

Virginia-Grown Barley for Craft Brewing: Evaluation of Free Amino Nitrogen Content and Malt Sensory

Characteristics

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## **Technical Abstract**

Regionally-Grown barley is in demand for craft malting and brewing in Virginia. Barley lines suited to both Virginia's climate and craft brewing applications are currently under development. Free amino nitrogen (FAN) is a malt quality parameter that influences beer flavor directly and via yeast metabolism during fermentation. FAN and the individual amino acids making up FAN influence yeast health, beer color development, flavor, and flavor stability. Despite potential impacts on beer quality, individual amino acid concentrations in barley and malt are not generally measured or monitored. The objective of this project is to evaluate and assess the conversion of FAN and individual amino acid concentrations during the malting and mashing process of genetically distinct varieties. An additional objective is to evaluate their malt sensory characteristics, to understand genetic variability therein. Raw barley and malt samples were subject to low temperature aqueous extraction, and wort was produced using an isothermal hot water extract technique. FAN and amino acid composition were determined for each line for raw barley, malt and wort extracts, prepared as described. Statistical analysis revealed that these lines had significantly different changes in FAN and amino acid composition. Sensory characteristics of malt hot steep teas were evaluated by forty (N=40) panelists with brewing experience using a sorting task to group malts with similar sensory characteristics, and to assign descriptors to those groups. From the sixteen (N=16) breeding lines, five (5) distinct sensory groups were identified. Taken together, our findings will inform the selection process for barley

lines for craft brewing, and add to the knowledge of the extent to which free amino acid composition varies among eastern barley lines and along the malting and mashing processes.

# **Virginia-Grown Barley for Craft Brewing: Evaluation of Free Amino Nitrogen Content and Malt Sensory Characteristics**

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## **General Audience Abstract**

Regionally-grown barley is in demand for craft malting and brewing in Virginia, and barley lines suited to both Virginia's climate and craft brewing applications are of interest. The production of beer requires high quality malt, which is defined by many parameters; one of which is free amino nitrogen (FAN), which plays a critical role in yeast nutrition and beer flavor through yeasts' metabolism and chemical reactions. FAN is a general measurement of nitrogen and its individual components (including amino acids) are rarely measured, despite their major role in beer flavor. The objective of this project was to assess the development and changes in FAN and amino acids that occur over the course of the barley supply chain – which will better inform breeders, growers, maltsters, and brewers of the raw materials they use and how they can affect their processes. A second objective was to assess the flavor of newly developed barley lines and varieties, and compare them to established varieties currently being grown in Virginia through sensory evaluation methods designed to determine similarities between samples. Results from the analysis of FAN and the amino acid composition identified significant differences between varieties, and results from sensory evaluation identified 5 distinct groups with similar flavor characteristics. Taken together, these findings will help inform the selection process for barley lines for craft brewing, and add to the knowledge of the extent to which free amino acid composition varies among barley lines and along the brewing processes.

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# Table of Contents

|                                                                                              |           |
|----------------------------------------------------------------------------------------------|-----------|
| <b>CHAPTER 1: INTRODUCTION</b>                                                               | <b>1</b>  |
| IMMEDIATE OBJECTIVE                                                                          | 3         |
| SPECIFIC AIMS AND HYPOTHESES                                                                 | 4         |
| <b>CHAPTER 2: LITERATURE REVIEW</b>                                                          | <b>6</b>  |
| A BARLEY FOR VIRGINIA.                                                                       | 6         |
| BARLEY PROTEINS                                                                              | 11        |
| MALTING PROCESS                                                                              | 14        |
| MASHING PROCESS                                                                              | 19        |
| FREE AMINO NITROGEN                                                                          | 22        |
| <i>Absorption &amp; Transport</i>                                                            | 23        |
| <i>The Effect of Individual Amino Acids on Fermentation</i>                                  | 26        |
| <i>Amino Acids &amp; Beer Flavor Development during Aging</i>                                | 30        |
| SENSORY EVALUATION                                                                           | 30        |
| <b>REFERENCES</b>                                                                            | <b>34</b> |
| <b>CHAPTER 3: EVALUATION OF FREE AMINO NITROGEN CONTENT AND MALT SENSORY CHARACTERISTICS</b> | <b>41</b> |
| ABSTRACT                                                                                     | 41        |
| INTRODUCTION                                                                                 | 42        |
| MATERIALS AND METHODS                                                                        | 43        |
| <i>Plant Material for Chemical Analysis</i>                                                  | 43        |
| <i>Plant Material for Sensory Analysis</i>                                                   | 43        |
| <i>Sample Preparation for Chemical Analysis</i>                                              | 44        |
| <i>Sample Preparation for Sensory Analysis</i>                                               | 46        |
| <i>Chemical Analysis</i>                                                                     | 47        |
| <i>Sensory Analysis</i>                                                                      | 48        |
| <i>Survey of Sensory Panelists with Brewing Experience</i>                                   | 50        |
| <i>Statistical Methods &amp; Data Analysis</i>                                               | 50        |
| RESULTS AND DISCUSSION                                                                       | 52        |
| <i>Free Amino Nitrogen</i>                                                                   | 52        |
| <i>Individual Amino Acid Concentration Profiles in Barley, Malt and Wort</i>                 | 62        |
| <i>Free Sorting of Malt Hot Steeps</i>                                                       | 75        |
| <i>Sensory Survey Data</i>                                                                   | 84        |
| CONCLUSIONS                                                                                  | 85        |
| REFERENCES                                                                                   | 87        |
| <b>CHAPTER 4: CONCLUSIONS AND FUTURE WORK</b>                                                | <b>92</b> |
| <b>REFERENCES</b>                                                                            | <b>96</b> |
| APPENDIX A                                                                                   | 107       |

## **Chapter 1: Introduction**

Malted barley is often referred to as the soul of beer. As one of only four main ingredients used in brewing (along with water, hops, and yeast), its contribution to unique flavors and characteristics of different beer styles is very significant. Differences in malt characteristics allow for incredible variety in beer styles and create distinctions in color, sweetness, body, and quite obviously – taste.

Before brewing, barley must first be malted. Malting involves three major steps, including steeping, germination, and kilning. Together, these work to degrade the barley kernel and fix the enzymatic content, resulting in a raw ingredient that can easily be used by brewers. Germination is the process in which the cell walls, proteins, and large starch granules are degraded; and kilning halts this process by reducing the moisture content and stopping the enzymatic degradation of starches before the amount of carbohydrates used for plant metabolism is too high. The level of degradation can be described by the term, “modification.” Barley modification, although an imprecise term (Herb et al., 2017), can be measured quantifiably using standard parameters that are listed on the malt’s certificate of analysis (COA). Specifications included on a certificate of analysis include (but are not limited to): diastatic power, beta-glucan, extract, and the soluble to total protein ratio. Briefly, these analyses determine the level of starch degrading enzymes, the cell walls remaining, the soluble solids available to the brewer, and the ratio of proteins that are soluble compared to the total amount of protein present in the malt, respectively.

A malt COA gives brewers a good understanding of the relevant characteristics for the starting material they are working with, and allows them the opportunity to change processes or recipes according to those values (Schmitt & Budde, 2010). The aforementioned parameters on a

typical malt COA give a good indication of potential beer quality and fermentation performance, but a discussion of all of these parameters is beyond the scope of this project. This thesis will focus on free amino nitrogen (FAN) and specific amino acid compositions present in malted barley, and the effects these parameters have on sensory and quality characteristics of malt and beer.

Historically, macro-breweries such as Anheuser-Busch and MolsonCoors set the industry standards for barley breeders and maltsters in terms of the development of barley lines for brewing applications. Due to their goals for brewing styles and their high use of adjunct (sugar sources other than malted barley, such as rice or corn), these companies demanded malted barley with high levels of protein, diastatic power (DP), and FAN. As craft breweries have increased in popularity, their increased market share has allowed them to also make demands of the malting industry to provide a wider range of products better suited to the needs of regional craft breweries. The Brewer's Association released a white paper in 2014 requesting maltsters to release more malts with lower DP values, lower protein content, lower Kolbach ratios, lower FAN, and notably: "distinctive flavors and aromas," (Bouckaert et al., 2014).

Brewers are currently looking for new and distinct flavors from their malt. The brewing industry has recently placed a lot of emphasis on research and development into new hop varieties and yeast strains. These trends can be traced to the popularity of hoppy styles such as the India Pale Ale (IPA) and other craft styles that contain hops and/or prominent yeast-derived aromas. Brewers would like to see this same research and development effort placed into the development of new barley varieties uniquely suited to craft beer, giving them an additional variable to make their brands stand apart in such a competitive industry, and to potentially offer opportunities to source a greater proportion of the raw materials from local farms through the

development of new barley varieties for pioneering regions. Barley varieties such as ‘Maris Otter,’ ‘Golden Promise,’ and ‘Barke’ are noted for their distinct taste, and are popular in beer styles with an emphasis on malt flavor. However, these varieties do not currently meet many agronomic performance standards compared to modern varieties, and therefore are not popular among barley growers. These heirloom varieties’ distinct flavor profiles indicate that it may be possible to breed barley varieties that both satisfy the goal of distinct flavor profiles, while also providing growers with high performing crops.

One area of current and increasing research interest into the effect of malt’s impact on beer flavor is focused on FAN concentration and composition. Nitrogen plays a critical role in beer quality. Nitrogenous compounds affect physical characteristics of beer such as haze, head retention, and stability; but they also play a role in yeast metabolism during fermentation – serving as precursors to essential flavor components such as esters and vicinal diketones (VDK’s). Because of this, barley malt FAN and likely the amino acid profile (the composition of amino acids that make up FAN) are important indicators of fermentation performance and overall beer flavor and quality (Hill & Stewart, 2019)

As such, the proposed project will address the following objectives to further explore malt FAN concentration and composition, and its potential impact on beer flavor.

### **Immediate Objective**

The overall objective of this research project is to assess differences in free amino acid content and composition of Virginia raw barley and their respective malts, and relate their potential variation to beer quality and sensory characteristics.

### **Long-Term Objective**

The long-term objective of this project is to examine and understand the relationship between the conversion of protein to free amino nitrogen during the malting process, and its effect on beer's quality and sensory characteristics.

### **Specific Aims and Hypotheses**

#### **Objective 1.**

Compare malt sensory characteristics of Virginia-grown malt barley breeding lines from the Virginia Tech small grains breeding program with current Virginia-grown barley varieties such as 'Violetta,' 'Calypso,' and 'Endeavor.'

#### **Working Hypothesis 1.**

Malt sensory characteristics from Virginia-grown malt barley breeding lines will differ significantly from pre-established Virginia-grown malt barley varieties.

#### **Objective 2.**

Determine the extent to which free amino nitrogen (FAN) and free amino acid concentrations vary among the selected set of malt barley breeding lines and varieties, and assess the conversion of barley FAN to malted barley FAN to wort for each line/variety in the sample set.

#### **Working Hypothesis 2.**

Free amino acid concentration and composition vary significantly between selected malt barley breeding lines and varieties.

#### **Objective 3.**

Communicate the results of this project to stakeholders in the craft brewing industry in Virginia, highlighting the sensory characteristics of Virginia-bred and pre-established Virginia-

grown malt barley varieties, and the potential of variation in FAN and amino acid content of craft malts to influence beer quality and sensory characteristics.

## **Chapter 2: Literature Review**

### **A Barley for Virginia.**

Barley breeding is a time intensive and laborious process. Breeders can take upwards of 10 to 15 years to develop a single variety, discarding alternative candidates throughout the entire process (Schmitt & Budde, 2010). Much of breeders' focus lies in disease resistance, desirable agronomic traits, and increasing yield. A new barley variety will only be accepted and recommended to farmers if it is a competitive line, meaning it performs at the same level as established barleys, or if it has qualities that are superior or unique relative to currently offered varieties (Schmitt & Budde, 2010). Breeders perform quality testing on a limited number of advanced breeding lines, and are not able to perform all the quality testing required to make sound conclusions until after the next season's crops are planted. This causes bottlenecks and slowdowns for breeding programs, only making the process take longer. Breeders, including the Virginia Tech Small Grains Breeding Program, seek to speed this process up and are currently working on methods for early evaluation of malting quality through the use of genomics and high throughput phenotyping (Santantonio & Loeb, 2022).

As one of the first domesticated crops, barley was historically a significant crop for human consumption, but has since been grown primarily as feed for livestock or malted barley for the brewing industry (Ullrich, 2010). As the United States' feed industry has shifted to prefer corn as the primary grain (Hutchins, 2022) and with the beer industry experiencing a boom around the turn of the century, the proportion of barley's end use as malt has increased significantly (Jennifer Bond, 2015). Due to malting barley's strict quality requirements, farmers receive a premium price compared to that of feed barley. Varieties that are destined for feed typically do not have favorable qualities for malting, although malting varieties can be deemed

acceptable for feed (Fox et al., 2009). There are exceptions to this. ‘Thoroughbred’, a Virginia Tech developed feed barley, is currently recommended by AMBA as a malting barley variety due to its high yield and adaptation to the mid-Atlantic region. As craft beer has surged in popularity in the past decade, barley producers have seen the economic value of malting barley rise, and breeders have responded to this by continuing to develop new varieties. As such, the Brewers Association released a white paper in 2014 identifying key malting barley characteristics for craft brewers (The Brewers Association, 2014). In this paper, the Brewers Association requested breeders and growers produce barley that is more suitable for all-malt brewing, which roughly translated varieties with lower levels of protein and starch degrading enzymes.

There is an important distinction between all-malt craft brewers, and macro brewers - who are mainly producing lagers with a high ratio of adjuncts, such as rice or corn. Rice and corn contribute little other than a source of carbohydrates to these styles (in terms of yeast nutrition), and brewers who use them can achieve a healthier fermentation and higher quality beer through the use of higher protein malts (Chen et al., 2006). These higher protein malts are usually associated with higher levels of enzymatic activity and free amino nitrogen, something the Brewers Association is trying to advocate against because of the potential negative impacts they have on all-malt brewing, including over attenuation and a decrease in flavor stability of packaged beer resulting from excess amino acids reacting to form Strecker aldehydes. The difference in needs of all-malt brewers and adjunct brewers is recognized by AMBA (American Malting Barley Association), and they recommend different FAN and enzymatic activity values depending on the application (American Malting Barley Association, 2021). According to

AMBA, all-malt brewers should seek a free amino nitrogen value in wort between 140 and 190 mg N/L, while adjunct brewers would benefit from a value above 210 mg N/L.

There are two types of barley grown in the United States: spring barley and winter barley. Spring barley has a shorter growing season and is typically grown in climates with harsh winters, whereas winter barley has a longer growing season and is sown in the fall instead of spring. Winter barleys are of significant interest for a number of reasons. Two specific reasons are an increased disease pressure in the summer as an effect of climate change (Stockinger, 2021), as well as the need for a “stable supply of high-quality malting barley,” (Stockinger, 2021). Climate change effects can allow for the overwintering of new pests with warmer winter temperatures, and conditions more favorable to the development of fungal diseases with excess rainfall during the growing season. Winter barleys also tend to be lower in protein, due to spring barley varieties having an increased development of protein when grain fill occurs during high temperatures in the hotter months and longer days (Stockinger, 2021). Winter barley’s lower protein content is advantageous to all-malt brewers, based on the Brewers Association’s objectives for breeders and growers (Bouckaert et al., 2014). Additionally, winter barley varieties are often used as cover crops and can offer additional income to those farmers who may choose to leave plots empty in the winter (Thomason et al., 2017). Despite the interest and clear benefits of the development of winter barleys, it remains a challenge due to the difficulty of combining winter hardiness and malt quality characteristics within one barley line (Muñoz-Amatriaín et al., 2010).

Virginia Tech and others have identified a gap in the barley to malt supply chain, as most producers and large malthouses are located in the northern part of the country (Thomason et al., 2017). These northern states typically produce spring barley varieties due to their harsh winters precluding winter cultivation of barley, although there have been recent efforts to develop winter

lines for these regions as global temperatures increase and Russian cold-adapted germplasm have become available (Sherman, 2020). Recently, the industry has seen a rise in craft malthouses, including Proximity Malt in Delaware, Murphy & Rude Malting in Virginia, and Epiphany Craft Malt in North Carolina. This, combined with industry advocates pitching winter barley as the perfect cover crop, sets the stage for the mid-Atlantic to enter a growing industry (Knadler, 2017).

Virginia Tech's small grains breeding program has previously released 'Thoroughbred,' and 'Avalon,' and is currently working on development of other winter barleys. Based on malt quality data from Virginia Tech's field trials in Warsaw in 2016, 'Thoroughbred' had excellent protein and FAN levels, but less than ideal percent extract compared to other varieties (Griffey et al., 2019). This is a problem with many six-row barleys, like 'Thoroughbred', which due to their morphology have higher protein contents and lower extract (total wort-solubles), and are thus better suited for the adjunct brewer (Turner). Despite its shortcomings, 'Thoroughbred' is still an AMBA recommended malting barley, mainly because it was the only variety specifically adapted to the east. 'Avalon,' a 2-row winter malt barley variety, was more recently released by Virginia Tech and demonstrated both excellent malt quality and flavor – in addition to disease resistance. As lines develop further and faster using double haploid technology, it is expected that superior malting barley will be available for Virginia in the near future (Thomason et al., 2017).

Despite modern advances in barley breeding, many brewers still prefer to use old heirloom varieties. Brewers often report that these heirloom varieties contribute more to beer flavor than other, contemporary barleys (Morrissey et al., 2021). Breeders continue to focus on malt quality as it relates to COA targets, rather than flavor development – resulting in an

underrepresentation of malt flavor in lighter beer styles (Morrissy et al., 2021). Challenges in conducting flavor chemistry and/or sensory evaluations of breeding lines for craft malting include the small quantity of grain available to work with for breeding lines, and the relative difficulty and expense of micro- or nano-malting these small quantities for sensory or flavor chemistry analysis. Furthermore, actually brewing beer from such small quantities is not practical. This is, however, an area of current research interest, and many authors including Bettenhausen et al. and Windes et al. have been using a metabolomics approach to find a link between malts produced from different barley varieties and their respective volatile compounds (Bettenhausen et al., 2020). Metabolomics, paired with hot steep and beer sensory evaluation has proved to be a useful tool in tracing barley varieties’, “contributions to malt and beer flavor,” (Morrissy et al., 2021).

