recommended depending on the initial nitrogen concentrations in the juice, the initial sugar content, the fermentation temperature, the yeast strain, the turbidity of the juice, and the amount of dissolved O₂²⁷.

One of the most common, commercially available and inexpensive YAN sources is Diammonium Phosphate (DAP), an inorganic nitrogen source. Commercially available organic nitrogen sources recommended for cider fermentations such as Fermaid O™ (Scott Laboratories, Petaluma, CA) or “Yeast Nutrient,” which is a combination of DAP and Urea (L. D. Carlson Company, Kent, OH), can be used in conjunction with DAP to provide a balance of YAN from both organic and inorganic sources^{27,35}. Too much YAN can result in altered, negative flavor profiles³³. Considering all of the factors that impact the utilization of nitrogen during fermentation, and all that remains unknown regarding these phenomena in cider fermentation, further research is needed to determine optimal YAN levels for cider making, rather than relying on a recommended minimum total YAN concentration developed through wine research.

2.6 Phenolic Compounds

Apples contain a high level of phenolic compounds, which play a major role in the quality and sensory complexity of a finished cider. Cider apples contain much higher levels of polyphenols than dessert apples, in which bitter and astringent qualities have been bred out over

generations¹⁰, with some cider varieties containing up to 10-fold more polyphenols³⁶ than dessert or processing varieties. Particularly, levels of the phenolic acids (chlorogenic and *p*-coumaroyl quinic) may be ten-fold higher in bittersweet cider cultivars than in dessert apples¹⁰. This high level of polyphenols in apples has also been associated with positive human health outcomes³⁷.

2.6.1 Tannins vs. Phenolic Compounds

Tannin is a term commonly used to describe phenolic compounds found in cider. The term is frequently used to refer to the bitter and astringent qualities conferred by the phenolic compounds in apples. According to Lea, tannin has now been established as a “range of oligomeric procyanidins based on a flavanoid (-)epicatechin structure.”¹⁰ These phenolic compounds, particularly the procyanidins, are also responsible for the brown color that apples turn as they oxidize³.

2.6.2 Polyphenol Composition in Apple Cultivars

In general, phenolic compounds can be divided into two categories, the flavonoids and the non-flavonoids³⁸. The main subclasses of flavonoids are the flavones, flavonols, flavan-3-ols, isoflavones, flavanones, and anthocyanidins³⁸. In apples, the main classes of flavonoids are flavanols, which can be further divided into monomers and oligomers, flavonols, dihydrochalcones, and anthocyanins³⁷ as seen in Figure 3. Non-flavonoids of low molecular weight are simple phenolic derivatives. There are many groups of phenolic derivatives including hydroxycinnamic acids which is the main class of non-flavonoids found in apples.^{39,40} Typical concentrations of the most predominant phenolic compounds varies greatly between apple cultivars and are not agreed upon in the literature^{36,37,41,42}. Structures and classifications of

common phenolic compounds found in apples are presented in Figure 3.

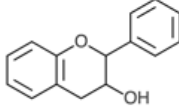
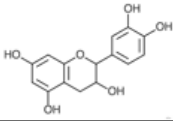
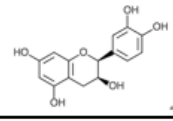
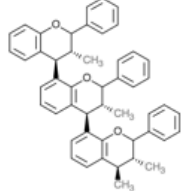
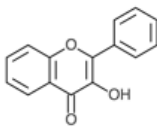
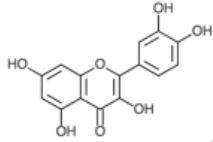
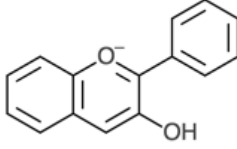
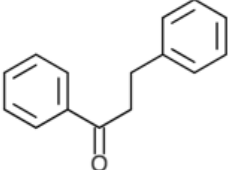
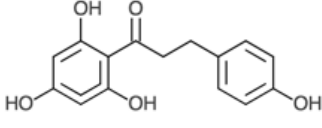
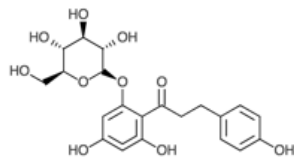
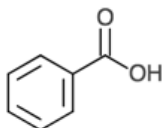
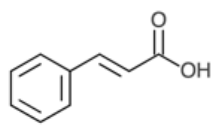
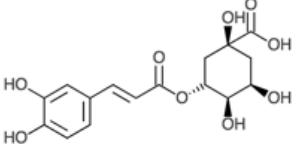
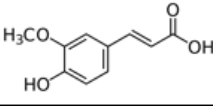
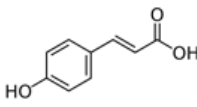
Flavonoids	Flavanols 	Monomers	Catechins	Catechin 
				(-)-Epicatechin 
	Condensed Tannins (Polymers)	Proanthocyanidins		
	Flavonols 		Quercetin 	
	Anthocyanidin			
	Dihydrochalcone 	Phloretin 	Phloridzin 	
Phenolic acids 	Hydroxycinnamic acids 	Chlorogenic acid 		
		Ferulic Acid 		
		p-coumaric Acid 		

Figure 3: Phenolic compounds typical of apples and their classifications^{38-40,43-47}

2.6.3 Role in Cider/Influence on Sensory Properties

Phenolic compounds impart bitterness and astringency to cider, are responsible for the color of cider, are precursors for volatile constituents that contribute to aroma, and contribute to “the overall mouthfeel” of the cider. Bitterness is associated with oligomeric procyanidins, up to tetramers in size, while astringency is related to higher molecular weight procyanidins⁴⁸. As molecules increase in size, astringency increases, until the molecules become too large to dissolve in saliva. They also lose bitterness with increasing degree of polymerization (increasing size), with monomers being the most bitter of phenolic compounds⁴⁹.

2.7 Yeast

Fermentation can be driven by the growth and metabolism of either environmental microbiota or an inoculum of selected yeast. Several factors influence the yeast strains that will be present in the environment, including climate, apple cultivar, fermentation equipment, and geographic location, but typically *Saccharomyces* is the predominant genus during alcoholic fermentation⁵⁰, whether inoculated or not. In inoculated fermentations, the desired *Saccharomyces* yeast strain will generally quickly dominate the fermentation, resulting in a more consistent, fermentation free from of odors. Since the use of a yeast inoculum, or starter culture, has become common practice, many efforts have been made to enhance the performance of particular yeast strains. For winemaking yeast strains these efforts have aimed to improve the efficiency of nutrient utilization, increase ethanol tolerance, reduce wine acidity, increase sugar uptake, and to optimize flocculation⁵¹ for a wide range of yeast strains. The yeast used for a particular fermentation is often selected based on its performance in relation to the initial juice composition (brix, pH, YAN), as well as fermentation temperature and the desired characteristics of the final cider²⁷. Although the vast majority of the research on relationships between juice

chemistry and yeast performance was performed in a wine matrix rather than a cider matrix, yeast selection is very often intended to (and expected to) impact the final sensory profile of ciders⁵² in the same manner as it would in wine. Much work has been done to optimize yeast strains for aroma production in beer⁵³ and wine, but these wine yeast strains are also marketed for cider production, with few commercially available yeast strains developed for aroma production in cider to utilize the known phenolic aroma precursors in apple.^{5,6,53–56}

2.7.1 Inhibition of Yeasts by Phenolic Compounds

Certain phenolic compounds, particularly the hydroxycinnamic acids, have been shown to inhibit growth of microorganisms including *Saccharomyces cerevisiae*⁴. Ethyl *p*-methoxycinnamate (EPMC) has been shown to inhibit *S. cerevisiae* growth at a minimum inhibitory concentration of EPMC at 10µg/mL⁵⁷. Caffeic acid had been shown to increase the lag phase of *S. cerevisiae*, and chlorogenic acid has been shown to have no effect on a freeze-dried Champagne strain of *S. cerevisiae* in model solution⁴. Other phenolic compounds including *p*-coumaric acid and ferulic acid have been shown to inhibit *S. cerevisiae* growth⁴. *P*-coumaric, caffeic, and ferulic acids have also been shown to inhibit the growth of other spoilage microorganisms in wine including *Lactobacillus collinoides* and *Lactobacillus brevis*⁵⁸.

2.7.1.1 Chlorogenic Acid

Chlorogenic acid is one of the phenolic compounds present in high concentrations in apples⁴⁶. It is a derivative of caffeic acid and plays a role in non-enzymatic browning reactions⁵⁹. The concentration of chlorogenic acid in apples has been shown to correlate well with total polyphenols in apples⁵⁹, although recent work has shown that chlorogenic acid is not the most prevalent phenolic compound in all apples.⁶⁰ No inhibitory effects on *S. cerevisiae* have been

observed due to chlorogenic acid when tested in an unspecified freeze-dried Champagne strain of *S. cerevisiae*⁴. While chlorogenic acid has been reported by many to be the most prevalent phenolic compound in apples, more recent work has shown that polyphenol composition is actually highly variable among apple cultivars^{37,42,46,60–62}. Chlorogenic acid has been reported at concentrations ranging from 10 to 1195 mg/L in apples and has been shown to have no inhibitory effects on *S. cerevisiae* growth at concentrations up to 1000 mg/L in model solution^{4,36,60,62,63}.

2.7.1.2 Ferulic and p-coumaric Acids

Ferulic and *p*-coumaric acids have shown inhibitory effects against an unspecified freeze-dried Champagne strain of *S. cerevisiae* in a model solution. *p*-Coumaric acid increased the lag phase of yeast growth at a concentration of 100 mg/L in model solution, and above 250 mg/L inhibition of *S. cerevisiae* growth after 72 hours was proportional to the concentration of *p*-coumaric acid present, with complete inhibition of *S. cerevisiae* at 1000 mg/L of *p*-coumaric acid. Complete inhibition of *S. cerevisiae* was observed at 250 mg/L ferulic acid in model solution⁴. Ferulic acid and *p*-coumaric acid have also been shown to inhibit the enzyme apple *o*-diphenol oxidase found in apples.⁶⁴

In most of the studies examining the phenolic composition of apples, concentrations of ferulic and *p*-coumaric acid have not been reported. In the few studies that did determine concentrations of these compounds, ferulic acid concentration was reported from 2 to 95 mg/L in apples. This is of note, as ferulic acid at concentrations as low as 50 mg/L have shown inhibition of fermentation, with complete inhibition at 250 mg/L. Concentrations of *p*-coumaric acid from 1 to 460 mg/L have been reported in apples, but this compound has shown less potent inhibition of fermentation than ferulic acid.^{4,63,65} The basic hydroxycinnamic acid structure, seen in Figure 4,

with the presence of the unsaturated side-chain is what is thought to be necessary to bring about inhibition⁶⁴.

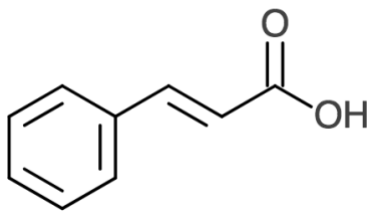


Figure 4: Hydroxycinnamic acid structure

Ferulic and *p*-coumaric acid concentrations have also been shown to change drastically within apples based on their maturity and harvest date. Ferulic acid concentrations decreased by 90 mg/L over the course of maturation, and *p*-coumaric acid decreased by approximately 450 mg/L⁶⁵. Hydroxycinnamic acid derivatives are formed early in the fruit development of both apples and pears, and concentrations decline sharply during the growth of the fruit⁶⁵. If apples are harvested too early this could impact fermentation and cider production due to the higher levels of potentially inhibitory (to *S. cerevisiae*) compounds found early on in the fruit development. Strategies to harvest apples early for increased acid and phenolic content for cider making have been the subject of recent research.⁶⁶ In the future, the concentration of these potentially inhibitory compounds in apples harvested early should also be evaluated, due to potential inhibitory effects.

Because polyphenol composition varies widely among apples, and these concentration ranges are based on very few prior reports, it is reasonable to expect that broader ranges would be observed in a broader survey of apples. Furthermore, even fewer reports provide information on inhibitory concentrations of these phenolic compounds in fermentation. With such little data reported of these compounds in apples it is entirely possible that many cultivars currently used in

cider production may have higher and potentially inhibitory concentrations of these phenolic compounds, which would be of interest to cidermakers.

