Impact of Juice Clarification Processes on Chemical Composition of Hard Cider

Sihui Ma

This thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of
Master of Science
in
Life Science
in
Food Science and Technology

Amanda C Stewart
Andrew P Neilson
Sean F O’Keefe
Gregory M Peck

Blacksburg, VA

Keywords: apple juice; cider; clarification; yeast assimilable nitrogen; polyphenols
**Impact of Juice Clarification Processes on Chemical Composition of Hard Cider**

Sihui Ma

**Abstract** (Academic)

Cider production volume has increased over 800% in the past 5 years in North America. This rapid growth of the cider industry coupled with traditional craft approach to cider making necessitates increased research on apple chemistry, processing, and fermentation strategies for cider production. A common problem in cider is the sulfur off-aromas production by yeast during fermentation. Fermentation of cloudy juice is often associated with sulfur off-aromas in white wine production; therefore, pre-fermentation juice clarification is an important and routine step in white winemaking practice. However, cider makers are often reluctant to clarify juice pre-fermentation due to beliefs that pre-fermentation juice clarification will reduce the concentration of yeast assimilable nitrogen (YAN) and polyphenols; thus, negatively impacting cider quality. In this study, different clarification methods were applied on York apple juice, and both raw and clarified juices were fermented into cider. The impact of pre-fermentation juice clarification treatments on the juice and finished cider chemistry was assessed by comparing the primary juice and cider chemistry, YAN concentration and amino acid composition in juice, polyphenol concentration and composition in juice and cider. Different clarification treatments affected the YAN concentration and amino acid composition differently. Polyphenol concentration in juice was decreased and individual polyphenol composition was different after the clarification (p<0.05), but these changes did not persist into the finished cider. The effect of pre-fermentation juice clarification on sensory properties of cider warrants further investigation. Future research should also include the development of appropriate analysis for polyphenol measurement in apple juice and cider.
Impact of Juice Clarification Processes on Chemical Composition of Hard Cider
Sihui Ma

General Audience Abstract

Cider production volume has increased over 800% in the past 5 years in North America. This rapid growth of the cider industry coupled with a traditional craft approach to cider making necessitates increased research on apple chemistry, processing, and fermentation strategies for cider production. A common problem in cider is the production of sulfur off-aromas by yeast during fermentation. Fermentation of cloudy juice is often associated with sulfur off-aromas in white wine production; therefore, pre-fermentation juice clarification is an important and routine step in white winemaking practice. However, cider makers are often reluctant to clarify juice pre-fermentation due to beliefs that pre-fermentation juice clarification will reduce the concentration of yeast assimilable nitrogen (YAN, serving as yeast nutrients) and polyphenols (a category of compounds that impact the color and sensory property of cider, as well as potential health benefits); thus, negatively impacting cider quality. In this study, different clarification methods were applied on York apple juice, and both raw juice and clarified juices were fermented into cider. The impact of pre-fermentation juice clarification treatments on the juice and finished hard cider chemistry was assessed by comparing the primary juice and cider chemistry, YAN concentration and amino acid composition in juice, polyphenol concentration and composition in juice and cider. Different clarification treatments affected the YAN concentration and amino acid composition differently. Polyphenol concentration in juice was decreased and individual polyphenol composition was significantly different after the clarification (p<0.05), but these changes did not persist into the finished cider. The effect of pre-fermentation juice clarification on sensory properties of cider warrants further investigation. Future research should also include
the development of more appropriate analytical methods for total polyphenol measurement in apple juice and cider.
Acknowledgement

My greatest gratitude is to my beloved parents, Dad Heng Ma and Mom Junli Xie and family for the infinite love, support, and encouragement, allowing me to experience the diversity of the beautiful world.

I am deeply grateful for the opportunity given by Professor Dr. Amanda Stewart for my graduate education in the United States. Thank you for your wise and insightful guidance for research, teaching, and my career development. I sincerely appreciate my committee: Drs. Sean O’Keefe, Andrew Neilson, and Greg Peck for your invaluable perspective and insight.

I would like to thank the faculty, staff, and students in the Department of Food Science and Technology for your help and support. I am glad to stay a few more years with you for my PhD study.

I would like to thank my friends for simply being with me: Jiajun Li, Minzhen Lu, Evelien Schilder, Xingrun Li, Jana Viatte, Changle Jiang and Emily Chen.

Finally, I would like to thank all the educators who claim the excellence and believe in me along the way: Mr. Rongguo Jin at Jiaozuo No.1 High School, Dr. Zhenyu Wang and Dr. Xiucheng Zhang at Northeast Forestry University, Dr. Kari Steffen at University of Helsinki, Mr. John Boyer, Dr. Amy Nelson, and Dr. Carrie Kroehler at Virginia Tech.
Table of contents

Abstract (academic) ........................................................................................................... ii
General Audience Abstract .............................................................................................. iii
Acknowledgment ................................................................................................................ v
Table of contents .............................................................................................................. vi
List of figures ..................................................................................................................... vii
List of tables ....................................................................................................................... ix

Chapter 1: Introduction....................................................................................................... 1
  1.1 Long term objective .................................................................................................... 1
  1.2 Overall objective ....................................................................................................... 1
  1.3 Specific objectives ..................................................................................................... 2
  1.4 Hypotheses ................................................................................................................. 2

Chapter 2: Literature review .............................................................................................. 4
  2.1 Development of cider industry in the U.S. and around the world ......................... 4
  2.2 From apple to cider .................................................................................................... 5
    2.2.1 Apple selection and preparation ......................................................................... 6
    2.2.2 Apple Processing: preparation, crushing, and pressing ..................................... 7
    2.2.3 SO2 addition ......................................................................................................... 8
    2.2.4 Yeast and fermentation ....................................................................................... 9
    2.2.5 Storage and packaging ....................................................................................... 9
  2.3 Primary chemistry of apple juice (SSC, pH, TA) .................................................... 10
  2.4 Yeast assimilable nitrogen (YAN) ............................................................................. 11
    2.4.1 YAN concentration in cider apple juice and its influence on cider fermentation ................................................................................................................................. 11
    2.4.2 Amino acid profiles in cider apple juice and their influences on cider fermentation ................................................................................................................................. 12
    2.4.3 Yeast nutrient supplements in cider making ....................................................... 15
  2.5 Polyphenols ................................................................................................................. 15
    2.5.1 Influence in cider making ................................................................................. 15
    2.5.2 Influence of polyphenols on the sensory properties of cider ............................ 16
    2.5.3 Concentration and composition of polyphenols in cider apples ....................... 17
    2.5.4 Health benefits of polyphenols ........................................................................ 20
  2.6 Impact of pre-fermentation clarification treatments on juice .................................... 22
    2.6.1 Juice turbidity ..................................................................................................... 22
    2.6.2 Clarification methods and effects ....................................................................... 22

Chapter 3: Materials and methods ................................................................................... 25
  3.1 Sample collection and preparation .......................................................................... 25
  3.2 Juice clarification treatments used in this study ....................................................... 26
    3.2.1 Static settling ....................................................................................................... 26
    3.2.2 Centrifugation .................................................................................................... 26
    3.2.3 Pectinase treatment followed by static settling ............................................... 26
    3.2.4 Turbidity measurement ..................................................................................... 27
    3.2.5 Pectinase dosage trial ....................................................................................... 27
    3.2.6 Pectin test ......................................................................................................... 28
  3.3 Primary juice chemistry ............................................................................................. 28
    3.3.1 Soluble solid concentration ............................................................................... 28
4.4.18 Phenylalanine ................................................................. 64
4.4.19 Total amino acid ............................................................. 65
4.5 Fermentation curves ............................................................. 70
4.6 Primary chemistry in cider ..................................................... 71
4.7 Total polyphenols in juice and cider ......................................... 73
4.8 Total proanthocyandinins in juice and cider .............................. 75
4.9 Individual polyphenols in juice and cider ................................. 77
  4.9.1 Procyanidin B1 (PC B1) ..................................................... 78
  4.9.2 Catechin ................................................................. 79
  4.9.3 Epicatechin ............................................................... 80
  4.9.4 Procyanidin B2 (PC B2) .................................................. 81
  4.9.5 Procyanidin B5 (PC B5) .................................................. 82
  4.9.6 Phloretin ............................................................... 83
  4.9.7 Chlorogenic acid ........................................................ 85
  4.9.8 Quercetin ............................................................... 86
  4.9.9 Total polyphenols by UPLC/MS ...................................... 87
4.10 Comparison of results by different analytical methods ................ 91
  4.10.1 YAN by enzymatic kit and total amino acids by UPLC .......... 91
  4.10.2 Total polyphenols by Folin and by UPLC/MS ...................... 92
  4.10.3 Total proanthocyandinins by DMAC and by UPLC/MS .......... 95
Chapter 5 Conclusion and future work ........................................ 98
  5.1 Hypotheses ...................................................................... 98
  5.2 Future work ..................................................................... 99
    5.2.1 Clarification of Brown Snout (or other highly tannic) apple juice and pectinase treatment on apple juice ..................................... 99
    5.2.2 Sensory analysis on finished cider .................................... 100
    5.2.3 Degree of polymerization of procyanidins .......................... 100
    5.2.4 Analytical method development ..................................... 100
Appendix ................................................................................. 101
  Appendix A .......................................................................... 101
  Appendix B .......................................................................... 102
  Appendix C .......................................................................... 104
  Appendix D .......................................................................... 106
  Appendix E .......................................................................... 109
  Appendix F .......................................................................... 110
  Appendix G .......................................................................... 111
  Appendix H .......................................................................... 112
Reference .................................................................................. 113
List of Figures

Figure 2.1 USA Current Cider Producer Starts by Year.................................................4
Figure 2.2 US volume sales of hard cider (2.25-gallons cases), 2010-2020......................4
Figure 2.3 Process flow diagram of cider production......................................................5
Figure 2.4 Goodnature SX-200 Hydraulic Juice Press......................................................7
Figure 2.5 Classification and structures of apple polyphenols........................................21
Figure 3.1 Flow chart outlining experimental approach employed in this project.............25
Figure 3.2 Principle of amino acid derivatization in Waters AccQ•Tag method..............30
Figure 4.1 Comparison of juice turbidity with same pectinase dosage in trial and in experiment.................................................................44
Figure 4.2 Time course of turbidity of ripe Granny Smith apple juice during pectinase treatment........................................................................................................46
Figure 4.3 YAN concentrations in York apple juice treated with different clarification methods........................................................................................................48
Figure 4.4 Concentrations of histidine in York apple juice treated with different clarification methods..................................................................................................51
Figure 4.5 Concentrations of serine in York apple juice treated with different clarification methods.................................................................................................51
Figure 4.6 Concentrations of asparagine in York apple juice treated with different clarification methods.................................................................52
Figure 4.7 Concentrations of glutamine in York apple juice treated with different clarification methods...............................................................................................52
Figure 4.8 Concentrations of arginine in York apple juice treated with different clarification methods..........................................................................................53
Figure 4.9 Concentrations of aspartic acid in York apple juice treated with different clarification methods......................................................................................54
Figure 4.10 Concentrations of glutamic acid in York apple juice treated with different clarification methods......................................................................................55
Figure 4.11 Concentrations of threonine in York apple juice treated with different clarification methods......................................................................................56
Figure 4.12 Concentrations of alanine in York apple juice treated with different clarification methods..........................................................................................57
Figure 4.13 Concentrations of γ-aminobutyric acid in juice treated with different clarification methods......................................................................................57
Figure 4.14 Concentrations of proline in York apple juice treated with different clarification methods......................................................................................58
Figure 4.15 Concentrations of lysine in York apple juice treated with different clarification method..........................................................................................59
Figure 4.16 Concentrations of tyrosine in York apple juice treated with different clarification methods......................................................................................60
Figure 4.17 Concentrations of methionine in York apple juice treated with different clarification methods......................................................................................61
Figure 4.18 Concentrations of valine in York apple juice treated with different clarification methods......................................................................................62
Figure 4.19 Concentrations of isoleucine in York apple juice treated with different clarification methods

Figure 4.20 Concentrations of leucine in York apple juice treated with different clarification methods

Figure 4.21 Concentrations of phenylalanine in York apple juice treated with different clarification methods

Figure 4.22 Concentrations of total amino acids in York apple juice treated with different clarification methods

Figure 4.23 Fermentation curves based on CO₂ loss during the fermentation of ciders made from juices treated with different clarification treatments

Figure 4.24 Standard curve of total polyphenols measurement by Folin assay

Figure 4.25 Total polyphenols in (A) York apple juice treated with different pre-fermentation clarification methods and (B) in cider made from these juices

Figure 4.26 Standard curve of total proanthocyanidin measurement by DMAC method

Figure 4.27 Total proanthocyanidins in (A) York apple juice treated with different pre-fermentation clarification methods and (B) in cider made from these juices

Figure 4.28 Concentrations of PC B1 in (A) York apple juice treated with different pre-fermentation clarification methods and (B) in cider made from these juices

Figure 4.29 Concentrations of catechin in (A) York apple juice treated with different pre-fermentation clarification methods and (B) cider made from these juices

Figure 4.30 Concentrations of epicatechin in (A) York apple juice treated with different pre-fermentation clarification methods and (B) cider made from these juices

Figure 4.31 Concentrations of PC B2 in (A) York apple juice treated with different pre-fermentation clarification methods and (B) cider made from these juices

Figure 4.32 Concentrations of PC B5 in (A) York apple juice treated with different pre-fermentation clarification methods and (B) cider made from these juices

Figure 4.33 Concentrations of phloretin in (A) York apple juice treated with different pre-fermentation clarification methods and (B) cider made from these juices

Figure 4.34 Concentrations of chlorogenic acid in (A) York apple juice treated with pre-fermentation different clarification methods and (B) cider made from these juices

Figure 4.35 Concentrations of quercetin in (A) York apple juice treated with different pre-fermentation clarification methods and (B) cider made from these juices

Figure 4.36 Concentrations of total polyphenols by UPLC/MS in (A) York apple juice treated with different pre-fermentation clarification methods and (B) cider made from these juices

Figure 4.37 Comparison of total amino acid concentrations in juice by UPLC/PDA and enzymatic kit

Figure 4.38 Comparison of total polyphenol concentrations in juice by UPLC/MS and Folin assay

Figure 4.39 Comparison of total polyphenol concentrations in cider by UPLC/MS and Folin assay

Figure 4.40 Comparison of total proanthocyanidin concentrations in juice by UPLC/MS and DMAC assay

Figure 4.41 Comparison of total proanthocyanidin concentrations in cider by UPLC/MS and DMAC assay
List of Tables

Table 2.1 Classification of cider apples ...............................................................6
Table 2.2 Amino acids, corresponding yeast metabolites, and the relating aromas ..........13
Table 2.3 Amino acids and their metabolites via Ehrlich pathway ................................14
Table 3.1 UPLC gradients for amino acid analysis ..............................................33
Table 3.2 UPLC gradients for the separation of polyphenols ................................39
Table 3.3 Retention times, molecular weights, and SIR channels for polyphenols .........40
Table 4.1 Turbidity of juices of cultivars Brown Snout and York treated with different clarification methods ..............................................................42
Table 4.2 Trial of pectinase dosage vs York juice turbidity ....................................43
Table 4.3 Juice turbidity of York apple juice treated with different clarification methods ......43
Table 4.4 Primary chemistry in juice of York apples treated with different clarification methods ..............................................................47
Table 4.5 Individual amino acid concentrations in York juice treated with different clarification methods ..............................................................50
Table 4.6 Chemical structures of amino acids .....................................................67
Table 4.7 Amino acids classification based on tendency to react with water, their solubility in water (g/100 mL H₂O at 25 °C), pKa values, and their pH at the isoelectric point (pI) .......69
Table 4.8 Primary chemistry in cider made from York apple juice treated with different pre-fermentation clarification methods ..................................................72
Table 4.9 Calibration curves of individual polyphenol standards ...............................77
Table 4.10 P-values of comparisons on mean concentration of individual polyphenols in juices treated with different clarification methods and the corresponding cider .................88
Chapter 1: Introduction

Cider production volume has increased over 800% in the past five years in North America, however best practices in cider making have not been well established. This rapid growth of the cider industry\textsuperscript{1, 2} necessitates increased research on apple chemistry, processing, and fermentation strategies for cider production. A common problem in both cider and wine fermentation is the production of sulfur off-aromas produced by yeast during fermentation, which can also be a problem in wine fermentation. In the wine industry, fermentation of cloudy juice is often associated with sulfur off-aromas in white wine production, therefore pre-fermentation juice clarification is an important and routine step in white winemaking practice. However, in the cider industry, cider makers are often reluctant to clarify the juice pre-fermentation due to beliefs that juice clarification will reduce the concentration of yeast assimilable nitrogen (YAN) and polyphenols; thus, negatively impacting cider quality. YAN are the soluble nitrogen that can be assimilated by yeast from the surrounding juice medium at the onset of fermentation. Polyphenols are also of great interest to cider makers as the compounds not only impact the sensory qualities of apple and hard cider, but are also associated with human health benefits. While many researchers have studied the effects of various juice clarification techniques on apple juice chemistry and quality (where juice itself is the final product), few studies have assessed the impact of pre-fermentation juice clarification treatments on finished hard cider chemistry and quality.

1.1 Long term objective

The long-term objective of this work is to define targeted apple processing and fermentation practices for improved hard cider quality.

1.2 Overall objective
The overall objective of this project is to investigate the impact of pre-fermentation juice clarification on YAN and polyphenols concentration and composition in apple juice and hard cider.

1.3 Specific objectives

Specific Objective 1:

Determine how pre-fermentation clarification treatments, such as static settling, centrifugation, and pectinase treatment followed by static settling, can change the chemical characteristic of apple juice, especially the YAN concentration and composition.

Specific Objective 2:

Assess the effect of clarification treatments, such as static settling, centrifugation, and pectinase treatment followed by static settling on the polyphenol concentration and composition in the apple juice and the corresponding cider.

1.4 Hypotheses

Working Hypothesis 1:

H₀: Pre-fermentation clarification treatments do not reduce the YAN concentration in the pre-fermentation apple juice.

Working Hypothesis 2:

H₀: Pre-fermentation clarification treatments do not change the composition of individual amino acids in the pre-fermentation apple juice.

Working Hypothesis 3:

H₀: Pre-fermentation clarification treatments do not reduce the total polyphenol concentration in the pre-fermentation apple juice.
**Working Hypothesis 4:**

$H_0$: Pre-fermentation clarification treatments do not change the composition of individual polyphenols in the pre-fermentation apple juice.

**Working Hypothesis 5:**

$H_0$: Pre-fermentation clarification treatments do not reduce the total polyphenol concentration in the post-fermentation hard cider.

**Working Hypothesis 6:**

$H_0$: Pre-fermentation clarification treatments do not change the composition of individual polyphenols in the post-fermentation hard cider.
Chapter 2: Literature review

2.1 Development of cider industry in the U.S. and around the world

Figure 2.1 USA current cider producer starts by year
(reproduced from Cyder Market LLC, 2015)

Cider has revitalized as an alcoholic beverage and becomes more favorable by consumers. Cider production started to increase in a clearly noticeable manner in the 1980/90s, with 77% of new production beginning substantially since 2008, as indicated by the sharp increase in new cider producers annually in the past decade (Figure 2.1).