Over the past decade, AMBA’s list of recommended malting barley varieties has markedly increased from eleven (six 2-rows and five 6-rows) in 2001, to 41 (34 2-rows and seven 6-rows) in 2022. Winter barleys have increasingly been recommended, with the first recommendation ‘Charles,’ in 2009 and a total of eight being recommended in 2022 (American Malting Barley Association, 2022). One notable winter barley is ‘Maris Otter,’ a malt variety developed in 1965, which is still used by brewers today due to its “superior malting and brewing qualities,” (Stockinger, 2021) even though it falls below modern “agronomic and malt quality standards,” (Morrissy et al., 2021). Its distinct flavor may be due more to its typical downstream processing more than its plant genetics, as it is often kilned to higher degrees of color (2.2-3.5 SRM). This is more in line with a Vienna style malt (2.7-3.8 SRM) rather than a Pilsen malt (1.4-2.1 SRM), although both can be used as base malts. The kilning step of the malting process allows for development of substantial flavor via Maillard reactions, and allows maltsters to

create much more variety between their products – including specialty malts. Maillard reactions occur between amino acids and reducing sugars, creating roasted, bready, or even meaty flavors. Because amino acids are involved in this flavor-forming reaction, research into different amino acid compositions and concentrations between barley varieties are of significant interest in their properties as potential Maillard reaction precursors (Wong et al., 2008). Researchers have also shown interest in determining whether there are genomic sequences responsible for ‘Maris Otter’s’ renowned flavor profile by comparing its sensory qualities to that of its progeny (Morrissy et al., 2021). ‘Maris Otter’s’ role in the pedigree of contemporary varieties and continued production as a malt barley variety shows that breeders and growers recognize brewer’s desire for unique flavor profiles.

### **Barley Proteins**

Acceptable total protein contents for 2-row malting barley varieties fall between 9.5% to 12.5% dry basis (Macleod, 2016). Reaching (and not exceeding) the target protein content can be a challenge if the crop is not managed correctly in the field.

Barley proteins were originally classified based on solubility, including water soluble albumins, salt soluble globulins, alcohol soluble prolamins, as well as alkali soluble glutelin (Arendt & Zannini, 2013; Briggs, 1978). These proteins can be subdivided further based on the amino acids they contain, their size, as well as their major functions and other physical/chemical characteristics.

For example, the protein class named hordeins can be subdivided into A, B, C, D, and  $\gamma$ -hordeins (Jaeger et al., 2021). Hordeins are storage proteins consisting of both prolamins and glutelin, and although claims on their exact percentage of the total protein fraction vary (Hill & Stewart, 2019; Kerpes et al., 2017; Qi et al., 2005), it is well understood that they represent the

largest portion. Hordeins contribute to the gluten found in beer, which represents a health risk for those with celiac disease (Kerpes et al., 2017). A subdivision of hordein, known as D-hordein, has a significant correlation with wort extract – a key malt quality metric. It is rapidly degraded during the malting process, and along with  $\gamma$ -hordein they represent the main fractions in beer, despite only comprising 1% to 5% of the total hordein fraction found in barley (Kerpes et al., 2017). This is attributed to the poor solubility of B- and C-hordeins, despite B-hordeins' disulfide bonds being enzymatically cleaved during the malting process (Kerpes et al., 2017). While all hordeins are rich in proline and glutamine, these two (D- and  $\gamma$ -) also contribute a high amount of glycine, leucine, and valine (Kerpes et al., 2017) which have significant effects on fermentation performance, serving as precursors for esters generated through yeast metabolism during fermentation. By targeting the breeding of barley varieties with lower levels of D- and  $\gamma$ -hordeins, breeders may be able to develop a lower soluble to total protein ratio - although the Brewers Association warns this may have negative effects due to the potential cascading effect of  $\beta$ -glucans (Bouckaert et al., 2014).

The water soluble portion of barley proteins consists mainly of albumins – comprising approximately 20% of barley proteins, namely LTP1 (lipid transfer protein 1) and protein Z which largely contribute to the foaming properties of a beer (Perrocheau et al., 2005). LTP1 has been identified to be involved in foam formation, while protein Z contributes to foam stability (Jégou et al., 2001) - both being important drivers of quality to consumers (Donadini et al., 2011). Both of these proteins demonstrate high heat stability, and LTP1 also shows resistance to proteases (Perrocheau et al., 2006). Proteomics research has recently explored the complete protein map of both barley and malt, however, Perrocheau et al. (2005) states that because that research has investigated the total protein composition, it is not relevant to barley's end use in

the brewing industry. The protein content and composition of a barley variety and the respective malt and beer produced from it is dependent on genetic and environmental factors (Luo et al., 2019). In addition to the complexity of the differential expression of endoproteases and exopeptidases between barley varieties (Jones & Budde, 2005; Muñoz-Amatriaín et al., 2010), this can make the relationship between a malt's total protein content, Kolbach ratio, and FAN concentration a confusing metric without further discussion of the barley variety. By targeting the expression of genes responsible for certain proteases and protease inhibitors, breeders might be able to create varieties with lower Kolbach ratios and FAN (Jones, 2005), partially satisfying the needs of current craft brewers.

It is important to note the difference between a barley's protein content, and a barley's FAN value, or any other value related to the concentration of nitrogenous compounds present in barley, malt and/or wort (such as total protein or the Kolbach ratio). A high protein content in barley does not necessarily mean that the malt produced from that barley will have high FAN values, unless properly modified (Thompson-Witrick & Pitts, 2020). Additionally, due to genetic and environmental impacts on the protein profile of a barley, total protein cannot serve as an indicator for yeast health as not all proteins are equally digested (Luo et al., 2019). Although these values are closely related, ultimately the level of proteolytic conversion during the malting and mashing process is in control of their relationship. In a study performed by Witrick and Pitts, they observed that although Canadian malt produced worts with the highest total nitrogen (909.9 mg/L compared to Texas' 686.5 mg/L), Texas malts produced worts with the highest FAN concentration (467.5 mg/L compared to Canada's 347.3 mg/L) in wort (Thompson-Witrick & Pitts, 2020). This example can be used to help identify the difference and variation in total and soluble nitrogen content among malts made from barleys of a similar protein content. This also

suggests a need to characterize FAN in malt and/or wort in addition to the barley itself in breeding programs. When selecting a malt, brewers must not only pay attention to the protein content, but also the ratio of soluble to total nitrogen. This ratio is influenced by the malting process, and is critical in determining the modification of the malt (Schoales & Heinrich, 2020). The soluble to total nitrogen ratio can influence beer quality due to the release of free amino nitrogen from larger proteins in the malting process, and their downstream effect in the fermentation performance, flavor development, and shelf stability (Yin, 2021). What is most important to understand about barley proteins is their range of properties and differing amino acid compositions, and how that translates to beer quality. Different barley protein fractions add certain foaming properties (Jaeger et al., 2021), amino acids (Hill & Stewart, 2019), and contribute to haze (Wang & Ye, 2021) in the finished beer.

### **Malting Process**

The malting process cannot begin until after the barley has been sorted and cleaned. The sorting step is critical because size differences between barley kernels can lead to differences in hydration, and therefore germination. It is also important not to blend varieties of barley prior to the malting stage, due to differences in genetics and protein content affecting rate of modification (Macleod, 2016). In analytical labs, multiple varieties may be malted at once through the use of stainless steel mesh tea balls (Schmitt & Budde, 2011), or through the use of individual steeping baskets (Turner et al., 2019). Although this keeps the malts separate, it does not optimize the process for each individual variety, which may lead to inconsistent or non-representative results compared to what would be done by a commercial production maltster, who would tailor the malting process to obtain the best quality for each variety. However, the purpose of malting labs is not to provide the best quality malt, rather to, “consistently

discriminate the potential malt quality of each line,” (Turner et al., 2019). Micro-malted samples should therefore be used as a guide, and malts made using large-scale malthouses may have differing quality compared to those produced at the lab scale (Karababa et al., 1993).

The malting process for a base malt involves a series of water steeps and air rests, followed by kilning. When barley is steeped in water, the grain rapidly begins to hydrate, which triggers germination (Turner et al., 2019). As the grain absorbs water, it not only activates the enzymes within the aleurone layer, but it also increases their mobility (Kuusela et al., 2004). During the air rest, the grain undergoes respiration and will begin to germinate. A maltster may choose to aerate during steeping, following claims that it improves homogeneity in the system and encourages faster germination. However, continuous aeration may lead to excessive germination and therefore increase malting losses, resulting in a poor-quality malt (Kelly & Briggs, 1992). Air rests seem to allow for adequate germination speed, and malts that go without an air rest in a process called, “immersion steeping,” achieve slower hydration and germination (Turner et al., 2019). Air rests have been shown to have a positive impact on the protein Z content, the protein fraction that impacts foam stability (Luo et al., 2020). Turner describes five different malting practices: immersion steeping, steeping under flowing water, flush steeping, spray steeping, and multisteeping (Turner et al., 2019). Each method utilizes unique steep to air rest ratios, intervals, and temperatures, each with their own advantages and disadvantages (Turner et al., 2019). Steeping is complete once the barley grain has reached the target moisture level, typically near 45% (Agu, 2012). Hydration varies by kernel size, kernel composition, and steeping temperature/regime. Once the cycle of steeps and air rests is completed, the next step is germination.

During germination, the barley is in a growth phase where enzymes are being made and diffused throughout the kernel (Boivin & Macleod, 2018). The endosperm is comprised of starches enveloped by proteins, and those proteins are also enveloped by beta-glucans. In order for diastatic enzymes to access starches, the beta-glucan and protein fractions must first be catabolized. Proteolysis is critical because it releases amylases and creates soluble nitrogen in the kernel, allowing for further enzyme synthesis (Agu, 2012). Gibberellic acid is a natural plant hormone, but may also be used as an additive to increase enzyme production and germination rate of the kernel (Tuomi et al., 1995). Absciscic acid is another additive used to inhibit germination, particularly to reduce growth of roots and shoots, therefore reducing germination losses (Tuomi et al., 1995). Germination time depends on germination vigor and water-sensitivity of the specific barley, but generally takes between 96 and 120 hours (Craine et al., 2021). The germinating barley is sprayed and mixed to keep moisture high, and CO<sub>2</sub> concentrations low, allowing it to respire. An added benefit of mixing is that it helps to prevent hot spots and matting, which increase germination rate and decrease homogeneity. Germination is halted based on mealiness and extent of acrospire growth. At this time, between 35% to 50% of proteins have been solubilized in the kernel (Yin, 2021).

The final stage in the malting process is kilning. Kilning is the drying process, where the green malt's moisture content will slowly reduce from 45% to below 5%. This is essential for the shelf stability of malt as a product, but it also is critical to fixing the enzymes and composition of the malt (Rani & Bhardwaj, 2021). To best preserve enzymes, kilning occurs at "low" temperatures, with four stages (free drying, intermediate drying, forced drying, and curing) ranging from 50 to 105 °C (Yin, 2021). During this process, non-enzymatic browning reactions known as Maillard reactions occur between amino acids and reducing sugars in the presence of

heat. Through deamination/deamidation and glycation of amino acids during the kilning process, amino acids are lost as they are converted into ammonia and  $\alpha$ -keto acids, and MRP's (Maillard reaction products) respectively (Hellwig et al., 2018; Sohn & Ho, 1995). These MRP's are often desirable, and although they are key in the development of flavor, kilning at high temperatures has been observed to impact malt FAN concentration negatively, through their reaction with sugars (Coghe et al., 2005). Total malt protein was not expected to vary based on differences in kilning procedures, however, Karababa et al. observed that the soluble protein in malt increased with increasing kiln temperatures, attributing it to an acceleration of proteolytic activity below 85 °C (Karababa et al., 1993). This shows the importance that malting practices have on FAN content, given the strong positive correlation between FAN and SP (Yin, 2021).

There are many different malting systems, just as there are different malting techniques. Floor malting is often used by craft malthouses due to its lower cost compared to higher technology malting equipment. Despite the ability to create malts with similar COA values, the malting systems can impart their own flavor effects, likely due to the microbial ecology that is influenced by the raw barley materials and malting practices (Bokulich & Bamforth, 2013; Yin, 2021). The impact of microbial populations on barley, and those developed during malting is another area of research into malt flavor and the concept of terroir, which is critical for creating a Virginia-distinct malt. This area has not been well-investigated, but is receiving increasing interest with the advent of increasingly affordable molecular techniques that can be used to address these research questions.

There has been interest into the use of raw (unmalted) barley as a brewing ingredient (Kok et al., 2019; Steiner et al., 2012; Zhuang et al., 2017). Most of these propositions have been made due to the potential effect the malting process has on the environment - from the massive

amount of water and heat it uses, to supply chain concerns as barley is shipped to and from malting facilities. Others have quoted cost concerns, as beer is taxed based on the percentage of malt used in a recipe in certain countries (Kok et al., 2019). Raw barley does not have many active enzymes, because they are developed and diffused through the kernel during the malting process. Because of this, researchers have worked on developing enzyme packages to mimic those developed during malting (Steiner et al., 2012). Despite the use of exogenous enzymes, quality markers were significantly lower between the wort made from raw barley to that made from malt (Steiner et al., 2012), especially the total and free amino nitrogen. Because of this, Steiner et al. observed that the beer made from raw barley had higher levels of diacetyl, as well as a lighter color due to the lack of Maillard reactions from the malting process. Jones et al. have investigated the role of protease inhibitors found in barley, malt, and beer; and although there were differences in the type and number of inhibitors present – their complicated relationship makes it so that researchers have as yet been unable to determine whether the differences affect proteolysis during the malting process. Interestingly, the protease inhibitors and associated enzymes form a complex once submerged in water, and do not dissociate until the temperature reaches near 100°C. This means that the enzyme-inhibitor complex is still active during mashing temperatures, and remains so until the enzymes are denatured during the boil (Jones, 2005). Jones and Marinac found that malt contains roughly 2.5 to 3 times more inhibitor than that of raw barley (1.8x based on proportional malting losses from 1 kg of barley) (Jones & Marinac, 1995), although Jones (2005) goes on to describe that due to the greater number of enzymes present in malt, all of the enzyme inhibitors can be found bound to proteases - whereas because of the lower enzymatic content of raw barley, the protease inhibitors are free in solution. Previous authors observed that the inclusion of various percentages of raw barley to the mash

resulted in a wort with lower levels of protein and amino acids (Enari et al., 1964; Yano et al., 2008), however it is unclear whether this would be due to the presence of enzyme inhibitors, or if it would be an effect of dilution due to the lower free amino nitrogen and soluble protein content found in raw barley. Evans et al. (2014) found that wort made from raw barley and a source of exogenous enzymes had a negative correlation between beta-glucan and FAN, all highlighting the importance of using a well modified malt.

### **Mashing Process**

After the barley finishes its malting process and has been subjected to appropriate malt analysis procedures, it is now an ingredient ready for the brewer to use. The brewer must first mill the malt, turning it into grist, a coarse flour with husk material still intact. After milling, the brewer will add the grist to the strike water, which is typically a few degrees higher than the intended mash temperature. This process is called mashing, and is necessary to extract and enzymatically convert starches and proteins into sugars and smaller peptides. Mashing in at a slightly higher temperature allows for the room temperature grist to reduce the mash temperature to the intended set point.

There are multiple different mashing styles used in craft brewing. Three common ones are the single infusion mash, the step mash, and the decoction mash. Adjunct brewers may also perform a third type of mash, which uses a separate vessel for gelatinizing other cereals with higher gelatinization temperatures (Cadenas et al., 2021). The decoction mash and step mash are somewhat similar in purpose; however, the step mash uses a more modern approach. Both the decoction and step mash reach a range of temperatures, each being the ideal temperature for different enzymatic processes. The difference between the two is the method used to achieve those different temperature rests. In the decoction mash, part of the mash is brought off and

raised to a boil in a separate vessel. After reaching a boil it is redirected to the original mash vessel, bringing the whole mash to the next temperature rest. This is repeated until the mash reaches its final mash out temperature. The decoction mash is claimed to provide a desirable malt characteristic to the wort, likely due to increased Maillard reactions and tannin extraction that occur during the boils. The step mash is an improved method of reaching multiple temperature profiles because it doesn't require boiling to raise the temperature – which denatures critical enzymes, and it doesn't require a separate vessel. The single infusion is the simplest of the three mashing methods, and occurs at a single set temperature, typically in the “brewer's window,” which is a compromise in temperature between the optimal temperature for activity of proteolytic and diastatic enzymes (Palmer, 2006). The brewer's window is the optimal temperature for beta and alpha amylase activity, which is ideal for wort extract levels.

Lekkas et al. estimated that nearly 30% of FAN found in wort is produced during the mash, the rest of it being attributed to that found in raw barley and generated through the malting process (Lekkas et al., 2014). Proteolytic enzymes that were formed during malting are heat stable (Yin, 2021), and survive the kilning step. Despite being most active at 50-55 °C (Briggs et al., 2004), these enzymes are still active in typical single infusion mash temperatures, and do not denature until around 70 °C (Briggs et al., 2004). Two important classes of proteolytic enzymes present in the mash are endoproteases and exopeptidases. Endoproteases cleave proteins at random location creating smaller peptides, whereas exopeptidases cleave peptides near the end of the peptide chain – breaking off small amino acid fragments (van der Velden & Hulsmann, 1999). Jones and Budde (2005) found that approximately a third of the wort's soluble protein content can be attributed to that present in the raw barley, one half can be attributed to the malting process, and approximately one fifth to the mashing process; whereas the same

relationship was not observed for FAN (Jones & Budde, 2005). As the enzymes responsible for the release of soluble protein and FAN are different, the concentration of FAN in a wort is largely dependent on the concentration of the SP, the substrate for the release of FAN. The FAN percentage of the barley grain is initially very low, and only increases as the soluble protein content is allowed to rise through enzymatic action (Jones & Budde, 2005). Based on this information, it is apparent that the combination of both endoproteases and exopeptidases are necessary for the majority of the FAN produced during malting and mashing. The concentration of total soluble nitrogen reaches its maximum after approximately 100 minutes of proteolytic activity (Briggs et al., 2004).