2.7.2 Metabolic Pathways in Yeast Affected by Phenolic Compounds

Yeasts of the genus *Saccharomyces*, particularly *S. cerevisiae* and *S. bayanus*, are responsible for the alcoholic fermentation of wines and ciders via the conversion of glucose and fructose to ethanol and carbon dioxide.⁶⁷ The main pathway through which glucose and fructose are metabolized is via glycolysis. In *Saccharomyces* glucose is converted through glycolysis to pyruvate which is then converted to acetaldehyde which is then reduced to ethanol.⁶⁷ Typically, glucose is fermented faster than fructose because yeast predominantly metabolizes sugars via the glycolytic pathway.^{27,67} Phosphorylation of glucose to glucose-6-phosphate is the first step in this pathway and is catalyzed by one of three enzymes, hexokinase PI, hexokinase PII, or glucokinase. Phosphorylation of fructose however is only catalyzed by the hexokinases and is typically metabolized after glucose has been depleted.⁶⁷

When choosing a yeast strain for a particular cider fermentation it is important to select an inoculum that maximizes the desired characteristics of the target cider style as determined by the cidemaker. Yeast strains have been selected and commercialized based on many attributes including alcohol tolerance, nitrogen needs, optimal growth temperature, fermentation kinetics, sensory characteristics imparted via yeast metabolism (such as esters), and competitiveness against other microorganisms.²⁷ Some strains of *S. cerevisiae* and *Brettanomyces* yeasts and have also been shown to synthesize certain volatile phenols that contribute to the aromas of wines from phenolic precursors present in grape juice.^{5,6}

2.7.3 Generation of Aromas

As one of the most abundant hydroxycinnamic acids (a class of phenolic acids) in the plant world, ferulic acid is of utmost interest in aroma generation because it is a precursor to vanillin, as well as a few other aroma compounds.⁶⁸ The hydroxycinnamic acids are closely related and are produced in plants via the shikimate pathway from L-phenylalanine or L-tyrosine. The bioconversions between compounds are illustrated in Figure 5.⁴⁰

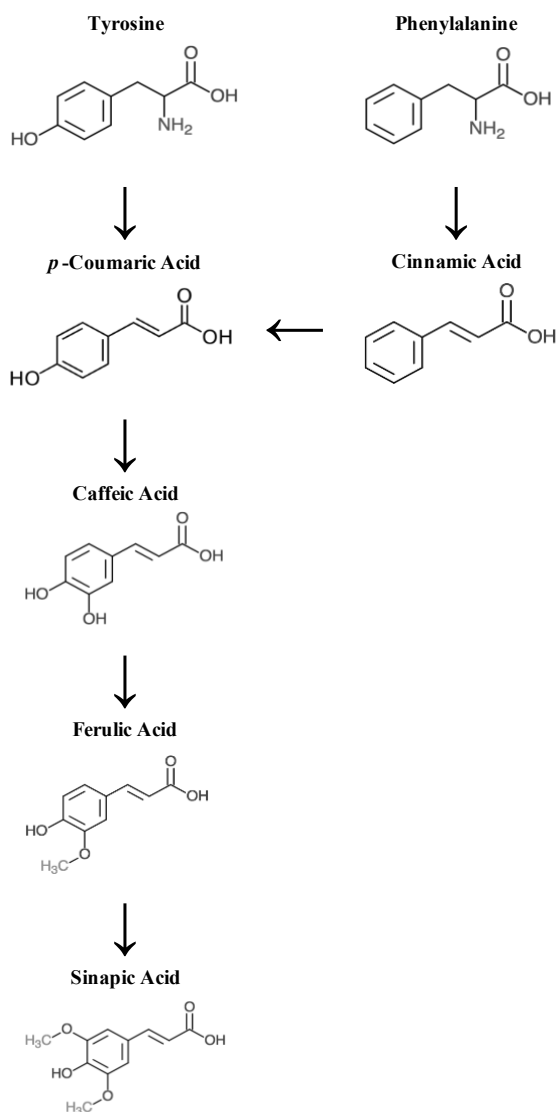


Figure 5: Biosynthetic pathways of hydroxycinnamic acids⁴⁰

The structures and antioxidant activities of these compounds are related. The antioxidant activity of monophenols, like *p*-coumaric acid and ferulic acid, increases substantially with methoxy substitutions in positions *ortho*- to the OH group. This correlates with the increasing inhibition of *S. cerevisiae* that has been shown by these compounds.⁴ The diphenolics like chlorogenic acid, however have been shown to have a higher radical scavenging ability than the monophenolics, although they have not been shown to inhibit fermentation.⁴⁰

The hydroxycinnamic acids can be metabolized by *Brettanomyces* yeast into volatile phenolic compounds with distinctive aromas and low sensory thresholds.⁶ *Brettanomyces* have long been recognized as spoilage organisms in wine, beer and cider, due to their ability to impart undesirable odors and flavors to the beverage, however these characteristics have increasingly been reported as desirable to some beer, wine, and cider styles and *Brettanomyces* can be used to increase the flavor complexity and generate these aromas.⁶⁹ Belgian wheat and German Rauch beers have desirable aromas described as clove, medicinal, spice-like or phenolic that are characteristic of 4-vinyl guaiacol's aromas.⁶⁸ Spiced ciders also have these characteristic aromas.⁷⁰ These odors have been described as “cider-like, spicy, clove-like or phenolic” along with a ‘mouse-like’ odor and flavor, and hydroxycinnamic acids have been demonstrated to serve as precursors to these largely undesirable volatile aroma compounds. Aroma compounds and their related aromas are shown in Figure 6.

Volatile Phenols	Structure	Phenolic Precursor	Aromas
4-vinyl guaiacol		ferulic acid	Flowery, Spicy
4-ethyl guaiacol		ferulic acid	Spicy, clove-like
4-vinyl phenol		p-coumaric acid	Heavy, Phenolic, Medicinal
4-ethyl phenol		p-coumaric acid	Spicy, smoky, woody, medicinal, phenolic
4-vinyl syringol		sinapic acid	weak odors

Figure 6: Volatile phenolic compounds, their structures, and the aroma descriptors associated with them^{5,6,71}

In white wines fermented with *Brettanomyces anomalus* and *B. intermedius* large amounts of 4-ethyl phenol and 4-ethyl guaiacol as well as trace amounts of 4-vinyl guaiacol were produced.⁶ The wine high in 4-ethyl guaiacol was strongly spicy and clove-like while the wine high in 4-ethyl phenol was spicy but more smoky. The aromas were identified via comparison with authentic reference samples through GC with effluent sniffing. It is thought that the typical smoky or medicinal aroma characteristics of wine and cider infected with *Brettanomyces* are the result of high concentrations of 4-ethyl phenol (Figure 6).⁶ Other yeasts, including *S. cerevisiae* have also been shown to generate these phenolic compounds from hydroxycinnamic acid precursors via metabolism.^{5,6,68,72} Vinylphenols, with characteristic clove, spice, phenolic, and medicinal aromas^{5,6} (Figure 6) can also be synthesized by *S. cerevisiae* via a phenylpropanoic acid intermediate, however since the phenylpropanoic acid is not detectable in the acellular

extract of yeast, the reaction is a direct decarboxylation in *S. cerevisiae*.⁵ It is thought that decarboxylation of cinnamic acids first results in vinyl phenols which are then reduced to yield the ethyl phenols.

The gene POF1 is believed to control the decarboxylation of ferulic acid to 4-vinyl guaiacol in *S. cerevisiae* by controlling production of a cellular decarboxylase. The decarboxylase enzyme synthesis has been shown to be constitutive of the yeast strain, as it is not induced by the presence of the substrate to be decarboxylated.⁵ POF1 has been eliminated from many yeast strains intended for brewing. It is possible that the POF1 gene and the ability of a yeast strain to decarboxylate the hydroxycinnamates may confer resistance to their inhibitory effects on yeast growth.⁷² In a survey of 14 different phenolic acids that are frequently found in wine or possibly in oak used in winemaking, only the cinnamic acids could be decarboxylated into volatile phenols by both *S. cerevisiae* and *S. bayanus*.⁵

Vinylphenol synthesis by yeasts can itself be inhibited by various other polyphenolic compounds. In a study related to wine fermentation conditions, *p*-coumaric acid decarboxylation was tested in a model medium supplemented with various purified phenolic compounds. Decarboxylation of 10 mg/L of *p*-coumaric acid was 100% inhibited by 1.6 g/L (+) catechin and 1.6 g/L polymeric procyanidins. It was inhibited 93% by 1.6 g/L (-) Epicatechin and 62% by 1.6 g/L Oligomeric procyanidins. It was not inhibited by 500 mg/L anthocyanins.⁵ These values are higher than those reported in apples, with (+) catechin ranging from 3-60 mg/L and (-) Epicatechin ranging from not detectable to 822 mg/L.³⁷ Similar results were seen in fermentation mediums of both red and white grape musts supplemented with *p*-coumaric acid and ferulic acid at 10 mg/L with different phenolic extracts from grape seeds, skins, and a combination of the two.⁵

Of particular interest to cidermakers, since similar aromas are generated via metabolism of hydroxycinnamic acids by both *Brettanomyces* and *S. cerevisiae*, some fermentations thought to be infected with *Brettanomyces* might only have a high level of hydroxycinnamates and a Pof⁺ yeast strain. *Brettanomyces* might not be present in the cidery at all.

In addition to the decarboxylation of ferulic acid into 4-vinyl guaiacol, five other major pathways in yeast that are relevant to food and flavor development have been identified. These include side chain reduction, coenzyme A, independent and dependent deacetylation, demethylation, and oxidative coupling. Ferulic acid transformations are of major interest to the food industry as a whole, not just the cider industry because of the possibility of transformation of ferulic acid, which is highly abundant in plants into vanillin, a highly valuable flavor and aroma compound used in the food, beverage, pharmaceutical, and perfume industries.⁶⁸

2.8 Sensory

In the food and beverage industries the sensory characteristics of a company's products are of the utmost importance in developing and marketing products, maintaining quality control parameters, as well as in retaining customers.⁷³ Traditionally, sensory characteristics of a product can be described by selecting a small group of panelists and training them in the main sensory characteristics of that product. There are a variety of recognized methods with which to train panelists that are efficient at identifying and quantifying the main sensory dimensions of a product, however they are time consuming and labor intensive, and therefore expensive. This makes them impractical in many industries for frequent use.^{73,74}

Novel sensory methods that are faster, cheaper, and easier have recently been developed. These include the flash profile, free choice profile, projective mapping, and sorting tasks. The sorting task is a simple method that is particularly suited for large groups of samples or fatiguing

or complex samples such as wine or cider.⁷³⁻⁷⁶ The sorting task has shown to provide similar results to trained panelists, and can provide global descriptive information about common characteristics of products.^{73,76,77} The simplicity of the sorting task has also been combined with the descriptive nature of other rapid methods to provide information about both the product space and the sensory properties that describe these groups without requiring any training of the panelists.⁷⁵

Sorting tasks generate collected data as difference matrices that can be analyzed with two primary sets of methods. The first set includes techniques such as DISTATIS⁷⁶, multidimensional scaling analysis, and common components and specific weight analysis. The second set is based on clustering techniques shown in tree representations.⁷³ Sorting tasks have been successfully used in beer, Amari, wines, and whiskeys and show potential for use in cider evaluation and description.^{73,75,76}

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Chapter 3: Effects of Hydroxycinnamates and Exogenous Yeast Assimilable Nitrogen on Cider Aroma and Fermentation Performance

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3.1 Abstract

Heritage apple cultivars for cider-making are often distinguished by a high concentration of tannins (phenolic compounds) and/or acids. The phenolic content of some cider apples far exceeds that of white wine, yet most cider fermentation practices are directly taken from white winemaking, not accounting for effects of high concentrations of phenolic compounds on fermentation. The objective of this study was to determine the impact of ferulic acid, *p*-coumaric acid, and chlorogenic acid—at concentrations reported in apples—and their interactive effects with yeast assimilable nitrogen (YAN) on fermentation kinetics and cider aroma. Our hypothesis was that phenolic compounds present in high-tannin cider apples would negatively impact fermentation kinetics, but not alter the aroma, and that added YAN would reduce these effects. Ferulic acid negatively affected fermentation performance ($p < 0.05$), but *p*-coumaric and chlorogenic acids did not. A sensory sorting task showed that *p*-Coumaric acid led to distinct differences in grouping by aroma, while ferulic acid resulted in distinct aroma descriptors. Chlorogenic acid did not affect aroma. Yeast strain influenced fermentation performance and aroma. Finally, addition of YAN improved fermentation performance for the low concentration ferulic acid condition, but not for the high concentration and affected *p*-coumaric acid-amended cider aroma.

3.2 Introduction

Craft cider consumption in the United States is rapidly increasing, with sales of craft cider from small producers sold locally or regionally up 39% in 2016 compared to 2012.¹ Cider is currently being produced commercially in 48 states and the District of Columbia. As of 2018, over 20% of United States cidemakers reported use of heritage apple cultivars, with increasing usage observed in recent years.^{2,3} The remaining majority of cider is made from dessert or processing apples and/or apple juice concentrate.⁴ Heritage apple cultivars are characteristically bittersharp and bittersweet, and possess levels of phenolic compounds up to ten-fold higher than those found in traditional dessert apples.^{5,6}

Typical concentrations of phenolic compounds vary greatly between apple cultivars with reported ranges of 570 to 13600 mg/L in the literature.^{5,7-9} The phenolic content of traditional bittersharp and bittersweet cider apples can far exceed that found in white wine, which is reported in the literature as 0 to 435 mg/L.¹⁰⁻¹² Despite this, most fermentation management strategies used for cider making are taken directly from white winemaking, and do not account for the inhibitory effects that high concentrations of some phenolic compounds can have on yeast during cider fermentation.

There are five main groups of phenolic compounds typically found in apples: flavanols (can be further divided into monomers and oligomers), hydroxycinnamic acids, dihydrochalcones, flavonols, and anthocyanins.⁷ The hydroxycinnamic acids in particular have been shown to inhibit growth of microorganisms, including *Saccharomyces cerevisiae*;^{13,14} however the impact of high phenolic compound concentration on fermentation rate when using heritage apple cultivars has not yet been investigated. Fermentation rate is of economic

importance to cideries because it can greatly influence production capacity, which is directly related to the financial return of a cidery.