Figure 2.2 US volume sales of hard cider (2.25-gallons cases), 2010-2020
(adapted from 2015 Beer Handbook by The Beverage Information & Insight Group, Mintel)

In 2013, the total value of US cider sales increased by 69% to reach US $1.3 billion since 2012. Cider sales grew 5-fold to 6.75 billion gallons between 2010 and
2015\(^5\) (Figure 2.2). In 2014, the Euromonitor International Country Report Cider /Perry predicted the cider/perry sales in the US would grow at a total volume Compound Annual Growth Rate (CAGR) by 35% over a 5 year forecast period starting 2013, reaching 785 million liters in 2018 by in the US. Mintel predicts that US cider sales will continue to grow at a stable rate, reaching 11 billion gallon/year at 2020\(^5\) (Figure 2.2). Increased consumption of hard cider has been attributed to new product development to meet the preferences of a new demographic of alcoholic beverage consumers, millennials\(^5\).

2.2 From apple to cider

![Figure 2.3 Process flow diagram of cider production](image)

Cider is an alcoholic drink made from fermented apple juice, generally following the process flow outlined in Figure 2.3. Because of its similarity to wine, many wine-making practices in are easily transferable to cider practices\(^6\). From 1903 to 2003, the Long Ashton Research Station (LARS) in the UK had researched extensively on cider production from the breeding of cider apple cultivars, to the chemistry of cider apple
juice, to cider production processes. The extensive and useful findings of their cider work still guide current research.

2.2.1 Apple selection and preparation

Traditional English cider apples were classified into 4 categories (Table 2.1) by Professor BTP Barker, the first director of LARS. The acid was determined as titratable acidity by straightforward titration with strong base, and the tannin was determined by Lowenthal Permanganate titration.

<table>
<thead>
<tr>
<th>Table 2.1 Classification of cider apples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid (% w/v)</td>
</tr>
<tr>
<td>Sharp</td>
</tr>
<tr>
<td>Bittersharp</td>
</tr>
<tr>
<td>Bittersweet</td>
</tr>
<tr>
<td>Sweet</td>
</tr>
</tbody>
</table>

Compared to dessert apples, traditional European cider apples tend to contain more sugar, are more easily pressed, yield higher amounts of juice, and have greater polyphenol concentrations. Cider is seldom made from a single apple cultivar; instead, it is oftentimes made by blending apples of different cultivars in order to balance acidity, sweetness, bitterness and astringency for a craving flavor. Apples availability is another reason for using apples from multiple cultivars for making cider. In fact, multiple apple cultivars are grown in the same orchard to generate the maximum economic value while avoiding harvest collision. The process of selecting and mixing apple juices from various cultivars to obtain desired blends can be seen as masterpieces of art for traditional cider makers. Additionally, many cider producers supplement their production with concentrated apple juice in order to continue cider production all year round.
2.2.2 Apple Processing: preparation, crushing, and pressing

Unlike grapes, apples can be stored after harvest for days, weeks or even months before processing. Prior to processing, apples are sorted to exclude rotten and moldy fruits. They are washed to remove leaves, branches, spray residues, insects, and any contamination from the orchard soil, transportation and storage.

Once cleaned, desirable apples are then ground to fine pulp called pomace. Crushing tools include a range of implements from hand-grinding mills to high-speed stainless steel graters. With increased reduction of apple size prior to pressing, increased yields of apple juices can be obtained.

A common pressing system for medium-scale cider producers is the hydraulic press known as the Squeezebox, a semi-continuous system (Figure 2.4). Other commercial scale pressing techniques including screw presses, rack and cloth presses, and bladder presses. Depending on production scale and equipment availability, the latter is often used in cider making operations.

![Hydraulic Juice Press](image copied from www.goodnature.com)

**Figure 2.4 Goodnature SX-200 Hydraulic Juice Press**

Rice hulls are commonly used as a pressing aid to increase the juice yield (personal communication, Jocelyn Kuzelka, cider making consultant). Treatment
of milled pulp, using commercial pectolytic enzyme Ultrazyme 100 (Ciba-Geigy) demonstrated considerable gains in juice yields, with minimal decreases in tannin content as long as SO$_2$ was used in the previous milling procedure to prevent the oxidation\textsuperscript{12}. Total polyphenols in pulp were 28% higher with milled pulp extracted at 40 °C before pressing and after milling than in pulp extracted at 12 °C\textsuperscript{12}.

As mentioned previously, a large proportion of commercially available cider is made with juice concentrate supplement. The concentrate may or may not have been produced with the intent of cider making. All of these extraction methods are expected to yield juices of vastly different turbidity and chemistry. This project focuses on approximating juice extracted using hydraulic presses (Figure 2.4), as these are the most common type of press used by mid-size cider making operations, which this research aims to inform.

2.2.3 SO$_2$ addition

Sulfur dioxide is an important additive in both winemaking and cider making. As an antioxidant, SO$_2$ reacts with excess oxygen molecules in wine and prevents enzymatic degradation by inhibiting the polyphenol oxidase that is naturally present in apples. Additionally, SO$_2$ exhibits antimicrobial activity against bacteria and non-Saccharomyces yeasts. Potassium metabisulfite is generally used as the source of SO$_2$ added for wine and cider production, although in some cases producers use an aqueous solution made from SO$_2$ gas. The amount of free SO$_2$ required is calculated based on the pH of apple juice\textsuperscript{13}. SO$_2$ has been shown to interfere with the results of the Folin-Ciocalteu assay\textsuperscript{14} and this must be
taken into consideration in the interpretation of Folin-Ciocalteu results where the samples may have been treated with SO₂, such as most commercially produced wines or ciders.

2.2.4 Yeast and fermentation

Traditional cider is fermented by wild yeast and other microflora on the apple skin, or resident on the cidery equipment. Currently, cider makers prefer to use similar/same commercial yeasts used in white wine making in order to control the production process. Commercially available selected active dry yeasts provide reliable and consistent fermentation. Lalvin EC1118 (Saccharomyces cerevisiae bayanus) is a common yeast strain used in white wine and cider commercial production as well as research due to its neutral aromas, strong competitive advantage in fermentation, and ease of use. Moreover, EC1118 can ferment wine/ciders at lower temperatures. The resulting wine/cider settles quickly after fermentation ends and is easy to rack since this yeast strain flocculates into compact lees.

If the SO₂ concentration is not high enough during the fermentation and storage, an undesirable reaction, malo-lactic fermentation, can occur after yeast fermentation. Since lactic acid produced by this reaction gives a smoother, rounder, and more complex mouthfeel than malic acid, this type of fermentation may be favored when the acidity of cider from yeast fermentation is very high. Addition of SO₂ can prevent malo-lactic fermentation.

2.2.5 Storage and packaging
After the yeast fermentation, cider is racked off from the lees (dead yeast and solids) by static settling, centrifugation, fining agent, or a combination of methods. Sweeteners, color, acid, and preservatives can be added depending on stylistic goals. Cross-flow membrane filtration is a popular system used for the final cider filtration. Cider is pasteurized and/or carbonated before packaging.

While fresh raw juice is often preferred for cider making by craft cider producers, there is a risk for pathogenesis in the fresh juice and undesired microbial growth during fermentation due to possible contamination during harvest and processing. Some cider makers prefer pasteurized juice as a starting material for their cider. Pasteurization of the bottled cider is also a common means for ensuring post-bottling microbial stability, especially in operations where sterile filtration is not an option.

2.3 Primary chemistry of apple juice (SSC, pH, TA)

Primary juice chemistry parameters are: soluble solids concentration (SSC), pH, titratable acidity (TA). SSC of apple juice is an approximate value of the total sugar content in the juice. Sugars in the juice will be converted into alcohol during fermentation. The sugar content in the juice is directly correlated with the percentage of alcohol that will be present in the finished cider. pH measures the strength of acid in the apple juice and allows cider makers to determine of the concentration of SO₂ to add. The pH of juice is measured before the fermentation. Juice with lower pH favors the growth of yeasts and inhibits the (undesirable) growth of bacteria. If the pH is too high, it is generally considered a good practice to add malic acid to lower the pH of the juice to < 3.5, inhibiting bacterial growth and conferring a further competitive advantage to yeast.
TA is the total amount of acid in the juice, most often expressed in malic acid equivalents.

2.4 Yeast assimilable nitrogen (YAN)

2.4.1 YAN concentration in cider apple juice and its influence on cider fermentation

Yeast assimilable nitrogen (YAN) concentration and composition in the pre-fermented apple juice is one of the rate controlling factors of cider fermentation\textsuperscript{17, 18}. YAN is composed of free amino acids and ammonium ions, which are soluble in the juice and can be utilized by the yeast. YAN concentration in the starting juice regulates yeast growth. The fermentation rate and the final yield of alcohol is low at the deficiencies of soluble nitrogen concentration\textsuperscript{19}. YAN deficiencies are also associated with slow and stuck fermentation and sulfur off-aromas\textsuperscript{20}. The stuck fermentation is the yeasts stop converting sugar into alcohol before the fermentation is completed. The kinetics of aroma production and microbiological instability of finished cider are also influenced by the nitrogen concentration of apple juice\textsuperscript{17}.

YAN concentration in fresh apple is the primary determinant of YAN in apple juice; but, post-harvest maturation, storage, transportation, and juice processing can lower YAN concentrations in apple juice\textsuperscript{21}. Thus, the analytical measure of YAN in pre-fermented apple juice is imperative for reliable for fermentation management.

YAN includes the concentration of primary amino acids (primary amino nitrogen or PAN), ammonium ions, and the contribution from the side chain of L-
arginine that can be hydrolyzed to ornithine and urea by yeast arginase. They are the forms of nitrogen that can be taken up and metabolized by yeast. No correlation between the contents of PAN and ammonium ions were found in grape musts²², as a result, the two measurements must be carried out separately and added together to obtain the YAN concentration.

2.4.2 Amino acid profiles in cider apple juice and their influences on cider fermentation

The amino acid profiles of apple juices are also of concern in fermentation management. Yeast strains consume amino acids in different patterns²³. Wine yeast stains primarily prefer to utilize arginine, then serine, glutamate, threonine, aspartate, and lastly lysine.

The amino acids are also precursors to some of the volatile compounds formed during fermentation²⁴. A study on wine grape must demonstrated that the concentration of many volatile compounds were significantly different between fermented musts with different amino acid compositions²⁵. More specifically, γ-aminobutyric acid (GABA), serine, and methionine in Chardonnay must has shown close relationship to fatty acids and their ethyl esters in finished wine²⁶. Statistical models have been built to further explain the observed relationship between the wine aromas to amino acid composition in wine must. The concentration of threonine and phenylalanine in wine must is strongly related with aromas involved in the fatty acid synthesis and portions of higher alcohols in finished wine²⁵.
Valine, leucine, and isoleucine were found to be the precursors of higher alcohols and volatile fatty acids\(^27\) (Table 2.2). The metabolism pathway of these 3 amino acids by wine yeast has been well-explained\(^28,29\). Amino acids were first transformed into the \(\alpha\)-keto form via the Ehrlich pathway, and then were converted to other volatile compounds (Table 2.3). The transamination is favored under low nitrogen concentration in the juice. Thus, fermentation of juice with higher nitrogen content does not produce an increased concentration of fusel acids and fusel alcohols (as is promoted by the Ehrlich pathway)\(^28\). For these reasons, grapes grown for products such as cognac in which these volatile compounds listed in Table 2.3 are highly favored are produced under extremely high crop load conditions, resulting in low fruit YAN and promotion of the Ehrlich pathway in yeast during fermentation.
Table 2.3 Amino acids and their metabolites via Ehrlich pathway

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>α-Keto acid</th>
<th>Aldehydes</th>
<th>Higher alcohols</th>
<th>Volatile fatty acids</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu 30</td>
<td>α-Ketoisocaproate</td>
<td>Isovaleradehyde</td>
<td>Isoamyl alcohol</td>
<td>Isovaleric acid</td>
<td></td>
</tr>
<tr>
<td>Ile 31</td>
<td>α-Keto-β-methylvalerate</td>
<td>2-Methylbutyraldehyde</td>
<td>Amyl alcohol</td>
<td>Isobutanol</td>
<td>Isobutyric acid</td>
</tr>
<tr>
<td>Val 32</td>
<td>α-Ketoisovalerate</td>
<td>Isobutyraldehyde</td>
<td>Phenylethanol</td>
<td>Phenylacetic acid</td>
<td></td>
</tr>
<tr>
<td>Phe 33</td>
<td>Phenylpyruvate</td>
<td>Phenylacetaldheyde</td>
<td>p-OH-phenylethanol</td>
<td>p-OH-phenylacetic acid</td>
<td>p-Cresol</td>
</tr>
<tr>
<td>Tyr 34</td>
<td>p-OH-phenylpyruvate</td>
<td>p-OH-phenylacetaldheyde</td>
<td>3-Methylthiopropional</td>
<td>3-Methylthiopropionic acid</td>
<td>Methanethiol</td>
</tr>
<tr>
<td>Trp 34</td>
<td>Indole pyruvate</td>
<td>Indole-3-acetaldehyde</td>
<td>Tryptophol</td>
<td>Indol-3-acetic acid</td>
<td>Skatole</td>
</tr>
<tr>
<td>Met 34</td>
<td>α-Ketobutyrate</td>
<td>3-Methylthiopropional</td>
<td>3-Methylthiopropionic acid</td>
<td>Malate</td>
<td>Diacetyl, acetoin</td>
</tr>
<tr>
<td>Asp 34</td>
<td>Oxaloacetate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sensory properties of amino acid metabolites were also reported in Ylva Ardo’s review. The flavor of aldehydes from branched-chain amino acids (leucine, isoleucine, valine) are malty, alcohols are fruity and alcoholic, while acids can produce flavors from sweaty, sour, rancid, rotten, fruity, and butty depending upon catabolism of leucine, isoleucine or valine. The flavor of metabolites from aromatic amino acids (phenylalanine, tyrosine, tryptophan) are associated with roses, flowers, bitter almonds, chemicals and putridity.

The concentration and composition of amino acids in apples differ across cultivars. Asparagine, aspartic acid, and glutamine are the principal amino acids in most apple juices. Low to medium concentration of serine, α-alanine, GABA, valine, isoleucine, and methylhydroxyproline are also common in apple juices. Trace amount of other amino acids are sometimes present. Lysine and sulphur-containing amino acids, which are usually limiting in protein of plants, are found in Red Delicious, Golden Delicious, Ralls, Fuji, QinGuan, Jonagold, Granny Smith, and Orin. From preliminary data collected by our group,
asparagine was the most prevalent amino acid in Blacktwig and Empire apples grown in Virginia in 2014 growing season (Ma 2015, unpublished).

2.4.3 Yeast nutrient supplements in cider making

In order to improve cider quality and to prevent slow fermentation and the incidence of sulfur off-aromas and off-flavors, nitrogen supplements (i.e.: inorganic diammonium phosphate (DAP) and organic nitrogen from autolyzed yeasts) are often added to the pre-fermented apple juice. Yet, research on Chardonnay juice has shown that despite consistent concentrations of added nitrogen, ester formation during fermentation may vary significantly depending on whether the added nitrogen source was rich in DAP or amino acids, even when the resulting total YAN concentration in juice is equivalent.

Over-addition of nitrogen can cause undesirable aromas and flavors and less production of desired aroma compounds during fermentation. In addition, excess residual nitrogen content of the corresponding fermented cider can promote unwanted microbial growth during storage.

2.5 Polyphenols

Apples are a source of polyphenol compounds. Polyphenol compounds are important determinants of apple and hard cider sensory characteristics, and are also closely associated with the potential health benefits of many plant foods.

2.5.1 Influence in cider making

Polyphenols can regulate the rate of yeast metabolism in cider making through the removal of oxygen via polyphenol oxidation. Polyphenols influence the clarification procedure during cider making by interacting with proteins.
through hydrogen and/or hydrophobic bonding. Flavan-3-ols dimers of (+)-catechin and (−)-epicatechin are involved in the formation of haze in apple juice and cider; the degree of cloudiness is related to the concentration and the ratios of polyphenols and proteins. In the clarification process, polyphenols act as pectic enzyme inhibitors. The polyphenols are oxidized to produce the brown color. For cider production, apples with a relatively high phenolic concentration are preferred for their sensory characteristics. On the other hand, cider apples such as Granny Smith and Orin with low concentration of catechin and chlorogenic acid produce light-colored juice and cider. Very low concentrations of polyphenols could allow lactic acid bacteria growth, thus leading to de-acidification, mannitol taint, and instability.

2.5.2 Influence of polyphenols on the sensory properties of cider

Polyphenols in apples play an important role in apple’s sensory qualities (i.e.: flavor, bitterness, astringency, and color). The total polyphenol concentration of the juices is relating to the taste of cider apples. For example among French cider apple varieties, the highest concentrations are found in bitter varieties (Kermorrien, Chevalier Jaune, and Jeanne Renard), intermediate polyphenol concentration are found in sweet (Antoinette, Bedan, and Douce Coel Ligne) and bittersweet varieties (Binet Rouge, Clozette, Douce Moen, and Dabinett), and the lowest concentrations are found in sharp (Avrolles and Guillevic) and acidulous (Petit Jaune, Juliana, and Judor) varieties. Astringency and bitterness are both evoked by polyphenol compounds; paired-comparison testing shows that the balance of bitterness and astringency can differ in ciders.
with the same total polyphenol content\textsuperscript{49}. Smaller oligomeric procyanidins pass into the taste papillae membrane\textsuperscript{50} and act appropriately upon the taste receptors, resulting in the sensation of bitterness perceived mostly at the back and sides of the tongue. Pure bitterness is the taste of certain plant-based alkaloids such as caffeine and quinine. Similarly, pure astringency is the taste of certain unripe fruits such as sloes (Prunus spinosa), quinces (Cydonia oblonga), and perry pears. Astringency has been defined as “the complex of sensations due to shrinking, drawing or puckering of epithelium as result of exposure to substances such as alums or tannins”\textsuperscript{51}. Larger procyanidins have a greater capacity to form nonspecific and semi-irreversible hydrogen bonds between o-diphenolic groups and proteins in the mouth\textsuperscript{52,53} resulting in the distinctive drying and puckering astringency sensation experienced uniformly across the tongue.

Flavan-3-ol derivatives are major contributors to cider flavor. The intensity of bitterness and astringency is related to the degree of polymerization of procyanidins\textsuperscript{54}. Certain hydroxycinnamic acids cause the bitterness of cider\textsuperscript{55}. Similarly, (+)-catechin contributes to bitterness and astringency. The larger the degree of polymerization of procyanidin in cider, the less bitter and the more astringent the cider will be\textsuperscript{56}.

2.5.3 Concentration and composition of polyphenols in cider apples

The concentrations of polyphenols in apples differ by variety, cultivation practices, fruit maturity, storage and transportation conditions, and many other factors. Research on Dabinett apple trees have shown that low nitrogen fertilizer application leads to the synthesis of increased quantities of fruit polyphenols
during maturation\textsuperscript{57}. Polyphenol composition of apples is difficult to express. Polyphenol profiles vary largely with different apple cultivars, growing region, climate, maturity, cultural practices, and storage conditions\textsuperscript{58}. Additionally, polyphenols are sensitive to light and temperature; exposure to light and high temperature can lead to degradation.