Research, or bench scale, mashes are used in a laboratory setting. There are two main research mash procedures, the congress mash developed by the American Society for Brewing Chemists (ASBC), and the isothermal mash developed by the European Brewing Convention (EBC). There exists a third called the TEPRAL mash, however it is rarely used in the literature. All of these mashes have their advantages and disadvantages, just as an industrial mash would. The congress mash consists of an initial 45°C protein rest, followed by a gradual increase in temperature (1°C per minute), with a final mash out temperature of 70°C. This is comparable to a step mash, and seems to be the most commonly used method in both commercial and research laboratories. The isothermal mash uses a single temperature, 65°C, similar to a single infusion mash. The single infusion mash is widely used in craft breweries due to the availability of well modified malt, and because of this researchers have called to have it replace the congress mash in laboratory analysis (Evans et al., 2011). However, all of the different methods achieve similar malt quality parameters when using the well modified malt (Evans et al., 2011; Kühbeck et al., 2005; Schmitt & Budde, 2010). That being said, some authors find that various extraction

methods such as the congress mash have better discriminatory power for the analysis of  $\beta$ -glucan, extract, and FAN (Moll & Flayeux, 1986), a malt quality parameter that will be discussed in detail in the next section.

### **Free Amino Nitrogen**

Free amino nitrogen (or FAN) is the measure of nitrogenous compounds that are assimilable by yeast during fermentation (Hill & Stewart, 2019). This measure includes ammonia, alpha amino acids, and di- and tri-peptides (Stewart et al., 2013). The total amount of free amino nitrogen is increased throughout the malting and mashing steps in the brewing process. A majority of free amino nitrogen in the barley kernel is developed during malting, which contributes to roughly 70 percent of the FAN, with the other 30 percent coming from mashing (Barrett & Kirsop, 1971). This exact figure has been disputed by Stewart et al. and Lekkas et al., claiming that a larger percentage is derived from the malting process. Lekkas et al. observed that nearly 88% of yeast utilizable nitrogen comes from the malting stage, and 12% from the mashing stage (Lekkas et al., 2014). The relative percentages could likely be an effect of the modification of the malt used, the mashing temperatures, and genetic factors. Free amino nitrogen can also be released as an effect of fermentation through yeast's secretion of proteolytic enzymes (Dreyer et al., 1983), the addition of hops, and the biosynthesis of amino acids within the cell (Bruner et al., 2022; Gibson et al., 2009; O'Connor-Cox & Ingledew, 1989). The increase in FAN from proteolysis is due to the presence of oligopeptides and higher molecular weight proteins still in the wort following the brewing process. Without further degradation, these higher molecular weight proteins and oligopeptides are not consumed by yeast due to their size (Ingledew & Patterson, 1999), and contribute to beer's foam stability (Stewart et al., 2013). Despite their resistance to proteolytic enzymes in barley (Perrocheau et al., 2005), foam

promoting proteins such as modified LTP1 may be degraded by yeast protease A, especially under conditions where the yeast are stressed (Leisegang & Stahl, 2005). Thus, providing the proper nutrition to yeast is critical not only for avoiding off flavors related to nitrogen starvation, but to make a visually appealing beer.

After absorption, small peptides and amino acids will undergo transamination reactions, where the amino group is stripped from the carbon skeleton. The yeast will use this amino group to build essential amino acids and structural proteins (O'Connor-Cox & Ingledew, 1989), and the carbon skeletons will be released and involved in growth and flavor forming reactions – creating aldehydes, fusel alcohols, and esters (James & Stahl, 2014). Depending on the structure of the amino acids, they will be involved in specific reactions as building blocks of structural proteins (O'Connor-Cox & Ingledew, 1989) and as precursors to unique aromas.

### **Absorption & Transport**

Different amino acids are consumed at different rates depending on the yeast's requirements, and yeast strains' nitrogen requirements may vary (Brice et al., 2014). However, a general consumption order has been established. Jones and Pierce first observed this in 1964, creating their own amino acid classification system based on uptake speed. Multiple changes and edits have been proposed over the years (Lekkas et al., 2014), although Pierce's classification system remains the standard comparison. Pierce's classification system consists of four groups, labeled A through D. Group A amino acids are absorbed nearly immediately, while group B amino acids are absorbed gradually, and group C amino acids are absorbed upon near complete utilization of group A amino acids (Jones & Pierce, 1964). Group D amino acids have little to no absorption, and remain in the highest concentrations in finished beer. This classification by uptake grouping should not be confused with Pierce's essential-ness grouping, made by Pierce in

1987. The classification of arginine, valine, phenylalanine, tryptophan and tyrosine varies between authors (Crépin et al., 2012; Stewart et al., 2013), although Crépin goes on to explain that this is due to the specifications under which the classifications were made, and that the studies used few strains in determining their grouping orders. Differences in FAN, pH, fermentation temperature, yeast strain, among other factors may influence this, and these effects are the topic of current research interest.

| <b>Group A</b>  | <b>Group B</b>          | <b>Group C</b>  | <b>Group D</b>       |
|-----------------|-------------------------|-----------------|----------------------|
| Fast Absorption | Intermediate Absorption | Slow Absorption | Little/No Absorption |
| Glutamic Acid   | Valine                  | Glycine         | Proline              |
| Aspartic Acid   | Methionine              | Phenylalanine   |                      |
| Asparagine      | Leucine                 | Tyrosine        |                      |
| Glutamine       | Isoleucine              | Tryptophan      |                      |
| Serine          | Histidine               | Alanine         |                      |
| Threonine       |                         | Ammonia         |                      |
| Lysine          |                         |                 |                      |
| Arginine        |                         |                 |                      |

**Table 1: General classification of amino acids based on uptake order by *Saccharomyces cerevisiae* Guinness No. 4200. From (Jones & Pierce, 1964).**

Yeast bring free amino nitrogen into the cell through a variety of transport systems. Most transporters are located in the mitochondria, vacuoles, and plasma membrane. Transporters inside the cell are typically specific for certain amino acids, although specificity does overlap between them (Bianchi et al., 2019). Those located in the plasma membrane are less selective than the other transport systems, because they are responsible for the amino acids entering the cell, rather than redirection to vacuoles or the mitochondria (Bianchi et al., 2019).

The least selective transporters are the Gap1 and the Agp1 systems, the latter of which selects for all protein building amino acids except lysine and arginine (Bianchi et al., 2019). The general amino acid permease system (GAP) has more significance, because although it functions as a high-affinity transporter in nitrogen starvation conditions – it also acts as a “nutrient sensor,” which communicates to other systems in the cell about nitrogen availability (Bianchi et al., 2019;

Kriel et al., 2011). The GAP system and other transporter's activity is affected by nitrogen catabolite repression (NCR), which allows yeast to assimilate amino acids based on a preferential order (James & Stahl, 2014). The absorption and utilization of preferred and non-preferred amino acids plays a large role on the aroma character of a beer, since many high aroma-impact compounds are generated via yeast metabolism during fermentation. Absorption is controlled by multiple factors, including the concentration of the medium, the ratio of individual amino acids, and the amino acid composition (Hill & Stewart, 2019; Jones & Pierce, 1964).

Many transporters are responsible only for a few or even single amino acids. The transport of amino acids specific to a given transporter can be repressed based on the presence of other amino acids in the medium. For example, the transporters Tat1p and Tat2p which select for the aromatic amino acids tryptophan, phenylalanine, and tyrosine preferentially uptake tryptophan and phenylalanine before tyrosine (Crépin et al., 2012). Similarly, the transporters Bap2p and Bap3p preferentially select for leucine and isoleucine before the uptake of valine (Crépin et al., 2012).

Certain yeast cells overexpressing genes for transporters, both general and specific, can experience toxicity and inhibitory growth due to the presence of amino acids in large quantities. This was observed most in mediums with single amino acids as opposed to mixtures (Risinger et al., 2006). Some transporters may be up or downregulated in response to the extracellular concentration of their amino acid substrates through their removal from the plasma membrane. This is not specific for only amino acid transporters - as Ptr2, a peptide transporter, also experiences this type of regulation (Ruiz et al., 2020). Ruiz et al. found that only 50  $\mu\text{M}$  of lysine inhibited growth of cells with an overexpressed Lyp1 gene. After giving Lyp1 cells 500  $\mu\text{M}$  of lysine and only observing a 0.4 drop in the cytoplasmic pH, Ruiz et al. concluded that growth

inhibition is caused by high concentrations of single amino acids, not by proton influx as previously understood. Furthermore, based on observed differences in transfer rates and inhibition between experiments, Ruiz et al. suggested that differences in amino acid toxicity are also dependent on the specific amino acid.

Notably, malts with similar FAN contents can have small but significant differences in specific amino acid concentrations between barley varieties and growing environments (Yin, 2021), although a few prior reports show that they remain relatively proportional in wort (Gibson et al., 2009; O'Connor-Cox & Ingledew, 1989). However, these reports focused on a smaller range of plant genotypes than those included in current breeding programs such as the Virginia Tech small grains breeding program. This, and the complexity of amino acid utilization that arises from differences in fermentative conditions (Bruner et al., 2022; Dekoninck et al., 2012), makes any conclusions on expected flavor profiles formed from yeast amino acid metabolism in the production of beer purely speculative, and beyond the scope of this project. However, this area is of emerging research interest, and will be reviewed in the following section.

### **The Effect of Individual Amino Acids on Fermentation**

Pierce's classification of amino acids based on essential nature relates amino acids to their overall effect on fermentation performance and beer quality. Class 1 is considered not important because they are readily assimilable or easily synthesized once exhausted, with the exception of proline - which is "unimportant" because it cannot be used by yeast under anaerobic conditions, despite being involved in later flavor forming reactions (Procopio et al., 2013; Yin, 2021). Class 2 and 3 are more essential because their synthesis results in off-flavors such as diacetyl (Hill & Stewart, 2019). Transamination products from amino acid metabolism can also

lead to more off-flavors, such as fusel alcohols formed by the Erlich pathway (Hazelwood Lucie et al., 2008).

| Class 1       | Class 2       | Class 3   |
|---------------|---------------|-----------|
| Aspartic Acid | Isoleucine    | Lysine    |
| Asparagine    | Valine        | Histidine |
| Glutamic Acid | Phenylalanine | Arginine  |
| Threonine     | Glycine       | Leucine   |
| Serine        | Tyrosine      |           |
| Methionine    |               |           |
| Proline       |               |           |

Table 2: Classification of amino acids based on essential nature for yeast. From (Pierce, 1987).

Low FAN values are associated with slow and/or a stuck fermentation, as well as the development of off-flavors (Hill & Stewart, 2019). Low FAN values can occur due to overuse of adjuncts, or from the use of improperly modified malts. Nitrogen is rarely a limiting factor in all-malt brewing; however, many brewers will add exogenous sources of nitrogen. These are widely used as a case of “insurance” in small breweries; specifically, in the case of high gravity brewing where there is a risk of running out of a nitrogen source prior to completion of fermentation. Brewers may add organic or inorganic forms of nitrogen, the latter being more prevalent in the market. Diammonium phosphate (DAP) is a popular source of inorganic nitrogen and is widely used in the beer, wine, and cider industry. Addition of solely nitrogen containing compounds is not very common in the beer industry, rather, yeast nutrients are sold as a complete package – usually containing zinc, manganese, as well as other key vitamins and minerals.

Supplementation of wort with specific amino acids has shown that certain amino acids or combinations of amino acids have interesting effects on flavor (Procopio et al., 2013) and growth. Supplementation with methionine was shown to reduce levels of VDK’s, while lysine supplementation had the opposite effect (Stewart et al., 2013). Supplementation with lysine did however, significantly reduce fermentation time (96 hours to 48 hours) in addition to a significant increase in yeast biomass and cells in suspension (Stewart et al., 2013). With proper

conditioning time following initial fermentation, yeasts are able to reabsorb some of these flavor compounds, however it was not specified how long after fermentation this data was collected. O'Connor-Cox & Ingledew observed that synthesis of VDK precursors was dependent on the valine concentration in low FAN worts, and dependent on threonine concentration in high FAN worts (O'Connor-Cox & Ingledew, 1989). This was explained by valine's direct relationship in the production of VDK precursors (Krogerus & Gibson, 2013), and threonine's role as a repressor of valine assimilation (O'Connor-Cox & Ingledew, 1989). Supplementation of certain amino acids may have a positive effect on beer aroma through feedback inhibition of certain amino acid metabolic pathways in yeast; but it also can result in higher concentrations of other aroma compounds, which may or may not be desirable (James & Stahl, 2014). This is a complex system involving yeast strain genomics, regulation of gene expression for amino acid transporters in a given yeast strain, regulation of metabolic pathways in yeast, and the complex differences in free amino acids in malted barley. While these factors certainly have been shown to affect the aroma of beer, the interactions between these various factors remains an area of current research.

Excess free amino nitrogen can contribute to excess beer haze and the risk of possible contamination via ability for this excess FAN to serve as a nutrient source for spoilage organisms (Hill & Stewart, 2019). Saerens et al. performed a study investigating the concentration of FAN in similar gravity worts, and determined that high FAN worts produced higher levels of medium chain fatty acid esters (MFCA) (Thompson-Witrick & Pitts, 2020), and a greater increase in acetate esters. Esters play a large role in beer aroma, and have varying aroma thresholds. At low thresholds, many of these esters impart a pleasant fruity characteristic such as banana – however at higher concentrations these flavor compounds can be detrimental and overpowering in the

beer. This is an interesting finding, as ethyl esters and acetate esters are derived from MFCA and amino acid precursors, respectively – yet both were affected by the FAN concentration (admittedly not to the same degree). However, when the maltose concentration of the medium was increased and the FAN concentration was kept constant (150 mg N/L), only acetate esters had an appreciable increase (Saerens et al., 2008), which is generally accepted as a consequence of high gravity brewing (Verstrepen et al., 2003). Based on these findings, one may conclude that by providing an adequate carbon to nitrogen ratio, brewers can achieve a more balanced and complex flavor profile. Excess FAN can also cause subsequent increases in levels of vicinal diketones (VDK's), likely as a result of its effects on fermentation rates and yeast's interactions with specific amino acids (Bettenhausen et al., 2018; James & Stahl, 2014). VDK's include 2,3-butanedione and diacetyl, which produce buttery aromas. These have very low sensory thresholds (James & Stahl, 2014), and are unacceptable in most beer styles. Excess FAN can also lead to a higher concentration of fusel alcohols as shown in lager and ale model fermentations (Procopio et al., 2013), which may lead to either a pleasant or solvent-like flavor and aroma in beer. However, the extent of yeasts amino acid metabolism and its contribution to the production of higher alcohols varies based on specific amino acid and sugar concentrations of the wort (Procopio et al., 2013).

| <b>Amino Acid</b> | <b>Higher Alcohols</b> | <b>Flavor or Aroma</b> |
|-------------------|------------------------|------------------------|
| Leucine           | Isoamyl alcohol        | Bitter                 |
| Isoleucine        | Amyl alcohol           | Alcohol, vinous        |
| Valine            | 2-Methylpropanal       | Alcohol                |
| Phenylalanine     | 2-Phenylethanol        | Rose, perfume          |
| Tryptophan        | Tryptophol             | Almonds                |
| Tyrosine          | Tyrosol                | Bitter                 |
| Methionine        | Methionol              | Cooked vegetable       |
| Isoleucine        | 2-Methylbutanol        | Alcohol                |
| Leucine           | 2-Methylbutanol        | Fusel, pungent         |
| Threonine         | Propanol               | Alcohol                |

**Table 3: Higher alcohols and their respective aromas formed from amino acids in the Erlich pathway, a metabolic pathway in yeast. Sourced from (James & Stahl, 2014).**

## **Amino Acids & Beer Flavor Development during Aging**

Worts with a high initial concentration of free amino nitrogen may not have complete utilization of FAN by the end of fermentation (Paik et al., 1991). Excess free amino acids (including proline) may still be present, and will continue to participate in chemical reactions which have the potential to impact beer flavor, after packaging. Higher levels of amino acids in finished beer can contribute to spoilage, either by the formation of off-flavors, or reduction of microbiological stability. This concept was understood prior to the 21<sup>st</sup> century, and as late as 1957 brewers sought malted barley with the lowest level of nitrogen possible (Pierce, 1987). The development of Strecker aldehydes is a main concern when discussing shelf stability as it relates to malt. Strecker aldehydes are the product of reactions between dicarbonyls and amino acids, and can lead to off-flavors that are described as pungent, overripe fruit, or meaty; and are formed by the amino acid precursors alanine, valine, and methionine, respectively (Yin, 2021). These off-flavors can have very low thresholds, with pyrazine formed by the amino acid precursor glycine having an odor detection threshold of just 0.002 ppm (Yin, 2021). These reactions are accelerated by a higher pH in the packaged beer, warm conditions, and higher concentrations of oxygen (Ferreira & Guido, 2018; Yin, 2021).

## **Sensory Evaluation**

The overall objective of this research is to identify barley breeding lines that have high potential for brewing quality, and to raise awareness of these varieties and malt's impact on flavor to stakeholders in Virginia's brewing industry. Despite base malt's flavor contribution to beer, other ingredients such as yeast, hops, and specialty malts have compounding flavor effects that lead to an entirely different sensory experience (Stewart et al., 2022). Sensory evaluation of

malts is therefore a more focused method of analysis compared to evaluation of beer, and an excellent method to assist craft brewers in ingredient selection.