Phenolic compounds have also been shown to contribute to cider flavor and aroma development via fermentation.¹⁵⁻¹⁷ As one of the most abundant hydroxycinnamic acids in plants, ferulic acid is of great interest in aroma generation because it is a precursor to vanillin¹⁸ (a valuable product of the food ingredient and flavor industry), as well as other aroma compounds, including 4-vinyl guaiacol and 4-ethyl guaiacol, which impart strong spicy, smoky, clove-like odors (Figure 6).^{16,18} *p*-Coumaric acid is a precursor to 4-ethyl phenol, which has a strong, woody, phenolic aroma with more smoky tones.¹⁶ The hydroxycinnamic acids are closely related to each other structurally and are produced via the shikimate pathway from L-phenylalanine or L-tyrosine.¹⁹

Yeast assimilable nitrogen (YAN) is one of the main classes of nutrients required for yeast growth and metabolism. It is one of the major factors influencing yeast growth and fermentation rate, as well as a factor in sulfurous off-flavor production.²⁰⁻²⁴ In cider production, YAN primarily comes from the apples themselves in the form of proteins, peptides, alpha amino acids, and ammonium ions, but these compounds are present in apples at much lower concentrations than in grapes.^{23,25} Exogenous YAN, or YAN derived from sources other than the fruit being fermented, is composed of two parts: alpha amino acids (assimilable organic nitrogen) and ammonium ions (inorganic nitrogen), which are both recommended by yeast nutrient manufacturers for a healthy fermentation.²⁵ Yeast nutrient manufacturers typically recommend YAN levels of 100 to 200 mg N/L for cider making, but there is little published research on optimum YAN levels for cider production.^{23,25}

The vast YAN levels found in apples range from 40 to 330 mg N/L.^{20,22,23} Thus, supplementation with additional YAN sources is typically recommended depending on the initial nitrogen concentrations in the juice, the initial sugar content, the fermentation temperature, the yeast strain, the turbidity of the juice, and the amount of dissolved oxygen (O₂).²⁵ However, too much YAN can result in altered, negative flavor profiles and microbiological instability in the product due to excess nutrients promoting unwanted microbial growth during and after fermentation by *S. cerevisiae*.²¹ Considering the multiple factors that impact the utilization of nitrogen during fermentation, further research is needed to determine optimal YAN levels for cider making, rather than solely relying on a recommended minimum total YAN concentration.

Our hypothesis was that the type and concentration of individual phenolic compounds present in a typical high-tannin cider apple cultivar would negatively impact fermentation kinetics compared to low-tannin juice fermentation, but not alter the aroma, and that added YAN would reduce both of these effects. The objective of this study was to evaluate the extent to which individual phenolic compounds found in apples (ferulic acid, *p*-coumaric acid, and chlorogenic acid) impact cider fermentation performance and aroma development. Furthermore, we tested whether there is an interactive effect between YAN concentration and these phenolic compounds on fermentation performance and aroma development.

3.3 Experimental

3.3.1 Apple Juice

White House Fresh Pressed 100% All Natural Apple Juice (National Fruit Product Co., Inc., Winchester, VA, USA) was selected for this study because it contains no added preservatives, antimicrobials, or ingredients such as ascorbic acid that are often added for

stability of commercially available apple juice. This juice is thus suitable for fermentation to cider. The individual containers of juice were mixed together to obtain a homogeneous lot of sufficient volume for this experiment, then transferred back to their original containers. The headspace was flushed with nitrogen and stored at -20°C until ready for use.

The following parameters were quantified in the initial, unfermented apple juice: pH (Accumet Ultra Triode Electrode Model 13-620-631, Thermo Fisher Scientific, Waltham, MA, USA); soluble solids (Brix Refractometer Model RF10, Extech Instruments Corporation, Nashua, NH, USA); titratable acidity (standard method as reported previously by others)²⁶; total YAN consisting of primary amino nitrogen (Megazyme PANOPA Enzymatic Kit, Megazyme International, Wicklow, Ireland) and ammonium ion (Megazyme Ammonia (Rapid) Enzymatic Kit, Megazyme International, Wicklow, Ireland). Total polyphenols in the pre-fermented juice were measured by the Folin-Ciocalteu method as described previously by others.^{27,28}

3.3.2 Fermentations

Fermentation treatments were carried out in triplicate. Juice was thawed at 4°C and treated with potassium metabisulfite prior to fermentation to inhibit spoilage organism growth.²⁹ A sanitizing solution consisting of 11.36 L of water, 8 g of potassium metabisulfite, and 12 g of citric acid was made up daily to sanitize equipment. Rubber stoppers and airlocks were sanitized in this solution and air dried before use. Two hundred milliliter aliquots of juice were allocated into previously autoclaved 250 mL Erlenmeyer flasks containing autoclaved magnetic stir bars. Yeast strain, nitrogen, and phenolic compound treatments were applied in a full-factorial experimental design as outlined in Table 4. Three phenolic compounds (ferulic acid, *p*-coumaric acid, and chlorogenic acid) were added to apple juice at two concentration levels each and there were also control groups with no phenolic compounds added (0, 150, and 300 mg/L for ferulic

acid and *p*-coumaric acid and 0, 250, and 500 mg/L for chlorogenic acid). These concentrations have previously shown inhibition of *S. cerevisiae* and/or are close to levels found in apples.^{5,7,13,30,31} Three YAN concentrations were also evaluated for each phenolic treatment (0, 100, and 200 mg N/L added). These levels of YAN addition span ranges currently recommended by yeast nutrient manufacturers.²⁵ The source of added YAN was a combination of diammonium phosphate (DAP), an inorganic nitrogen source, and Fermaid O™, an organic nitrogen source (Lallemand, Inc., Montreal, Canada).²⁵ Each phenolic and YAN treatment was subject to fermentation by two different yeast strains, EC1118 and W15 (Lallemand, Inc., Montreal, Canada). Under similar conditions, these two yeast strains are expected to exhibit different fermentation kinetics and nitrogen requirements and to produce different volatile aromas.

Table 4: Cider treatments with different phenolic compounds, yeast strains, and yeast assimilable nitrogen (YAN) levels. All combinations were conducted in triplicate. (½ of the YAN addition was added to the juice before yeast inoculation, and the other ½ of the YAN was added, according to YAN supplier recommendations, 1/3 - ½ of the way through fermentation. The YAN addition level is only the total of what was added, not including the YAN level of the juice prior to addition.)

Phenolic Compounds: Tannin Types (concentration added)	Yeast Strains					
	EC1118			W15		
	YAN Addition Level (mg N/L)			YAN Addition Level (mg N/L)		
Control (0 mg/L)	0	100	200	0	100	200
Ferulic Acid (150 mg/L)	0	100	200	0	100	200
Ferulic Acid (300 mg/L)	0	100	200	0	100	200
<i>p</i> -Coumaric Acid (150 mg/L)	0	100	200	0	100	200
<i>p</i> -Coumaric Acid (300 mg/L)	0	100	200	0	100	200
Chlorogenic Acid (250 mg/L)	0	100	200	0	100	200
Chlorogenic Acid (500 mg/L)	0	100	200	0	100	200

The sanitized stoppers and airlocks were placed onto each flask after inoculation with yeast at the supplier recommended inoculation rate.²⁵ Each flask was mixed at 1000 rpm for 5 minutes using a magnetic stir bar and the weight at time zero was recorded. All flasks were then

placed into a temperature-controlled chamber at 18°C. Each flask was mixed at 1000 rpm for 5 minutes, twice daily to resuspend the yeast. The weight was recorded after each mixing to monitor fermentation rate. Fermentations were considered complete when weight measurements were consistent for four data points (2 days). Samples from each flask were then collected in 50 mL centrifuge tubes, flushed with nitrogen, and frozen at -80°C for further analysis. The remaining cider sample replicates from each treatment were pooled, flushed with nitrogen, and stored in wine bottles at 4°C for future sensory analysis of cider aroma.

Prior to chemical analysis, finished cider was degassed in a 45°C shaking water bath for 10 minutes and purged with nitrogen for 10 seconds. Cider pH, titratable acidity, residual sugar (Megazyme FRUGL Enzymatic Kit, Megazyme International, Wicklow, Ireland), total YAN and total polyphenols were determined using the same methods as applied to the apple juice.

3.3.2.1 Assessment of Fermentation Performance

Maximum fermentation rate (r_{\max}) was determined by calculating the maximum slope of the exponential phase of each fermentation curve (Figure 7) using the secant method over three time points using R, an open source statistical computing environment.³² The slope of the section of the line with the minimum secant value was determined to be r_{\max} for each treatment.

Residual sugar in the form of glucose, sucrose, and total residual sugar (glucose + fructose) was evaluated to determine if the fermentation completed to dryness. Fermentations were considered incomplete, or stuck, if they had reached a stable weight as described in the previous section yet contained more than 2 g/L total residual sugar.

3.3.2.2 Statistical Analysis

For all chemical analyses of juice, results are reported as mean \pm standard error of $n = 3$ analytical replicates. For all chemical analyses of cider, the mean \pm standard error of $n = 3$ fermentation replicates are reported, with each of those values having been calculated as the mean of $n = 3$ analytical replicates. 4-way ANOVAs with all interactions were performed in R³² to evaluate whether the treatments and/or interactions thereof affected r_{\max} or residual sugar concentration (glucose, fructose, total sugar). Significance was defined as $p < 0.05$. Results of chemical analyses in which levels present were not detectable were treated as zero for statistical analysis.

A regression tree was fitted to describe which treatments had the greatest influence on r_{\max} . Tree-based methods have been used to help automate and aid in decision making in many fields, including machine learning and engineering.³³ This method takes one continuous dependent variable and many independent variables, decides which independent variable can account for the most variance in each subgroup, and splits the data accordingly. In this case, the dependent variable was r_{\max} and the independent variables were yeast strain, nitrogen level, type of phenolic compound (ferulic acid, *p*-coumaric acid, chlorogenic acid, and control), and phenolic compound level. The aim is to partition the data to optimally reduce variation. The R³² package *rpart*³⁴ was used to fit the regression tree by partitioning the r_{\max} data for all fermentations using the dependent and independent variables defined above into the most homogeneous partitions possible.³⁵

3.3.3 Assessment of Cider Aroma

Sensory analysis of cider aroma was conducted to determine how the interactive effects of phenolic compound addition and YAN contributed to the aromas generated in cider during

fermentation. We used a free-sort method combined with free-text description to rapidly investigate the differences between cider aromas and collect descriptions of these differences.

3.3.3.1 Panelists

All 25 panelists were untrained, at least 21 years of age, and had consumed hard cider on at least one prior occasion. Panelists received 12 to 18 samples per session, and the same 25 panelists participated in all three sessions.

3.3.3.2 Samples

The cider samples were stored in full 375 mL wine bottles with screw cap closures, headspace flushed with nitrogen, in the dark at 4°C for seven months prior to sensory evaluation. Each of the 42 ciders was presented as a 148 mL aliquot in a black wine glass to prevent visual differences such as color from impacting panelist perception. Each glass was covered with a watch glass to ensure uniform volatiles in every glass. Samples were served at room temperature. All ciders were coded with a random 3-digit number.

3.3.3.3 Sorting Task Procedure

The sensory study was completed over three days with the panelists each participating in all three sessions. Chlorogenic acid amended ciders and control ciders were presented together as Session 1 (18 ciders total), *p*-coumaric acid amended ciders were presented separately as Session 2 (12 ciders), and ferulic acid amended ciders were presented separately as Session 3 (12 ciders). Panelists were instructed to group the samples according to their similarity by smell alone. Panelists were free to group the ciders according to any criteria they chose, into at least two (2) groups and no more than $n - 1$ groups where n is the number of ciders presented in a given

session. They were then asked to label each group they formed with descriptive term(s) of their own choice.

3.3.3.4 Data Analysis

The results of the sorting task were analyzed using DISTATIS (through the *DistatisR*³⁶ package)^{37,38} to obtain product maps for the cider sorting data. DISTATIS is an extension of multidimensional scaling (MDS) that accounts for the individual variation among panelists by calculating the R_v coefficients between matrices and weighting them based on their similarity. The specific calculations have been previously described in the literature.^{37,39} Panelist assigned descriptors for sorted groups were first stemmed and synonymized, and then grouped into categories of similar descriptors.^{39,40} Descriptor categories were then projected onto the product maps generated using DISTATIS for the cider sort data to illustrate the sensory characteristics of the cider groups. For an overall analysis of the text descriptions, a contingency table of products by descriptors for all three days (42 ciders) was analyzed by Correspondence Analysis using the ExPosition package.⁴¹