Apple cultivar seems to be the most important determining factor of polyphenol concentration in fruits. According to Guyot \textit{et al.} (2003), French cider apples across three harvest seasons, polyphenol profiles were not observed to be significantly different year-to-year, and the polyphenols concentrations decreased slightly during the starch regression period of apple maturation\textsuperscript{59}.

The classification and structures of polyphenols that have been found in apples were specified in Figure 2.5. Among all the polyphenols in apple, chlorogenic acid is commonly known as the predominant class (~50\% of total polyphenols)\textsuperscript{58, 60-62}. The reported levels of chlorogenic acid can range from 0 to 506 mg/L in apple juices from different cultivars and geographic origins\textsuperscript{55, 63, 64}. Chlorogenic acid is the predominant compound within the category of hydroxycinnamic acid, accounting for 9-38\% of polyphenols in apple depending on the variety\textsuperscript{48}. The second-most common compound within the hydroxycinnamic acid category is p-coumaroylquinic acid. The ratio of these two compounds varies greatly among cultivars, and contributes to the oxidation process, color development, and overall flavor during cider making. At low concentrations, chlorogenic and p-coumarylquinic acids act as the precursors of the volatile phenols produced by \textit{Brettanomyces} yeast\textsuperscript{65}, which can be seen as
sensory faults or stylistic character of cider, depending on the producer’s stylistic goals.

In a few apple varieties (i.e.: Granny Smith, Delicious and Orin⁹⁰) grown in China and some cultivars grown in Canada⁶⁶, epicatechin and procyanidin B2 concentrations were higher than chlorogenic acid. Apple cortex and red grapes used in wine making contain similar amount of proanthocyanidins, while grape proanthocyanidins are centralized in the seeds and skins and apple proanthocyanidins are dispersed in the entire fruit, with a huge proportion in the cortex⁶⁷,⁶⁸.

The constitutive flavan-3-ol units are the basic structure for apple proanthocyanidins in all cultivars, with 90% of the units being (−)-epicatechin and a small fraction of catechin being the terminal units. The degree of polymerization (DPs) of procyanidins affects the balance of bitterness to astringency, which present the “mouth feel” and “body” of ciders⁶⁹. Oligomeric procyanidins (DP 2 to 5) contributes to bitterness, while more polymerized structures (DP 6 to 10) provide astringency⁷⁰. The DPs of most apple procyanidins range from 4 to 8, with some exceptions up to DP 50⁴⁸. Moreover, the existence of polymerized procyanidins can form haze and precipitation in apple juice due to their interaction with proteins and polysaccharides. Centrifugation can be used to reduce the average DPs in juice⁷¹. Polyphenoloxidase can partially act upon cathechins to form brown oxidation products, the yellow-orange color of which is sometimes seen as beneficial to cider quality.
The third class of polyphenols is dihydrochalcones, including phloridzin and phloretin xyloglucoside. Dihydrochalcones represent 4.8% of the average total polyphenols in apple\textsuperscript{22}. Even at such low concentrations, they greatly contribute to cider quality, and they are characteristic of apples\textsuperscript{45}. Together with epicatechin, phloridzin may preferentially accounts for the orange color of the oxidation products, which are responsible for half of the juice color\textsuperscript{73}. The last polyphenols class is flavonols, including quercetin-3-O-rutinoside, quercetin 3-O-glucoside, and quercetin 3-O-galactoside.

2.5.4 Health benefits of polyphenols

The health benefits of polyphenols have been extensively studied. The functional and biological properties of polyphenols are strongly determined by their structure and the degree of polymerization. Research has shown that polyphenols can help to reduce the risk of chronic diseases, such as diabetes and obesity\textsuperscript{74,75}. Animal and \textit{in vitro} cell models are extensively utilized in the research of polyphenols and human health\textsuperscript{76,77}, and the health effects of food polyphenols have also been confirmed via \textit{in-vivo} human intervention studies\textsuperscript{78}. They are known to act as natural antioxidants, as well as anti-mutagenic, and anti-carcinogenic compounds\textsuperscript{79,80}. However, the correlation of polyphenols and antioxidant capacity can poorly explain the health benefits because of the low bioavailability of polyphenols\textsuperscript{81,82}. While, the effects and mechanisms of polyphenols relating to gastrointestinal systems are a topic of current research\textsuperscript{75,83,84}; the interactions between polyphenols and gut microbiota remain a very promising new area to explore\textsuperscript{85,86}.

20
Figure 2.5 Classification and structures of apple polyphenols

---

21
2.6 Impact of pre-fermentation clarification treatments on juice

2.6.1 Juice turbidity

Proteins, pectins, hemicellulose, and solubilized starch compose the colloids in apple juice. The stable colloidal dispersion in cloudy juice is believed to be formed by positively charged core (protein) surrounded by negatively charged shell (pectin). This model has been reported by several researchers\textsuperscript{88, 89}. Heating can increase the interaction between proteins and polyphenols, forming large precipitated particles resulting in more turbid juices\textsuperscript{90}. On the other hand, pasteurization temperatures can degrade the thermo-labile proteins and decrease turbidity. Ascorbic acid addition diminishes polyphenol oxidation and promotes polyphenol polymerization and reaction with the proteins, resulting in turbidity increases of 13.4% on average\textsuperscript{91}.

2.6.2 Clarification methods and effects

In white wine production, juice clarification is always considered an important step in the winemaking process, as it functions to prevent or lessen the negative affects of H\textsubscript{2}S production during fermentation\textsuperscript{92}. However, over-clarification of juice, resulting in an extremely low turbidity/low solids juice, is believed to cause poor yeast growth\textsuperscript{93, 94}.

Filter cloth is commonly used as a conventional clarification method in the press. Some larger cider makers use centrifugation process to obtain clear juice, while smaller cider makers will leave the raw cloudy juice at low temperature allow for self-clarification via static sedimentation. The industrial centrifugation can be expected to generate greater shear force than the lab scale centrifugation.
which we applied in this study, since the diameter of the industrial centrifuge is much bigger than a lab-scale centrifuge. In one study conducted with Golden Delicious apples, centrifugation of the raw juice slightly altered the polyphenol composition\textsuperscript{59}. The concentration of flavanols remained the same but their average DPs was lower in the centrifuged juices, due to the removal of particles associated with the procyanidins suspended in apple juice. But the concentrations of catechins and hydroxycinnamic derivatives stayed the same, due to their small molecules that cannot be absorbed to the particles in crude juices.

Commercial cider makers also use fining agents such as gelatin to clarify the juice. Gelatin is a protein that will coagulate with soluble polyphenols and clear the un-dissolved particles in apple juice by co-precipitation\textsuperscript{12}. This treatment is detrimental to total polyphenol contents. Research has been done on pectinase treatment\textsuperscript{95}, honey treatment\textsuperscript{96}, flocculation with gelatin-bentonite, chitosan treatment\textsuperscript{97} and ultrafiltration\textsuperscript{58, 98} for apple juice processing, but not with the specific intent of cidermaking applications. After pectinase treatment, the viscosity of apple juice decreased and the suspended materials in raw cloudy juice coagulated. The loss of phenolics is more severe after gelatin-bentonite treatment (28.2\%) than ultrafiltration (7.2\%)\textsuperscript{58}.

Study on grapes showed that static settling with and without clarifying agents such as gelatin decreased the YAN content by 20\%-50\% in grape juice; while, the YAN concentration was not as affected by settling without gelatin. However, not all amino acids suffered decreased concentration in grape juice treated with the clarifying agent as compared to untreated juice. No research
regarding the effect of clarification on YAN or polyphenol concentration and composition in apple juice pre-fermentation and the accompanying cider post-fermentation has been conducted.

In white wine making, the general recommended turbidity of grape juice pre-fermentation is 100 to 250 Nephelometric Turbidity Units (NTU)\textsuperscript{94}. Over-clarification with resulting turbidity below 50 NTU will decrease the fruity aroma in white wine, while grape juice turbidity over 250 NTU is often associated with sulfur off-aroma production during fermentation\textsuperscript{94}. No conclusive turbidity recommendation has been made for cider making, although many of the same yeast strains are used across white winemaking and cider making\textsuperscript{15}. As an additional settling and racking step follows the fermentation process in cidermaking (Figure 2.3), we hypothesized that any differences in juice chemistry following pre-fermentation juice clarification would not persist into the finished cider.

This study was designed to investigate the impact of different clarification methods on the chemical composition in apple juice and whether those impacts persist into the finished cider.
Chapter 3: Materials and methods

A diagram outlining the overall experimental approach employed in this project is depicted in Figure 3.1.

![Flow chart](image)

Figure 3.1 Flow chart outlining experimental approach employed in this project

3.1 Sample collection and preparation

York apples were harvested from the Virginia Tech Alson H. Smith, Jr. Agricultural Research and Extension Center (AREC, Winchester, VA, USA) in September, 2015 and stored at 4 °C in ambient atmospheric conditions for approximately two weeks prior to the experiment.

Brown Snout apples were harvested from the Lansing Research Farm of Cornell University (Lansing, NY), in Fall 2015 and stored at 4 °C for approximately two weeks prior to the experiment.

Apples were sliced and juiced by Champion Juicer (Lodi, CA, USA).
3.2 Juice clarification treatments used in this study

3.2.1 Static settling

Immediately after juicing, aliquots of 3000 mL of apple juice were evenly distributed and stored in three 1000 mL flasks at 18 °C overnight for static settling. Juice and sediment were separated by a peristaltic pump (Manostat, NY, USA). A 400 mL of juice were transferred to individual 500 mL flasks. The remainder was collected in centrifuge tubes and stored at −80 °C until the time of analysis.

3.2.2 Centrifugation

Three thousand mL juice was aliquoted into 50 mL centrifuge tubes and centrifuged at 2301 x g for 3 minutes (Thermo Scientific Sorvall Legend X1 centrifuge, Thermo Scientific, Waltham, MA, USA). A supernatant fraction totaling 1200 mL for each treatment was collected by combining the supernatant from each 50 mL tube. Four hundred mL of the supernatant fraction was then distributed into each 500 mL flasks for fermentation (three flasks containing 400 mL of supernatant each, 1200 mL in total). The remaining supernatant was collected in centrifuge tubes and stored at −80°C for future analysis.

3.2.3 Pectinase treatment followed by static settling

Pectinase PEC5L commercial enzyme preparation (Scottzyme, Petaluma, CA, USA) was diluted 10X in water. The diluted pectinase was then added to raw juice at the recommended concentration of 0.1 µL/L in 500 mL flasks. Juice was covered by rubber stoppers and settled at 18 °C overnight. The supernatant was
separated by a peristaltic pump (Manostat, NY, USA) to approximate the racking
process used in commercial cider production.

3.2.4 Turbidity measurement

Juice turbidity was measured using Thermo Scientific Orion AQ4500
turbidity meter (Thermo Scientific, Waltham, MA, USA) and expressed in
Nephelometric Turbidity Units (NTU). The turbidity meter measures the light
from the source beam that has been scattered by the suspended particles in the
solution, with a detector on the side of the beam pathway. The light from the
source beam that passes through the solution directly will not be measured. With
the additional help from the reference beam, the interference from the colored
constituents in the solution to skew the results is eliminated.

3.2.5 Pectinase dosage trial

A trial was conducted to investigate the effect of pectinase concentration
on finished juice turbidity in York apples. The first trial was to determine
whether juice clarified by pectinase can achieve the same turbidity as juice
clarified by physical clarification methods (static settling and centrifugation). This
scenario would be ideal for our experiment in that it would allow direct
comparison of chemical and physical means of clarification to the same turbidity.
Pectinase of different concentrations (0.1, 0.01, and 0.001 µL/L) were added to
both Brown Snout and York apple juice. Juice was covered by rubber stoppers
and settled at 18 °C overnight. Supernatant was taken for turbidity measurement.

A narrower pectinase concentration range was determined (0.001 to 0.01
µL/L) for further investigation. A second trial was conducted to determine the
pectinase dosage that can make the juice clarified by pectinase the same turbidity as juice clarified by centrifugation. Pectinase was added to York apple juice at the concentration from 0.001 µL/L to 0.01 µL/L with an increment of 0.001 µL/L. Flasks were covered with rubber stoppers and settled at 18 °C overnight. Supernatant was taken for turbidity measurement.

3.2.6 Pectin test

Pectin tests were conducted on Brown Snout juice since the juice was still cloudy after the pectinase treatment. A 3 mL aliquot of juice was added with 6 mL of 95% ethanol : 1% HCl. Reactions were read by visual observation after a few minutes. Gel formation is an indicator of the presence of pectin. This test is qualitative and cannot distinguish between types of pectins, but it is a reliable indicator of the presence of pectin, nonetheless.

3.3 Primary juice chemistry

3.3.1 Soluble solids concentration (SSC)

Soluble solid content in raw juice, settled juice, centrifuged juice, and juice after pectinase treatment was measured by a portable °Brix refractometer RF15 (Extech Instrument, Nashua, NH, USA). This model RF15 has auto temperature compensation and can show the corrected readings by automatically adjusting the ambient temperature to 20 °C. All results were reported as °Brix at 20 °C.

3.3.2 pH and TA

A 10 mL aliquot of juice was pipetted into 100 mL beaker containing 75 mL of degassed distilled water. pH was measured with an Orion VERSA STAR
pH/ISE/Conductivity/Dissolved Oxygen Multiparameter Benchtop Meter with ROSS Ultra pH/ATC Triode Gel-filled electrode (Thermo Scientific, Waltham, MA, USA). TA was measured by titrating the diluted juice against 0.1 mol/L NaOH to the end point of pH 8.2. Initial and final burette reading were recorded to two decimal place and used to calculate the TA.

3.4 YAN in juice

The concentration of primary amino nitrogen (PAN) in juice after application of different clarification treatments was measured by enzymatic kit (K-PANOPA kit, Megazyme, Wicklow, Ireland). In this method, primary amino nitrogen groups of free amino acids react with N-acetyl-L-cysteine (NAC) and o-phthaldialdehyde (OPA) to produce a isoindole derivative. The isoindole derivative is measured spectrophotometrically by the increase in absorbance at 340 nm. Its concentration is stoichiometric and dependent on the amount of free amino nitrogen available, thus allowing quantification of free amino nitrogen.

The concentration of ammonia in juice with different clarification treatments was measured using the Megazyme Ammonia Assay kit (K-AMIAR, Megazyme, Wicklow, Ireland). In this method, NH$_4$ reacts with 2-Oxoglutarate and NADPH under glutamate dehydrogenase to produce L-glutamic acid, NADP$^+$, and H$_2$O. NADPH consumption can be measured spectrophotometrically by the decrease in absorbance at 340 nm, and the amount of NADP$^+$ produced is stoichiometric with the amount of ammonia, thus allowing quantification of ammonia.

Concentrations of YAN in juice with different clarification treatments were quantified by adding the concentration of PAN and ammonia together.
3.5 Amino acid profile in juice

Twenty amino acids in juice treated with different clarification methods were analyzed and quantified (Waters UPLC® Amino Acid Analysis (AAA) Solution, Milford, MA, USA) using the AccQ•Tag Ultra method\textsuperscript{103}. This method has been validated by several analysis labs on samples with various matrixes\textsuperscript{104-107}. The AccQ•Tag Ultra reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, or AQC) is a compound that can specifically derivatize amines. The principle of the derivatization on amino acids is illustrated in Figure 3.2. Both primary and secondary amino acids in the juice were derivatized by the AccQ•Tag Ultra reagent. The stable derivatives were separated by reverse phase UPLC (Waters ACQUITY UPLC H-Class quaternary system with PDA detector) and quantified with UV absorbance. The excess reagent was hydrolyzed into 6-aminoquinoline (AMQ), which does not interfere with the reaction.

![Figure 3.2 Principle of amino acid derivatization in Waters AccQ•Tag method](image)

3.5.1 Standard preparation
L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), L (+)-norvaline (Acros Organics, New Jersey, USA), γ-aminobutyric acid (GABA) (Sigma-Aldrich, St. Louis, MO, USA), and L-asparagine (Sigma-Aldrich St. Louis, MO, USA) were dissolved individually in 0.1 N HCl solution (Fisher Scientific, Fair Lawn, NJ, USA) to make separate 5 mM stock solution. A 50 µL aliquot of each stock solution and 100 µL of Waters Amino Acid Hydrolysate Standard (the mixture contains 2.5 Mm of each of the dissolved 0.1 N HCl hydrolysate amino acids, histidine, glycine, methionine, proline, valine, serine, isoleucine, glutamic acid, threonine, lysine, leucine, arginine, alanine, tyrosine, phenylalanine, with the exception of cysteine, which is 1.25 Mm) (Milford, MA, USA) were mixed with 700 µL ultrapure water from Millipore Milli-Q water purification system (Millipore, Bedford, MA, USA) to make the working standard which contained 250 pmol/µL of each of the amino acid, except for cysteine (125 pmol/µL).

3.5.2 Sample preparation and derivatization

All samples were filtered through PTFE 0.22 µm membrane filter with luer lock (Micro Solv, Eatontown, NJ, USA), and spiked with 2.5 Mm internal standard Norvaline prior to derivatization.

Filtered samples were derivatized before injection by AccQ•Tag Ultra Derivatization Kit (Waters Corporation, Milford, MA, USA), which contained the AccQ•Tag Ultra Reagent Powder (Vial 2A), AccQ•Tag Ultra Borate Buffer (Vial 1), and AccQ•Tag Ultra Reagent Diluent (Vial 2B). The AccQ•Tag Ultra reagent was reconstituted according to the method provided with the kit, by dissolving the AccQ•Tag Ultra Reagent Powder to 1Ml of the AccQ•Tag Ultra Reagent Diluent.
The reconstituted AccQ•Tag Ultra reagent can be stored in a desiccator at room temperature for up to one week. Reagents were discarded when they turned yellow or green.

For pre-column derivatization, 70 μL of AccQ•Tag Ultra Borate buffer was added to a clean total recovery vial (Waters Corporation, Milford, MA, USA). A 10 μL aliquot of calibration standard/samples were delivered to the same vial. The mixture was vortexed briefly using Fisher Mini Vortexer (Fisher Scientific, Waltham, MA, USA). A 20 μL aliquot of reconstituted AccQ•Tag Ultra reagent was added to the vial, and vortexed immediately for several seconds. The mixture stood for one minute at room temperature, and was then heated for 10 minutes at 55 °C in an Isotemp analog dry bath incubator (Fisher Scientific, Waltham, MA, USA).

3.5.3 Chromatography conditions

Mobile phase A: AccQ•Tag Ultra Eluent A (Waters Corporation, Milford, MA, USA)
Mobile phase B: 90: 10 ultrapure water/ AccQ•Tag Ultra Eluent B (Waters Corporation, Milford, MA, USA)
Mobile phase C: ultrapure water
Mobile phase D: AccQ•Tag Ultra Eluent B
Purge solvent: 50:50 acetonitrile: water
According to the standard conditions prescribed by the kit, derivatized amino acid standard and juice samples were injected, at 1 μL injection volume onto the AccQ•Tag Ultra Column 2.1×100 mm, 1.7 μm (Waters Corporation, Milford, MA, USA) with AcQuity UPLC BEH C18, 2.1×5 mm, 1.7 μm VanGuard Pre-column (Waters Corporation, Milford, MA, USA). Solution was eluted at 43 °C at a flow rate of 0.7 mL/min according to the following gradient (Table 3.1).