The free-sorting task is a rapid sensory evaluation method that can be used to help researchers understand similarities between samples. Simply, the free-sorting task instructs panelists to place samples into groups based a criterion of their choosing, typically by similarities in flavor or aroma when applied to the evaluation of food products. One benefit of a sorting task is the ability to evaluate many samples at once (Chollet et al., 2014). Sorting tasks are useful in creating “perceptual maps of a large set of products,” (Lelievre-Desmas et al., 2008) which is ideal for assisting breeders and providing them additional selection criteria for the quality of a large set of malt barley breeding lines.

Beyond the ability to show similarities between products, the free-sorting task also allows panelists to add descriptors to the groups they make (Chollet et al., 2011). This can be done through the use of word banks, the free-sorting task also allows panelists to use self-generated terms.

Another benefit to free-sorting tasks is the ability to use either trained or untrained assessors. Unlike other methods of sensory evaluation, such as descriptive analysis, which usually consist of long training sessions to familiarize panelists with product lexicon – the free-sorting task is able to achieve similar results through both trained and untrained panelists. Although trained and untrained panelists have been shown to sort samples similarly, their use of descriptors that they apply does vary. When products were analyzed by both trained and untrained panelists, untrained panelists used a broader vocabulary accompanying their groups, which led to issues in data analysis (Lelievre-Desmas et al., 2008). The use of non-standard or diverse vocabulary for descriptors makes product mapping difficult to interpret, and fixing this

by re-categorization of similar descriptors leads to descriptions being manufactured by the sensory analyst rather than the panelist (Chollet et al., 2011).

Lelievre-Desmas et al. performed a sorting study with beer, and noted that providing a list of descriptors to untrained panelists was beneficial for obtaining a larger number of acceptable descriptors, but that it may have influenced their choices as shown by their overuse (or inappropriate use) of descriptors such as “hop” or “malt” (Lelievre-Desmas et al., 2008). Providing a list of descriptors to untrained panelists can also detract from panelist responses depending on the length of the list, and whether the descriptors provided include definitions or terms that panelists understand (Chollet et al., 2011). Confusion and over selection of terms is a concern when providing lists of descriptors to untrained panelists, so it may not be beneficial to some studies, especially when panelists are already familiar with the product space being evaluated.

Due to the typical beer consumer’s limited familiarity with malted barley itself, results obtained from these panelists would likely not reflect those from panelists who work in the brewing industry and therefore have more familiarity with evaluation of brewing ingredients, including malt. Those involved with the brewing industry may have more experience in malt literacy, and could be one step below trained panelists in their accuracy of descriptors, but a step above an untrained beer consumer. This, combined with the reality that purchasers of malt barley are almost exclusively maltsters and brewers, makes them ideal panelists to contribute to malt sensory evaluation for research purposes.

The ASBC’s malt hot steep is a popular method used in sensory evaluation of malts, and is a shortened and isothermal alternative to the popular congress mash method of laboratory wort production. Bettenhausen et al. used the hot steep as a method of wort preparation for a trained

panel performing a Check All That Apply (CATA) exercise (Bettenhausen et al., 2021). Stewart et al. used a modified hot steep method to collect ranking data for three descriptors, also using a trained panel (Stewart et al., 2022). This sample preparation method is quick, and despite not exactly mimicking the brewing process it can give breeders, maltsters, and brewers a base understanding of similarities between samples.

Sorting tasks generate data that can be analyzed using various approaches, including mapping representations and clustering techniques (Chollet et al., 2011). One method of interest is called DISTATIS, which is a form of multidimensional scaling. This method will create a map of the samples based on their similarities, as well as confidence ellipses around those points based on panelist's agreement (Chollet et al., 2011).

Compared to other common methods of sensory evaluation, such as descriptive analysis, the free-sorting task is quick and can be performed in a single session. The ability to use untrained panelists allows for more panelists to participate as well – which can lead to more stable results. Additionally, the ability of the free-sorting task to evaluate a large number of products makes it a high-throughput method and ideal for breeding programs which often have a very large number of breeding lines to be assessed.

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### **Chapter 3: Evaluation of Free Amino Nitrogen Content and Malt Sensory Characteristics**

#### **Abstract**

Regionally-grown barley is in demand for craft malting and brewing in Virginia, and barley lines suited to both Virginia's climate and craft brewing applications are currently under development. Free amino nitrogen (FAN) is a malt quality parameter that influences beer flavor directly and via yeast metabolism during fermentation. FAN and the individual amino acids making up FAN influence yeast health, beer color development, flavor, and flavor stability. Despite potential impacts on beer quality, individual amino acid concentrations in barley and malt are not generally measured or monitored. The objective of this project is to evaluate and assess the conversion of FAN and individual amino acid concentrations during the malting and mashing process for genetically distinct varieties, in addition to evaluation of their malt sensory characteristics. Raw barley and malt samples were subject to low temperature aqueous extraction, and wort was produced using an isothermal hot water extract technique. FAN and amino acid composition were determined for each line for raw barley, malt and wort extracts, prepared as described. Statistical analysis revealed that these lines had significantly different changes in FAN and amino acid composition. Sensory characteristics of malt hot steep teas were evaluated by forty (N=40) panelists with brewing experience using a sorting task to group malts with similar sensory characteristics, and to assign descriptors to those groups. From the sixteen (N=16) barley lines, five (5) distinct sensory groups were identified. Taken together, our findings will inform the selection process for barley lines for craft brewing, and add to the knowledge of the extent to which free amino acid composition varies among barley lines and along the brewing processes.

## Introduction

Regionally grown barley is becoming of interest for Virginia craft brewers, and barley varieties suited for both Virginia's climate and craft brewing applications may spark a revival for Virginia's agricultural industry. Approximately 90% of barley grown in the US comes from states with cool, arid climates such as Montana, North Dakota, and Idaho. The Virginia Tech small grains breeding program has been developing winter malt barley (*Hordeum vulgare*) varieties suitable for the Eastern US since 2010. By establishing winter barley varieties in the Eastern US, Virginia brewers would benefit from a local supply chain – reducing transportation costs and their associated environmental impacts.

A recent and growing area of research is the development of barley varieties more suited to the craft brewing industry. While historically macro-breweries were interested in higher protein barley due to their heavy use of nutrient-poor adjuncts, craft brewers using all-malt brewing methods are more interested in lower protein varieties – in addition to unique and flavorful profiles (Bouckaert et al., 2014). Free amino nitrogen (FAN), or the concentration of small peptides, amino acids, and ammonia, is a malt quality parameter that directly affects beer flavor, and has a large impact on a beer's aroma via yeast metabolism (Stewart et al., 2013). FAN develops during both the malting and brewing processes, where enzymes present in the kernel hydrolyze proteins into smaller peptides and amino acids. Little prior work has investigated the role that barley genotype plays on the development of this key parameter, or whether differences may influence the flavor of beer via yeast metabolism. Therefore, the objectives of this project were to: (1) compare malt sensory characteristics of Virginia-grown malt barley breeding lines from the Virginia Tech small grains breeding program with established Virginia-grown malt barley varieties such as Violetta, Calypso and Endeavor, (2)

determine the extent to which free amino nitrogen (FAN) varies among the selected set of malt barley breeding lines and varieties, and (3) assess the conversion of barley FAN to malted barley FAN for each line/variety in the sample set.

## **Materials and Methods**

### **Plant Material for Chemical Analysis**

Sixteen barley lines from the Virginia Tech barley breeding program were included in the chemical analysis portion of this study. Samples were grown at the Virginia Tech Agricultural Research and Extension Center (AREC) in Warsaw, VA, and at Kentland Farm located in Blacksburg, VA in replicate field trials. A total of four biological replications were included in the study, two per location. Raw barley was sent to the Montana State Barley, Malt & Brewing Quality Lab (Bozeman, MT, USA) and micro malted using a CLP Steep/Germ Combination Kiln (Curio Malting, UK). The standard malting protocol used in this project is typical for the production of Pilsen malts, and is described in detail in Appendix A. Both protein and moisture content for the raw barley and malt were determined by the Montana State Barley, Malt & Brewing Quality Lab (Bozeman, MT, USA) using near-infrared (NIR) spectroscopy (Infratec™ NIR Grain Analyser, FOSS Analytical, Hillerød, DK).

### **Plant Material for Sensory Analysis**

Of the sixteen barley lines included in the chemical analysis, only thirteen barley lines from the Virginia Tech barley breeding program were included in the sensory analysis portion of this study due to the quantity of sample needed from a single location. Because of the quantity needed, the three barley lines ('Hirondella,' 'VA20BFHB-18DH352,' 'VA20BFHB-18DH534')

were removed from sensory evaluation. Samples were grown at the Virginia Tech Agricultural Research and Extension Center (AREC) in Warsaw, VA. All thirteen samples from the Virginia Tech breeding program were also included in the chemical analysis portion of the study. Three additional, commercially available malts representative of lightly kilned base malts were included, as reference to examples of commercially successful malts. Available certificate of analysis (COA) data for a limited number of commercially available and Virginia-bred malts used in this part of the project is provided in Appendix A (Tables 19 & 20).

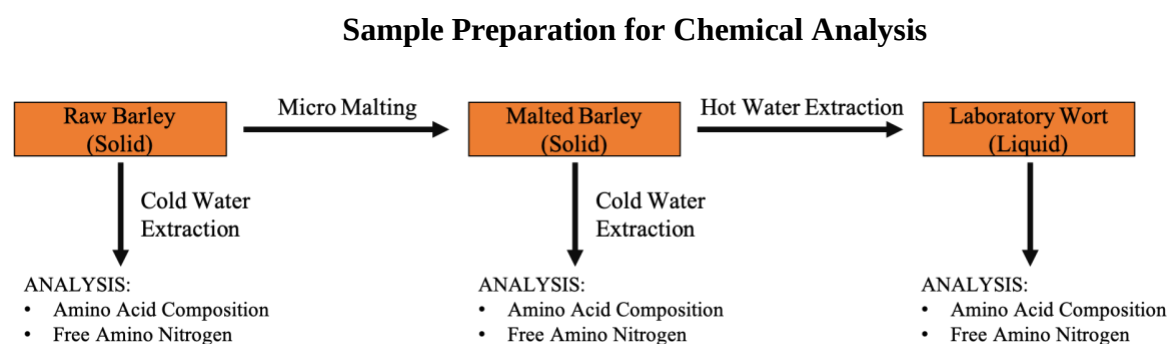


**Figure 1: Experimental design for the analysis of the development of barley FAN and amino acid composition throughout the malting and brewing processes.**

For chemical analysis, two separate extraction methods were performed based on the stage of the sample along the malting process, going from raw barley to malted barley, to wort. Raw barley and malt samples were coarsely ground using a Perten Laboratory Mill 3310 (PerkinElmer, Inc., Waltham, MA). A variety of grind sizes were tested for mill standardization, which resulted in the mill's largest grind setting being selected for all samples. A comparison of malt specifications and a particle size distribution for the mill standardization can be found in Appendix A (Tables 17 & 18). The mill was brushed clean after each sample.

The first extraction method was performed on both raw barley and malted barley samples to obtain liquid samples required for spectrophotometric and chromatographic analysis. Raw and

malted barley samples were ground as previously described, and five grams were added to 50 mL centrifuge tubes. A 40 mL aliquot of deionized water were added to each tube, and the tubes were quickly placed in an ice bath. Samples remained in the ice bath for 2 hours and were briefly vortexed every 15 minutes. Samples were then centrifuged for 15 minutes at  $4752 \times g$ . Previous authors researching the development of FAN during the malting and mashing process examined the effect of extraction temperature, length, and the particle size of the grist (Kühbeck et al., 2005; Osman et al., 2002). Osman et al. (2002) reported that despite differences in particle size, fine ground and coarse ground samples reach an equivalent concentration of FAN after two hours. Authors such as (Lekkas et al., 2014) have also explored the development of FAN through the malting process, however they elected to use a much shorter extraction time. These methods informed our choice of extraction method for the raw barley and malted barley.

In order to obtain a wort sample for evaluation, a laboratory wort was generated using a modified version of the American Society of Brewing Chemists Methods of Analysis Malt-4 (ASBC Malt-4, 2011). First, 100 ml of deionized water was added to pre-weighed and labeled 250 ml Erlenmeyer flasks. Flasks were placed in the 5-liter chamber of the Fisherbrand™ Isotemp™ 215 (Fisherbrand™, Hampton, NH) and allowed to preheat to 67°C. Ground malt (25 g) was added to the flasks, which were immediately stirred to avoid dough-balls, and transferred to the 10-liter chamber of the water bath set to 65°C. Flasks were then covered with aluminum foil, and swirled manually every 10 minutes to thoroughly mix. After 1 hour, the flasks were removed, cooled, and deionized water was added to adjust the mash to the target mash weight of 225 grams – accounting for natural differences in the Erlenmeyer flask weight. The mashes were then poured over a pre-wet filter (Fluted Filter Paper Grade 597 ½, Ahlstrom-Munksjö, Helsinki, Finland) and drained into a 1000 mL beaker. The first 100 mL of filtrate was poured back over,

and the rest of the contents were allowed to drain. The filtrate transferred into a 50 mL centrifuge tube and centrifuged for 15 minutes at 4752 x *g* (Thermo Scientific™ Sorvall™ Legend™ X1 Centrifuge Series with TX-400 Swinging Bucket Rotor, Thermo Fisher Scientific Inc., Waltham, MA).

The pH of each extract was determined at room temperature using an Orion™ Versa Star Pro™ pH/ISE/Conductivity/Dissolved Oxygen Multiparameter Benchtop Meter with an Orion™ ROSS Ultra™ Low Maintenance pH/ATC Triod™ Combination Electrode (Thermo Fisher Scientific Inc., Waltham, MA). The specific gravity was determined using a DMA 35 Basic handheld density meter (Anton Paar GmbH, Graz, Austria). Aliquots of all three extraction methods (raw barley extract, malted barley extract, and laboratory wort) were transferred into mini-centrifuge tubes and promptly frozen at -20°C until analysis. Prior to analysis, samples were thawed at room temperature, vortexed, and once again centrifuged for 5 minutes at 3000 x *g* prior to FAN and amino acid analysis.

### **Sample Preparation for Sensory Analysis**

For sensory analysis, hot steep extracts were prepared according to a modified version of the American Society of Brewing Chemists Methods of Analysis Sensory Analysis-14 (ASBC Sensory Analysis-14, 2017). Using a manual coffee-style ceramic burr grinder, malt samples (52 g) were ground and added to 20 oz insulated metal water bottles (Neihepal, purchased on Amazon.com). Distilled water (Kroger®, Cincinnati, OH) was pre-heated to approximately 67°C using an Avantco WB19L 5 Gallon 128 Cup (19 L) Water Boiler - 120V, 1500W (Avantco Equipment, Meridian, ID), and 400 ml were added to each insulated bottle. The bottle caps were tightened and the contents vigorously shaken to ensure complete hydration of the grist. After 15

minutes, the contents were poured over a pre-wet filter (Fluted Filter Paper Grade 597 ½, Ahlstrom-Munksjö, Helsinki, Finland) and the first 100 ml of filtrate collected were poured back over the filter. After the contents were drained, 15 to 20 ml of sample were poured into 5.5 oz black soufflé cups with clear plastic lids (Choice® Foodservice Products, Layton, UT) and labeled with randomly generated blinding codes prior to presentation to the panelists.

### **Chemical Analysis**

Free amino nitrogen (FAN) was measured using the Primary Amino Nitrogen Assay Kit (PANOPA); (Megazyme International, Wicklow, Ireland) and the K-LARGE (Rapid) Kit (Megazyme International, Wicklow, Ireland). The PANOPA kit is used to determine the amount of primary amino nitrogen in the sample, and a similar method is described in the American Society of Brewing Chemists Methods of Analysis Wort-12C (ASBC Wort-12C, 2020) following validation by (Otama, 2013). The K-LARGE kit is used primarily to determine the amount of L-arginine present in the sample, due to the inability of the PANOPA kit to measure the nitrogen contribution of its side chain (Dukes & Butzke, 1998; Mira de Orduña, 2001). This value can be significant in substrates with high concentrations of arginine relative to other free amino acids, such as some *Vitis vinifera* wine grapes. Based on prior reports of free amino acid concentrations in barley, we expect arginine to be present, but in variable relative concentrations (Shewry & Tatham, 1990).

The amino acid composition of the samples was identified using ultra-high performance liquid chromatography (UPLC), with a photodiode array detector (PDA), as described in detail by (Ma et al., 2018); (Waters AcQuity UPLC/PDA, Waters Corporation, Milford, Massachusetts). The amino acid analysis kit with cell culture media method modification was

used. Small modifications were made to the procedure used by Ma et al., (2018) for free amino acid quantification in apple juice, such as the addition of the internal standard *L*-tryptophan (Thermo Fisher Scientific Inc. Waltham, MA) – which was dissolved in 0.1 M HCl solution prior to addition to the amino acid standard mix provided with the analysis kit.

### Sensory Analysis

This study was approved by the Virginia Tech Institutional Review Board (IRB 22-457) to ensure safe protocols for human subject research. Malt samples were sent to the Montana State Barley, Malt & Brewing Quality Lab (Bozeman, MT, USA) for deoxynivalenol (DON) analysis, and DON levels were determined to be safe for human consumption (Table 4). This mycotoxin produced by *Fusarium* infections is heat-stable and can survive the malting process, causing vomiting and gastrointestinal distress in minute doses.