3.4 Results & Discussion

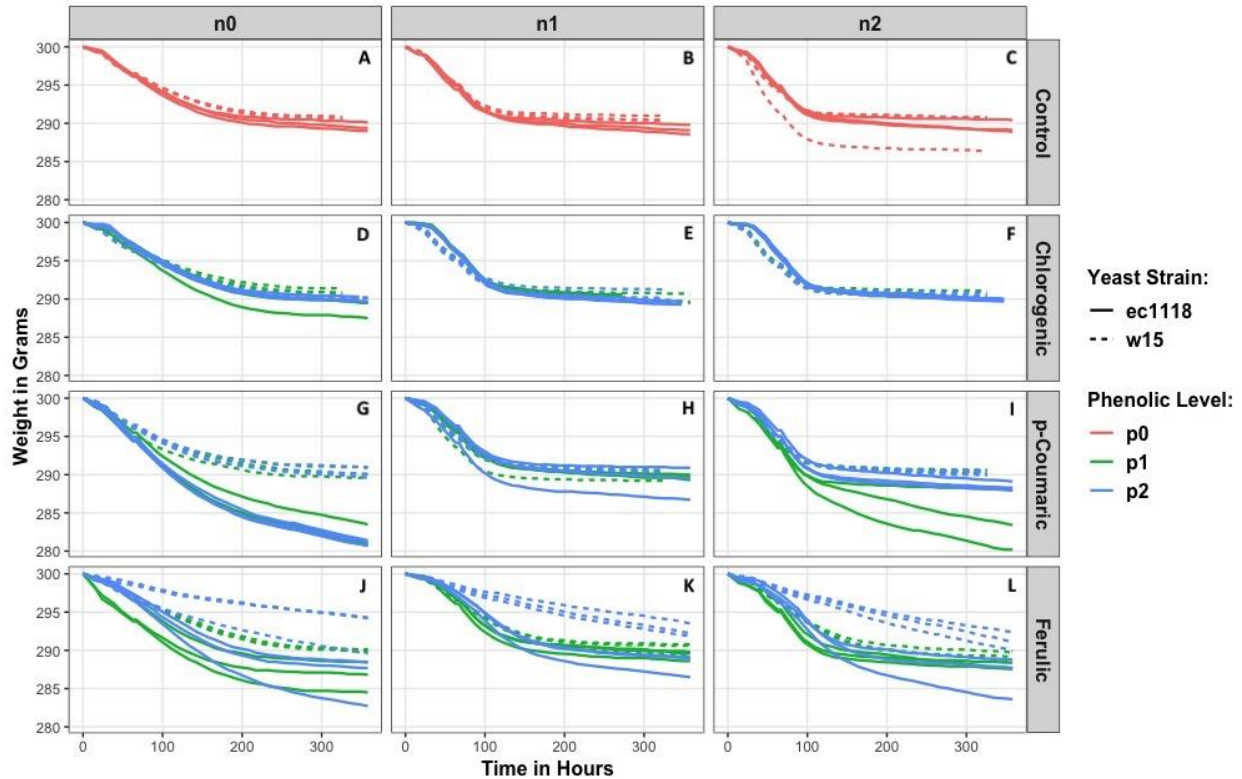


Figure 7: Fermentation rate curves measured as weight lost as CO₂ is generated during fermentation. Dashed lines indicate W15 yeast was used and solid lines indicate EC1118 yeast. Red indicates no phenolic compound was added. Green indicates the lower phenolic compound addition level, which was 150 mg/L for ferulic and *p*-coumaric acids and 250 mg/L for chlorogenic acid. Blue lines indicate the high level of phenolic compound addition, which was 300 mg/L for ferulic and *p*-coumaric acids and 500 mg/L for chlorogenic acid. Each column indicates a different nitrogen addition level, n0 = 0 mg N/L added to the starting juice; n1 = 100 mg N/L added; and n2 = 200 mg N/L added. Each row represents a phenolic compound that was added to the ciders, or control where no phenolic compound was added.

3.4.1 Impact of Phenolic Compounds on Fermentation Performance

Fermentation curves for each treatment are shown in Figure 7. Of the three phenolic compounds tested, only ferulic acid had an inhibitory effect on r_{max} , and only when fermented by W15 yeast ($p < 0.05$). For the W15 yeast strain, the higher concentrations of 300 mg/L ferulic acid (with no added nitrogen) resulted in the lowest value of r_{max} observed in the treatments with no added nitrogen (-0.040 g CO₂/hour) (Figure 8). Furthermore, this treatment resulted in the only stuck fermentation observed, as determined by the residual sugar concentration of 2.2 g/L,

which is more than 2 g/L, our criterion for fermentation to dryness. The r_{\max} for the treatment with the lower concentration of ferulic acid (150 mg/L) fermented by W15 yeast did not differ from the control. For treatments with the higher level (300 mg/L) of ferulic acid, the fermentation did not proceed following a typical 1st order reaction curve when fermented by W15 yeast (Figure 7J - 7L). This pattern was not observed with EC1118 yeast.

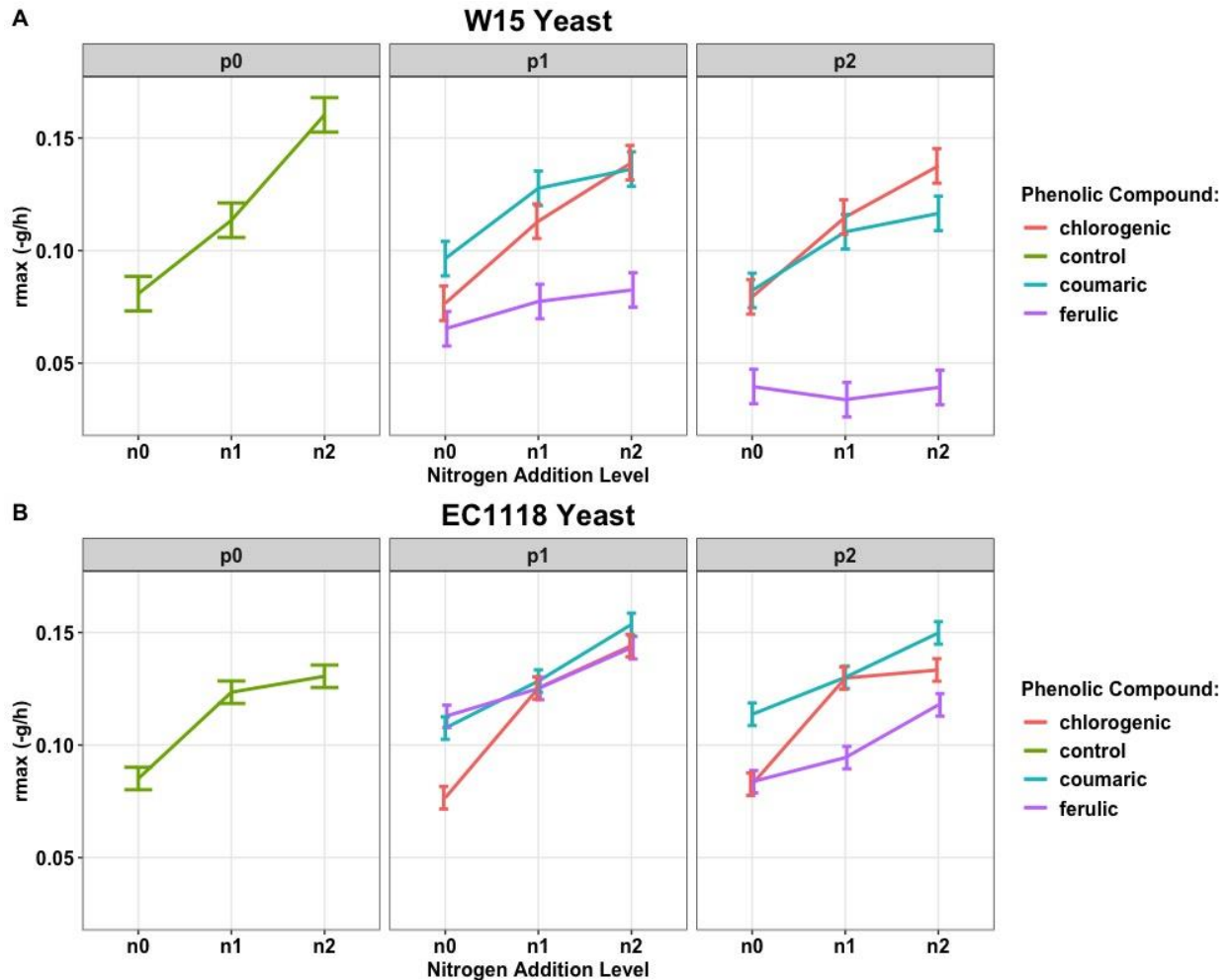


Figure 8: Interaction plots showing the maximum fermentation rate (r_{\max}) (\pm standard error) differences for each treatment interaction, separated by yeast strain. Each line on the x-axis represents a nitrogen addition level where n0 = 0 mg N/L added; n1 = 100 mg N/L added; and n2 = 200 mg N/L added to the fermentations. p0 was the control treatment with no phenolic compound added to the pre-fermentation juice. p1 was 150

mg/L for ferulic and *p*-coumaric acids and 250 mg/L for chlorogenic acid. p2 was 300 mg/L for ferulic and *p*-coumaric acids and 500 mg/L for chlorogenic acid. Non-parallel lines indicate an interaction effect.

The r_{\max} values were not influenced by *p*-coumaric acid or chlorogenic acid at either the low or high concentrations tested in either yeast strain (with no added nitrogen). No stuck fermentations resulted from the *p*-coumaric or chlorogenic acid treatments.

Our findings for r_{\max} differ from previous findings in industrial fermentation applications which reported inhibition by ferulic acid at 150 mg/L and by *p*-coumaric acid at 300 mg/L.¹³ These discrepancies highlight the importance of conducting this work in the relevant matrix conditions.

Of note, for both the low and high concentrations of chlorogenic acid, significantly ($p < 0.05$) more glucose remained after completion of fermentation compared to the other treatments, indicating that the treatments may have altered the balance of glucose to fructose metabolism. However, the maximum residual glucose concentration detected was 0.12 ± 0.03 g/L, which is likely not of practical significance. Residual sugar values for all treatments are provided in Supplementary Information Table 1 (Appendix A).

3.4.2 Influence of YAN on Fermentation Performance in the Presence of Phenolic Compounds

When YAN was added to our only stuck fermentation, (300 mg/L ferulic acid fermented by W15 yeast with 2.2 g/L residual sugar), the fermentation rate did not recover to that of the control (without ferulic acid). However, increased YAN in the presence of ferulic acid with W15 yeast resulted in significantly lower ($p < 0.05$) total residual sugar levels, below the threshold for fermentation to dryness (0.7 g/L and 0.6 g/L for the high and low YAN addition levels, respectively). This indicates that addition of YAN resulted in more complete fermentations in the presence of high concentrations of ferulic acid.

In the control treatments, with no added phenolic compounds, the effects of YAN addition were in agreement with prior work by others,^{20,22,24,42} where addition of nitrogen generally increases the fermentation rate, and the extent of these effects differ among yeast strains. For yeast strain EC1118, adding nitrogen resulted in increased r_{\max} ($p < 0.05$, Figure 8), although r_{\max} for the low and high concentrations of nitrogen fermented by EC1118 did not differ. For yeast strain W15, the high concentration of added nitrogen resulted in increased r_{\max} , although r_{\max} for the low concentration of added nitrogen did not differ from the control.

Since yeast strain, phenolic compound addition, and YAN addition were all found to affect r_{\max} to varying degrees, and interactions between all three main effects were determined to be significant by ANOVA ($p < 0.05$), a regression tree approach was employed to visualize the relative impacts of these treatments on r_{\max} . These interactions are not unexpected and are likely due to differences in metabolic pathways between the yeast strains.

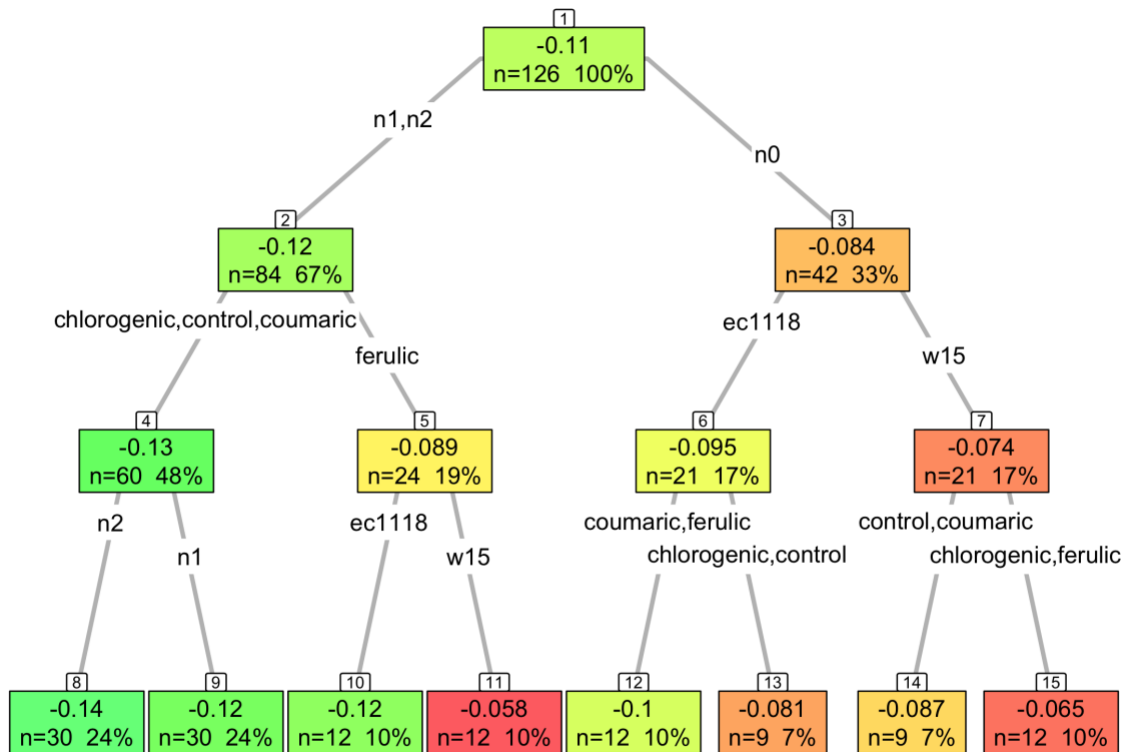


Figure 9: Regression tree for r_{max} . Larger negative numbers indicate higher r_{max} . The criteria by which data were split at each divergence are labeled on each connecting line of the tree. For example, the first split is based on nitrogen level. All fermentations at levels n1 and n2 are on the left and all fermentations with no added nitrogen are represented on the right. The fastest fermentations are represented by green shading in the boxes, and the slowest by red. In each box of the regression tree ($n = \#$) indicates the number of fermentations represented in that group. The number at the top of the box indicates the mean r_{max} of that group, and the % is the percent of fermentations explained by this grouping.