<table>
<thead>
<tr>
<th>Time/minute</th>
<th>%A</th>
<th>%B</th>
<th>%C</th>
<th>%D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>10.0</td>
<td>0</td>
<td>90.0</td>
<td>0</td>
</tr>
<tr>
<td>0.29</td>
<td>9.9</td>
<td>80.0</td>
<td>90.1</td>
<td>0</td>
</tr>
<tr>
<td>5.49</td>
<td>9.0</td>
<td>15.6</td>
<td>11.0</td>
<td>0</td>
</tr>
<tr>
<td>7.10</td>
<td>8.0</td>
<td>15.6</td>
<td>57.9</td>
<td>18.5</td>
</tr>
<tr>
<td>7.30</td>
<td>8.0</td>
<td>0</td>
<td>57.9</td>
<td>18.5</td>
</tr>
<tr>
<td>7.69</td>
<td>7.8</td>
<td>0</td>
<td>70.9</td>
<td>21.3</td>
</tr>
<tr>
<td>7.99</td>
<td>4.0</td>
<td>0</td>
<td>36.3</td>
<td>59.7</td>
</tr>
<tr>
<td>8.59</td>
<td>4.0</td>
<td>0</td>
<td>36.3</td>
<td>59.7</td>
</tr>
<tr>
<td>8.68</td>
<td>10.0</td>
<td>0</td>
<td>90.0</td>
<td>0</td>
</tr>
<tr>
<td>10.20</td>
<td>10.0</td>
<td>0</td>
<td>90.0</td>
<td>0</td>
</tr>
</tbody>
</table>

Peaks were identified and quantified by the Apex Track function of Empower™ Software (Waters Corporation, Milford, MA, USA).

3.6 Fermentation apparatus and procedures

3.6.1 Sanitizing solution

The sanitizing solution was prepared as follows: two teaspoons of potassium metabisulfite and one tablespoon of citric acid was added to 11.36 L of water. Solution was mixed until dissolved and stored at room temperature for up to 24 hours. New sanitizer solution was made daily, as needed. The rubber stoppers and airlocks were sanitized using this solution.

3.6.2 SO₂ addition
In order to obtain the generally recommended amount of 0.8 mg/L molecular SO\textsubscript{2} in solution, free SO\textsubscript{2} required for apple juice with pH 3.4 is 33 mg/L. Due to the much greater potential for oxidation as a result of extremely high surface area to volume ratio existing in the microscale fermentation conditions employed in this experiment, a higher dosage of free SO\textsubscript{2} was applied in this experiment. Potassium metabisulfite (Presque Isle Wine Cellars, North East, PA, USA) was added at 100 mg/L to yield 58.8 mg/L (58.8 ppm) of SO\textsubscript{2} in the juice.

3.6.3 Yeast nutrient addition

Fermaid K (Scott Laboratories Inc., Petaluma, CA, USA) was added into each flask at the recommended dosage of 25 g/hL, resulting in a final juice concentration of 25 mg N/L added.

Diammonium phosphate (DAP) was added into each flask at the concentration of 0.50 g/L for an additional 100 mg N/L in the juice.

YAN concentration in York juice treated with different clarification methods was around 50 mg N/L, so with addition of Fermaid K and DAP, the final nitrogen concentration will be approximately 50+25+100=175 mg N/L, which is above the minimum general recommendation of 140 mg N/L. Nitrogen additions were made to alleviate any effect of nitrogen deficiency on completion of fermentation.

3.6.4 Yeast inoculation

Commercially available active dry yeast, strain EC1118 (Scott Laboratories Inc., Petaluma, CA, USA) was rehydrated and inoculated at the
concentration of 25 g/hL into each flask. Sanitized rubber stoppers and airlocks were autoclaved and put onto each flask. Flasks were refrigerated at 18 °C for the duration of fermentation.

3.6.5 Fermentation

Over the total duration of the fermentation process, fermentation flasks were stirred with octagon magnetic stir bars on stir plates (Corning Inc., New York, USA) every 12 hours at the rate of 1000 rpm for 5 minutes. The weight of each flask was measured and recorded every 12 hours until the differences between measurements were consistent at four data points (2 days). At the end of fermentation, samples were taken from each flask to measure the residual sugars using an enzymatic kit. The fermentations were considered complete when the residual sugar reached 0.2 g/L (<0.2% w/w%). Samples of cider from each flask were taken and stored at −80 °C for future analysis.

3.7 Primary cider chemistry

3.7.1 Residual sugar

Residual sugar was measured by the Megazyme D-fructose/D-glucose assay kit (Wicklow, Ireland)\textsuperscript{108}. The sum of D-fructose and D-glucose represented the sugar remaining in the juice that could potentially be utilized by the yeast to produce ethanol.

D-glucose can react with adenosine-5’-triphosphate (ATP) and be phosphorylated into glucose-6-phosphate (G-6-P) under the presence of hexokinase (HK) to produce adenosine-5’-diphosphate (ADP) as shown in the following reaction scheme (1):

\[
\text{HK} \quad \text{ADP} \quad \text{G-6-P} \quad \text{ATP} \quad \text{G-6-P} \quad \text{ADP}
\]
D-glucose + ATP $\rightarrow$ G-6-P + ADP (1)

Catalyzed by glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidized to gluconate-6-phosphate, while nicotinamide-adenine dinucleotide phosphate (NADP$^+$) is reduced to nicotinamide-adenine dinucleotide phosphate (NADPH).

G-6-P + NADP$^+$ $\xrightarrow{\text{G6P-DH}}$ gluconate-6-phosphate + NADPH + H$^+$ (2)

The amount of NADPH formed in this reaction is stoichiometric with the amount of D-glucose, and can be measured spectrophotometrically by the increase in absorbance at 340 nm, allowing quantification of D-glucose.

D-fructose can react with adenosine-5'-triphosphate (ATP) and be phosphorylated into fructose-6-phosphate (F-6-P) under the presence of hexokinase (HK) to produce adenosine-5'-diphosphate (ADP) following the reaction scheme outlined below (3).

D-fructose + ATP $\xrightarrow{\text{HK}}$ F-6-P + ADP (3)

Then F-6-P is converted to G-6-P by phosphoglucone isomerase (PGI).

F-6-P $\xrightarrow{\text{PGI}}$ G-6-P (4)

The formed G-6-P participates in reaction (2) forming NADPH, leading to a further increase in absorbance at 340 nm that is stoichiometric with the amount of D-fructose, thus allowing quantification of D-fructose.

3.7.2 pH and TA

Cider samples were degassed to remove any CO$_2$ gas by microwaving 10s (Model SMW777, Curtis International LTD., Etobicoke, Canada), and pH and TA were measured using the same method described for juice analysis in 3.3.2.
3.7.3 Free and total SO$_2$

Cider samples were sent to the Enology Service Lab at Virginia Tech for free and total SO$_2$ analysis using the Aeration-Oxidation method$^{109}$, a standard wine analysis method described by others.

3.7.4 Ethanol content

Cider samples were sent to the Enology Service Lab at Virginia Tech for ethanol content analysis by gas chromatography-mass spectroscopy (GC-MS) analysis$^{110}$.

3.8 Total polyphenols in juice and cider

Total polyphenols in juice and cider samples were measured by the Folin-Ciocalteu assay$^{111}$, a standard Food Analysis method described by others. Folin & Ciocalteu’s phenol reagent (Sigma-Aldrich, St. Louis, MO) was used in this analysis and results were expressed in gallic acid equivalent using gallic acid standard (Sigma-Aldrich, St Louis, MO).

3.9 Total proanthocyanidins in juice and cider

Total proanthocyanidins in juice and cider samples were measured with the 4-dimethylaminocinnamaldehyde (DMAC) method$^{112}$. DMAC chromogenic reagent for indoles and flavanol (Sigma-Aldrich, St. Louis, MO) was used in this analysis and analytical standard procyanidin B2 (Sigma-Aldrich, St. Louis, MO) was used as the standard. Samples were centrifuged for 5 minutes at 9600 x g in a Thermo Scientific™ Sorvall™ Legend™ Micro 17 micro centrifuge (Thermo Scientific, Waltham, MA, USA). Absorbance was determined using G10S UV-Vis spectrometer (Thermo Scientific, Waltham, MA, USA). Results were expressed as procyanidin B2 equivalent.
3.10 Individual polyphenols in juice and cider

Individual polyphenol compounds were analyzed in hydrolyzed juice and cider samples by Waters UPLC-QDA system.

3.10.1 Standard preparation

Standards used in this analysis were authentic analytical standards of catechin, epicatechin, chlorogenic acid, quercetin, phloretin (Sigma-Aldrich, St. Louis, MO), procyanidin B1, B2, B5, C1, and cinnamtannin A2 (Planta Analytica, Danbury, CT). Prior to use, all standards were stored at −80 °C. Individual 1 mg/mL polyphenols standards were prepared in methanol. The standard mixture stock was made by mixing 1 µL of each of those individual standards. The mixed stock (100 µg/mL per analyte) was further diluted with 95% mobile phase A (0.1% formic acid in water) and 5% mobile phase B (0.1% formic acid in acetonitrile) to provide 10 individual calibration standards (10, 1, 0.1, 0.01, 0.001 µg/mL per analyte).

3.10.2 Sample preparation

0.2 g/L of ascorbic acid and 0.83 M of acetic acid were added as preservative solution at ¼ of the juice/cider volume. A 0.5 mL aliquot of preservative solution was added to each 2.0 mL of juice/cider sample.

*Hydrolysis Method Development.* Samples were flushed under nitrogen for 15 s before the addition of acid for acid hydrolysis to remove sugar moieties. 1 mol/L HCl was used to adjust the pH of samples to 2 and Hydrion pH test strips (Micro Essential Lab, NY, USA) ranging from 1-3 were used to determine the end point. Samples were flushed with nitrogen for 15 s before being heated at 100
in the oven for 10, 20, 30, 40, 50, and 60 minutes. All samples were immediately cooled on ice for 1 hour before extraction. Optimized heating time of 10 minutes was chosen from the trial and used in future hydrolysis. This duration allowed for the greatest hydrolysis of sugar moieties with minimal oxidation of polyphenols.

*Extraction.* Two times the sample volume of ethyl acetate was used for extraction. The ethyl acetate layer was collected and the water layer was re-extracted three times. The extracted samples (the combined ethyl acetate layers) were dried under nitrogen and re-dissolved in 1 mL 95% mobile phase A (0.1% formic acid in water) and 5% mobile phase B (0.1% formic acid in acetonitrile). Dissolved samples were filtered through PTFE 0.22 µm membrane filters with luer lock (Micro Solv, Eatontown, NJ, USA) before injection.

### 3.10.3 UPLC conditions

A 10 µL aliquot of each sample was injected into an Acquity UPLC HSS T3 column (2.1 mm×100 mm, 1.8 µm) with AcQuity UPLC HSS T3, 2.1×5 mm, 1.7 µm VanGuard Pre-column both (Waters Corporation, Milford, MA, USA). Samples were then eluted at 43 °C with a flow rate of 0.6mL/min according to the following gradient in Table 3.2.

<table>
<thead>
<tr>
<th>Time/minute</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>0.5</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>6.5</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>7.5</td>
<td>65</td>
<td>35</td>
</tr>
</tbody>
</table>

Mobile phase A: 0.1% formic acid in water

Mobile phase B: 0.1% formic acid in acetonitrile
8.6  
8.7  
14.6

Sample temperatures were maintained at 4 °C pre-injection. QDA detector conditions were as follows:

Ionization mode: ESI-

Probe temperature: 600 °C

Capillary voltage: 0.8 kV

Mass range: m/z 200 to 1250 (centroid) and select SIRs (Table 3.3)

Cone voltage: 15 V

The actual sampling rate of mass scan was 4.8 points/sec. The target m/z ratios were set based on the molecular weight of each compound (Table 3.3).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>Molecular Weight (g/mol)</th>
<th>SIR (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC B1</td>
<td>2.57</td>
<td>578.07</td>
<td>577.13</td>
</tr>
<tr>
<td>Catechin</td>
<td>2.89</td>
<td>290.092</td>
<td>289.09</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>2.93</td>
<td>354.31</td>
<td>353.06</td>
</tr>
<tr>
<td>PC B2</td>
<td>3.28</td>
<td>578.04</td>
<td>577.13</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>3.55</td>
<td>290.092</td>
<td>289.09</td>
</tr>
<tr>
<td>PC C1</td>
<td>3.81</td>
<td>866.218</td>
<td>865.21</td>
</tr>
<tr>
<td>Cinn A2</td>
<td>3.97</td>
<td>1154.808</td>
<td>576.4</td>
</tr>
<tr>
<td>PC B5</td>
<td>4.65</td>
<td>578.136</td>
<td>577.13</td>
</tr>
<tr>
<td>Quercetin</td>
<td>6.57</td>
<td>302.23</td>
<td>301.01</td>
</tr>
<tr>
<td>Phloretin</td>
<td>7.34</td>
<td>274.26</td>
<td>273.05</td>
</tr>
</tbody>
</table>

All compound peaks were processed and quantified using the Apex Track function of Empower software. Peaks were smoothed using a mean smoothing method with smoothing level of 9.

3.11 Statistical analysis
Results were statistically analyzed with Prism v6.0e (GraphPad Software, Inc., La Jolla, CA). Data were analyzed by one-way ANOVA followed by Tukey’s HSD multiple comparisons test. Summary statistics were expressed as mean ± standard deviation for n = 3 replicates for all analyses. Significance was defined as p < 0.05.
Chapter 4: Results and discussion

4.1 Juice turbidity after clarification

<table>
<thead>
<tr>
<th>Juice treated with different clarification methods</th>
<th>Juice turbidity/NTU</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brown Snout</td>
</tr>
<tr>
<td>Raw juice</td>
<td>13030</td>
</tr>
<tr>
<td>Settled juice</td>
<td>7140</td>
</tr>
<tr>
<td>Centrifuged juice</td>
<td>3370</td>
</tr>
<tr>
<td>Pectinase-treated juice followed by settling with different pectinase dosage/(µL/L)</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
</tr>
</tbody>
</table>

Based on the results of both Brown Snout and York apple juices (Table 4.1), all clarification methods worked well on York juice but retained cloudiness in Brown Snout apple juice. None of the clarified juices from Brown Snout apples fell within the recommended turbidity of pre-fermentation juice in white wine making, 100-250 NTU. With the addition of pectinase at the recommended dosage from the manufacturer, the Brown Snout apple juice was still too cloudy (2305 NTU) for fermentation. Thus, all of the following tests were performed on York apple juice only. The effect of cultivar on juice clarification is very important and certainly merits further investigation.

From the results of the pectinase dosage trial (Table 4.2), three pectinase dosage rates (0.003 µL/L, 0.006µL/L, and 0.01 µL/L) were chosen. At the first dosage of pectinase, the turbidity of clarified juice was 542 NTU, which was closest to the turbidity of centrifuged juice at 2301 x g (580 NTU, Table 4.1). Using juices of similar turbidity prepared by these 2 clarification methods, we aimed to compare the impact of physical clarification (centrifugation) and biochemical clarification (pectinase) on the chemical composition of the juice and the finished cider. The second pectinase dosage of 0.006
μL/L was chosen because the turbidity of the clarified juice fell in the range of 100-250 NTU. The third pectinase dosage (0.01 μL/L) was chosen because it was the recommended dosage by the manufacturer of the commercially available enzyme preparation used in this study.

Table 4.2 Trial of pectinase dosage vs York juice turbidity (one sample measured per treatment)

<table>
<thead>
<tr>
<th>Pectinase dosage / (μL/L)</th>
<th>Juice turbidity / NTU</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>1295</td>
</tr>
<tr>
<td>0.002</td>
<td>963</td>
</tr>
<tr>
<td>0.003</td>
<td>542</td>
</tr>
<tr>
<td>0.004</td>
<td>339</td>
</tr>
<tr>
<td>0.005</td>
<td>242</td>
</tr>
<tr>
<td>0.006</td>
<td>198</td>
</tr>
<tr>
<td>0.007</td>
<td>141</td>
</tr>
<tr>
<td>0.008</td>
<td>111</td>
</tr>
<tr>
<td>0.009</td>
<td>95.4</td>
</tr>
<tr>
<td>0.01</td>
<td>78.8</td>
</tr>
<tr>
<td>0.1</td>
<td>16.2</td>
</tr>
</tbody>
</table>

Table 4.3 Juice turbidity of York apple juice treated with different clarification methods. Values are reported as mean ± SEM for n=3 replicates and different superscripts indicate significant difference (P <0.05) between treatments by Two-way ANOVA and Tukey’s HSD test.

<table>
<thead>
<tr>
<th>Juice treated with different clarification methods</th>
<th>Juice turbidity/NTU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw juice</td>
<td>3827±0.65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Settled juice</td>
<td>1162±18&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Centrifuged juice</td>
<td>581±3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pectinase-treated juice with different pectinase dosage/(μL/L)</td>
<td>0.1 28±2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.003 56±6&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.006 26±1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Raw juice, settled juice, centrifuged juice, and pectinase-treated juices treated with the three chosen pectinase dosages were prepared. Apples used were from a mixture of York apples harvested at different time points with different storage periods ranging from a period of a two to three weeks to two months. The turbidity of all resulting juices
was measured. As evident from the values reported in Table 4.3, the turbidity of the experimental York juices was different from the turbidity values obtained in the preliminary trials with York apples from the Winchester AREC, presented in Table 4.1 and Table 4.2. The turbidity of raw juice, settled juice, centrifuged juice, and pectinase-treated juices were significantly different between the preliminary trials and the experimental work. The turbidity of centrifuged juice did not match the turbidity of the pectinase-treated juice treated with pectinase of 0.03 µL/L. The turbidity of pectinase juice treated with pectinase of 0.06 µL/L was below 100 NTU. Thus, only the raw juice, settled juice, centrifuged juice and pectinase-treated juice treated with the recommended pectinase dosage were further analyzed and fermented into cider. Figure 4.1 shows a comparison of the turbidity difference of juice treated with the same amount of pectinase in the preliminary trial and in the first experiment.

Figure 4.1 Comparison of juice turbidity with same pectinase dosage in trial and in experiment (only one sample per treatment was run for this preliminary dosing trial)
There were two major challenges encountered during apple juice clarification: (a) the pectinase did not function well on clarifying Brown Snout apple juice; and (b) the turbidity of pectinase-treated juice was not consistent across the trial and for the experiment for York apple juice.

Some prior work investigating the stability of apple juice turbidity and the principle of pectinase clarification of apple juice can help to explain these unexpected results.