Table 4: Deoxynivalenol (DON) concentrations reported by MSU’s Barley, Malt & Brewing Quality Lab for breeding lines included in sensory evaluation.

| Breeding Line       | DON (ppm) |
|---------------------|-----------|
| VA20MFHB-18D349     | <0.3      |
| VA20MFHB-18DH533    | 0.48      |
| VA17M-13DH1720 LX   | <0.3      |
| Flavia              | <0.3      |
| VA20MFHB-18DH541    | <0.3      |
| Violetta            | <0.3      |
| VA16M-84            | <0.3      |
| Endeavor            | <0.3      |
| Avalon              | <0.3      |
| VA20BFHB-18DH417 2R | <0.3      |
| VA20MFHB-18DH350    | 0.33      |
| Thoroughbred        | <0.3      |
| Calypso             | <0.3      |

Due to the large number of malted barley lines being evaluated in this project (N=16), a sorting task with labeling was selected (Chollet et al., 2014). The sorting task is described in detail by (Abdi et al., 2007; Chollet et al., 2011; Chollet et al., 2014; Lahne, 2020), and is a rapid sensory method that can be used to show similarities between products. Groups can also be labeled, generating general descriptive terms for each sample. Despite achieving similar product

mapping results with the use of untrained and trained panelists (Chollet et al., 2014), Lelievre-Desmas et al. (2008) found that the use of untrained panelists may result in issues with data analysis, specifically due to the use of non-standard descriptors, leading to descriptors having to be remanufactured by the sensory analyst rather than using the panelist's original descriptors (Lelievre-Desmas et al., 2008).

Because of the limitations that can be caused through the use of untrained panelists, panelists were recruited by contacting several breweries in Virginia due to their employee's familiarity and experience with malt as a brewing ingredient. While not trained, these panelists were assumed to be fluent in malt lexicon and it was expected that there would be more similarity between, and specificity within panelist generated terms (Chollet et al., 2011; Koenig et al., 2020). For this study, 6 sensory sessions consisting of 6 to 9 panelists were performed. Panels took place at the breweries, and panels were set up in rooms that did not have aromas of wort present. Panelists (N=46) evaluated all malt hot steep samples (N=16). Six panelists' responses were removed from the dataset for a variety of reasons – such as not evaluating all samples, misnaming blinding codes, and including duplications of samples between the grouping.

Panelists were presented with a randomized order of the sample set (n=16), and were asked to place the samples into groups based on their similarities. Detailed instructions were provided on paper and reiterated vocally, explaining the purpose behind the sensory panel, as well as instructions on how to sort the samples and conditions that must be met for the sorting task (i.e. samples must be placed in mutually exclusive groups, and that there may be no less than 2 groups and no more than 15 ( $K - 1$ )). Panelists were asked to group these by hand using pen and paper. Following, or concurrent with grouping (Hamilton & Lahne, 2020), panelists

were instructed to label these groups with descriptors that reflected their criteria used for grouping the samples.

### **Survey of Sensory Panelists with Brewing Experience**

Panelists who participated in the sorting task were also asked to complete a brief survey about their outlook on malt purchasing decisions and the use of yeast supplements in the brewing process. The questions were printed on the back of the sorting task worksheet with the instructions that were provided to them. Survey questions can be found in Appendix A.

### **Statistical Methods & Data Analysis**

Two-way ANOVA with interaction was performed using R software (R version 4.1.2 (2021-11-01)) to identify whether the factors barley line (n=16 lines) and sample type (raw barley, malted barley, wort) and the interaction of barley line\*sample type affected the FAN concentration.

Amino acid data was analyzed using two technical (analytical) replicates per biological replicate (n=4 biological reps). Average amino acid concentrations were calculated for each breeding line (n=16) and each sample type along the process (barley, malt, wort) (n=3), along with the standard error of the mean (SEM), which was performed using R software (R version 4.1.2 (2021-11-01)). Relative amino acid concentrations were determined for each sample by calculating the ratio of individual amino acids (n=21) to the total amino acid concentration. The averages of the sample's relative concentrations were calculated from the replicates (n=4) and plotted as a boxplot using R software (R version 4.1.2 (2021-11-01)). Differences in the relative

amino acid composition between samples were determined for each sample type (barley, malt, wort) using discriminant correspondent analysis (DICA) (Ma et al., 2018).

Data from sensory evaluation was analyzed using DISTATIS, described in detail by other authors (Abdi et al., 2007; Abdi et al., 2012; Cairns et al., 2022; Kessinger et al., 2021). Briefly, through the *DistatisR* (Beaton, 2019) package, a distance matrix was created for the panelists' grouping data. Hierarchical cluster analysis was used to determine the relationship between both panelists and samples. Compromise matrices, or the weighted average of individual distance matrices (Abdi et al., 2005), were created for the sample set, which was then projected onto the DISTATIS plot creating a two-dimensional map representing similarity between samples. Contingency tables were created to analyze the descriptors used for the sample set, and correspondence analysis using the *ExPosition* (Beaton, 2014) package in R was used to project the descriptors onto the DISTATIS plot. Confidence ellipses (95%) were projected around the samples.

Survey data from sensory panelists obtained using the survey questions included in Appendix A was analyzed manually to determine trends and demographic data.

## Results and Discussion

### Free Amino Nitrogen

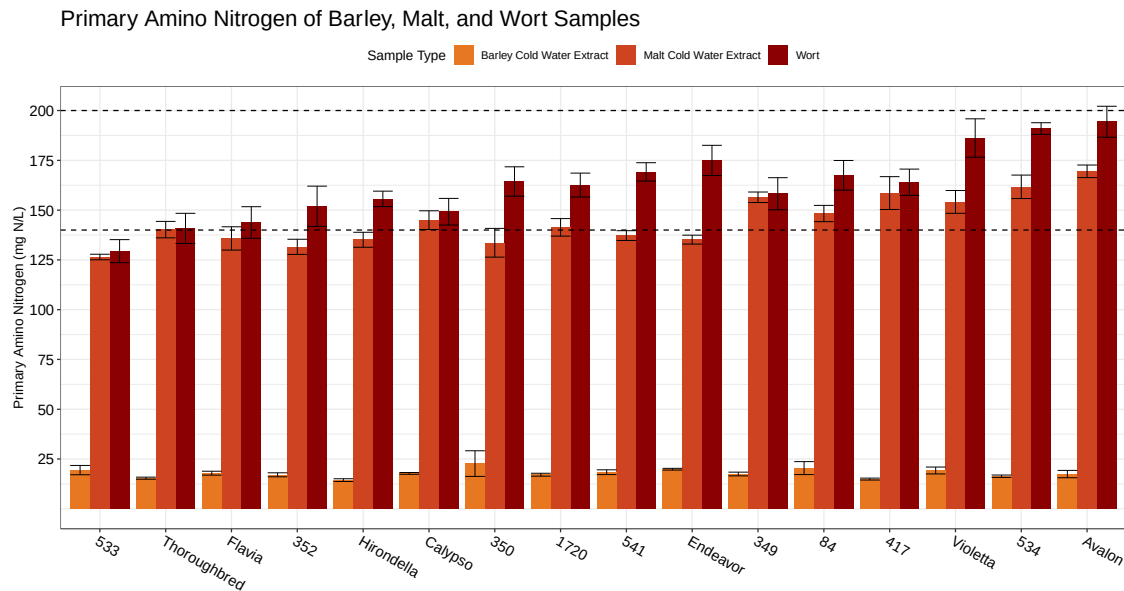


**Figure 2: FAN (reported as primary amino nitrogen) for barley varieties and their step in the malting and brewing process. Error bars represent standard error for n=4 biological replicates per barley line for each sample type.**

All samples fell within the recommended FAN range for all-malt brewing as suggested by the Brewer's Association (Bouckaert et al., 2014). Figure 2 shows the average FAN values for all breeding lines (n=4 biological replicates, two from Blacksburg, VA and two from Warsaw, VA, locations pooled for this data set and analysis). To first probe whether location influenced FAN, ANOVA with Tukey's HSD post hoc analysis was conducted on a linear model with location, variety, and the interaction of location and biological replicates (block) as well as the interaction of location and variety as predictor variables. This was performed individually for the three separate sample types – with barley, malt, and wort FAN values as response variables. Results from ANOVA showed a significant genetic by environmental (GxE) effect for barley FAN, but not for the other sample types.

Table 5: ANOVA table for a linear model using location, variety, the interaction of location and variety, as well as the interaction between location and biological replicate (block) as predictors for FAN. Significance codes: 0 ‘\*\*\*’ 0.001 ‘\*\*’ 0.01 ‘\*’ 0.05 ‘.’ 0.1.

|                    | Analysis of Variance Table: Barley |        |         |         |          |              |
|--------------------|------------------------------------|--------|---------|---------|----------|--------------|
|                    | df                                 | Sum Sq | Mean Sq | F value | Pr(>F)   | Significance |
| Location           | 1                                  | 100.39 | 100.388 | 13.2453 | 1.10E-03 | **           |
| Variety            | 15                                 | 278.06 | 18.538  | 2.4459  | 1.98E-02 | *            |
| Location X Block   | 4                                  | 55.55  | 13.887  | 1.8322  | 0.15064  |              |
| Location X Variety | 15                                 | 478    | 31.867  | 4.2045  | 5.16E-04 | ***          |
| Residuals          | 28                                 | 212.22 | 7.579   |         |          |              |

|                    | Analysis of Variance Table: Malt |        |         |         |          |              |
|--------------------|----------------------------------|--------|---------|---------|----------|--------------|
|                    | df                               | Sum Sq | Mean Sq | F value | Pr(>F)   | Significance |
| Location           | 1                                | 463.3  | 463.27  | 6.3004  | 1.81E-02 | *            |
| Variety            | 15                               | 9251   | 616.73  | 8.3875  | 8.92E-07 | ***          |
| Location X Block   | 4                                | 300.2  | 75.04   | 1.0206  | 0.41377  |              |
| Location X Variety | 15                               | 1437.7 | 95.85   | 1.3035  | 0.26383  |              |
| Residuals          | 28                               | 2058.8 | 73.53   |         |          |              |

|                    | Analysis of Variance Table: Wort |         |         |         |          |              |
|--------------------|----------------------------------|---------|---------|---------|----------|--------------|
|                    | df                               | Sum Sq  | Mean Sq | F value | Pr(>F)   | Significance |
| Location           | 1                                | 3818.2  | 3818.2  | 25.053  | 2.73E-05 | ***          |
| Variety            | 15                               | 19568.6 | 1304.6  | 8.56    | 7.22E-07 | ***          |
| Location X Block   | 4                                | 200.5   | 50.1    | 0.3289  | 0.8562   |              |
| Location X Variety | 15                               | 1446.7  | 96.4    | 0.6328  | 0.8231   |              |
| Residuals          | 28                               | 4267.3  | 152.4   |         |          |              |

ANOVA results indicate that GxE effects were significant for barley, however, not for malt or wort samples (Table 5). Post hoc analysis with Tukey’s HSD was performed on the model for barley (Table 6), and show that this is likely attributed to samples ‘VA16M84,’ and ‘VA20MFHB18DH350’ (Table 6). For sample ‘VA20MFHB18DH350,’ this difference was likely caused by human error in measuring the free amino nitrogen (data not shown).

**Table 6: Results from Tukey's HSD of ANOVA for a linear model using location, variety, the interaction of location and variety, as well as the interaction between location and biological replicate (block) as predictors for FAN.**

| Variety            | Location   |        |
|--------------------|------------|--------|
|                    | Blacksburg | Warsaw |
| Avalon             | abc        | c      |
| Calypso            | bc         | bc     |
| Endeavor           | abc        | abc    |
| Flavia             | abc        | bc     |
| Hirondella         | c          | bc     |
| Thoroughbred       | bc         | c      |
| Violetta           | abc        | bc     |
| VA16M84            | ab         | c      |
| VA17M13DH1720 LX   | bc         | bc     |
| VA20BFHB18DH352    | bc         | bc     |
| VA20BFHB18DH417    | c          | bc     |
| VA20BFHB18DH534 2R | bc         | bc     |
| VA20MFHB18DH349    | bc         | bc     |
| VA20MFHB18DH350    | a          | c      |
| VA20MFHB18DH533    | bc         | abc    |
| VA20MFHB18DH541    | abc        | bc     |

To further investigate location's role in the differences caused by sample type (barley, malt, and wort), another model was run that included the interaction effect of location and sample type.

**Table 7: ANOVA table for a linear model using location, variety, sample type, as well as the interaction between location and sample type and the interaction between variety and sample type as predictors for FAN. Significance codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1.**

| Factor                 | Analysis of Variance Table |        |         |          |          |              |
|------------------------|----------------------------|--------|---------|----------|----------|--------------|
|                        | df                         | Sum Sq | Mean Sq | F value  | Pr(>F)   | Significance |
| Location               | 1                          | 2904   | 2904    | 39.154   | 4.41E-09 | ***          |
| Variety                | 15                         | 15882  | 1059    | 14.277   | <2.2e-16 | ***          |
| Sample Type            | 2                          | 796040 | 398020  | 5366.860 | <2.2e-16 | ***          |
| Location X Sample Type | 2                          | 1478   | 739     | 9.965    | 8.96E-05 | ***          |
| Variety X Sample Type  | 30                         | 13215  | 441     | 5.940    | 1.13E-13 | ***          |
| Residuals              | 141                        | 10457  | 74      |          |          |              |

**Table 8: Results from Tukey's HSD of ANOVA for a linear model using location, variety, sample type, the interaction of location and sample type, as well as the interaction between variety and sample type as predictors for FAN.**

| Location   | Free Amino Nitrogen |      |      |
|------------|---------------------|------|------|
|            | Barley              | Malt | Wort |
| Blacksburg | d                   | c    | a    |
| Warsaw     | d                   | c    | b    |

Results from ANOVA revealed significant treatment effects at  $p < 0.001$  for all factors, including both two-way interactions (Table 7). Post hoc testing using Tukey's HSD (honest

significant difference) revealed significant differences in wort FAN concentrations between locations – with Warsaw, VA having lower FAN concentrations on average. The interaction effect of location and sample type showed that location had a significant influence on the primary amino nitrogen content for the wort samples (hot water extract), but not for the malt and barley (cold water extracts of the solids) (Table 8).

This may be due to many factors, however, one possible explanation could be the effect that growing location has on the enzyme limit dextrinase, a starch debranching enzyme, which has been observed to be positively associated with the Kolbach index of malt – the ratio of soluble to total protein (Wang et al., 2006).

Genetic by environmental effects have also been observed for  $\beta$ -glucan contents (Choi et al., 2020), a main constituent of the cell walls in barley. Wort  $\beta$ -glucan content has a negative correlation with FAN (Yin, 2021), and due to the absence of a clear correlation between the  $\beta$ -glucan content of raw barley and wort (Choi et al., 2020), this indicates that FAN and other malt quality parameters must be determined in wort and that any analysis performed on raw barley or malt samples without the preparation of a laboratory wort may not provide accurate discrimination in a breeding line's wort FAN content.

Genetic and environmental effects play a large role in the development of FAN during the mashing process, likely due to confounding effects of other malt quality parameters such as  $\beta$ -glucan and differences in the enzymatic content of the breeding lines – however further genomic analysis would be required to determine this relationship. Nonetheless, our results indicate that there is a significant GxE interaction effect that influence's a barley variety's conversion of FAN from malt to wort. This interaction effect should be considered when drawing conclusions as to the likely wort FAN to be expected from a given barley line.

There was also a significant interaction effect between barley variety and the sample type (Table 7), meaning that the increase of FAN from barley to malt to wort was not consistent for all barley varieties evaluated. To further evaluate this, the increase in FAN from the malting and mashing processes were calculated as a percentage, with the malting process increase calculated as the difference between malt FAN and barley FAN, divided by the malt FAN – and similarly – the increase due to the mashing process was calculated as the difference between the wort FAN and malt FAN, divided by the wort FAN. A linear model was created for this, and ANOVA results indicated significant interaction effects between the different processes and the variety (Table 9).

**Table 9: ANOVA table for a linear model using process, variety, location, and the interaction between process and variety as predictors for FAN development during the malting and mashing processes. Significance codes: 0 ‘\*\*\*’ 0.001 ‘\*\*’ 0.01 ‘\*’ 0.05 ‘.’ 0.1.**

| Analysis of Variance Table |    |        |         |          |          |              |
|----------------------------|----|--------|---------|----------|----------|--------------|
| Factor                     | df | Sum Sq | Mean Sq | F value  | Pr(>F)   | Significance |
| Process                    | 1  | 19.061 | 19.061  | 5067.382 | <2E-16   | ***          |
| Location                   | 1  | 0.014  | 0.014   | 3.665    | 5.86E-02 | .            |
| Variety                    | 15 | 0.144  | 0.01    | 2.547    | 0.00318  | **           |
| Process X Variety          | 15 | 0.201  | 0.013   | 3.569    | 7.08E-05 | ***          |
| Residuals                  | 95 | 0.357  | 0.004   |          |          |              |

**Table 10: Results from Tukey’s HSD of ANOVA for a linear model using process, variety, location, the interaction of process and variety as predictors for FAN development during the malting and mashing processes.**

| Variety            | Process |         |
|--------------------|---------|---------|
|                    | Malting | Mashing |
| Avalon             | a       | bcde    |
| Calypso            | a       | cde     |
| Endeavor           | a       | b       |
| Flavia             | a       | cde     |
| Hirondella         | a       | bcde    |
| Thoroughbred       | a       | e       |
| Violetta           | a       | bcd     |
| VA16M84            | a       | bcde    |
| VA17M13DH1720 LX   | a       | bcde    |
| VA20BFHB18DH352    | a       | bcde    |
| VA20BFHB18DH417    | a       | cde     |
| VA20BFHB18DH534 2R | a       | bcde    |
| VA20MFHB18DH349    | a       | de      |
| VA20MFHB18DH350    | a       | bc      |
| VA20MFHB18DH533    | a       | de      |
| VA20MFHB18DH541    | a       | bc      |

Results from ANOVA showed that there were significant effects due to the process (malting and mashing), variety, and that there was also a significant interaction effect between process and variety. Location did not have a significant effect at a  $p < 0.5$ , however it still appeared to have some effect on the development of FAN – giving more context to Table 8 and the discussion following it. Significant differences were identified for the varieties' FAN development during the mashing process, but not for the malting process. These differences are illustrated in Figure 3, Larger differences between varieties were identified following the mashing process, which could be explained by potential differences in enzymatic activity that can vary due to the influence of both genetic and environmental factors (Arends et al., 1995), as well as malting's major role in the development of these enzymes and free amino nitrogen.