Figure 9 illustrates that adding nitrogen had the biggest impact on r_{max} , with the largest variation between r_{max} explained by the difference between no nitrogen added and the higher addition levels, as shown at the first split (Figure 9, Boxes 2 and 3, the top of the regression tree). When no nitrogen was added (right side of regression tree, after first split, Box 3), the next largest impact on r_{max} was due to yeast strain (Boxes 6 and 7), followed by type of phenolic compound. The impact phenolic compounds had on r_{max} was different in each yeast strain. In W15 yeast with no added nitrogen (Box 7), chlorogenic acid and ferulic acid resulted in slower

fermentations (Box 15) than the control and *p*-coumaric acid fermentations (Box 14). However, in the EC1118 fermentations with no added nitrogen (Box 6), the control and chlorogenic acid fermentations (Box 13) were slower than the ferulic acid and *p*-coumaric acid fermentations (Box 12).

When nitrogen was added to the fermentations (left side of regression tree, after first split, Box 2) ferulic acid had the next largest impact on r_{\max} (Box 5) with a slower fermentation rate than all other phenolic compound additions or the control (Box 4). For the ferulic acid ciders with nitrogen added (Box 5) yeast strain had the next biggest impact on r_{\max} with W15 resulting in the slowest fermentations (Box 11) and EC1118 having a much larger r_{\max} (Box 10). When nitrogen was added to ciders other than ferulic acid amended ciders, the level of nitrogen also impacted r_{\max} (Boxes 8 and 9).

3.4.3 Effects of tannin and YAN on cider aroma as determined through sensory evaluation

The grouping maps in Figures 10-12 show more obvious separations between groupings in ferulic acid amended (Figure 10) and *p*-coumaric acid amended (Figure 11) ciders than in the chlorogenic acid amended and control cider maps (Figure 12). In these maps, larger first eigenvalues of the panelist-agreement matrices³⁷ indicate that more of the variation is described by the groups assigned. The amount of variation described by each dimension (τ) for ferulic acid-amended ($\tau_1 = 28\%$ of variation, $\tau_2 = 7\%$ of variation, 35% explained by the first two dimensions) and *p*-coumaric acid-amended ($\tau_1 = 37\%$ of variation, $\tau_2 = 7\%$ of variation, 44% explained by the first two dimensions) ciders compared to chlorogenic acid-amended and control ciders ($\tau_1 = 21\%$ of variation, $\tau_2 = 6\%$ of variation, 27% explained by the first two dimensions) indicates that there are more obvious sensory differences in aromas between the

ferulic acid and *p*-coumaric acid amended ciders than there are between the chlorogenic acid amended and control ciders.

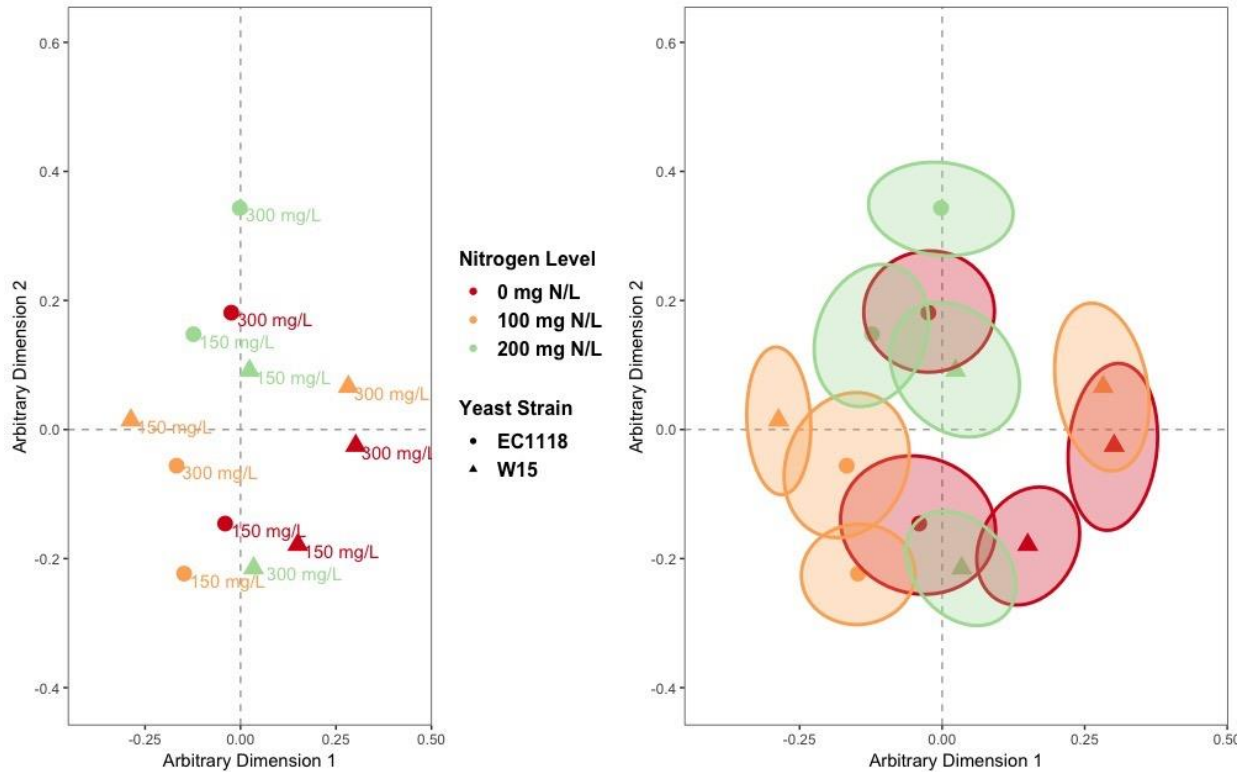


Figure 10: Ferulic acid sensory study results as product maps generated in DISTATIS. The left-hand side shows the cider treatments with yeast strain illustrated by shape; EC1118 is represented by circles and W15 is represented by triangles. Nitrogen addition level is differentiated by color; where red is no nitrogen added, orange is the low level (100 mg N/L) addition, and green is the high level (200 mg N/L) addition. Phenolic level is labeled on the plot. 95% confidence ellipses are illustrated by the ovals in the right-hand panel, and clustering of the confidence ellipses indicates similarities between cider groupings.

Panelists detected differences in aroma amongst ferulic acid amended ciders (Figure 10), with some clustering based on YAN additions and yeast strain *within* the ferulic acid ciders (Figure 10). Based on Correspondence Analysis of the aroma descriptors for all three days of the sensory study, ferulic acid-amended ciders overall were the most different from the other ciders (Figure 13). However, the ciders with *p*-coumaric acid supplementation had the most perceptually distinct sensory differences among themselves, with panelists clearly grouping the ciders based on *p*-coumaric acid level as well as yeast strain and nitrogen level (Figure 11).

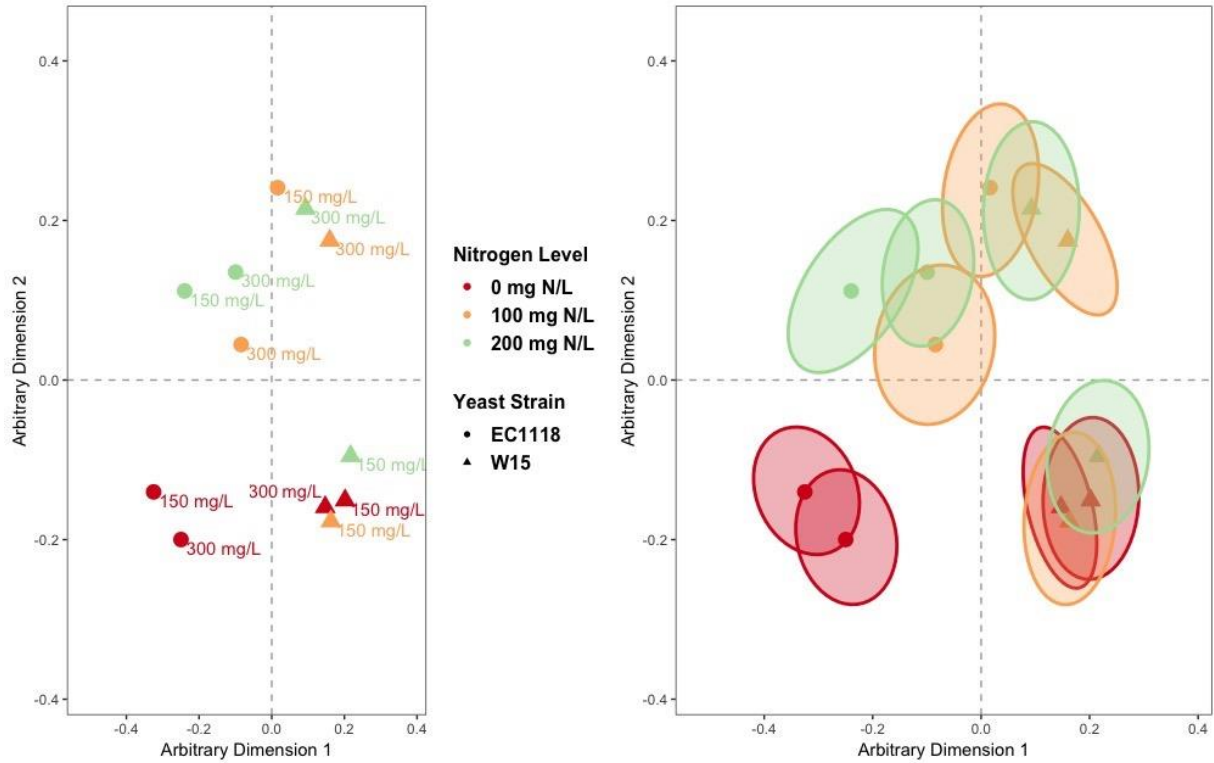


Figure 11: *p*-Coumaric acid sensory study results as product maps generated in DISTATIS. The left-hand side shows the cider treatments with yeast strain illustrated by shape; EC1118 is represented by circles and W15 is represented by triangles. Nitrogen addition level is differentiated by color; where red is no nitrogen added, orange is the low level (100 mg N/L) addition, and green is the high level (200 mg N/L) addition. Phenolic level is labeled on the plot. 95% confidence ellipses are illustrated by the ovals in the right-hand panel, and clustering of the confidence ellipses indicates similarities between cider groupings.

Control ciders and ciders amended with chlorogenic acid could not be distinguished from each other by aroma based on nitrogen level or concentration of chlorogenic acid added (Figure 6). However, there is a potential sample size effect, since panelists sorted 12 ciders each for the ferulic acid and *p*-coumaric acid sorting tasks and sorted 18 ciders for the control and chlorogenic acid treatments combined.

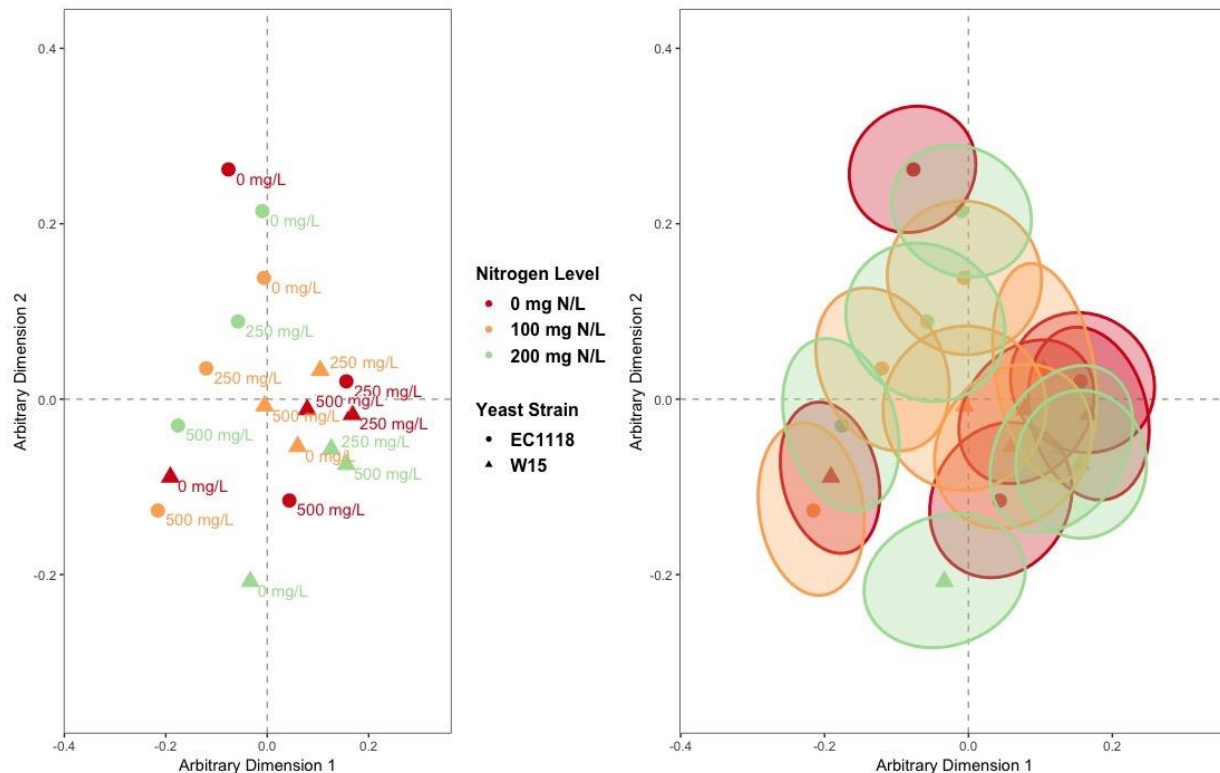


Figure 12: Control and chlorogenic acid sensory study results as product maps generated in DISTATIS. The left-hand side shows the cider treatments with yeast strain illustrated by shape; EC1118 is represented by circles and W15 is represented by triangles. Nitrogen addition level is differentiated by color; where red is no nitrogen added, orange is the low level (100 mg N/L) addition, and green is the high level (200 mg N/L) addition. Phenolic level is labeled on the plot. 95% confidence ellipses are illustrated by the ovals in the right-hand panel, and clustering of the confidence ellipses indicates similarities between cider groupings.