The stability of cloudy apple juice is maintained by the pectin binding the particles in the juice. A simplified model of this colloid is illustrated as the positively charged protein-carbohydrate complex at the core, and surrounded by the negatively charged pectin. Through significant electrostatic repulsion, the pectin coating prevents the particles from coagulating too much. Under transmission electron microscopy, the mechanism of pectinase (Solvay 5XLHA) destroying the apple juice (Granny Smith) colloid resulting in pectin degradation can be observed. Sorrivas et al. (2006) measured the turbidity of apple juice over time after adding pectinase to the juice. The inconsistent and fluctuating turbidity results were unexpected (Figure 4.2). One reason for the inconsistencies of the turbidity measured in both the pectinase-treated juice in the trial and in the experiment may result from the juices having settled for different time periods after the pectinase addition, resulting in samples being taken at different times.
Figure 4.2 Time course of turbidity of ripe Granny Smith apple juice during pectinase treatment (adapted from V. Sorrias, 2006\textsuperscript{114})

A study of commercially available cherry juice (Agrana, Vallø, Denmark) demonstrated that pectinase did not greatly impact the contiguous turbidity, the turbidity measured at time 0 after pectinase addition. The clarification effect did not happen immediately after the pectinase addition. However, juices treated with pectinase did clarify much more during cold storage\textsuperscript{115}.

No optimal temperature was specified by the manufacturer for the pectinase used in this project, although it was recommended that longer settling time be allowed at colder temperatures. While temperature strongly affects the activity of enzymes, pH is another factor that influences the enzymatic activity of pectinase\textsuperscript{89}. Unfortunately, enzymatic activity of the product used in this study was not available from the manufacturer (upon contacting the manufacturer we were told that this is a proprietary blend of enzymes and all technical information beyond that which is available on the technical information sheet is a trade secret).
The change of polyphenols concentration during post-harvest storage likely impacted the efficiency of pectinase clarification. How polyphenols affect the juice clarification has been explained in previous section 2.5.1.

The pectinase used in this project was a blend of enzymes specifically designed for wine making; but according to the manufacturer’s technical data sheet, this blend can also be used on apple cider making. The pectins in apples and grapes are noticeably different. Apples contain 0.5-1.6% of pectin by wet weight\textsuperscript{1/6}. Because the subunits of pectin are very complex, there are various of pectinases depending on how the enzymes degrade the substrates\textsuperscript{1/7}. It is possible that the pectinase used in this project did not work well on the pectin in Brown Snout apples. Further investigation is required to identify effective juice clarification treatments across cultivars.

4.2 Primary juice chemistry

The SSC, pH, and TA of raw juice and clarified juices are listed in Table 4.4.

<table>
<thead>
<tr>
<th>Experimental treatment</th>
<th>SSC (°Brix)</th>
<th>pH</th>
<th>TA (g L\textsuperscript{-1} malic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw juice</td>
<td>10.9±0.1\textsuperscript{a}</td>
<td>3.59±0.02\textsuperscript{a}</td>
<td>4.04±0.14\textsuperscript{a}</td>
</tr>
<tr>
<td>Settled juice</td>
<td>10.4±0.4\textsuperscript{a}</td>
<td>3.59±0.01\textsuperscript{a}</td>
<td>3.89±0.27\textsuperscript{a}</td>
</tr>
<tr>
<td>Centrifuged juice</td>
<td>9.7±1.5\textsuperscript{a}</td>
<td>3.60±0.01\textsuperscript{a}</td>
<td>3.42±0.15\textsuperscript{a}</td>
</tr>
<tr>
<td>Pectinase-treated juice</td>
<td>11.1±0.7\textsuperscript{a}</td>
<td>3.59±0.02\textsuperscript{a}</td>
<td>3.66±0.73\textsuperscript{a}</td>
</tr>
</tbody>
</table>

No significant differences were found for SSC, pH or TA between the raw juice and the clarified juices, regardless of clarification method. As these three parameters relate to soluble solids content in the juice, the results indicate that the content of soluble solids in juice was not drastically affected by the clarification treatment.
4.3 YAN in juice

**Figure 4.3 YAN concentrations in York apple juice treated with different clarification methods** (Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by one-way ANOVA and Tukey’s HSD test)

YAN concentration in the raw juice is 57±2.35 mg of N/L. No previous research data on YAN concentration in York apple juice has been found (Figure 4.3). The YAN concentration in pectinase treated juice was lower than the concentration in the raw juice, settled juice, and centrifuged juice. Although solids were lost during the physical clarification (settling and centrifugation), the pectinase treated juice (28±2 NTU) was much clearer than the other juices (3827±0.65 NTU for raw juice, 1162±18 NTU for settled juice, and 581±3 NTU for centrifuged juice, reported in Table 4.3). YAN loss in the pectinase treated juice may be due to the extensive loss of solids during the clarification by pectinase.

Apple fruit YAN concentrations are relatively lower than those found in European wine grapes (*Vitis vinifera*). There are very few studies reporting YAN for cider apples; the majority of apple chemistry research has been on non-alcoholic apple juice, rather
than fermented cider, and YAN is not an important consideration for apple juice quality. The few studies available report individual amino acid concentrations but not YAN values\textsuperscript{17,39}. The primary body of cider research was performed at Long-Ashton research station before the concept of YAN was widely recognized and studied in beverage fermentation, and thus reported results for total nitrogen or total soluble nitrogen are by Kjeldahl\textsuperscript{17}. These values include forms of nitrogen such as that present in proteins that are not assimilable by yeast. Total nitrogen values by Kjeldahl cannot be directly compared to YAN values reported in the wine grape literature. A range of 25-172 mg/L on YAN was observed in cider apples grown in Virginia in the 2014 growing season (Boudreau 2015, unpublished). The amount of nitrogen in apple juices is largely dependent on the age of the orchard and the amount of fertilizers applied on the apple trees\textsuperscript{118}.

A YAN concentration of 140 mg/L in grape musts is often considered the minimum requirement to reliably complete fermentation in wine making with the low solid content and normal sugar content\textsuperscript{119,120}. However, an analogous minimum recommended concentration of YAN for cider fermentation has not been established. Further study on initial YAN concentration and the accompanying fermentation will provide a frame of reference for cider makers on nitrogen supplementation. An optimal concentration and composition of YAN will promote yeast health and achieve desired sensory profiles.

4.4 Amino acid profile in juice

In this section, the concentrations of 20 individual amino acids and total amino acid concentration in the raw juice and clarified juices will be reported as a function of
juice clarification technique (Table 4.5).

### Table 4.5 Individual amino acid concentrations in York juice treated with different clarification methods

(Values are reported as mean ± standard deviation for n=3 replicates and different superscripts indicate significant difference (P<0.05) on the mean concentration of individual amino acids between treatments by one-way ANOVA and Tukey’s HSD test)

<table>
<thead>
<tr>
<th>Concentration of amino acid (mg/l)</th>
<th>Raw juice</th>
<th>Settled juice</th>
<th>Centrifuged juice</th>
<th>Pectinase-treated juice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>3.572±0.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.011±0.285&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.370±0.916&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.160±0.204&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.988±0.341&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.465±0.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.203±0.251&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.091±0.604&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tyrosin</td>
<td>0.683±0.235&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.119±0.610&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.471±0.264&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.558±0.183&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serine</td>
<td>4.617±0.158&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.735±0.765&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.979±0.459&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.415±0.713&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>55.397±1.908&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53.152±10.318&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85.170±7.532&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48.097±7.858&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Asparagine</td>
<td>40.771±1.923&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.225±6.845&lt;sup&gt;a&lt;/sup&gt;</td>
<td>58.146±0.684&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38.006±5.308&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>29.947±1.442&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.123±5.388&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.582±0.391&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.105±4.184&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutamine</td>
<td>3.694±0.143&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.369±0.524&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.622±0.339&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.271±0.553&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.051±0.037&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.076±0.195&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.566±0.138&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.981±0.166&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.198±0.145&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.176±0.540&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.725±0.113&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.860±0.422&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>γ-Aminobutyric acid</td>
<td>0.838±0.042&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.806±0.101&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.079±0.028&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.754±0.124&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Proline</td>
<td>0.801±0.028&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.815±0.111&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.060±0.113&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.753±0.088&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.247±0.078&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.404±0.124&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.549±0.066&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.266±0.005&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.894±0.038&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.187±0.158&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.213±0.288&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.848±0.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Valine</td>
<td>0.000±0.000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.366±0.071&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.559±0.507&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.000±0.000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.970±0.048&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.094±0.024&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.854±0.181&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.851±0.132&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.777±0.027&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.121±0.065&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.769±0.028&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.756±0.107&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.264±0.125&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.821±0.244&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.242±0.140&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.077±0.403&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

4.1 Histidine

[Image of Histidine concentration graph]
Figure 4.4 Concentrations of histidine in York apple juice treated with different clarification methods (Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by one-way ANOVA and Tukey’s HSD test)

No significant difference on the concentration of histidine was found among the raw juice and the clarified juice with different clarification methods (Figure 4.4). The concentration of histidine in the raw juice was 3.57±0.49 mg/L.

4.4.2 Serine

![Graph showing concentration of serine in different juice treatments](image)

Figure 4.5 Concentrations of serine in York apple juice treated with different clarification methods (Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by one-way ANOVA and Tukey’s HSD test)

The concentration of serine was significantly higher in centrifuged juice than its concentration in the rest of the juices (Figure 4.5). The concentration of serine was 4.62±0.16 mg/L in the raw juice and 6.98±0.46 mg/L in the centrifuged juice.

4.4.3 Asparagine
Figure 4.6 Concentrations of asparagine in York apple juice treated with different clarification methods (Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by one-way ANOVA and Tukey’s HSD test)

The concentration of asparagine was significantly higher in the centrifuged juice than its concentration in the rest of the juices (Figure 4.6). The concentration of asparagine was 55.40±1.91 mg/L in the raw juice and 85.17±7.53 mg/L in the centrifuged juice. Asparagine was the most abundant amino acid in York apple juice. In the raw juice, it made up 35.8% of the total amino acids analyzed.

4.4.4 Glutamine
The concentration of glutamine in the centrifuged juice was significantly higher than its concentration in the rest of the juices (Figure 4.7). The concentration of glutamine was $3.69 \pm 0.14$ mg/L in the raw juice and $5.62 \pm 0.34$ mg/L in the centrifuged juice.

4.4.5 Arginine
Figure 4.8 Concentrations of arginine in York apple juice treated with different clarification methods (Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by one-way ANOVA and Tukey’s HSD test)

No significant difference was found on the concentration of arginine among the raw juice and the clarified juice with different clarification methods (Figure 4.8). The concentration of arginine was 3.99±0.34 mg/L in the raw juice. Arginine was reported to be one of the most prevalent amino acid in grapes, taking up 6-44% of total nitrogen in several grape juices\textsuperscript{121}. The concentration of arginine was much lower than reported arginine concentration (107±26 mg/L to 1646±141 mg/L) in white grape Vitis vinifera cultivars grown in Washington State\textsuperscript{122}, mean arginine concentration of Chardonnay juices from California (550mg/L) and Washington State (389 mg/L)\textsuperscript{123}. This is an important difference between apple and grape juice chemistry, as the vast majority of the beverage fermentation literature available has been conducted with grape as the substrate, where arginine is usually by far the most prevalent yeast assimilable form of nitrogen (proline is also very prevalent in grape, but is not yeast assimilable). The impact of differences in amino acid profile in apple versus grape juice on yeast metabolism during fermentation warrants further research.

4.4.6 Aspartic acid
The concentration of aspartic acid in the centrifuged juice was significantly higher than its concentration in the rest of the juices (Figure 4.9).

The concentration of aspartic acid was 40.77±1.92 mg/L in the raw juice and was 58.15±0.68 mg/L in the centrifuged juice. Aspartic acid was the second most abundant amino acid in York apple juice and it made up 26.4% of all amino acid present in the raw juice.

4.4.7 Glutamic acid
Figure 4.10 Concentrations of glutamic acid in York apple juice treated with different clarification methods (Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by one-way ANOVA and Tukey’s HSD test)

The concentration of glutamic acid in the centrifuged juice was significantly higher than its concentration in the rest of the juices (Figure 4.10).

The concentration of glutamic acid was 29.95±1.44 mg/L in the raw juice and 43.58±0.39 mg/L in the centrifuged juice. In raw juice, glutamic acid contributed to 19.4% of the total amino acids present.

4.4.8 Threonine
Figure 4.11 Concentrations of threonine in York apple juice treated with different clarification methods (Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by one-way ANOVA and Tukey’s HSD test)

The concentration of threonine in the centrifuged juice was significantly higher than its concentration in the rest of the juices (Figure 4.11). The concentration of threonine was 1.05±0.04 mg/L in the raw juice and 1.57±0.14 mg/L in the centrifuged juice.

4.4.9 Alanine
Figure 4.12 Concentrations of alanine in York apple juice treated with different clarification methods (Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by one-way ANOVA and Tukey’s HSD test)

The concentration of alanine in the centrifuged juice was significantly higher than its concentration in the rest of the juices (Figure 4.12). The concentration of alanine was 3.20±0.15 mg/L in the raw juice and 4.73±0.11 mg/L in the centrifuged juice.

4.4.10 γ-aminobutyric acid

Figure 4.13 Concentrations of γ-aminobutyric acid in juice treated with different clarification methods (Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by one-way ANOVA and Tukey’s HSD test)

The concentration of γ-aminobutyric acid in the centrifuged juice was significantly higher than its concentration in the rest of the juices (Figure 4.13). The concentration of γ-aminobutyric acid was 0.84±0.04 mg/L in the raw juice and 1.08±0.03 mg/L in the centrifuged juice.
4.4.11 Proline

![Bar chart showing concentration of proline in different apple juice treatments](chart.png)

**Figure 4.14 Concentrations of proline in York apple juice treated with different clarification methods** (Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by one-way ANOVA and Tukey’s HSD test)

The concentration of proline in the centrifuged juice was significantly higher than its concentration in the rest of the juices (Figure 4.14). The concentration of proline was 0.80±0.03 mg/L in the raw juice and 1.06±0.11 mg/L in the centrifuged juice. Proline made up 0.5% of the amino acids observed in the raw juice, which is much lower than the relative concentration of proline observed in grape juice. Proline has been found to be the predominant amino acid in several grape juices, ranging from 4.3% to 32.2% of the total nitrogen\(^{124}\); another notable difference in grape vs. apple juice chemistry, with the potential to impact the interpretation of YAN data and post-fermentation microbial stability.

4.4.12 Lysine
The concentration of lysine in the centrifuged juice was significantly higher than its concentration in the raw juice and centrifuged juice (Figure 4.15). The concentration of lysine was 0.25±0.08 mg/L in the raw juice and 0.55±0.07 mg/L in the centrifuged juice.

4.4.13 Tyrosine
Figure 4.16 Concentrations of tyrosine in York apple juice treated with different clarification methods (Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by one-way ANOVA and Tukey’s HSD test)

No significant difference was found in the concentration of tyrosine in the raw juice and clarified juices (Figure 4.16). The concentration of tyrosine was 0.68±0.24 mg/L in the raw juice.

4.4.14 Methionine

![Bar chart showing concentrations of methionine in different clarified juices](image)

Figure 4.17 Concentrations of methionine in York apple juice treated with different clarification methods (Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by one-way ANOVA and Tukey’s HSD test)

The concentration of methionine in the settled juice was significantly higher than its concentration in the rest of the juices (Figure 4.17). The concentration of methionine was 0.89±0.04 µg/mL in the raw juice and 2.19±0.16 µg/mL in the settled juice. All of these concentrations of methionine are within the previously reported range of apple juice methionine concentration (0-43 mg/L)\(^{19, 39, 125}\). But they are below the minimum value reported (20 mg/L)\(^{126}\) to
prevent H₂S production during fermentation, and lower than typical grape juice methionine concentrations which range from 0 to 52 mg/L methionine⁹²⁻¹²⁹.

4.4.15 Valine

Figure 4.18 Concentrations of valine in York apple juice treated with different clarification methods (Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by one-way ANOVA and Tukey’s HSD test)

The concentration of valine in the settled juice, 1.37±0.07 µg/mL, was significantly higher than its concentration in the rest of the juices (Figure 4.18). The concentration of valine was below the limit of detection in raw and pectinase-treated juice.

4.4.16 Isoleucine
The concentration of isoleucine in the settled juice was significantly higher than its concentration in the rest of the juices (Figure 4.19). The concentration of isoleucine was 0.97±0.05 µg/mL in the raw juice and 2.09±0.02 µg/mL in the settled juice.

4.4.17 Leucine
Figure 4.20 Concentrations of leucine in York apple juice treated with different clarification methods (Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by one-way ANOVA and Tukey’s HSD test)

The concentration of leucine in the settled juice was significantly higher than its concentration in the rest of the juices (Figure 4.20). The concentration of leucine was 0.78±0.03 µg/mL in the raw juice and 2.12±0.07 µg/mL in the settled juice.

4.4.18 Phenylalanine

Figure 4.21 Concentrations of phenylalanine in York apple juice treated with different clarification methods (Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by one-way ANOVA and Tukey’s HSD test)

The concentration of phenylalanine in the settled juice was significantly higher than its concentration in the rest of the juices (Figure 4.21). The concentration of phenylalanine was 3.26±0.13 µg/mL in the raw juice and 5.82±0.24 µg/mL in the settled juice.
4.4.19 Total amino acid

![Bar chart showing amino acid concentrations in different juices](image)

**Figure 4.22 Concentrations of total amino acids in York apple juice treated with different clarification methods** (Values are reported as mean ± SEM for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by one-way ANOVA and Tukey’s HSD test)

Concentrations of total amino acids in juice treated with different clarification methods were shown in Figure 4.22. Total amino acid values were obtained by adding the concentrations of all of the target individual amino acids together. Total amino acid was 154.71±6.31 µg/mL in the raw juice and 225.16±7.72 µg/mL in the centrifuged juice. Cysteine and glycine were not found in any apple juices in this study. The total amino acid in the raw juice was within the previously reported range of apple amino acid, 27 to 574 mg/L\(^{130}\). Aspartic acid, asparagine, and glutamic acid together made up 81.5% of the total amino acids. This was consistent with previous findings by Lequere and Drilleau (1998)\(^ {130}\), Su´arez Valles et al. (2005)\(^ {31}\), Garai-Ibabe et al. (2013)\(^ {132}\), Ye et al (2014)\(^ {125}\), and Eleutério dos Santos et al. (2015)\(^ {133}\). The amino acid concentration
and composition can change depending on the apple cultivars, climate and nitrogen fertilizers\textsuperscript{134, 135}.

In summary (Table 4.5 summarizes this information with structures), the concentration of histidine, arginine, and tyrosine were not different between the raw juice and the clarified juices.

The concentration of serine, aspartic acid, asparagine, glutamic acid, glutamine, threonine, alanine, γ-aminobutyric acid, and proline were higher in the centrifuged juice than the remaining juices.

The concentration of methionine, valine, isoleucine, leucine, and phenylalanine were higher in the settled juice than in other treatments.

The concentration of lysine was significantly higher in the centrifuged juice than the raw juice and pectinase treated juice, but not different than in the settled juice.

To summarize the clarification treatment effects on individual amino acid concentrations, there was not a consistent trend across all of the analytes. The different trends observed in concentration changes of individual amino acids by clarification treatments may be due to differences in amino acid structures (Table 4.6), chemistry and related solubility in juice. In addition, the solubility reported in Table 4.6 is in water at neutral pH, but the solubility of amino acids changes at various pH, due to the fact that amino acids have various pKa’s \textsuperscript{136}. Amino acids are also believed to be involved in the non-enzymatic browning process, reacting with polyphenols, reducing sugars, and organic acids\textsuperscript{137, 138}. Research has shown that lysine and GABA are high browning producing amino acids\textsuperscript{137, 139, 140}, while asparagine and aspartic acid are less preferred in
the browning process. Findings on the involvement on glutamic acid in the browning process are contradictory\textsuperscript{78, 141, 142}.