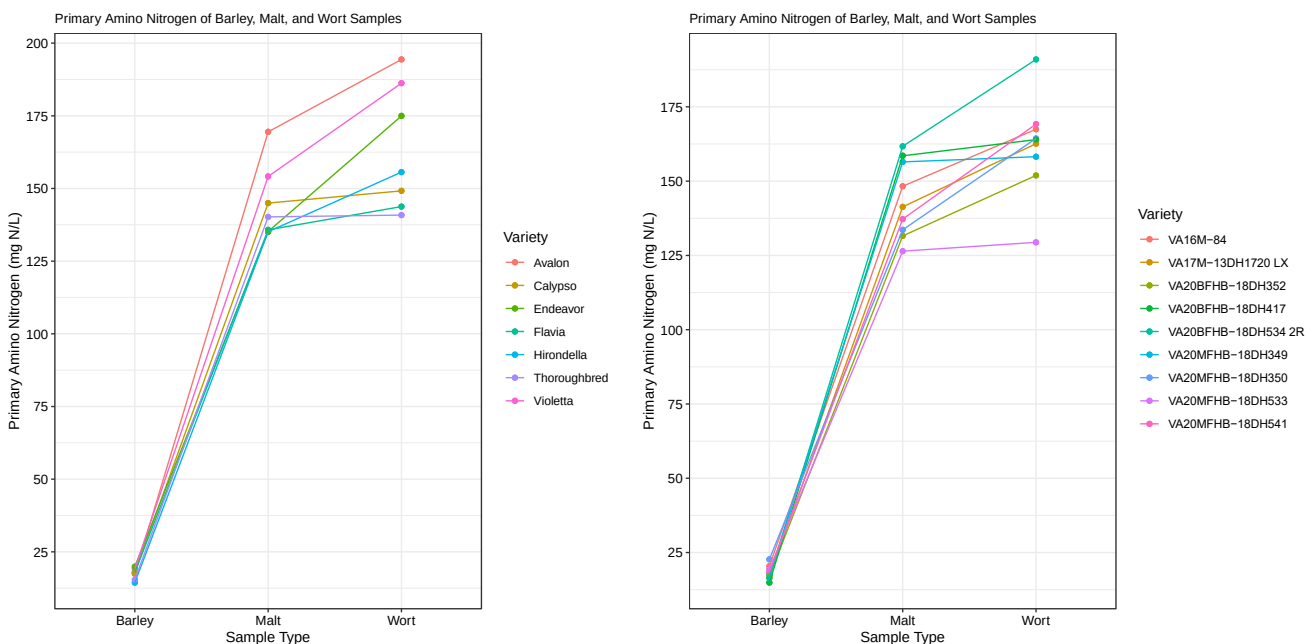


**Figure 3: Primary amino nitrogen of barley, malt, and wort samples. Diagram on the left includes barley varieties currently being used in brewing. Diagram on the right includes Virginia Tech experimental barley breeding lines.**

In Figure 3, an interaction plot showing the evolution of FAN for each barley line as the samples are processed from barley to malt to wort is presented. An average of 87.5% ( $\pm 3\%$ ) of the total FAN in our samples was determined to evolve as a result of the malting process

(conversion from raw to malted barley) and 10.3% ( $\pm 10.2\%$ ) of FAN evolved during the mashing process (conversion from malted barley to wort) – with about 2.2% loss of the total free amino nitrogen conversion (Figure 3), likely due to error and losses due to the growth and removal of the acrospire after malting (Yin, 2021).

As previously noted, these data were obtained from a sample set with a 16 different barley genotypes, and barley genotype or line, was the treatment within a given sample type. The observed difference in standard deviation between the development of FAN in the malting (conversion from barley to malt) and mashing (conversion from malt to wort) process indicates that barley genetic factors may have a larger impact during the malting process than mashing – one reason being genetic differences in the changes of soluble protein during the germination phase of malting, another due to the low thermostability of proteolytic enzymes, some of which can be deactivated at 55°C (Yin, 2021), a temperature which is generally exceeded in the mashing process. Therefore, the variability in the development of FAN in the mashing process may be a result of some breeding lines having different rates and degrees of modification, and this may be due to a number of factors (Agu & Palmer, 2001).

Our findings on FAN conversion along the process of malting and mashing are similar to previous research done by Lekkas et al. (2014), where a total of 88% of FAN was determined to come from the malting process and 12% from the mashing process using 28 barley varieties and similar extraction methods. Similarly, Lekkas et al. (2014) observed that differences in FAN development occurred between barley varieties, where 94% of the total FAN was already present in 10 of the malt samples, and 80% was already present in the other 18 samples – the rest being developed during the mashing process. The current research adds to the knowledge of free amino nitrogen development in barley over the malting process by including raw barley in this

calculation, whereas other studies have not determined the FAN contribution coming from the raw barley itself.

These results demonstrate that to determine the FAN content in wort of a new barley line, it is necessary to take the sample all the way through the process of malting and generation of wort in order to obtain a sample representative of the barley line's potential performance in the brewhouse. It is not sufficient to simply look at FAN in raw or malted barley to predict wort FAN, since our results indicate that there is a significant interaction between barley variety and sample type (Table 7, and the intersecting lines in Figure 3).

Some varieties did not have a significant development of FAN during the mashing process, such as 'VA20MFHB-18DH349,' 'VA20MFHB-18DH533,' 'VA20MFHB-18DH417,' Calypso, Thoroughbred, and Flavia; all of which had a relatively low difference between the malt cold water extract and the wort sample (Figure 3). Based on data collected by Virginia Tech's small grains breeding program (Table 11), this again may be explained due to high levels of  $\beta$ -glucan, in which some of these samples had nearly four times the recommended limit (<100 ppm) by AMBA (American Malting Barley Association, 2021). Excess  $\beta$ -glucan is undesirable in malted barley due to its negative effects during lautering, causing slow runoff of wort into the boil kettle, in addition to its negative effect on wort clarity.

**Table 11: Malt analyses performed by USDA-ARS for Virginia Tech's Barley Breeding Program for the Warsaw, VA 2022 crop year.**

| Line              | Rowed | Malt Extract (%) | Barley Protein (%) | Kolbach Index | Diastatic Power (°L) | β-Glucan (mg/L) | FAN (mg/L) |
|-------------------|-------|------------------|--------------------|---------------|----------------------|-----------------|------------|
| Calypso           | 2     | 80.24            | 11.27              | 34.31         | 141.15               | 139.89          | 105.18     |
| Violetta          | 2     | 79.85            | 11.49              | 36.91         | 156.95               | 205.25          | 133.05     |
| Flavia            | 2     | 78.37            | 10.87              | 35.62         | 88.86                | 395.42          | 114.01     |
| Avalon            | 2     | 80.17            | 11.63              | 43.42         | 156.37               | 141.79          | 160.91     |
| VA16M-84          | 2     | 81.43            | 11.76              | 34.65         | 98.03                | 431.07          | 120.54     |
| VA20MFHB-18DH349  | 2     | 80.65            | 11.51              | 31.72         | 108.33               | 504.9           | 123.68     |
| VA20MFHB-18DH350  | 2     | 81.16            | 10.01              | 36.78         | 121.91               | 242.86          | 123.9      |
| VA20MFHB-18DH533  | 2     | 80.87            | 11.12              | 31.89         | 135.73               | 493.3           | 95.75      |
| VA20MFHB-18DH541  | 2     | 82.22            | 9.68               | 38.06         | 123.34               | 251.27          | 129.72     |
| Thoroughbred      | 6     | 79.78            | 10.23              | 37.45         | 113.72               | 505.96          | 113.31     |
| Hirondella        | 6     | 80.2             | 9.28               | 35.97         | 101.09               | 547.58          | 97.63      |
| VA17M-13DH1720 LX | 6     | 78.7             | 9.93               | 34.88         | 109.6                | 577.96          | 101.37     |

While it is possible to relate these effects to the lack of genetic specificity provided by a micro-malting protocol used to analyze these parameters (Schmitt & Budde, 2011; Turner et al., 2019), the Kolbach index – another measure of modification – fell within recommendations for most of the evaluated breeding lines (although on the low end of the scale). Calypso, ‘VA20MFHB-18DH349,’ and ‘VA20MFHB-18DH533,’ did have S/T ratios (Kolbach Index) below the recommended range (Table 11), which in turn would result in a lower FAN content due to the positive correlation between soluble protein and FAN. As expected from this data, these samples had poor development of FAN during the mashing process, as shown in Figure 2 and 3. This indicates that other COA parameters such as the β-glucan content or Kolbach index, as well as the malting practice used for the production of these malts may have negatively impacted the mashing process and conversion of FAN in this study, and points to the importance of malting processes that are optimized for the barley variety being malted and the malt style target. Smaller differences in the FAN content of malted barley and wort samples can indicate under modification – and although the micro-malting procedure that was used in this study included checks or standards to track and determine modification throughout the malting process, the variety used was a Montana grown spring barley variety, which likely had different malting behavior.

This further complicates the goal of evaluating a wide range of barley genotypes for craft brewing potential. It should be noted that the data collected by the Virginia Tech small grains breeding program and the data collected by the current research may not be directly comparable, due to potential differences in the micro-malting procedures between MSU and the USDA-ARS labs, as well as other factors such as an increase in FAN during grain storage (Herb et al., 2017). Herb et al. (2017) found that FAN increased 4.3% after 5 months, and 15.3% after 10 months of grain storage, although storage temperature was not specified.

Fitting a linear model with only variety as a predictor variable and FAN values within a single sample type (wort, malt, raw barley) as the response, we found no significant differences in FAN for raw barley. However, treatment means did differ in the malt samples. In malt samples, Endeavor, Hirondeella, Flavia, and 'VA20MFHB-18DH541,' were not different from each other; and lines 'VA17M-13DH1720 LX,' Calypso, and Thoroughbred were also not significantly different from each other. The treatment means for FAN in wort samples also differed, however Violetta and Endeavor were not different. Three groups were found not be not significantly different from each other with respect to wort FAN concentration, the first group being lines 'VA20MFHB-18DH541,' 'VA16M-84,' and 'VA20MFHB-18DH350;' another was lines 'VA20BFHB-18DH417,' 'VA17M-13DH1720 LX,' and 'VA20MFHB-18DH349;' and lastly the varieties Calypso, Flavia, and Thoroughbred were not significantly different from each other with respect to wort FAN, the value most relevant to the FAN that the yeast will have available for fermentation of wort and/or that will end up in the beer if not fully utilized by yeast.

## Individual Amino Acid Concentration Profiles in Barley, Malt and Wort

Twenty-one amino acids were analyzed for the cold-water extractions of barley and malt, and the laboratory wort samples. A total of sixteen genetically unique barley varieties were included in this study, identical to the samples used in the evaluation of free amino nitrogen. Biological samples (n=4) were analyzed using two technical replicates. Total amino acid concentrations for each variety and sample type can be found in Figure 4. Individual amino acid compositions can for the three sample types can be found in Figure 5, 6, and 7. All twenty-one amino acids were detected. The values in these figures are averages and error bars represent the standard error of the mean.

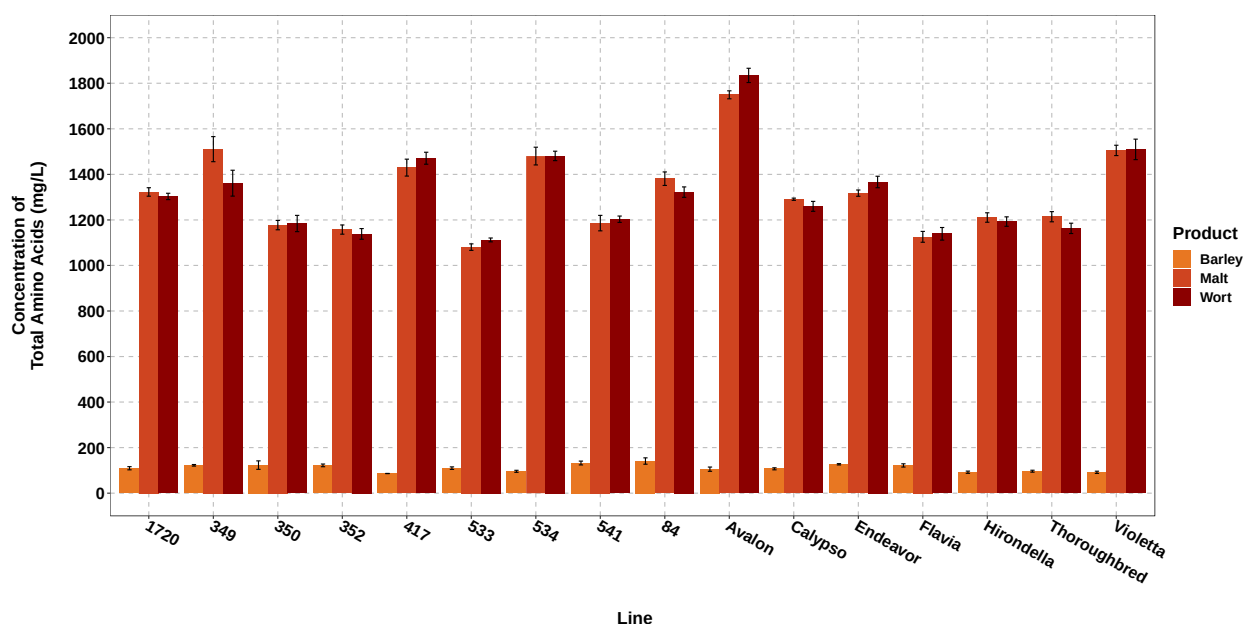


**Figure 4: Total amino acid concentrations of all barley varieties assessed in this study and their respective step in the brewing process. Values are average concentrations of all replicates and sub-replicates (biological and technical). Error bars represent the standard error of the mean.**

Briefly, total amino acid concentrations for each sample were tested for GxE factors. Based on GxE results from the analysis of free amino nitrogen, significant interaction effects were expected for raw barley samples. Results from ANOVA on the total amino acid concentration of each sample indicated significant location, variety, and GxE effects, and

interestingly also showed significant interaction effects even within biological replicates.

Although this may be of some interest to breeders, the effects may not be significant to the point that they play a role in malt flavor and fermentation performance. Additionally, due to maltsters frequently blending lots of malt from different locations and/or varieties, these effects will be ignored for the rest of the discussion.

**Table 12: ANOVA table for a linear model using location, variety, the interaction of location and biological replicate (block), as well as the interaction between location and variety as predictors for FAN. Significance codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1.**

| <b>Analysis of Variance Table: Total Amino Acids Barley</b> |           |               |                |                |                  |                     |
|-------------------------------------------------------------|-----------|---------------|----------------|----------------|------------------|---------------------|
|                                                             | <b>df</b> | <b>Sum Sq</b> | <b>Mean Sq</b> | <b>F value</b> | <b>Pr(&gt;F)</b> | <b>Significance</b> |
| <b>Location</b>                                             | 1         | 1527          | 1527.1         | 5.229          | 2.45E-02         | *                   |
| <b>Variety</b>                                              | 15        | 31794         | 2119.6         | 7.257          | 3.02E-10         | ***                 |
| <b>Location X Block</b>                                     | 4         | 3162          | 790.5          | 2.706          | 0.035            | *                   |
| <b>Location X Variety</b>                                   | 15        | 23785         | 1585.7         | 5.429          | 1.06E-07         | ***                 |
| <b>Residuals</b>                                            | 92        | 26871         | 292.1          |                |                  |                     |

| <b>Analysis of Variance Table: Total Amino Acids Malt</b> |           |               |                |                |                  |                     |
|-----------------------------------------------------------|-----------|---------------|----------------|----------------|------------------|---------------------|
|                                                           | <b>df</b> | <b>Sum Sq</b> | <b>Mean Sq</b> | <b>F value</b> | <b>Pr(&gt;F)</b> | <b>Significance</b> |
| <b>Location</b>                                           | 1         | 33648         | 33648          | 7.082          | 9.19E-03         | **                  |
| <b>Variety</b>                                            | 15        | 3842954       | 256197         | 53.918         | <2E-16           | ***                 |
| <b>Location X Block</b>                                   | 4         | 33211         | 8303           | 1.747          | 0.14633          |                     |
| <b>Location X Variety</b>                                 | 15        | 161339        | 10756          | 2.264          | 9.17E-03         | **                  |
| <b>Residuals</b>                                          | 92        | 437145        | 4752           |                |                  |                     |

| <b>Analysis of Variance Table: Total Amino Acids Wort</b> |           |               |                |                |                  |                     |
|-----------------------------------------------------------|-----------|---------------|----------------|----------------|------------------|---------------------|
|                                                           | <b>df</b> | <b>Sum Sq</b> | <b>Mean Sq</b> | <b>F value</b> | <b>Pr(&gt;F)</b> | <b>Significance</b> |
| <b>Location</b>                                           | 1         | 40081         | 40081          | 9.344          | 2.93E-03         | **                  |
| <b>Variety</b>                                            | 15        | 4310308       | 287354         | 66.989         | <2E-16           | ***                 |
| <b>Location X Block</b>                                   | 4         | 76152         | 19038          | 4.438          | 2.52E-03         | **                  |
| <b>Location X Variety</b>                                 | 15        | 215485        | 14366          | 3.349          | 1.71E-04         | ***                 |
| <b>Residuals</b>                                          | 92        | 394638        | 4290           |                |                  |                     |

The total concentration of amino acids found in barley is quite low, with a total of only 111.5 mg/L (SEM = 2.31). Compared to malt and wort samples, only 8.5% of total free amino acids were already present and detected in the sample, with the malting and mashing processes leading to the rest of the approximately 91.5% found in those samples.