Since the control ciders were not presented consistently each day of sensory evaluation, the ciders from each day of evaluation cannot be directly compared to each other. Text analysis was employed to help overcome this limitation by allowing comparison across all samples by sensory descriptors, since all samples were evaluated by the same panelists, if not in one session (Figure 13).

The aroma descriptors assigned to ciders amended with either ferulic acid or *p*-coumaric acid by our panelists (Figure 13 and Supplementary Figures) are similar to descriptors previously used to describe vinylphenols (Figure 6): spicy, phenolic, clove, and medicinal.^{15,16,18} Prior reports have demonstrated that ferulic acid and *p*-coumaric acid are precursors to vinylphenols (Figure 6), as they can be decarboxylated by certain strains of *S. cerevisiae* to form

vinylphenols.^{15,16,43} This hypothesis is further supported by the observations that the descriptor “clove” was *only* used to describe the ferulic acid amended ciders (Figure B1), and “medicinal” and “artificial” descriptors were *only* used to describe *p*-coumaric acid amended ciders (Figure B2), indicating that these compounds are being converted into their respective volatile metabolites. The chlorogenic acid ciders (along with the control ciders) were generally described with “apple” and “sweet” descriptors (Figure B3).

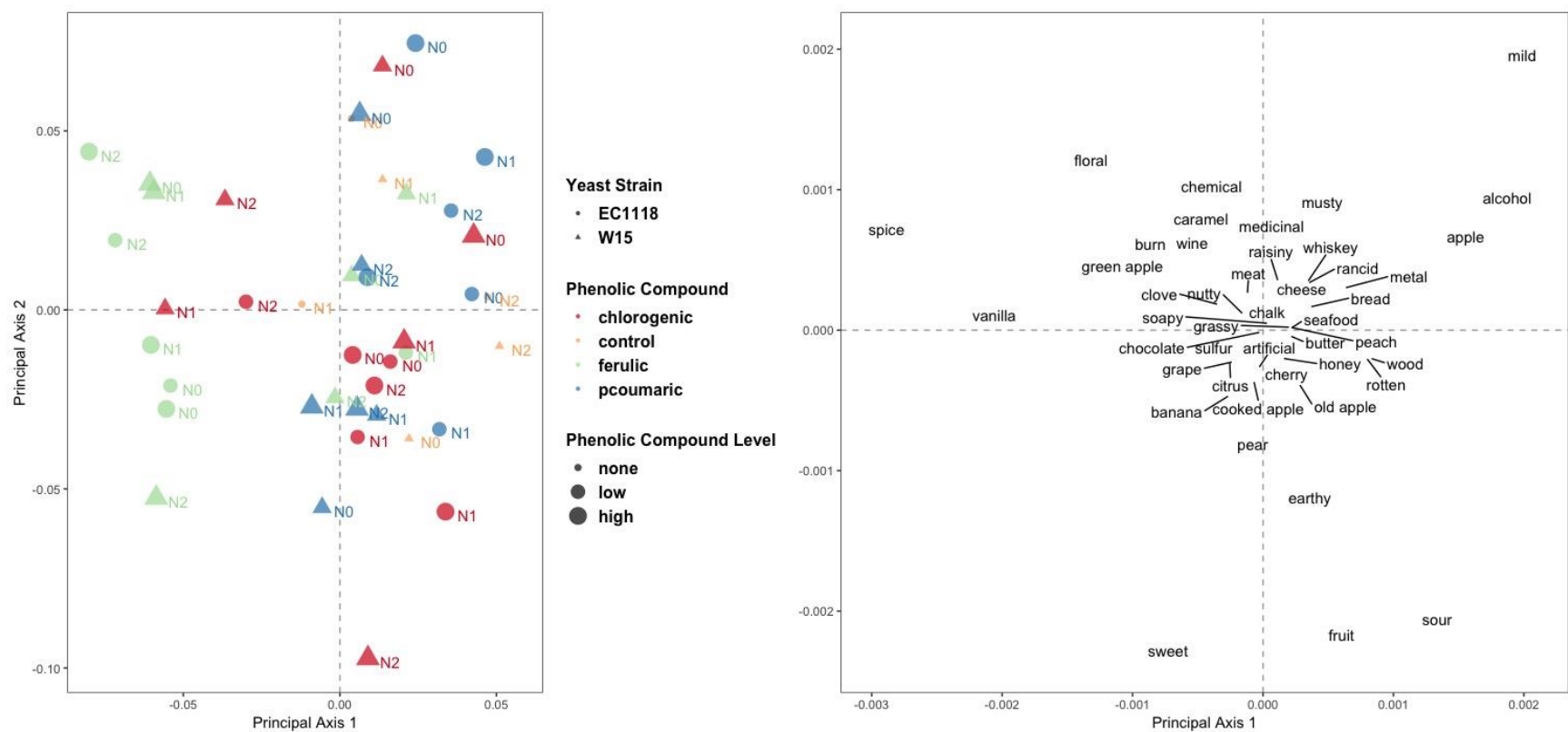


Figure 13: Aroma descriptors for all three sessions of the sensory study combined. The left-hand panel shows the correspondence analysis of all three sessions, and the right-hand panel shows the descriptors for the panelists groupings projected onto the same space. Location of descriptors on the right-hand panel correspond to the ciders on the left-hand panel. Size of the shapes on the left indicate the phenolic compound addition level, shape indicates yeast strain with EC1118 represented by circles and W15 represented by triangles. Chlorogenic acid ciders are red, control ciders are orange, ferulic acid ciders are green, and *p*-coumaric acid ciders are blue. Nitrogen levels are labeled on the left-hand plot.

3.5 Conclusion

Overall, within the concentration ranges evaluated, adding YAN to cider fermentations will lead to a higher maximum rate of fermentation. Further investigation would be required to optimize YAN concentrations for the quickest fermentation to dryness. With high amounts of ferulic acid present, YAN can help the fermentation resolve to dryness, but yeast strain can influence the effectiveness of this strategy.

Furthermore, these results demonstrate that cider fermentation kinetics and cider aroma can be influenced by the type and concentration of phenolic compounds present in high tannin apples, and that addition of YAN can moderate these effects. Cidermakers can utilize apple cultivar selection to select for phenolic compounds that can influence the aromas generated during cider fermentation. Yeast strain selection is also an important consideration in this regard, as yeast strain will affect fermentation kinetics, nitrogen requirements, and the aromas generated during cider fermentation. Taken together, cidermakers can use our findings to inform fermentation strategies designed to develop the desired sensory characteristics and achieve stylistic goals in the finished cider.

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3.7 Declaration of Interest Statement

No potential conflict of interest was reported by the authors.

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Chapter 4: Survey of Ferulic Acid, *p*-Coumaric Acid, and Chlorogenic Acid in Apple Cultivars Grown in Virginia

4.1 Experimental

4.1.1 Juice Collection

A group of 58 samples representing different apple cultivars and orchards collected across Virginia were screened for the phenolic compounds used in the fermentation rate experiment in Chapter 3: ferulic acid, *p*-coumaric acid, and chlorogenic acid. Apples were harvested from August to November 2018 and stored at 4°C in ambient atmosphere conditions until pressing. Apples were sliced and juiced with a Champion Juicer (Lodi, CA, USA). The pulpy juice was then strained through a medium sieve (1/16 inch) metal kitchen strainer, and the juice was collected in 50mL tubes and frozen at -80°C for further analysis.

4.1.2 Juice Analysis

The juice was thawed at 4°C and centrifuged at 2300 x g for 5 minutes. Juice pH, titratable acidity, and soluble solids were measured using the same methods described in section 3.3.

4.1.3 Standard Preparation

Ferulic acid (99%), *p*-coumaric acid (≥98.0%, HPLC grade), and chlorogenic acid (≥95% titration) (Sigma Aldrich, St. Louis, MO USA) were dissolved in ultrapure water from Millipore Milli-Q water purification system (Millipore, Bedford, MA, USA) to make a stock solution with a concentration of 500 mg/L of ferulic acid, *p*-coumaric acid, and chlorogenic acid. This stock solution was then diluted to the following concentrations: 0.01 mg/L, 0.1 mg/L, 0.2 mg/L, 0.3

mg/L, 0.4 mg/L, 0.5 mg/L, 1 mg/L, 2mg/L, 4 mg/L, 8 mg/L, 16 mg/L, 32 mg/L, 62.5 mg/L, 125 mg/L, 250 mg/L, and 375 mg/L to make a standard curve.

4.1.4 Sample Preparation

Each sample was diluted two-fold with ultrapure water and then filtered through a 0.22 µm membrane PTFE filter with luer lock (Microsolv, Leland, NC USA) into a 12 x 32 mm glass screw neck vial (Waters, Milford, MA USA).

4.1.5 UPLC Conditions and Method

Waters Application Note 720004069EN⁷⁸ was followed using column ACQUITY UPLC HSS T3, 1.8µm, 2.1 x 100 mm at 45°C in the ACQUITY UPLC H-Class system. EmpowerTM software (Waters, Milford, MA USA) was used to identify and quantify the phenolic compound sample peaks.

4.2 Results & Discussion

Of the 58 apple cultivars surveyed, ferulic acid was only present above 0.01 mg/L in 3 cultivars, and the highest level was 2.8 mg/L in Dabinett, which is far below the low level of 150 mg/L that was used in this study. The lowest concentration of ferulic acid that has shown inhibition of *S. cerevisiae* in the literature is 50 mg/L⁶ so these low levels would need further investigation to determine their impact on r_{max} .

p-Coumaric acid was found in 37 of the cultivars, mostly at low levels, however in Dabinett and Tremlette's Bitters cultivars, *p*-coumaric acid was found at or above the high level (300 mg/L) we tested in this experiment, 294 ± 12.2 mg/L and 387 ± 19 mg/L, respectively. These cultivars could be used in cider to impact the sensory aroma characteristics, possibly

imparting some of the aroma descriptors in Figure 8. Nitrogen addition could help increase the maximum rate of fermentation.

Chlorogenic acid was found in every cultivar sampled, at levels below what was tested in this experiment except for that found in Tremlette's Bitters at 636.6 ± 32.2 mg/L.

4.3 Conclusions

These results indicate that phenolic composition of a Virginia apple can impact the aromas generated through cider fermentation. Of particular interest to cidemakers are that Dabinett and Tremlette's Bitters have higher levels of *p*-coumaric acid than other cultivars and could possibly result in more medicinal or phenolic aromas in the ciders made with these cultivars. The aroma compounds generated in this study were not identified chemically, just by their descriptors.

Chapter 5: Conclusions and Future Work

Overall, the goal of this work was to develop fermentation management strategies specifically for the cider industry that account for the chemical differences between cider apples and white wines. This was done by determining whether various concentrations of three phenolic compounds (ferulic acid, *p*-coumaric acid, and chlorogenic acid) influence fermentation rate and sensory attributes of cider, and by determining whether there is an interactive effect of total YAN and phenolic compound concentration on fermentation kinetics and cider aroma as determined by sensory evaluation. The measurable concentrations of inhibitory phenolic compounds (ferulic acid, *p*-coumaric acid, and chlorogenic acid) present in Virginia apples was also determined. A variety of techniques including fermentation, sensory, and UPLC methods were employed to achieve this goal.

5.1 Phenolic Compound and YAN Influence on Fermentation Performance

This fermentation study was designed to determine if ferulic acid and *p*-coumaric acid, at levels present in apples, would inhibit cider fermentation and/or change cider aroma. We also sought to understand whether any inhibition could be overcome with exogenous YAN additions, and if cider aroma would be affected by YAN addition. Fermentations were conducted using two yeast strains, EC1118 and W15 (Lallemand, Inc., Montreal, Canada), to determine the extent to which these effects varied among yeast strains.

5.1.1 Key Findings

5.1.1.1 Phenolic Compound and YAN Influence on Fermentation Performance

Our results indicate that ferulic acid significantly decreases the maximum fermentation rate, yet adding YAN to the stuck fermentation did not overcome this decrease in r_{\max} . *p*-Coumaric acid did not have as much of an impact on r_{\max} as expected. Chlorogenic acid also did not impact r_{\max} , as was expected. Since we did see that increasing YAN led to less inhibition overall, this discrepancy between our results and prior work with *p*-coumaric acid in model solution could be a result of differing nitrogen levels in the fermentation substrates, as well as other differences between apple juice and the model solutions in previous work. It has also been shown by others that *p*-coumaric acid and ferulic acid can be decarboxylated by yeast, however that decarboxylation can be inhibited by presence of other phenolic compounds that are found in apples, including catechin. This inhibition or non-inhibition of decarboxylation could have contributed to the lack of rate difference we saw. This could be further investigated by measuring other phenolic compounds such as (+) catechin, (-) epicatechin, oligomeric procyanidins (1-2),

and polymeric procyanidins (3-4) that have been shown to inhibit vinylphenols synthesis by *S. cerevisiae*.

5.1.1.2 Phenolic Compound Influence on Cider Aroma determined through Sensory

Evaluation

The outcomes of sensory evaluation of cider aromas indicate that the presence in apple juice of the phenolic compounds tested in our study may have a greater impact on sensory characteristics of cider than previously thought. Ferulic acid had an impact on cider aroma, although the effects were not as obvious to panelists as those observed as a result of *p*-coumaric acid addition to apple juice pre-fermentation. *p*-Coumaric acid had the greatest impact on cider aroma groupings as determined by sensory evaluation, with panelists clearly grouping the ciders based on level of *p*-coumaric acid added, nitrogen level, as well as by yeast strain. The panelists could not distinguish the ciders made with any level of chlorogenic acid from control ciders made from un-amended apple juice. The differences observed in samples made with pre-fermentation addition of *p*-coumaric acid and ferulic acid to levels observed in other apple juice could be due to the decarboxylation of these compounds into 4-ethyl phenol and 4-vinyl guaiacol respectively during the fermentation by the common *S. cerevisiae* strains EC1118 and W15. Future work needs to be done analytically to verify the presence of these compounds in cider made with these phenolic compounds.