<table>
<thead>
<tr>
<th>Amino acid which concentration remained unchanged after the clarification</th>
<th>Amino acid which concentration was higher in the \textit{settled \ and \ centrifuged juice} than the other juices</th>
<th>Amino acid which concentration was higher in the \textit{settled juice} than other juices</th>
<th>Amino acid which concentration was higher in the \textit{centrifuged juice} than other juices</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>Lysine</td>
<td>Phenylalanine</td>
<td>Serine</td>
</tr>
<tr>
<td><img src="image" alt="Histidine structure" /></td>
<td><img src="image" alt="Lysine structure" /></td>
<td><img src="image" alt="Phenylalanine structure" /></td>
<td><img src="image" alt="Serine structure" /></td>
</tr>
<tr>
<td>Arginine</td>
<td>Methionine</td>
<td>Aspartic acid</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Arginine structure" /></td>
<td><img src="image" alt="Methionine structure" /></td>
<td><img src="image" alt="Aspartic acid structure" /></td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Valine</td>
<td>Asparagine</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Tyrosine structure" /></td>
<td><img src="image" alt="Valine structure" /></td>
<td><img src="image" alt="Asparagine structure" /></td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Glutamic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Isoleucine structure" /></td>
<td><img src="image" alt="Glutamic acid structure" /></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>Glutamine</td>
<td></td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Leucine structure" /></td>
<td><img src="image" alt="Glutamine structure" /></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Threonine structure" /></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Amino acids are naturally quite a diverse group of compounds. This diversity in chemistry is what allows for the astounding diversity in protein structure observed in nature. It would not be an exaggeration to state that life itself is possible due to the diversity of chemistry across amino acids. Therefore, it is not surprising that these compounds did not react in a uniform fashion to the clarification treatments. Amino acids are distinguished by their unique side chains. Depending on the tendency of their side chains to react with water, they are further classified in Table 4.7. The hydrophobic amino acids are characterized by their reluctance to react with water. But the hydrophilic amino acids (polar or charged) are thermodynamically favored to interact with water, forming hydrogen bonds for polar amino acids and salt bridges by charged amino acids. The pH of amino acids at the isoelectric point affects their solubility. The solubility is the lowest at the isoelectric point. When the pH of the solution is less than the pI, the amino acids are positively charged; when the pH of the solution is greater than the pI, the amino acids are negatively charged. Based on the pH of juices in this study (3.60), the solubility of aspartic acid and glutamic acid should be much lower than the listed solubility in
Table 4.7. Aspartic acid and glutamic acid are negatively charged, and GABA, arginine, and lysine are positively charged in the apple juice.

Table 4.7 Amino acids classification based on tendency to react with water, their solubility in water (g/100mL H₂O at 25°C)¹⁴³, pKa values, and their pH at the isoelectric point (pI)¹⁴⁴

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Solubility in Water</th>
<th>pKa¹</th>
<th>pKa²</th>
<th>pKa³</th>
<th>pI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pKa¹</td>
<td>pKa²</td>
<td>pKa³</td>
<td></td>
</tr>
<tr>
<td>Hydrophobic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.0453</td>
<td>2.20</td>
<td>9.11</td>
<td>10.07</td>
<td>5.66</td>
</tr>
<tr>
<td>Alanine</td>
<td>16.65</td>
<td>2.35</td>
<td>9.87</td>
<td>6.00</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.117</td>
<td>2.32</td>
<td>9.76</td>
<td>6.02</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>2.426</td>
<td>2.33</td>
<td>9.74</td>
<td>5.98</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.965</td>
<td>2.58</td>
<td>9.24</td>
<td>5.48</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>162.3</td>
<td>2.00</td>
<td>10.60</td>
<td>6.30</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>8.85</td>
<td>2.29</td>
<td>9.72</td>
<td>5.96</td>
<td></td>
</tr>
<tr>
<td>Polar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>Soluble</td>
<td>2.28</td>
<td>9.21</td>
<td>5.74</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>2.5</td>
<td>2.17</td>
<td>9.13</td>
<td>5.65</td>
<td></td>
</tr>
<tr>
<td>Asparagine</td>
<td>3.35</td>
<td>2.02</td>
<td>8.80</td>
<td>5.41</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>5.023</td>
<td>2.21</td>
<td>9.15</td>
<td>5.68</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>very soluble</td>
<td>2.09</td>
<td>9.10</td>
<td>5.60</td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>4.19</td>
<td>1.77</td>
<td>9.18</td>
<td>6.10</td>
<td>7.59</td>
</tr>
<tr>
<td>Charged</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>15</td>
<td>2.01</td>
<td>9.04</td>
<td>12.48</td>
<td>10.76(+)</td>
</tr>
<tr>
<td>Lysine</td>
<td>very soluble</td>
<td>2.18</td>
<td>8.95</td>
<td>10.53</td>
<td>9.74(+)</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.778</td>
<td>2.10</td>
<td>9.82</td>
<td>3.86</td>
<td>2.77(−)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.864</td>
<td>2.10</td>
<td>9.47</td>
<td>4.07</td>
<td>3.22(−)</td>
</tr>
<tr>
<td>γ-aminobutyric acid</td>
<td>130</td>
<td>4.23</td>
<td>10.34</td>
<td>7.285(+)</td>
<td></td>
</tr>
</tbody>
</table>

*pKa¹, carboxylic acid; pKa², amino; pKa³, side chain

Based on the colloidal model described in 2.6.1, positively charged amino acids are in the core, surrounded by the negatively charged pectin. But it is also possible that the hydrophobic amino acids are freely present in the juice. In static settling, particles are moving gradually and mildly by gravitational force, so the core and shell system should remain integral. The concentration of all amino acids are expected to be the same in the settled juice with the raw juice. But the concentration of methionine, valine, isoleucine, leucine, lysine, and phenylalanine were higher in the settled juice comparing to the raw juice. In centrifugation, the particles are driving by brutal centrifugal force, thus the core
and shell system can be damaged, releasing amino acids in the core. A small portion of pectin in the juice was removed, causing the loss of amino acids in the juice. So the concentration of all amino acids are expected to be higher (releasing effect is bigger than the loss), unchanged (releasing effect is equal to the loss), and lower (releasing effect is smaller than the loss) in the centrifuged juice than the raw and settled juice. In the pectinase-treated juice, the pectin shell is broken, releasing the amino acids in the core. The concentration of amino acids in the pectinase-treated juice is expected to be higher comparing to the raw juice. The filtration through PTFE filters before loaded onto the UPLC column is another factor to be considered. Juices containing more pectin (raw juice, settled juice, and centrifuged juice) may have lost more amino acids in this filtration step. Higher concentrations of all amino acids are expected to be found in the pectinase-treated juice. But none of the amino acid has higher concentration in the pectinase-treated juice comparing to the raw juice.

Tyrosine has the lowest solubility (0.0453g/100 mL water at 25 °C) in water among all amino acids\textsuperscript{143}. When the solids are removed by the clarification, the relatively amount of free water increases in the clarified juice compared to the raw juice. It is likely that more tyrosine is pulled out from the solids and its concentration remains the same.

Besides all of the possible explanations above, it is difficult to explain the change of concentration of hydrophobic amino acids in the juice. Hydrophobic amino acids are driven to the most hydrophobic areas in the juice. Further research would be needed to understand the mechanisms behind the behaviors of each amino acid in apple juice after clarification treatments.

4.5 Fermentation curves
Figure 4.23 Fermentation curves based on CO₂ loss during the fermentation of ciders made from juices treated with different clarification treatments (Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by one-way ANOVA and Tukey’s HSD test)

The fermentation curve was developed based on the loss of mass due to CO₂ purged from the system during fermentation (Figure 4.23). The fermentation was allowed to proceed until the mass of each fermentation was consistent for 2 days, which was about one week. Although the initial YAN concentration in the pectinase treated juice was significantly lower than the rest of the juices, the fermentation of pectinase treated juice did not fall behind, as might be expected. This is most likely due to the routine addition of nitrogen supplement before the fermentation. As fermentation kinetics were not an outcome we evaluated in this experiment (only monitored to ensure that this processing step was proceeding appropriately), a commercial nitrogen supplement was added to supplement the low YAN concentration in the starting juice (~60mg/L), to promote complete fermentation, so that acceptable cider would be obtained for further evaluation.

4.6 Primary chemistry in cider
Table 4.8 Primary chemistry in cider made from York apple juice treated with different pre-fermentation clarification methods. Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by Two-way ANOVA and Tukey’s HSD test.

<table>
<thead>
<tr>
<th>Experimental treatment - cider made from</th>
<th>Residual sugar (g/L)</th>
<th>pH</th>
<th>TA (g L⁻¹ malic)</th>
<th>Free SO₂ (mg/L)</th>
<th>Total SO₂ (mg/L)</th>
<th>Ethanol content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw juice</td>
<td>0.000±0.004&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.51±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.94±0.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.3±0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38±2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.06±0.16&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Settled juice</td>
<td>0.012±0.003&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.53±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.80±0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41±3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.21±0.19&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Centrifuged juice</td>
<td>0.006±0.004&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.54±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.75±0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.7±1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48±4&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>5.94±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pectinase-treated juice</td>
<td>0.033±0.016&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.55±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.18±0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.0±0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52±4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.13±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Primary chemistry results for cider made from juice treated with different clarification methods are listed in Table 4.8. No significant difference was found in pH, TA, free SO₂, and ethanol content in cider fermented from raw juice and other clarified juices. But the residual sugar in the cider fermented from the pectinase-treated juice was significantly higher than than other ciders. One possible explanation for this observation is that even with the nitrogen supplementation of the pre-fermentation juice, since the initial YAN was 49% lower in the pectinase-treated juice than the other juices, the YAN concentration in the pectinase-treated juice before fermentation was still lower than the other juices. The final YAN in that treatment (153 mg N/L, while the final YAN in the raw juice is 182 mg N/L) may have still been too low to allow for complete fermentation, meaning that the yeast did not have adequate nutrients were not able to metabolize as much of the sugar in the juice. Total SO₂ was significantly different among all the ciders. There were no differences in free SO₂ concentration among the ciders, however cider fermented from the pectinase treated juice had a higher total SO₂ concentration than others. This may have been due to the fact that the juices were clarified before the SO₂ addition was made, and there were fewer solids in the pectinase treated juice, thus less of the bound SO₂ was lost through binding to solids and racking in subsequent steps. In the
future, in order to maintain consistent free and total SO₂ across all samples, SO₂ additions should be made before the clarification treatments are imposed. Nonetheless, the difference in total SO₂ (free and bound forms) is not expected to influence the oxidative state of the juice and our results, as the free SO₂ is that which is available to perform antioxidant function and those values were the same across treatments.

4.7 Total polyphenols in juice and cider

![Figure 4.24 Standard curve of total polyphenols measurement by Folin assay](image)

Linear regression was obtained by plotting the absorbance vs gallic acid with known concentration. The $R^2$ of the curve was 0.9996 (Figure 4.24).

![Figure 4.25 Total polyphenols in (A) York apple juice treated with different pre-fermentation clarification methods and (B) in cider made from these juices](image)

(Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by one-way ANOVA and Tukey’s HSD test)
The concentrations of total polyphenols in juice treated with different clarification methods and the finished cider were shown in Figure 4.25. The total polyphenols concentration was $0.630 \pm 0.02 g/L$ in the raw juice, $0.439 \pm 0.009 g/L$ in the settled juice, $0.342 \pm 0.007 g/L$ in the centrifuged juice, and $0.252 \pm 0.015 g/L$ in the pectinase treated juice. Total polyphenol concentration in clarified juice for each treatment was significantly different from all other treatments. Reported concentrations of total polyphenols ranged from 26.2 mg/L to 88.2 mg/L in certain apple cultivars grown in Shandong Province in China\textsuperscript{39}. Additional studies show that total polyphenol concentration varied up to 7-fold depending on genotype\textsuperscript{53}. Higher concentrations were observed at 1111.5 mg/L in raw apple juice\textsuperscript{145}, and around 1600 mg/L in Golden Delicious apple juice with minimal enzymatic oxidation\textsuperscript{55}. The common range of Spanish cider apples is considered to be 800-1300 mg/L\textsuperscript{45}. The total polyphenol concentration in the raw juice was within the reported range for apples, but lower than the reported range of Spanish cider apples.

The total polyphenols concentration was $0.236 \pm 0.015 g/L$ in the cider fermented from the raw juice. No significant difference was found among the ciders fermented from the raw juice and the clarified juices. But for each juice-cider pair, total polyphenol concentration was lower for ciders fermented from raw, settled, and centrifuged juices than in the corresponding juice. P-values for each comparison were: $<0.0001$, 0.0032, and 0.0020. Polyphenols interact with proteins to form sediments during cider fermentation and storage\textsuperscript{43, 146}, so this loss was expected. More loss was expected with cider fermented from more turbid juice.
Although the Folin-Ciocalteu assay is commonly applied in horticulture and food science, we would argue that these results did not represent the true total polyphenol concentration in the juices and ciders, since SO$_2$, sugars, and other reducing compounds have been shown to interfere with this measurement. Therefore we explored other methods for polyphenols analysis (DMAC for total procyanidins and UPLC/MS to individually quantify a set of target compounds).

4.8 Total proanthocyanidins in juice and cider

**Figure 4.26 Standard curve of total proanthocyanidin measurement by DMAC method**

A linear regression was obtained by plotting the absorbance vs PC B2 with known concentration, creating a standard curve. The $R^2$ of the curve was 0.9992 (Figure 4.26).

**Figure 4.27 Total proanthocyanidins in (A) York apple juice treated with different pre-fermentation clarification methods and (B) in cider made from these juices (Values are
The concentrations of total proanthocyanidins in apple juice treated with different clarification methods and the finished cider were shown in Figure 4.27. The total proanthocyanidin concentration was 0.035±0.000 g of PC B2 equivalent/L in the raw juice, 0.010±0.001 g of PC B2 equivalent/L in the settled juice, 0.016±0.003 g of PC B2 equivalent/L in the centrifuged juice, and 0.094±0.007 g of PC B2 equivalent/L in the pectinase treated juice. The total proanthocyanidin concentrations in the settled juice and centrifuged juice were significantly lower than the concentration in the raw juice. The concentration in the pectinase treated juice was significantly higher than the concentration in the raw juice. The pectin concentration in the raw juice, settled juice, and centrifuged juice was much higher than in the pectinase treated juice. This may unfortunately be an artifact of the analytical method, rather than an actual indication of the effect of our pre-fermentation juice clarification treatments on proanthocyanidin concentration in the juice. Our results for proanthocyanidin concentration in the raw juice, settled juice and centrifuged juice were likely not an accurate representation of the juice proanthocyanidin content due to the interference of pectin present in those samples (that had been removed by pectinase in the remaining treatments). Pectin cannot dissolve in methanol, and methanol is the solvent used in the DMAC assay. After adding the DMAC reagent, the proanthocyanidins attached to pectin were precipitated out. Since absorbance cannot be read on the unstable system with color particles floating inside, the samples were centrifuged to remove the insoluble particles. Thus, the absorbance of the supernatant was lower than the actual absorbance of the samples.
For this reason, we suggest that further research investigate the potential utility of pectinase treatment as a sample prep step when using the DMAC method for analysis of apples or other pectin-rich fruits.

The yield of proanthocyanidins through extraction in apple juice production is very low, having been reported at 32%⁵⁹,¹⁴⁷ of the proanthocyanidins present in fruit being transferred into the juice through pressing. Proanthocyanidins were lost through pressing because they are adsorbed to the cell-wall materials and other solids in the apple juice.

The total proanthocyanidin concentration was 0.056±0.004 g of PC B2 equivalent/L in the cider made from raw juice, and 0.042±0.001 g of PC B2 equivalent/L in the cider made from centrifuged juice. The total proanthocyanidins concentration in the cider made from centrifuged juice was significantly lower than the concentration in the rest of the juices. The loss of proanthocyanidins concentration in the cider fermented from pectinase treated juice has been observed in a previous study¹⁴⁸. This is believed to be caused by attachment of proanthocyanidins to (protein rich) yeast cell walls during the fermentation¹⁴⁹.

4.9 Individual polyphenols in juice and cider

Table 4.9 Calibration curves of individual polyphenol standards (Concentration ranged from 0.001 to 10 µg/mL)

<table>
<thead>
<tr>
<th>Name</th>
<th>Equation</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phloretin</td>
<td>( Y = 5.57\times10^6 , X - 4.66\times10^2 )</td>
<td>0.999197</td>
</tr>
<tr>
<td>Quercetin</td>
<td>( Y = 3.36\times10^6 , X - 3.33\times10^3 )</td>
<td>0.998628</td>
</tr>
<tr>
<td>PC B5</td>
<td>( Y = 6.05\times10^5 , X - 1.60\times10^3 )</td>
<td>0.999871</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>( Y = 2.41\times10^6 , X + 9.50\times10^2 )</td>
<td>0.988501</td>
</tr>
<tr>
<td>PC B2</td>
<td>( Y = 3.04\times10^5 , X - 8.91\times10^2 )</td>
<td>0.998149</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>( Y = 1.47\times10^6 , X + 1.10\times10^4 )</td>
<td>0.999603</td>
</tr>
<tr>
<td>Catechin</td>
<td>( Y = 1.82\times10^6 , X + 1.41\times10^3 )</td>
<td>0.985718</td>
</tr>
<tr>
<td>PC B1</td>
<td>( Y = 7.33\times10^5 , X - 1.18\times10^3 )</td>
<td>0.999989</td>
</tr>
</tbody>
</table>
Due to the limitation and high cost of commercially available polyphenol standards, not all individual polyphenols were quantified. Samples were hydrolyzed to remove the glycosides attached to the polyphenols, and only parent compounds were measured in this method. This allowed determination of the total polyphenols with a given parent compound, rather than attempting to make an exhaustive analysis of all possible glycosides, which is more difficult and costly and is impractical.

Since all samples were filtered through 0.22 µm filters, the following results cannot be expected to exactly represent the true concentration of individual polyphenols in the juice/cider. Especially for cloudy juice, the results may not reflect the true concentration of individual polyphenols that would be present in the product. However, filtration of the sample is absolutely necessary to protect the instrument used in this analysis. As all samples were treated the same throughout, we can make accurate comparisons across treatments with the former statements in mind. The concentration of each compound was quantified by the standard curve generated using authentic standards for each compound. The calibration curves are listed in Table 4.9 with corresponding R² (the concentration of Cinnamtannin A2 and procyanidin C1 were below the limit of quantification, thus they were not reported).

4.9.1 Procyanidin B1 (PC B1)

Apple juice treated with different clarification methods

Cider made from juice treated with different clarification methods
Concentrations of PC B1 in apple juice treated with different clarification methods and the finished cider are shown in Figure 4.28. Concentration of PC B1 was 0.0605±0.0358 µg/mL in the raw juice and 0.5895±0.1268 µg/mL in the pectinase treated juice. The concentration of PC B1 was significantly higher in the pectinase treated juice than the rest of the juices. The concentration of PC B1 in the raw juice was higher than the reported PC B1 concentration in the flesh of Newtown Pippin (0.02 µg/g)\textsuperscript{113}. This may be due to the fact that in this experiment the York juice was pressed with peel and peel contains more polyphenols than flesh, which could result in higher juice polyphenol than flesh polyphenol concentration. The difference could also be attributable to cultivar differences.