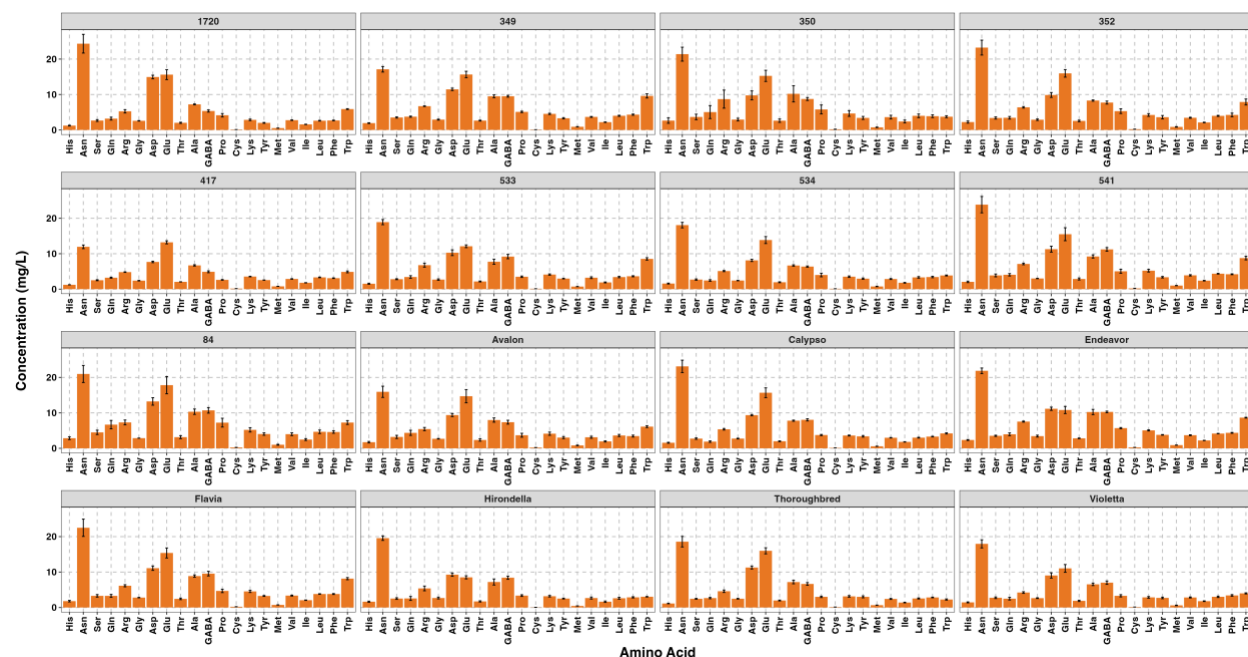


**Figure 5:** Bar graphs for each variety and their respective amino acid composition found in barley. Values shown are averages for all replicates and sub-replicates (biological and technical, respectively). Error bars represent the standard error of the mean.

The most predominant amino acids in raw barley were asparagine, glutamic acid, and aspartic acid, although the relative concentrations varied among barley varieties (Figure 5). These amino acids have been classified as Group A, which are preferentially and quickly absorbed by *Saccharomyces cerevisiae* (Crépin et al., 2012; Jones & Pierce, 1964), although this is strain dependent. Asparagine is known to form both acrylamide and 5-hydroxymethylfurfural during heating at high temperatures, which have been regarded to be carcinogens at high concentrations (Oddy et al., 2022). The concentration of asparagine increases markedly during sprouting that occurs during the malting process, however, the simultaneous increase of other amino acids will reduce the formation of these compounds by competing with reducing sugars to create Maillard reaction products (Yıltrık et al., 2021). Asparagine has been observed to increase in grains during stress, and may increase in lines with longer periods of senescence. Senescence progression also increased the ratio of asparagine to its precursor aspartic acid (similarly with the ratio of glutamine to glutamic acid) (Oddy et al., 2022). Differences in the

ratio of these amino acids and their precursors were observed in the samples evaluated, and overall, samples had a lower ratio of glutamine to glutamic acid and a higher ratio of asparagine to aspartic acid. Notably, ‘VA20BFHB-18DH417,’ had the lowest ratio of asparagine to aspartic acid in raw barley. Cysteine, methionine, histidine, and glycine concentrations were found in the lowest concentrations in the barley samples. Cysteine was detected only in trace amounts for all three sample types, and this is likely due to oxidation and the formation of cystine through a disulfide bond with another cysteine molecule (Krattenmacher et al., 2019).



**Figure 6:** Bar graphs for each variety and their respective amino acid composition found in malt. Values shown are averages for all replicates and sub-replicates (biological and technical, respectively). Error bars represent the standard error of the mean.

The total amino acid concentration increased by 91.6% during the malting process, reaching an average concentration of 1321.1 mg/L (SEM = 16.65). The amino acid concentration of malt is significantly higher than that of the barley samples. The relative amino acid composition also changed markedly, with the most predominant amino acids in malted barley samples being glutamine and proline. These are likely attributed to the hydrolysis of storage proteins such as prolamins during the malting process, which are rich in proline and

glutamine (Kerpes et al., 2017). Subdivisions of these storage proteins such as D- and  $\gamma$ -hordein are some of the main protein fractions found in beer, and also contribute a high amount of glycine, leucine, and valine (Kerpes et al., 2017). Similar to raw barley samples, malted barley samples had relatively low concentrations of cysteine, methionine, glycine, and histidine.

Unfortunately, there were no green malt samples analyzed prior to the kilning process. This does not allow for results to be drawn from the increase in amino acids during the germination process, and the subsequent decline in amino acids through thermal degradation and reaction during the kilning process.

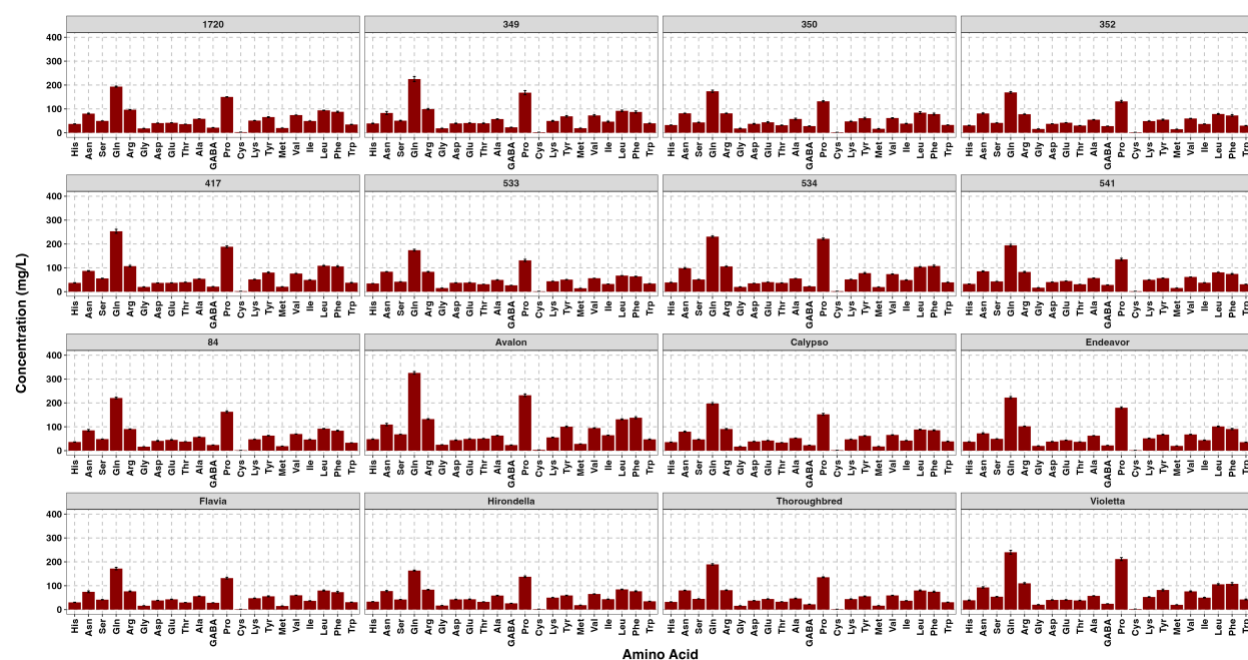


**Figure 7:** Bar graphs for each variety and their respective amino acid composition found in wort. Values shown are averages for all replicates and sub-replicates (biological and technical, respectively). Error bars represent the standard error of the mean.

The total amino acid concentration was similar between malt and wort samples, however the total amino acid concentration slightly decreased to 1315 mg/L (SEM = 17.6). This total “decrease” in amino acid compositions is mostly attributed to lower concentrations of glutamine and proline, which had an average concentration that were 29.8 mg/L and 32.9 mg/L lower than was present in malted barley samples, respectively. This may simply be due to the extraction

processes used to determine the FAN and amino acid compositions, however, the only other amino acids that had a lower concentration in wort samples was  $\gamma$ -aminobutyric acid, as well as a negligible decrease in isoleucine and asparagine.

Lysine, glycine, tryptophan, and tyrosine have been shown to cause the most non-enzymatic browning via the Maillard reaction, with lysine causing more browning than other amino acids at a pH of 6.0 (Ajandouz & Puigserver, 1999). These amino acids are involved in flavor development and reaction products such as pyridines, pyrrole, formylsine, maltosine provide characteristic malty flavors in beer. Barley variety and sample type had significant effects on lysine concentrations, and there was also significant interaction effect between variety and sample type (Table 13). However, average values for the lysine concentration of wort only ranged from 43.7 mg/L ('VA20MFHB-18DH533') to 55.5 mg/L (Avalon), which might be expected due to these lines having the lowest and highest wort FAN values.

**Table 13: ANOVA table for a linear model using variety, sample type, and the interaction between variety and sample type as predictors for FAN. Significance codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1.**

| Analysis of Variance Table: Lysine |     |        |         |          |          |              |
|------------------------------------|-----|--------|---------|----------|----------|--------------|
|                                    | df  | Sum Sq | Mean Sq | F value  | Pr(>F)   | Significance |
| Variety                            | 15  | 475    | 32      | 6.463    | 2.17E-16 | ***          |
| Sample Type                        | 2   | 70276  | 35138   | 7178.182 | <2E-16   | ***          |
| Variety X Sample Type              | 30  | 570    | 19      | 3.885    | 2.18E-08 | ***          |
| Residuals                          | 144 | 705    | 5       |          |          |              |

The relative proportion of amino acids were calculated by dividing individual amino acids by the total amino acid concentration for each sample type, and a boxplot was created for each sample type (Figure 8). Some amino acids had much higher of a range than others. Raw barley had a large range in the proportion of asparagine concentrations, as well as for glutamic acid and tryptophan. Asparagine accounted for over 20% of the total amino acid concentration in three samples, including Calypso, Hirondella, and 'VA17M-13DH1720LX.' Raw barley had a larger range than malted barley and wort samples for most minor amino acids, although this may

have not been significant. Cysteine and methionine had a very low range across all sample types. Results show that the relative proportion of malt or wort samples is likely the most influenced by the malt's glutamine and proline concentration, which again, can be caused by the malt's overall and relative protein fractions.

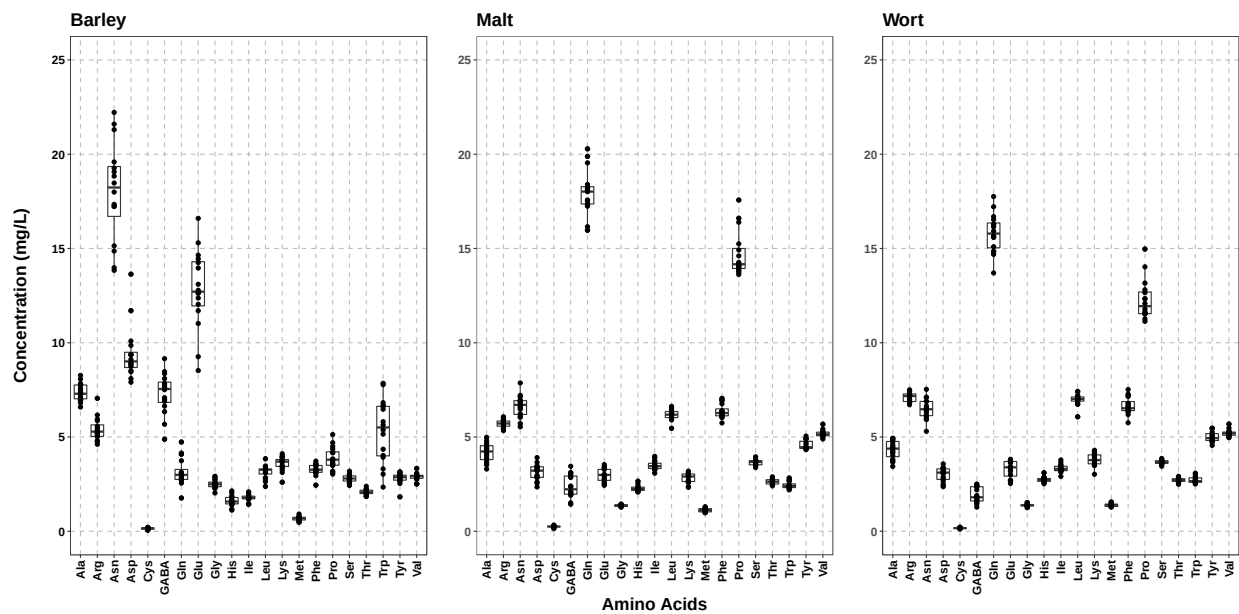


**Figure 8: Boxplot of the proportions of amino acid compositions found in each sample type.**

Principal component analysis (PCA) was performed on raw barley, malted barley, and wort samples to determine causes of variance in the amino acid composition (Figures 9, 10, 11). The data used in this analysis were the fixed effects of the lines, which were determined using a GxE model with Violetta as the reference.

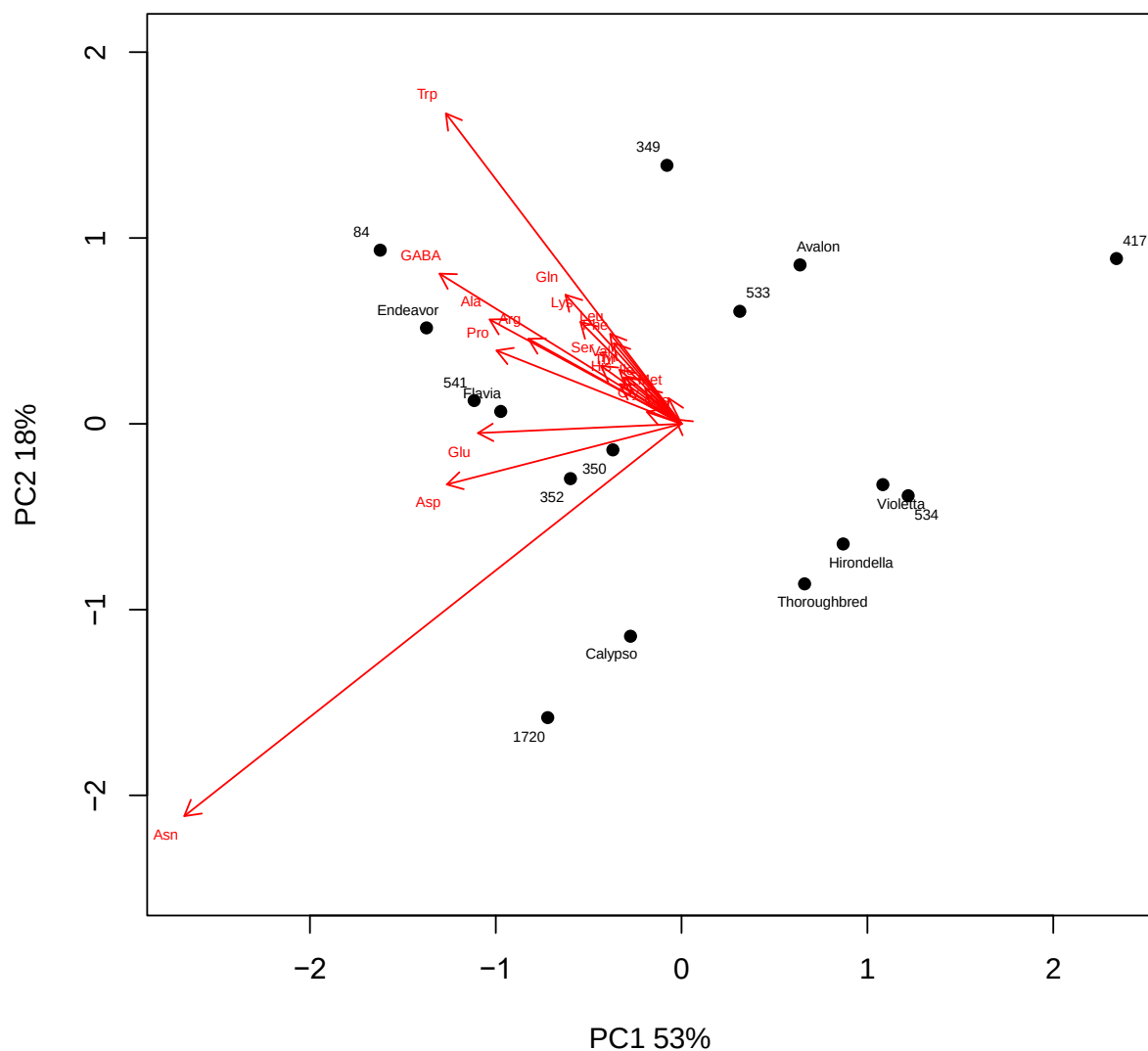


**Figure 9: Biplot of amino acid composition data for barley samples.**

For raw barley samples, the first principal component (PC1) explained 53% of variance, and the second dimension explained 18% (71% collectively). The breeding lines and varieties had somewhat of a large spread, although small clusters were spread within the plot, one of which including both Violetta and ‘VA20BFHB-18DH534,’ and another being both Flavia and ‘VA20MFHB-18DH541.’ Breeding line ‘VA20BFHB-18DH417,’ explained the most variance in the first dimension, and as seen in Figure 5 it was the only line with a higher concentration of

glutamine than asparagine, whereas asparagine was the predominant amino acid among all other samples. As mentioned previously, line 'VA20BFHB-18DH417,' also had the lowest ratio of asparagine to aspartic acid, which has been shown to increase during senescence. This and the low increase in free amino nitrogen in the sample 'VA20BFHB-18DH417,' may have some relationship due to agronomic factors, and it worth exploring more. Tryptophan and asparagine had explained the most variance, shown in both principal component 1 and 2. Many amino acids had similar relationships, indicated by the large cluster of vectors in the second quadrant. The amino acid  $\gamma$ -aminobutyric acid (GABA) had the third largest magnitude, followed by aspartic acid.