5.1.2 Limitations

5.1.2.1 Phenolic Compound and YAN Influence on Fermentation Rate Limitations

A limitation of this study was that following recommended wine fermentation practice, nitrogen was added about ½ of the way through fermentation. This is recommended by yeast

nutrient manufacturers, however it led to noise in the weight data collected to determine fermentation rate.

In one style of airlocks the water inside started to evaporate and had to be refilled, introducing error into the weight measurements used to monitor fermentation rate. For future studies, only the S style airlocks should be used.

5.1.2.2 Phenolic Compound and YAN Influence on Sensory Attributes Limitations

A limitation of this study was that there was not sufficient control cider to include in each day of sensory evaluation. If I were to re-do this study I would make more control cider to be able to include control sample with each sensory session. This would allow for direct comparison between days (as opposed to comparing through text analysis of descriptors only), as there would be a reference cider that was the same on all three days to compare the results back to.

5.1.3 Future Work

5.1.3.1 Phenolic Compound and YAN Influence on Fermentation Outcomes

Future studies should be directed towards examining the optimum level of YAN addition to ensure a complete, rapid fermentation to dryness. We have shown that addition of nitrogen can increase the max fermentation rate, however it has still not been determined what the optimum level of YAN addition is. This rate study should be combined with a sensory study to further develop the effect of YAN on sensory aspects of a cider.

5.1.3.2 Phenolic Compound and YAN Influence on Sensory Attributes

Future studies should explore the compounds produced via fermentation in the presence of *p*-coumaric acid in particular. *p*-Coumaric acid has been shown to be present at levels above

what we tested (300 mg/L) in certain Virginia apple cultivars, and it has also been shown to affect the aromas generated in the cider produced. Future work should explore what compounds are being produced in these fermentations, if they are truly 4-ethyl phenol and 4-vinyl guaiacol, and how they affect the sensory characteristics of the cider. Gas chromatography-Olfactometry (GC-O) is a technique that could be used to determine the identity of these compounds and the aromas they are associated with.

5.2 Survey of Apple Cultivars

5.2.1 Key Findings

In a survey of 58 different Virginia apple cultivars it was found that ferulic acid was present in 4 of them, the highest concentration found to be 2.8 mg/L. *p*-Coumaric acid was present in 37 of those cultivars at levels from not detectable to 386.79 mg/L, and chlorogenic acid was present in all of them at vastly different concentrations, from 0.69 to 636.57 mg/L. Ferulic acid was present in fewer of the cultivars evaluated than expected, however *p*-coumaric acid was found at higher levels than those shown to affect cider aroma in our study, (300 mg/L) in two of the cultivars. The highest concentrations of both ferulic acid and *p*-coumaric acid were found in Dabinette which is a U.K. cider apple that is gaining popularity in the U.S. The highest levels of chlorogenic acid and *p*-coumaric acid were found in Tremlette's Bitters which is another U.K. cider apple gaining in popularity in the U.S. As these cultivars continue to gain in popularity cidemakers should be aware of the potential for these compounds to be present in the apples and their potential to have an impact on the cider's fermentation and the aromas generated throughout.

5.2.2 Limitations

All of the apples for this study were collected throughout the 2018 harvest season and then pressed on the same day in November. It has been shown that hydroxycinnamic acid content in apples decreases as they ripen and age. This was a limitation of our study because these phenolic compounds might have been present, or present at higher levels upon harvest.

5.2.3 Future Work

It would be worth investigating whether these inhibitory phenolic compounds are present in apples right at harvest. Since these phenolic compounds can decrease in the apple with time, when an apple is pressed and utilized to make cider could greatly affect not only the fermentation kinetics but the sensory properties of the cider as well.

Overall, this study has increased our understanding of how some hydroxycinnamic acids influence fermentation kinetics and the interactive effects YAN can have with them. Fermentation rate as well as cider aroma are affected by the phenolic compound content of the apple juice to be fermented. These findings could be further understood through continued research into the optimal YAN addition levels to fermentation and the mechanisms behind aroma generation in cider fermentations. While many questions still remain regarding phenolic compound and nitrogen influence on fermentations and their products, these findings have motivated continued investigation into the complex interactions of phenolic compounds, nitrogen utilization, fermentation, and the sensory characteristics of Virginia ciders.

Appendix A

Fermentation Treatment				Max Fermentation Rate (g/hour)				Fermentation Performance Metrics						
Yeast	Phenolic Compound	Nitrogen Level	Phenolic Level	Max Fermentation Rate (g/hour)	Standard Error	Max Fermentation Rate Groups	Glucose Concentration mg/mL	Standard Error	Glucose Concentration Groups	Fructose Concentration mg/mL	Standard Error	Fructose Concentration Groups	Residual Sugars	
													Glucose Concentration	Fructose Concentration
w15	ferulic	n2	p2	-0.039	4.65E-04	a	0.028	0.006	cdef	0.696	0.069	b		
ec1118	chlorogenic	n2	p2	-0.133	9.63E-04	klmno	0.025	0.008	def	0.031	0.010	d		
ec1118	ferulic	n0	p2	-0.084	1.85E-03	bdefg	0	0	f	0	0	d		
ec1118	chlorogenic	n2	p1	-0.144	9.63E-04	lmno	0.045	0.009	bdef	0.071	0.013	d		
ec1118	control	n0	p0	-0.085	0.00E+00	bcdefgh	0	0	f	0	0	d		
w15	ferulic	n1	p2	-0.034	6.26E-04	a	0	0	f	0.613	0.081	bc		
ec1118	coumaric	n0	p1	-0.108	1.55E-03	cdefghijk	0	0	f	0	0	d		
ec1118	coumaric	n0	p2	-0.114	9.26E-06	efghijkl	0.008	0.004	ef	0	0	d		
ec1118	chlorogenic	n0	p1	-0.077	1.24E-03	bc	0	0	f	0	0	d		
ec1118	chlorogenic	n0	p2	-0.083	4.79E-04	bdefg	0.038	0.009	bdef	0	0	d		
ec1118	chlorogenic	n1	p1	-0.125	5.93E-04	ijklmn	0.019	0.006	def	0	0	d		
ec1118	chlorogenic	n1	p2	-0.130	5.49E-16	klmno	0.076	0.007	ab	0	0	d		
ec1118	control	n1	p0	-0.123	4.68E-06	ijklmn	0	0	f	0	0	d		
ec1118	control	n2	p0	-0.131	6.50E-04	klmno	0	0	f	0	0	d		
ec1118	coumaric	n1	p1	-0.128	7.62E-04	klmno	0	0	f	0	0	d		
ec1118	coumaric	n1	p2	-0.130	1.76E-03	klmno	0	0	f	0.088	0.002	d		
ec1118	coumaric	n2	p1	-0.154	3.40E-03	no	0	0	f	0	0	d		
ec1118	ferulic	n0	p1	-0.113	4.17E-03	defghijkl	0	0	f	0	0	d		
ec1118	ferulic	n1	p1	-0.125	4.40E-04	ijklmn	0	0	f	0	0	d		
ec1118	ferulic	n1	p2	-0.094	1.86E-03	bdefghi	0	0	f	0	0	d		
ec1118	ferulic	n2	p1	-0.143	2.52E-16	lmno	0	0	f	0	0	d		
ec1118	ferulic	n2	p2	-0.118	1.82E-03	hijklm	0.008	0.004	def	0	0	d		
w15	ferulic	n0	p1	-0.065	3.79E-04	ab	0	0	f	0.075	0.014	d		
w15	ferulic	n2	p1	-0.082	2.40E-04	bdef	0	0	f	0	0	d		
ec1118	coumaric	n2	p2	-0.150	8.80E-04	mno	0	0	f	0.098	0.003	d		
w15	ferulic	n0	p2	-0.040	2.67E-03	a	0	0	f	2.218	0.246	a		
w15	chlorogenic	n1	p1	-0.113	3.36E-04	defghijkl	0.056	0.007	bed	0	0	d		
w15	chlorogenic	n2	p1	-0.139	3.79E-04	klmno	0.045	0.007	bdef	0	0	d		
w15	coumaric	n0	p2	-0.082	3.79E-04	bdef	0	0	f	0.067	0.011	d		
w15	coumaric	n1	p2	-0.108	6.18E-04	cdefghijk	0	0	f	0.110	0.013	cd		
w15	coumaric	n2	p1	-0.136	6.57E-04	klmno	0	0	f	0.086	0.013	d		
w15	coumaric	n2	p2	-0.116	7.50E-04	ghijklm	0	0	f	0.112	0.015	cd		
w15	ferulic	n1	p1	-0.077	4.40E-04	bc	0	0	f	0.069	0.013	d		
w15	coumaric	n1	p1	-0.128	2.86E-03	ijklmno	0	0	f	0.092	0.011	d		
w15	coumaric	n0	p1	-0.096	2.49E-03	bdefghij	0	0	f	0.010	0.005	d		
w15	control	n2	p0	-0.160	5.35E-03	o	0	0	f	0	0	d		
w15	chlorogenic	n0	p1	-0.077	2.15E-16	bc	0.049	0.006	bede	0.148	0.005	cd		
w15	chlorogenic	n0	p2	-0.079	3.79E-04	bed	0.076	0.009	abc	0	0	d		
w15	chlorogenic	n1	p2	-0.115	6.57E-04	fghijkl	0.120	0.005	a	0	0	d		
w15	chlorogenic	n2	p2	-0.138	3.79E-04	klmno	0.086	0.008	ab	0	0	d		
w15	control	n1	p0	-0.113	3.79E-04	efghijkl	0	0	f	0	0	d		
w15	control	n0	p0	-0.081	6.57E-04	bede	0	0	f	0	0	d		

Residual Sugar				Residual Nitrogen				Total Polyphen		
Concentration	Standard	Concentration	Ammonia	Ammonia	PAN	PAN	Total Polyphenols	Standard		
(Glucose + Fructose)	Error	(Glucose + Fructose)	Concentration	Standard	Concentration	Concentration	(mg/L gallic acid	Error		
mg/mL		Groups	(mg N/L)	Error	Groups	(mg N/L)	equivalents)			
0.724	0.068	b	159.221	9.706	a	147.221	11.228	b	518.163	5.296
0.056	0.011	d	107.212	1.116	b	91.683	1.594	cde	550.306	3.187
0	0	d	0	0	f	31.478	0.407	klmnop	527.200	2.475
0.115	0.019	cd	85.576	2.573	c	59.927	0.893	efghijkl	388.222	9.508
0	0	d	0	0	f	5.156	1.194	p	262.459	4.715
0.613	0.081	bc	63.806	1.898	d	173.114	3.219	ab	457.956	5.864
0	0	d	0	0	f	22.507	0.620	mnop	390.163	1.609
0.008	0.004	d	0	0	f	27.927	0.500	lmnop	500.237	1.479
0	0	d	0	0	f	19.678	1.229	nop	446.163	8.186
0.038	0.009	d	0	0	f	61.054	2.237	efghijk	560.237	6.011
0.019	0.006	d	0	0	f	74.627	4.833	efgh	424.883	6.715
0.076	0.007	d	1.101	0.510	f	59.035	1.534	efghijkl	480.583	2.405
0	0	d	0	0	f	47.757	1.375	ghijklmno	222.756	2.235
0	0	d	1.226	0.567	f	54.483	0.845	fghijklm	252.681	2.546
0	0	d	0	0	f	37.181	0.980	jklmnop	385.274	3.371
0.088	0.002	d	0	0	f	60.101	1.076	efghijkl	500.385	1.602
0	0	d	0	0	f	67.389	1.125	efghij	396.533	2.551
0	0	d	0	0	f	15.038	1.347	op	426.311	3.214
0	0	d	0	0	f	44.247	1.182	hijklmno	395.348	3.136
0	0	d	0	0	f	50.773	0.983	ghijklmn	530.459	1.846
0	0	d	16.222	0.383	ef	70.768	0.683	efghi	429.570	2.805
0.008	0.004	d	26.535	3.076	e	80.098	1.291	defg	518.756	5.998
0.075	0.014	d	0	0	f	42.677	1.466	hijklmno	397.215	3.537
0	0	d	61.903	0.751	d	166.677	7.508	b	390.489	1.385
0.098	0.003	d	0	0	f	74.974	0.680	efgh	509.422	1.863
2.218	0.246	a	0	0	f	51.616	1.879	ghijklmn	493.956	1.550
0.056	0.007	d	0	0	f	78.788	1.470	defg	410.385	1.958
0.045	0.007	d	20.181	0.665	ef	69.596	1.419	efghij	413.941	5.705
0.067	0.011	d	0	0	f	51.010	2.011	ghijklmn	508.178	4.260
0.110	0.013	cd	0	0	f	108.333	2.309	cd	470.993	4.354
0.086	0.013	d	6.829	1.689	ef	110.034	1.711	cd	344.770	4.984
0.112	0.015	cd	12.854	2.079	ef	200.303	3.231	a	484.326	1.400
0.069	0.013	d	0	0	f	108.552	3.096	cd	384.918	1.762
0.092	0.011	d	0	0	f	38.165	1.750	ijklmno	381.659	2.808
0.010	0.005	d	0	0	f	32.737	1.243	klmnop	417.452	2.994
0	0	d	19.145	0.211	ef	50.702	0.770	ghijklmn	267.867	3.235
0.197	0.005	cd	0	0	f	52.481	1.597	ghijklm	432.755	4.933
0.076	0.009	d	0	0	f	70.404	1.893	efghi	517.200	3.502
0.120	0.005	cd	0	0	f	87.140	2.190	edef	598.533	2.116
0.086	0.008	d	18.203	0.514	ef	114.105	2.837	c	573.496	8.245
0	0	d	0	0	f	30.342	0.777	klmnop	288.459	2.011
0	0	d	0	0	f	25.187	0.408	mnop	276.459	4.204