Concentration of PC B1 was 0.0405±0.0198 µg/mL in the cider fermented from raw juice and 0.0589±0.0236 µg/mL in the cider fermented from the pectinase treated juice. No significant difference was found on the PC B1 concentrations in ciders.

### 4.9.2 Catechin
Concentrations of catechin in apple juice treated with different clarification methods and the finished cider are shown in Figure 4.29. Concentration of catechin was 0.1298±0.0338 µg/mL in the raw juice and 0.3843±0.0239 µg/mL in the pectinase treated juice. The concentration of catechin was significantly higher in the pectinase treated juice than the rest of the juices. The concentration of catechin in the raw juice was within the reported mean levels of catechin (0.0041 µg/g to 0.9787 µg/g) in apples.

Concentration of catechin was 0.0405±0.0198 µg/mL in the cider fermented from raw juice and 0.0589±0.0236 µg/mL in the cider fermented from the pectinase treated juice. No significant difference was found in the catechin concentrations in ciders. But the concentration of catechin was decreased from the pectinase treated juice to the finished cider (p=0.0002). An increase of catechin over the course of the cider production process has been previously reported by others on five cider apples grown in France, which was the opposite of our findings.

4.9.3 Epicatechin
Concentrations of epicatechin in (A) York apple juice treated with different clarification methods and the finished cider were shown in Figure 4.30. Concentration of epicatechin was 0.2955±0.0591 µg/mL in the raw juice and 0.8494±0.0117 µg/mL in the pectinase treated juice. The concentration of epicatechin was significantly higher in the pectinase treated juice than the rest of the juices. Concentration of (−)-epicatechin in the raw juice was within the reported range of mean levels of epicatechin (0.0484 µg/g to 3.885 µg/g) in apples, but lower than the mean concentration of (−)-epicatechin (38.37 mg/L) in 46 Spanish cider apples.

Concentration of epicatechin was 0.2797±0.0877 µg/mL in the cider fermented from raw juice and 0.3682±0.0492 µg/mL in the cider fermented from the pectinase treated juice. No significant difference was found on the epicatechin concentrations in ciders. But the concentration of epicatechin was decreased from the pectinase treated juice to the finished cider, which was consistent with previously observed results. The effect of juice clarification treatments on epicatechin in juice and cider followed the same trend as observed with catechin, as would be expected due to the similarity in structure of these two compounds (Figure 2.5)

4.9.4 Procyanidin B2 (PC B2)
Concentrations of PC B2 in apple juice treated with different clarification methods and the finished cider are shown in Figure 4.31. Concentration of PC B2 was 0.0910±0.0477 μg/mL in the raw juice and 0.8417±0.0962 μg/mL in the pectinase treated juice. The concentration of PC B2 was significantly higher in the pectinase treated juice than the rest of the juices. Concentration of PC B2 in the raw juice was within the reported range of mean levels of PC B2 (0.006 μg/g to 3.854 μg/g) in apples\textsuperscript{113}, and was higher than the reported mean concentration of PC B2 (44.27 mg/L) in 46 Spanish cider apples\textsuperscript{45}.

Concentration of PC B2 was 0.2165±0.0568 μg/mL in the cider fermented from raw juice and 0.2567±0.0689 μg/mL in the cider fermented from the pectinase-treated juice. No significant difference was found in the PC B2 concentrations in ciders.

4.9.5 Procyanidin B5 (PC B5)
Concentrations of PC B5 in apple juice treated with different clarification methods and the finished cider are shown in Figure 4.32. Concentration of PC B5 was 0.0407±0.0072 µg/mL in the raw juice and 0.1563±0.0237 µg/mL in the pectinase treated juice. The concentration of PC B5 was significantly higher in the pectinase treated juice than the rest of the juices. Concentration of PC B5 in the raw juice was within in the reported range of mean levels of PC B5 (0.001 µg/g to 1.262 µg/g) in apples.\(^\text{113}\)

Concentration of PC B5 was 0.0452±0.0140 µg/mL in the cider fermented from raw juice and 0.0525±0.0072 µg/mL in the cider fermented from the pectinase treated juice. No significant difference was found on the PC B5 concentrations in ciders.

4.9.6 Phloretin
Concentrations of phloretin in apple juice treated with different clarification methods and the finished cider are shown in Figure 4.33. Concentration of phloretin was 0.0015±0.0002 µg/mL in the raw juice and 0.0057±0.0027 µg/mL in the pectinase treated juice. The concentration of phloretin was significantly higher in the pectinase treated juice than the raw juice and the settled juice. Concentration of phloretin in the raw juice was within the reported mean levels of phloretin (0 µg/g to 309.444 µg/g) in apples.\(^\text{113}\)

Concentration of phloretin was 0.0398±0.0069 µg/mL in the cider fermented from raw juice and 0.0403±0.0022 µg/mL in the cider fermented from the pectinase treated juice. No significant difference was found on the phloretin concentrations in ciders. Alcohol produced from the fermentation may help with extraction and release more phloretin in the finished cider, since phloretin is soluble in ethanol, but insoluble in water.\(^\text{159}\) Further research is needed to explain why the concentration of Phloretin is higher in the finished ciders than in the juices.
4.9.7 Chlorogenic acid

Figure 4.34 Concentrations of Chlorogenic acid in (A) York apple juice treated with pre-fermentation different clarification methods and (B) cider made from these juices (Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by one-way ANOVA and Tukey’s HSD test)

Concentrations of chlorogenic acid in apple juice treated with different clarification methods and the finished cider are shown in Figure 4.34. Concentration of chlorogenic acid was 5.2806±0.6929 µg/mL in the raw juice. The mean concentration of chlorogenic acid was not significantly different among any of the juices, regardless of pre-fermentation juice clarification treatment applied. Concentration of chlorogenic acid in the raw juice was within the reported mean levels of chlorogenic acid (0.5878 µg/g to 6.1924 µg/g) in apples. Chlorogenic acid was the most abundant individual polyphenol compound quantified in this study and comprised 88.2% of the total individual polyphenols analyzed in the raw juice. Similar results have been reported that the chlorogenic acid accounted for 32.5% to 79.1% of the total polyphenols in five cider apple cultivars. The yield of chlorogenic acid is relatively high (65%) through the course of apple juice processing.
Concentration of chlorogenic acid was 5.1833±0.1037 µg/mL in the cider fermented from raw juice and 5.3867±0.2687 µg/mL in the cider fermented from the pectinase treated juice. No significant difference was found in the chlorogenic acid concentrations in ciders. No change of chlorogenic acid concentrations has been reported between juice and cider over the course of cider fermentations and our results are in agreement with this finding. Notably, an increase of the concentration of 4-pcoumaroylquinic acid, a sub-compound within the chlorogenic acid category, has been observed in grape wine fermentation.  

4.9.8 Quercetin

![Figure 4.35](image)

**Figure 4.35** Concentrations of quercetin in (A) York apple juice treated with different pre-fermentation clarification methods and (B) cider made from these juices (Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) between treatments by one-way ANOVA and Tukey’s HSD test)

Concentrations of quercetin in apple juice treated with different clarification methods and the finished cider are shown in Figure 4.35. Concentration of quercetin was 0.0200±0.0079 µg/mL in the raw juice. The concentration of quercetin was not significantly different among the juices. Concentration of quercetin in the raw juice was
markedly lower than the reported mean levels of quercetin (0.1447 µg/g to 2.2275 µg/g) in apples\textsuperscript{113}.

Concentration of quercetin was 0.0972±0.0136 µg/mL in the cider fermented from raw juice and 0.1372±0.0070 µg/mL in the cider fermented from the pectinase treated juice. No significant difference was found on the quercetin concentrations in ciders, although like phloretin, quercetin concentration increased from juice to fermented cider.

Quercetin together with phloretin comprises the dihydrochalcone class. They comprise 0.35\% of the total polyphenol in the raw juice in our study. The percentage we observed was lower than the previous reported percentages in apples (from 2.3 to 12\%)\textsuperscript{148}. This may be due to oxidation during the clarification process. The yield of dihydrochalcone compounds through extraction was high (80\%) in the apple processing\textsuperscript{151}. Research has shown that the concentration of dihydrochalcone did not vary during the fermentation\textsuperscript{148}.

4.9.9 Total polyphenols by UPLC/MS
Total polyphenols concentration was obtained by adding the concentration of all individual polyphenols by UPLC/MS (shown in Figure 4.36). The concentration of total polyphenols in the pectinase-treated juice (9.1945±0.6402 µg/mL) was higher than the rest of the juices. But no significant difference was found on the total polyphenols concentration in the finished cider.

Table 4.10 P-values of comparisons of mean concentration of individual polyphenols in juices treated with different clarification methods and the corresponding cider

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Raw juice vs paired cider</th>
<th>Settled juice vs paired cider</th>
<th>Centrifuged juice vs paired cider</th>
<th>Pectinase-treated juice vs paired cider</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC B1</td>
<td>0.4580</td>
<td>0.1767</td>
<td>0.2655</td>
<td>0.0159</td>
</tr>
<tr>
<td>Catechin</td>
<td>0.4955</td>
<td>0.2204</td>
<td>0.3638</td>
<td>0.0002</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>0.8313</td>
<td>0.2008</td>
<td>0.2732</td>
<td>0.1778</td>
</tr>
<tr>
<td>PC B2</td>
<td>0.0455</td>
<td>0.0482</td>
<td>0.1876</td>
<td>0.0016</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>0.8027</td>
<td>0.3552</td>
<td>0.2889</td>
<td>0.0023</td>
</tr>
<tr>
<td>PC B5</td>
<td>0.6505</td>
<td>0.2500</td>
<td>0.2560</td>
<td>0.0114</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.0026</td>
<td>0.0022</td>
<td>0.0521</td>
<td>0.0003</td>
</tr>
<tr>
<td>Phloretin</td>
<td>0.0107</td>
<td>0.0009</td>
<td>0.0346</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total polyphenols by UPLC/MS</td>
<td>0.8178</td>
<td>0.5630</td>
<td>0.3554</td>
<td>0.0155</td>
</tr>
</tbody>
</table>

Statistical analysis was also conducted on the mean values of individual polyphenol compounds in the juices treated with different clarification methods and the paired ciders. Table 4.10 lists all the p values from these comparisons. The concentration of PC B1, catechin, PC B2, epicatechin, PC B5 was lower in the cider fermented from the pectinase-treated juice than the pectinase-treated juice. This may due to the oxidation of
these compounds during the fermentation. We did not expect all the compounds and all the juice/ciders from each treatment being oxidized in the same way. Dimers with more hydroxyl groups are easier to be oxidized than the monomers with less hydroxyl groups. More oxidation on the juice/cider from the static settling and centrifugation are expected than the raw and pectinase-treated juice/cider, because the static settling and pectinase treatment take longer time than raw juice (without clarification treatment) and centrifugation. This issue may be solved by adding SO₂ into the juice before clarification treatments to protect the polyphenols in the juice from oxidation. The concentrations of Quercetin and Phloretin were higher in all the ciders than the paired juices.

Studies have shown that most of the polyphenols in apple were located in the peel on fresh weight basis. The higher concentrations of three individual polyphenols (PC B2, phloretin, and quercetin) in the ciders than in the juices may due to better extraction of the polyphenols compounds during the fermentation, especially with the help of ethanol produced during fermentation. Small particles of peels and seeds were present during the fermentation since we juiced the whole fruit for the juice. The juice did pass through a screen upon exiting the juicer, but small particles would be expected to pass through this macro screen. Polyphenols in the peels and seeds may be released into solution slower than those in the flesh. This may explain the fact that for quercetin and phloretin, lower concentrations were detected in the juice than in the cider. Quercetin and phloretin in York apple, as with polyphenols in most apple cultivars, are richer in the peel than in the flesh. We could reasonably expect that upon soaking in a solution with increasing ethanol concentration, the concentration of quercetin and phloretin would increase in the cider.
To summarize, the concentrations of individual polyphenols in apple juice treated with different pre-fermentation juice clarification treatments and in the corresponding cider did not follow a uniform trend across all compounds. Concentrations of PC B1, catechin, epicatechin, PC B2, PC B5, and phloretin were higher in the pectinase-treated juice than in juices from other treatments. Concentration of chlorogenic acid and quercetin remained the same in the juices treated with different clarification methods compared to the raw juice. The increase in the pectinase-treated juice may be due to the fact that pectinase breaks down more solids in the juice, releasing more polyphenol compounds that were originally bonded within the solids into the juice. Chlorogenic acid possibly bonds less to the solids and is mostly soluble in the juice, so its concentration was not changed by the clarification processes.

Concentration of quercetin and the total polyphenols concentration assessed by UPLC/MS were higher in the cider fermented from the pectinase-treated juice. But no difference was found in the concentrations of PC B1, catechin, chlorogenic acid, PC B2, epicatechin, PC B5, and phloretin among the ciders, even though differences were detected in the juices that were used to make these ciders. This finding indicates that research on the impact of juice clarification processes on apple juice chemistry may not be directly applicable to ciders that would be made from these juices. Cidermakers should look to research results specifically addressing cider chemistry, and not assume that findings in apple juice will hold true for ciders, even those made from the apple juices analyzed. Changes in chemistry from juice to cider may result from oxidation, extraction by ethanol, yeast metabolism, or further loss of polyphenols bound to yeast cell walls or other solids during the racking process.
Polyphenol concentration and composition impacts the sensory properties of cider, most importantly bitterness and astringency (although color would also be affected). Most of the individual polyphenol compound concentrations (8 out of 10 compounds, which making up 99.65% of the total polyphenols quantified by the UPLC/MS method) remained the same in the cider fermented from the clarified juice compare to cider fermented from the raw juice. If pre-fermentation juice clarification to 100 to 250 NTU is desired to prevent the formation of H₂S during cider fermentation (as is standard practice in white winemaking conditions), cider makers should not be concerned with the loss of polyphenols in the finished cider due to clarification by pectinase, centrifugation, or static settling of pre-fermentation apple juice. Sensory evaluation is warranted to determine whether our analytical findings are indeed indicative of sensory impact of these treatments.

4.10 Comparison of results by different analytical methods

4.10.1 YAN by enzymatic kit and total amino acids by UPLC/PDA

![Graph showing comparison of YAN and total amino acids across different clarification methods.](image-url)
Figure 4.37 Comparison of total amino acid concentrations in juice by UPLC/PDA and enzymatic kit (Values are reported as mean ± standard deviation for n=3 replicates and common superscripts indicate no significant difference (P>0.05) within each treatment by two analytical methods by one-way ANOVA and Tukey’s HSD test)

Figure 4.37 compares the YAN concentration by adding individual amino acid (in pmol/µL) quantified by UPLC/PDA amino acid analysis to the YAN concentration measured by enzymatic kit. In order to obtain results that could be directly compared, the results from the UPLC/PDA for individual amino acid concentration were multiplied by the proportion of the molecular weight of each compound made up of yeast assimilable nitrogen (one nitrogen per amino acid was assumed for the purposes of this comparison, as this is what the YAN assay also quantifies). Although Proline is not measured by the enzymatic method (Proline lacks a primary amino group that can be bonded by the OPA/NAC reagent), apple juice only contains a small amount of Proline (0.05% of the total amino acid by mg/L). UPLC/PDA amino acid analysis does not measure ammonia, but the present juice samples contain trace concentration of ammonia (below the limit of detection of the enzymatic kit, and considered as 0 when calculating YAN).

We expected to see similar results from these two methods. However, in raw and settled juices, the results by UPLC/PDA were lower than the enzymatic assay. This may be due to the loss of amino acids by the filtration required before the samples were injected into the UPLC/PDA system, while the samples were not filtered in the YAN enzymatic assay.

4.10.2 Total polyphenols by Folin and by UPLC/MS
Figure 4.38 Comparison of total polyphenol concentrations in juice by UPLC/MS and Folin assay (Values are reported as mean ± standard deviation for n=3 replicates and different superscripts indicate significant difference (P<0.05) within each treatment by two analytical methods by one-way ANOVA and Tukey’s HSD test)

Figure 4.38 compared the total polyphenol concentration in the juice by adding all individual polyphenol compounds quantified by UPLC/MS method (following acid hydrolysis) and the measurement by Folin assay. In all juice samples, the results from former method were significantly lower than from the latter method. This possibly was due to the oxidation of polyphenols during the acid hydrolysis applied in sample preparation for UPLC/MS method. This may be also due to the filtration step before loading samples on the UPLC column in the UPLC/MS method, removing much solids in the juice. Nonetheless, if these methods were perfect, we would have expected to see higher values for total polyphenols than for individual polyphenols, since the individual polyphenols
analysis only quantified a very limited set of target compounds, albeit the most prevalent compounds in apple. Next steps should compare these two methods where acid hydrolysis and all other sample prep steps are consistent for both analytical methods.

![Graph showing comparison of total polyphenol concentrations in cider by UPLC/MS and Folin assay](image)

**Figure 4.39 Comparison of total polyphenol concentrations in cider by UPLC/MS and Folin assay** (Values are reported as mean ± standard deviation for n=3 replicates and different superscripts indicate significant difference (P<0.05) within each treatment by two analytical methods by one-way ANOVA and Tukey’s HSD test)

Figure 4.39 compares the total polyphenol concentration in the cider by adding all individual polyphenol compounds quantified by UPLC/MS (following acid hydrolysis) method and the measurement by Folin assay. As with the juices, in all ciders, the results from the former method were significantly lower than from the latter methods. This possibly was due to the same reason explained in the previous section dealing with juices.
4.10.3 Total procyanidins by DMAC and by UPLC/MS

Figure 4.40 Comparison of total procyanidin concentrations in juice by UPLC/MS and DMAC assay (Values are reported as mean ± standard deviation for n=3 replicates and different superscripts indicate significant difference (P<0.05) within each treatment by two analytical methods by one-way ANOVA and Tukey’s HSD test)

Figure 4.40 compares the total procyanidin concentration in the juice by adding all individual procyanidin compounds quantified by UPLC method (PC B1, PC B2, and PC B5) and the measurement by DMAC assay. Only in the pectinase treated juice, the result from former method was significantly higher than from the latter method. Most of the pectin still remained in the raw juice, settled juice, and centrifuged juice, and procyanidins may bond with the pectin. After adding the DMAC reagent, which was methanol based, the pectin was precipitated. The color compounds produced from the reaction by DMAC and procyanidins were present in the sediment and removed by centrifugation, thus
procyanidin concentrations by DMAC method were underreported in this case. Since procyanidin dimers and polymers may be degraded into monomers during the hydrolysis we would expect that not all procyanidins would have been quantified by the UPLC/MS method either. The UPLC/MS method was limited by the fact that it was designed for quantification of only 8 target compounds (and glycosides of these compounds, due to the hydrolysis sample prep step), for which procyanidin standards were commercially available, the two results varied in juices (but only the pectinase treated juices).