ols		Acids				
Total Polyphenols		Titratable				
(mg/L gallic acid		Acidity (g/L as				
equivalents)		Standard		Standard		
Groups	pH	Error	pH Groups	malic acid	Error	TA Groups
			equivalents)			
cdef	3.89	0.003	a	3.90	0.00	a
abcd	3.79	0.003	b	3.80	0.00	b
bcde	3.75	0.001	cd	3.79	0.01	b
mno	3.77	0.008	bc	3.77	0.01	bc
pq	3.74	0.002	de	3.73	0.01	cd
ghijk	3.74	0.003	defg	3.73	0.01	cd
mno	3.74	0.002	def	3.72	0.01	d
defg	3.74	0.001	de	3.71	0.01	de
hijkl	3.71	0.002	fghij	3.70	0.00	def
abc	3.73	0.001	defgh	3.70	0.00	def
ijklmn	3.66	0.001	mn	3.70	0.00	def
efghi	3.70	0.001	kl	3.70	0.00	def
q	3.71	0.002	ghij	3.70	0.00	def
pq	3.73	0.000	defgh	3.70	0.00	def
mno	3.71	0.001	hijk	3.70	0.00	def
defg	3.70	0.001	jk	3.70	0.00	def
lmn	3.67	0.001	lmn	3.70	0.00	def
ijklmn	3.74	0.001	de	3.70	0.00	def
mn	3.69	0.001	klm	3.70	0.00	def
bcde	3.69	0.001	kl	3.70	0.00	def
ijklmn	3.68	0.002	klm	3.70	0.00	def
cdef	3.70	0.005	ijk	3.70	0.00	def
lmn	3.68	0.001	klm	3.70	0.00	def
mno	3.73	0.001	efghi	3.70	0.00	def
def	3.65	0.001	no	3.68	0.01	ef
efgh	3.67	0.003	mn	3.67	0.01	f
klmn	3.61	0.002	pq	3.60	0.00	g
klmn	3.58	0.001	rs	3.60	0.00	g
defg	3.60	0.001	q	3.60	0.00	g
fghij	3.60	0.001	qr	3.60	0.00	g
o	3.62	0.001	pq	3.60	0.00	g
efgh	3.63	0.001	op	3.60	0.00	g
mno	3.63	0.002	opq	3.60	0.00	g
no	3.56	0.001	st	3.59	0.01	g
klmn	3.55	0.001	st	3.58	0.01	g
pq	3.54	0.001	tu	3.51	0.01	h
ijklm	3.52	0.001	uv	3.50	0.00	h
cdef	3.52	0.002	uv	3.50	0.00	h
a	3.54	0.001	tu	3.50	0.00	h
ab	3.52	0.001	uv	3.50	0.00	h
p	3.52	0.002	uv	3.50	0.00	h
p	3.51	0.004	v	3.49	0.01	h

Appendix B

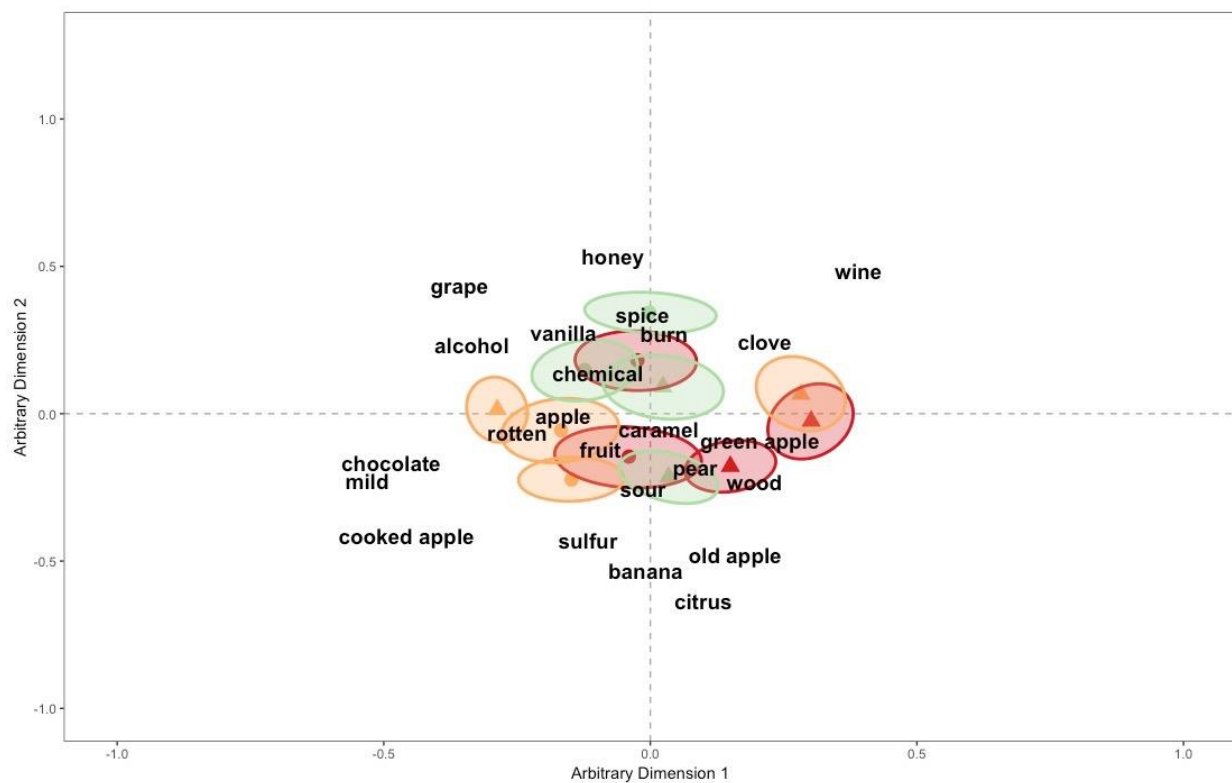


Figure B14: Ferulic acid aroma descriptors projected onto the 95% confidence ellipse map generated through DISTATIS. Color of the ellipses indicates nitrogen level. Red means no nitrogen was added, orange is n1 (100 mg N/L), and green is n2 (200 mg N/L). Circles indicate fermentations took place with EC1118 yeast and triangles used W15 yeast.

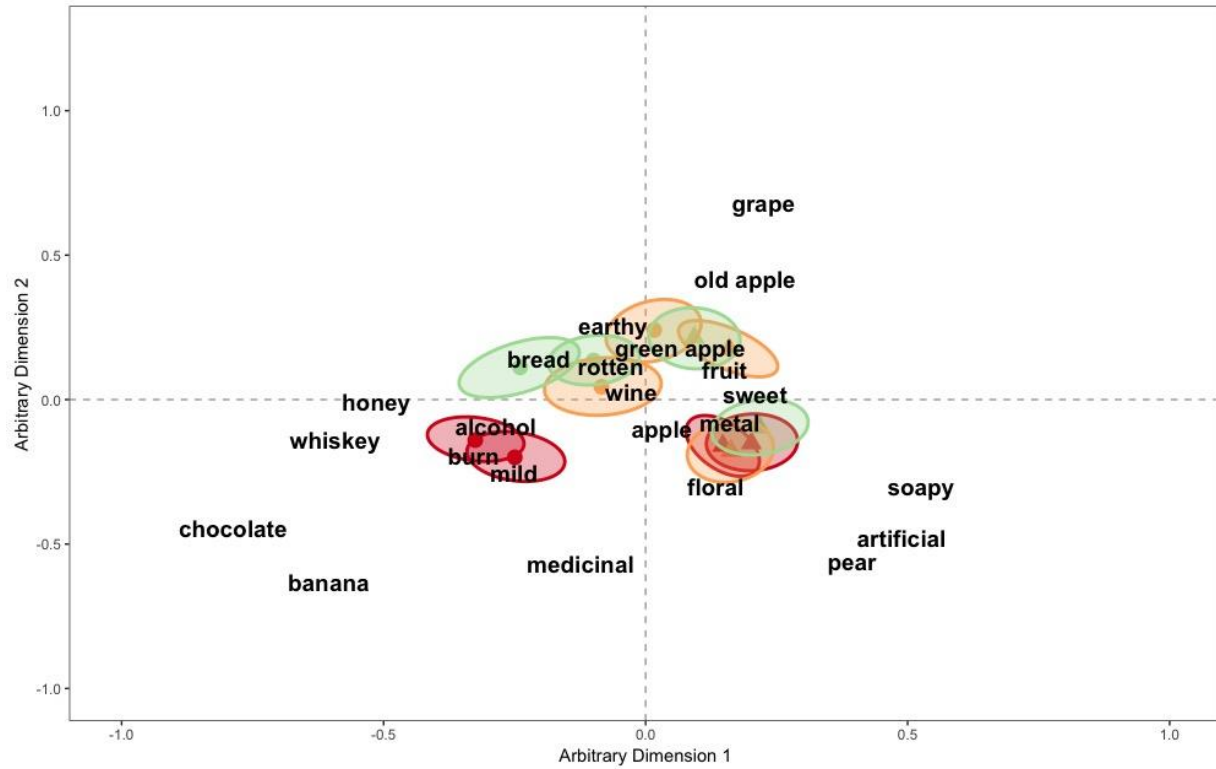


Figure B15: *p*-Coumaric acid aroma descriptors projected onto the 95% confidence ellipse map generated through DISTATIS. Color of the ellipses indicates nitrogen level. Red means no nitrogen was added, orange is n1 (100 mg N/L), and green is n2 (200 mg N/L). Circles indicate fermentations took place with EC1118 yeast and triangles used W15 yeast.

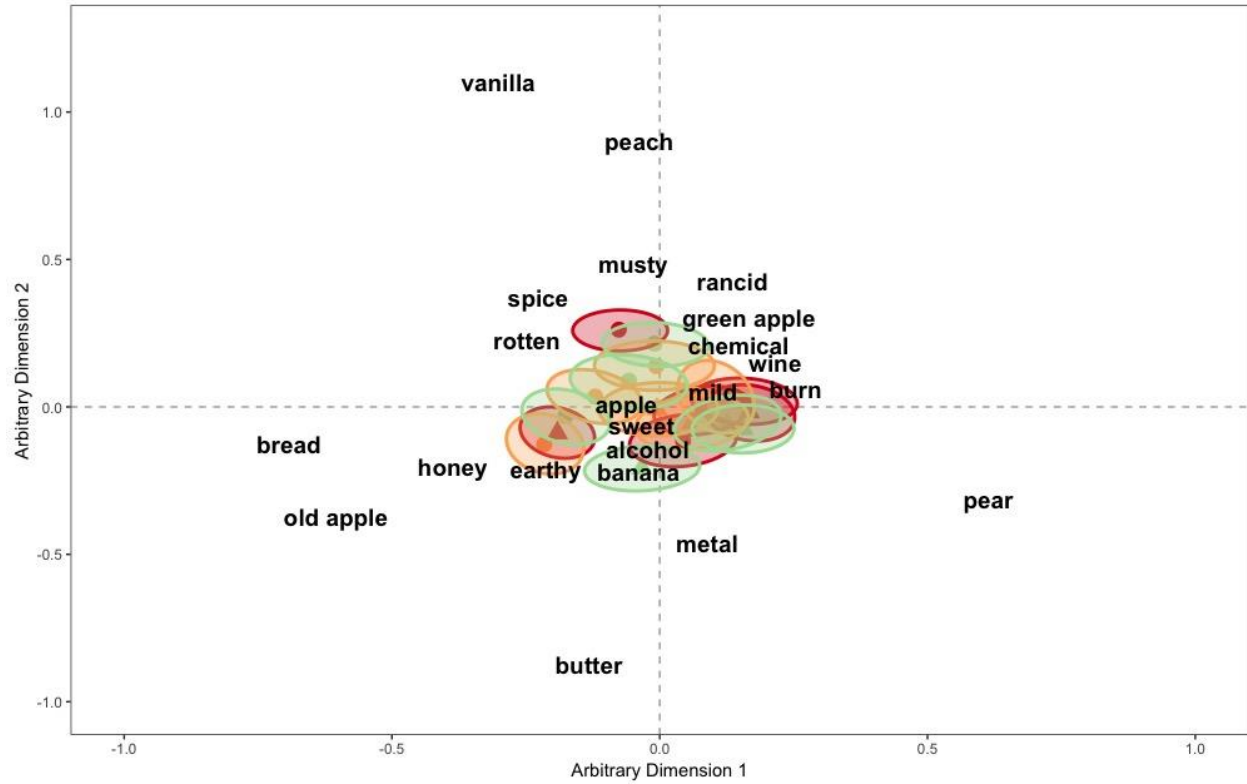


Figure B16: Control and chlorogenic acid aroma descriptors projected onto the 95% confidence ellipse map generated through DISTATIS. Color of the ellipses indicates nitrogen level. Red means no nitrogen was added, orange is n1 (100 mg N/L), and green is n2 (200 mg N/L). Circles indicate fermentations took place with EC1118 yeast and triangles used W15 yeast.