Figure 4.41 Comparison of total procyanidin concentrations in cider by UPLC/MS and DMAC assay (Values are reported as mean ± standard deviation for n=3 replicates and different superscripts indicate significant difference (P<0.05) within each treatment by two analytical methods by one-way ANOVA and Tukey’s HSD test)

Figure 4.41 compared the total procyanidin concentration in the cider by adding all individual procyanidin compounds quantified by UPLC method (PC
B1, PC B2, and PC B5) and the measurement by DMAC assay. Only in the cider fermented from centrifuged juice, the result from former method was significantly higher than from the latter method. For cider samples, pectin was no longer an interference in the DMAC method. As explained in 4.10.3, the results from UPLC method were expected to be different than from DMAC method.

As mentioned previously, Folin assay and DMAC method both have their limitations. Filtration of samples before injection is a required step to protect the UPLC system. Therefore, more research needs to be done on the validation of interference in the Folin and DMAC methods. Appropriate sample preparation needs to be developed to remove pectin prior to analysis for better quantification by the DMAC method.
Chapter 5: Conclusion and future direction

5.1 Hypotheses

5.1.1 Pre-fermentation clarification treatment by pectinase reduced the YAN concentration in the pre-fermentation apple juice. But static settling and centrifugation did not reduce the YAN concentration in the pre-fermentation apple juice. Cider makers are encouraged to measure YAN in the pre-fermentation apple juice to make sure the juice contains the appropriate concentration of YAN for fermentation, and to be aware that pectinase treatment may lead to improved clarification, but loss of YAN.

5.1.2 Pre-fermentation clarification treatments changed the composition of individual amino acids in the pre-fermentation apple juice. Different clarification methods impact the concentration of individual amino acids differently. Cider makers need to be aware that the amino acid composition will differ in juices clarified by different clarification treatments. These differences can be reasonably expected to impact cider aroma, but the extent of aroma differences imparted by the concentration changes observed in this study warrant further investigation.

5.1.3 Pre-fermentation clarification treatments reduced the total polyphenol concentration in the pre-fermentation apple juice, but these differences did not translate into differences in the total polyphenol concentration in the post-fermentation hard cider.

5.1.4 Pre-fermentation clarification treatments (static settling and centrifugation) reduced the total procyanidins concentration in the pre-fermentation apple juice but the total procyanidins concentration was higher in the pectinase treated juice
than the raw juice. Pre-fermentation clarification treatment (centrifugation) reduced the total procyanidins concentration in the post-fermentation hard cider, while other pre-fermentation clarification treatments (static settling and pectinase) did not. Therefore, cidermakers may wish to select a pre-fermentation juice clarification process other than centrifugation in order to maintain the highest concentration of procyanidins in the finished cider.

5.1.5 Pre-fermentation clarification treatments changed the composition of individual polyphenols in the pre-fermentation apple juice for all target compounds except phloretin, chlorogenic acid and quercetin, but did not change the composition of individual polyphenols in the post-fermentation hard cider, except for higher quercetin concentration observed in ciders that were treated with pectinase pre-fermentation. Future work should identify those compounds with the highest sensory impact (not necessarily those with the highest concentration) and use these findings to select pre-fermentation juice clarification strategies that will maintain the highest sensory impact of polyphenols in cider.

5.2 Future work

5.2.1 Clarification of Brown Snout (or other highly tannic) apple juice and pectinase treatment on apple juice

The effects of harvest time, post harvest storage, pectin type and polyphenol concentration in the juice on the clarification of Brown Snout (or other highly tannic) apple juice require further research. Targeted pectinase preparations, dosage rates and conditions should be developed based on specific apple pectin and tannin content.
5.2.2 Sensory analysis on finished cider

Due to small-scale fermentation, not enough cider was produced for sensory analysis. The total solids present in the wine contribute to the body of wine. The relationship between clarification treatments on pre-fermentation apple juice to sensory property of the finished hard cider is unknown, but remains exceedingly important.

5.2.3 Degree of polymerization of procyanidins

The intensity of bitterness and astringency in the cider is related to the degree of polymerization of procyanidins. Oligomeric procyanidins (DP 2-5) lead to bitterness, while more polymerized structures (DP 6-10) provide astringency. But signal for proanthocyanidins with higher DP is very small on QDA detector. Using a separate assay to determine mDP (mean degree of polymerization) would be a useful next step in understanding how analytical data on apple polyphenols can be best related to sensory character of cider.

5.2.4 Analytical method development

More accurate and faster analytical methods on total polyphenols concentration in apple juice and hard cider need to be developed. The extent of SO₂ and reducing sugars in the apple juice impacting the total polyphenols by Folin assay needs to be assessed. Pectinase clarification or other sample preparation steps to remove the pectin interference in the DMAC method need to be developed. Cone voltage optimization of MS on each compounds need to be studied to decrease the limit of detection and quantification on the MS detector.
Appendices

Appendix A

Protocols for measuring pH and titratable acidity

1. Calibrate the pH meter
2. Fill a burette with freshly made 0.1 mol/L NaOH
3. Add 75mL degassed distilled water to a 100mL beaker to ensure that the pH electrode will be adequately covered when it is immersed into the distilled water. Place a small stir bar in the beaker.
4. Rinse the pH electrode with distilled water. Position the electrode in the beaker so that it does not touch the side of the beaker, fully immersed in distilled water, and is above the level of the stir bar.
5. Position the burette vertically and the outlet is above the beaker.
6. Accurately pipet 10mL juice/degassed cider into the beaker containing the distilled water
7. Record the initial burette reading to 2 decimal place
8. Record the pH of initial juice
9. Slowly titrate the NaOH solution into the beaker with constant and gentle stirring.
   Observe the change in pH. As the pH approaches 7, slow down the addition rate of NaOH and continue to add dropwise until the pH of the solution is 8.2.
10. Record the final burette reading to 2 decimal place.
11. Calculation

   \[
   \text{Titratable acidity} = 67 \times \text{molarity of } \text{NaOH} \times \frac{\text{titre value (mL)}}{\text{volume of juice/wine (mL)}} [\text{g/L}] 
   \]
Appendix B

Primary Amino Nitrogen (PAN) Assay Procedure

Limit of detection:

2.59 mg/L, when the absorbance difference is 0.020 and the sample volume is 0.05 mL.

Linear range: 0.2 to 10 µg of amino nitrogen per assay

Procedures:

1. Warm Bottle 1 to room temperature and remove any moisture from the outside of the bottle. Dissolve the tablets (containing NAC) from Bottle 1 in distilled water as solution 1; 1 tablet per 3 mL of distilled water.

2. Add 1.5 mL solution 1 to each plastic cuvette with light path of 1 cm.

3. Add 0.025 mL of sample to each plastic cuvette, use distilled water instead for Blank, and Isoleucine standard solution (140 mg of nitrogen/L) from Bottle 3 for Standard.

4. Mix by gently inverting the cuvette with caps several times. Wait for 2 minutes and read the absorbance under wavelength of 340 nm. Record the reading as $A_1$.

5. In the dark, add 0.05 mL of solution from Bottle 2 (OPA in ethanol (96% v/v)) and mix by gently inverting the cuvette with caps several times. Wait for 15 minutes and read the absorbance under wavelength of 340 nm. Record the reading as $A_2$.

6. Calculate the concentration of PAN in the samples as following:

$$
\Delta A_{\text{PAN}} = (A_2, \text{ sample} - A_1, \text{ sample}) - (A_2, \text{ blank} - A_1, \text{ blank})
$$

$$
c = \frac{V \times MW \times 1000}{\varepsilon \times d \times V} \times \Delta A_{\text{PAN}} = 129.74 \times \Delta A_{\text{PAN}} \text{ [mg of N/L]}
$$

where:

$V = \text{final volume [mL]} = 1.575 \text{ mL}$

$MW = \text{molecular weight of nitrogen [g/mol]} = 14.01 \text{ g/mol}$
1000 = conversion from g to mg of N/L

$\varepsilon$ = extinction coefficient of isoindole derivative at 340 nm = 6803 l/(mol•cm)

d = light path [cm] = 1 cm

v = sample volume [mL] = 0.025 mL
Appendix C

Ammonia assay procedure

Limit of detection:
0.071 mg/L, when the absorbance difference of 0.020 in 2.00mL of sample.

Linear range: 0.2 to 7 μg of ammonia per assay

Procedures:

1. Dissolve the content of Bottle 2 in 12 mL of distilled water.
2. Add 2.00 mL of distilled water to each plastic cuvette with light path of 1cm.
3. Add 0.10 mL of sample to each plastic cuvette, use distilled water instead for Blank, and Ammonia standard solution in Bottle 4 (0.04mg/mL isoleucine in 0.02% (w/v) sodium azide) for Standard.
4. Add 0.30 mL of solution from Bottle 1 (buffer contains 2-oxoglutarate and sodium azide (0.02% w/v) as a preservative) and 0.20 mL of solution from Bottle 2 (NADPH) to each plastic cuvette.
5. Mix by gently inverting the cuvette with caps several times. Wait for 2 minutes and read the absorbance under wavelength of 340 nm. Record the reading as $A_1$.
6. Add 0.02 mL of solution from Bottle 3 (Glutamate dehydrogenase suspension) and mix by gently inverting the cuvette with caps several times. Wait for 5 minutes and read the absorbance under wavelength of 340 nm. Record the reading as $A_2$.
7. Calculate the concentration of ammonia in the samples as following:

\[
\Delta A_{\text{ammonia}} = (A_{2, \text{ sample}} - A_{1, \text{ sample}}) - (A_{2, \text{ blank}} - A_{1, \text{ blank}})
\]

\[
e = \frac{V \times MW}{\varepsilon \times d \times V} \times \Delta A_{\text{ammonia}} = 0.07082 \times \Delta A_{\text{PAN}} [\text{g/L}]
\]

where:
\[
V = \text{final volume [mL]} = 2.62 \text{ mL}
\]
\[
\text{MW} = \text{molecular weight of ammonia [g/mol]} = 17.03 \text{ g/mol}
\]
\[
\varepsilon = \text{extinction coefficient of NADPH at 340 nm} = 6300 \text{ l/(mol} \cdot \text{cm)}
\]
\[
d = \text{light path [cm]} = 1 \text{ cm}
\]
\[
v = \text{sample volume [mL]} = 0.10 \text{ mL}
\]
Appendix D

Fermentation protocol

1. Autoclave 18×500 mL flasks with the stirs bars, and prepare the sanitizer solution as following: Add 2 teaspoons of potassium metabisulfite and 1 tablespoon of citric acid in 3 gallon of water.

2. York apples are sliced, then juiced by table top juicer to get 18L juice. Samples are taken from the raw juice and freeze (500mL). Each 400 mL of raw juice will be placed in 500mL flask in triplicates for fermentation (raw juice fermentation 123).

3. Clarification treatment on raw juice
   a. 1000 mL of raw juice will be placed in 1L flask in triplicates for static settling at 10C overnight (total 3L). Each 400 mL of clarified juice will be placed in 500mL flask in triplicates for fermentation (settled juice fermentation 123). The rest of the settled juiced will be frozen for future analysis
   b. 3000 mL of raw juice will be centrifuged and each 400 mL of juice will be placed in 500mL flask in triplicates for fermentation (centrifuged juice fermentation 123). The rest of the centrifuged juiced will be frozen for future analysis.
   c. 1000 mL of raw juice will be placed in 1L flask in triplicates for pectinase treatment at 3 levels (total 9L).
      i. One is the recommended dosage as 1.0ul/100mL (10.0ul/1000mL in each flask). Each 400 mL of clarified juice will be placed in 500mL flask in triplicates for fermentation (pectinase-treated juice A fermentation 123). The rest of the clarified juiced will be frozen for future analysis.
ii. One is dosage of 0.03ul/100mL (0.3ul/1000mL in each flask), that the turbidity of clarified juice will match with the centrifuged juice. Each 400 mL of clarified juice will be placed in 500mL flask in triplicates for fermentation (*pectinase-treated juice B fermentation 123*). The rest of the clarified juiced will be frozen for future analysis.

iii. One is dosage of 0.06ul/100mL (0.6ul/1000mL in each flask), that the turbidity of clarified juice will fall within the range of 100-250 NTU. Each 400 mL of clarified juice will be placed in 500mL flask in triplicates for fermentation (*pectinase-treated juice C fermentation 123*). The rest of the clarified juiced will be frozen for future analysis.

4. Fermentation (6 treatment*3=18 total)
   a. Potassium metabisulfite is added at 100mg/L, which will yield 58.8 mg/L (58.8 ppm) of SO₂ in the juice. Free SO₂ required for apple juice with pH 3.4 is 33mg/L, but regarding to the SO₂ that bonds with solids in juice and bigger surface area to volume ratio in small scale fermentation, targeting SO₂ addition is more than 33mg/L. 1 g potassium metabisulfite is dissolved in 25 mL warm water to make 0.04g/mL solution. Add 1mL of 0.04g/mL solution to 400mL juice in each flask and mix.
   b. After a few hours, add Fermaid K as the recommended dosage 25g/hl. This will provide 25mg N/L. 2.5 g Fermaid K is dissolved in 25 mL room-temperature water to make 0.1g/mL solution. Add 1mL of 0.1g/mL solution to 400mL juice in each flask and mix.
   c. Add DAP.
Take 5.0 g DAP and dissolve in 25 mL of water to make 0.2g/mL solution. Add 1.0 mL of the solution to each 400mL juice, and the final concentration in juice is 0.50g/L. This will provide 100mg N/L to the juice.

(DAP, dosage 25g/hl=0.25g/L, will provide 50mg N/L according to the Cider Handbook, page 18). Based on Tom’s results, the YAN concentration in Old York is 65mg/L, so with addition of Fermaid K and DAP, the final nitrogen concentration will be 65+25+100=190 mg N/L, which is above the minimum limit 140 mg N/L and will make sure the fermentation can go to the end.

d. Add the yeast.

The EC1118 yeast (Scott Labs, Petaluma, CA, USA) is rehydrated and inoculate at the concentration of 25g/hL. 2.5 g yeast is dissolved in 25 mL 40°C water to make 0.1g/mL solution, and stir gently to break any clumps. Let the suspension stand for 20 minutes, then stir gently again. Take out 1mL of the yeast suspension, and slowly add 1mL juice to it. After 10 minutes, every 2 mL of juice-yeast suspension mixture is added to each of the flask.

e. The rubber stoppers and airlocks are sanitized and put on the flasks, and then flasks are put into a refrigerator at 18°C.

f. The weight of each flask are measured and recorded every 12 hours until the difference of measurement are consistent at 4 data points (2 days). Samples will be taken from each flask to measure the residue sugar by enzymatic kit. The fermentations will be considered dry when the residue sugar is 0.2 g/L (<0.2% by weight).

g. Samples will be taken and frozen from each flask for future analysis.
Appendix E

Residual Sugar

Enzymatic kit method

1. Shake bottle 3 and 4 gently to remove any enzymes that left on the robber stopper.

2. Transfer the all the solution in bottle 4 (PGI) into bottle 3 (HK/G69-DH) and gently swirling to form the mixture suspension of HK/G69-DH/PGI.

3. Pipette 2.00 mL room temperature distilled water into cuvette with 1cm light path, and mix the cuvette with sealing cap after adding 0.1 mL cider sample (0.1mL distilled water for blank), 0.1 mL solution I (buffer), and 0.1 mL solution II (NADP+/ATP) into the cuvette.

4. Read the absorbance ($A_1$) at 340 nm after 3 minutes. Add 0.04 mL of suspension HK/G69-DH/PGI and mix. Read the absorbance ($A_2$) at 340 nm after 10 minutes.

5. Calculation:

\[
\Delta A_{D\text{-glucose} + D\text{-fructose}} = (A_2, \text{sample} - A_1, \text{sample}) - (A_2, \text{blank} - A_1, \text{blank})
\]

\[
c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A \text{[g/L]}
\]

$c$=concentration of D-glucose and D-fructose[g/L]

$V$=final volume=2.34 [mL]

$MW$=molecular weight of D-glucose or D-fructose=180.16 [g/mol]

$\varepsilon$=extinction coefficient of NADPH at 340 nm=6300 [l×mol⁻¹×cm⁻¹]

$d$=light path=1 [cm]

$v$=sample volume=0.1 [mL]

For measuring D-glucose and D-fructose,

\[
c = \frac{2.34 \times 180.16}{6300 \times 1 \times 0.1} \times \Delta A_{D\text{-glucose} + D\text{-fructose}} = 0.6692 \times \Delta A_{D\text{-glucose} + D\text{-fructose}} \text{[g/L]}
\]
Appendix F

Total polyphenols by Folin method

1. Dilute 2N Folin-Ciocalteu Reagent from Sigma into 0.2 N.

2. Weight out 7.5g of sodium carbonate (Na$_2$CO$_3$) and dissolve in 100mL water to make 7.5% saturated Na$_2$CO$_3$ solution.

3. Weight out 0.1026g of garlic acid and dissolve in 100mL water to make 1.0 g/L gallic acid stock solution. Prepare 0.0, 0.1, 0.2, 0.3, 0.4, 0.5 g/L gallic acid standard solutions using the 1.0g/L stock standard solution.

4. Mix 50 µl apple juice/cider sample and the gallic acid standards with 450 µl H$_2$O in cuvette. Then add 1.25 mL 0.2 N Folin-Ciocalteau reagent into each cuvette and mix.

5. Add 1.0 mL saturated Na$_2$CO$_3$ solution and mix.

6. Read absorbance at 765 nm after 2 hr.

7. Conduct regression between the absorbance vs. concentrations of standard solutions. Determine the concentrations of the samples based on the regression
Appendix G

Total proanthocyanidins by DMAC method [12]

This colorimetric assay, utilized by Payne et al., will be performed with modifications to assess the total amount of proanthocyanidins in the apple juice and cider using the reagent 4-dimethylaminocinnamaldehyde (DMAC).

1. DMAC solution
   
   This solution will be prepared by combining 6.0 mL HCl and 54 mL methanol, which will cool for 15 min at 4°C before adding 0.06 g DMAC to the solution and mixing thoroughly, for a total of 60 mL DMAC solution.

2. The apple juice and cider samples will be compared to a standard curve using methanol as the blank and standard solutions of procyanidin B2 stock solution (0.1mg/mL in methanol) with concentrations of 1, 10, 50, and 100 ppm. The cuvette with 1 cm light path will be filled with either 200 μL apple juice/cider samples or 200 μL standard solutions for preparation of the standard curve.

3. 1mL DMAC solution will be pipetted into each well and the absorbance will be read at 640 nm.
Appendix H

Example chromatography of standard (top) and raw juice sample (bottom) from amino acid analysis
Reference


52. Joslyn, M. A.; Goldstein, J. L., Astringency of Fruit and Fruit products in Relation to Phenolic ContentS. *Advanced Food Research* 1964, 13, 179-217.


80. Miller, N. J.; Rice-Evans, C. A., The relative contributions of ascorbic acid and phenolic antioxidants to the total antioxidant activity of orange and apple fruit juices and blackcurrant drink. Food Chemistry 1997, 60, 331-337.


101. Primary amino nitrogen (PAN) assay procedure; Megazyme: 2014.

102. Ammonia (rapid) assay procedure; Megazyme: 2014.

103. ACQUITY UPLC H-Class and H-Class bio amino acid analysis system guide; Waters Corporation: 2012.
125. Ye, M.; Yue, T.; Yuan, Y., Changes in the profile of volatile compounds and amino acids during cider fermentation using dessert variety of apples. European Food Research and Technology 2014, 239, 67-77.
143. Anaspec General Properties of Amino Acids.