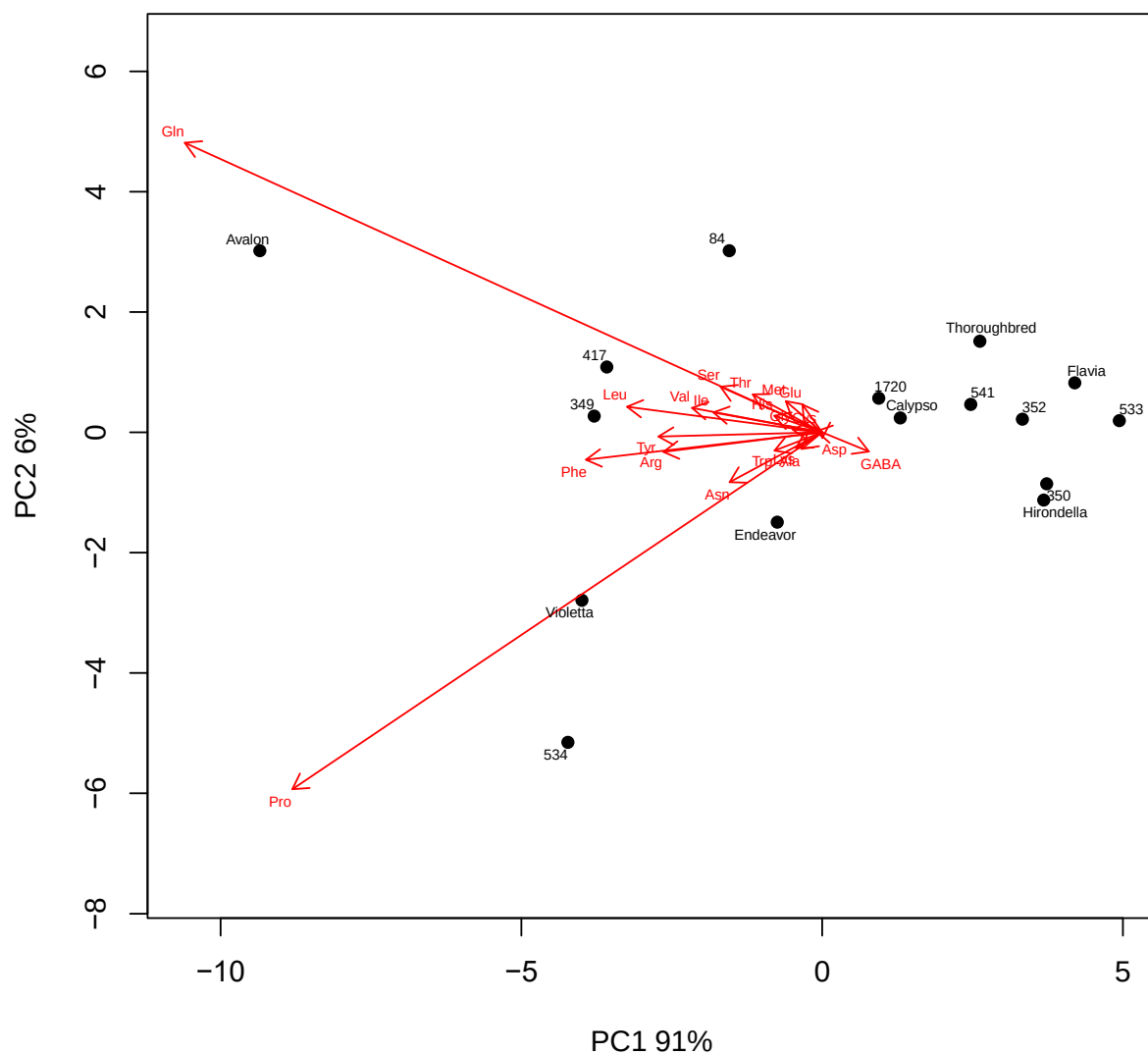


**Figure 10: Biplot of amino acid composition data for malt samples.**

For malted barley samples, the first principal component (PC1) explained 91% of the variance, and the second principal component (PC2) explained 6% (97% collectively). Malt samples had less of an overall spread than barley samples, with large clusters of breeding lines and varieties placed together. Unlike raw barley samples, most of the variance was explained by a single dimension. Overall, lines that were placed in close proximity in the raw barley samples were not in malted barley samples, one example being ‘VA20MFHB-18DH350,’ and Hirondeila.

Although, the two lines, ‘VA17M-13DH1720 LX,’ and Calypso, as well as Violetta and ‘VA20BFHB-18DH534,’ were placed near each other in both sample types. In malted barley samples, Avalon was observed to explain much of the variance, and was on the far left of the plot with no other varieties nearby.

Glutamine and GABA had an inverse relationship, and this may be explained by the fact that glutamine is a precursor in the pathway for the production of GABA in plants. Interestingly, proline also plays an intermediate role in the production of GABA, however the significance of its involvement has not been directly supported (Dabravolski & Isayenkov, 2023). There was a strong relationship between most other amino acids as many of them had similar vector magnitudes, including valine and isoleucine.

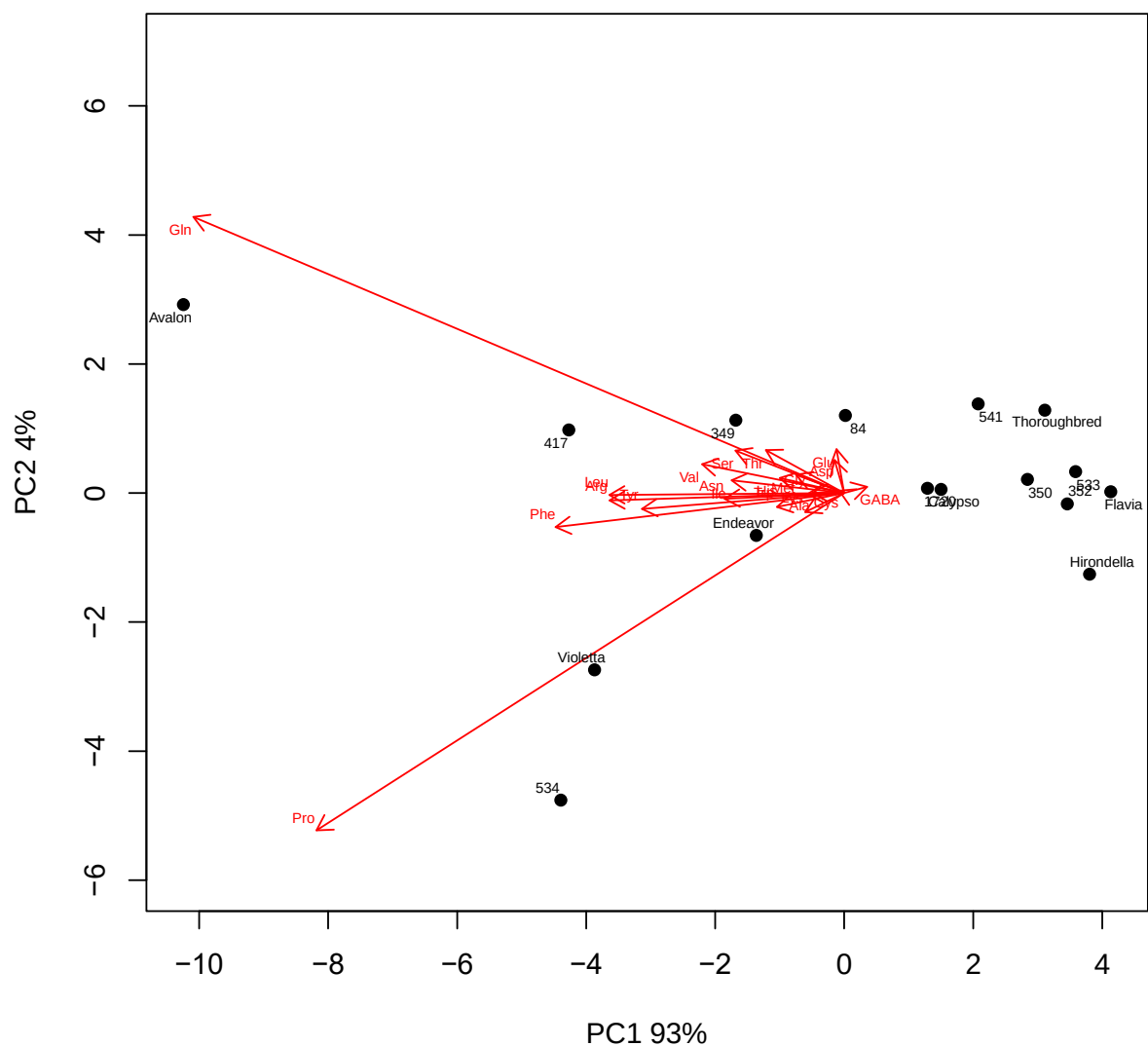


**Figure 11: Biplot of amino acid composition data for wort samples.**

For wort samples, the first principal component (PC1) explained 93% of the variance, and the second principal component (PC2) explained 4% (97% collectively). Similar to that of the malt biplot (Figure 10), Avalon was placed on the far left away from other samples. Violetta and ‘VA20BFHB-18DH534,’ were placed similarly to how they were in the malt biplot as well, and this was a general trend for the entire plot. Glutamic acid and aspartic acid had a very similar relationship, just as leucine and arginine did. As was the case for malt samples, again, GABA

had a negative relationship to almost all amino acids (less so in the case of glutamic and aspartic acid).

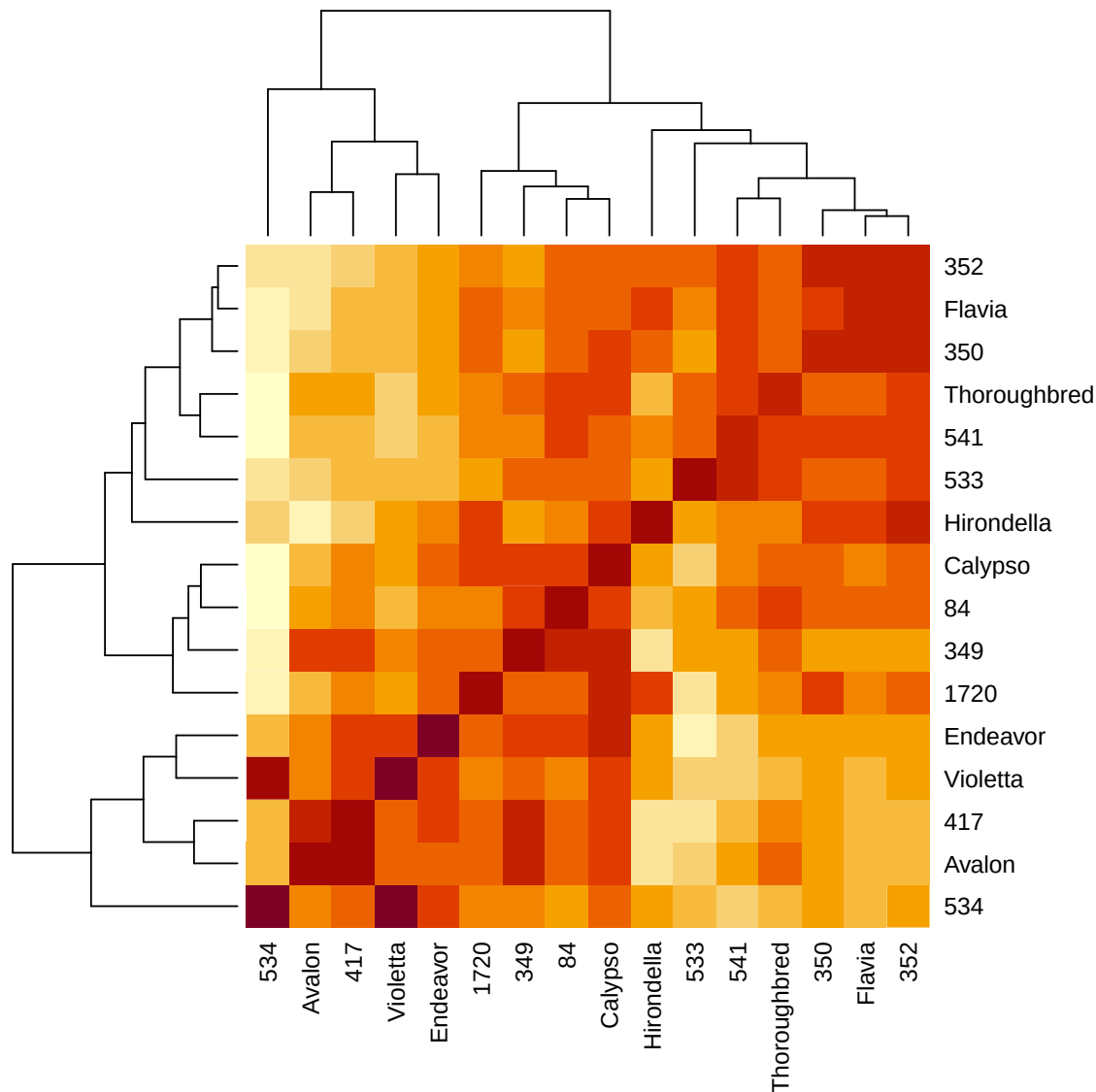


**Figure 12: Heat map of wort samples' amino acid composition.**

Overall, in raw barley samples most of the variance in the dataset can be explained by the two amino acids asparagine and tryptophan. The amino acid GABA explained more of the variance for barley samples and was placed in proximity to many other amino acids. GABA is an important amino acid in plants, and plays a role in stress response (Dabravolski & Isayenkov, 2023). However, GABA took on a negative relationship with other amino acids following the

malting and mashing processes. In malted barley and wort samples, most of the variance is explained by glutamine and proline, both major amino acids found in storage proteins.

Principal component analysis of the amino acid profiles indicated that Avalon, Violetta, and ‘VA20BFHB-18DH534,’ were most unique in their amino acid profile compared to other lines and varieties. Due to Violetta’s history of being grown in Virginia, amino acid data (if presumed to be a major factor in malt flavor) for ‘VA20BFHB-18DH534,’ may indicate that this breeding line may be a suitable replacement for Virginia. All three of these breeding lines and varieties also had the highest level of free amino nitrogen compared to others assessed in this study, and despite to Brewers Association’s suggestions for lower FAN varieties, these all fall within the recommended range for all-malt brewing. Despite having similar FAN concentrations, these lines differed in their total amino acid concentration and composition. Both Violetta and ‘VABFHB-18DH534,’ had similar total amino acid concentrations and correlated compositions (Figure 12), but Avalon had a higher concentration. Additionally, the change in the concentration of total amino acids from malted barley to wort was larger in Avalon, and increased by 85.1 mg/L. This may indicate that Violetta and ‘VA20BFHB-18DH534,’ have more wort soluble protein and small peptides that were detected as assimilable nitrogen, and because of their lower amino acid concentration this may result in less flavor from Maillard reactions and yeast metabolites.

### **Free Sorting of Malt Hot Steeps**

Data for analysis was collected from 46 panelists. Six panelists’ data were removed due to not assessing all samples (n=4), failure to label groups (n=1), and samples being placed in more than one group (n=1). Panelists placed the samples in 3 to 11 groups, with an average of 5 (4.5) and a mode of 3. Following, or concurrent with grouping, panelists labeled their groups

with descriptors or phrases that they believed most accurately characterized the samples. Group labels were tokenized, resulting in 473 descriptors, 305 of which were unique. Some terms (such as “expressive,” “round,” and “bright”) were removed due to concerns that they would not be accurately categorized. Terms describing the samples’ sweetness were carefully determined by the researcher to be sweet or not sweet, for example – terms such as “low sweet,” or variations thereof were removed, whereas “medium,” and “high,” were simply categorized as sweet. After manual text analysis, 60 original descriptors were removed, and descriptors were placed into 35 categories, 11 of which were omitted from text projection due to being used less than 10% of the time (count < 4). A contingency table showing the number of times panelists labeled samples with categorized descriptors is shown in Table 13. This can be used to help visualize similarities and differences in sensory characteristics for each sample and across the entire sample set. This contingency table was then used to determine coordinates for the descriptors that were projected onto the compromise map (Figure 15).

Table 14: Contingency table showing the count of categorized descriptors recorded for each sample. Cells with a value of zero were deleted for ease of reading.

| Sample            | Barnyard | Bready | Cereal | Grainy | Grassy | Herbaceous | Malt |
|-------------------|----------|--------|--------|--------|--------|------------|------|
| VA17M-13DH1720 LX | 2        | 5      | 1      | 6      | 5      | 1          | 2    |
| VA20MFHB-18DH349  |          | 8      | 3      | 10     | 4      | 1          | 5    |
| VA20MFHB-18DH350  |          |        |        | 1      |        |            |      |
| VA20BFHB-18DH417  | 1        | 1      |        |        |        |            |      |
| VA20MFHB-18DH533  |          | 4      | 2      | 8      | 6      |            | 4    |
| VA20MFHB-18DH541  | 2        |        | 2      | 1      | 5      |            | 2    |
| VA16M-84          | 1        | 5      | 1      | 3      | 3      |            | 2    |
| Avalon            |          | 1      |        | 2      | 2      |            | 1    |
| Bestmalz Pilsen   |          | 1      |        | 1      |        |            |      |
| Calypso           |          |        |        |        |        |            |      |
| Endeavor          |          |        |        |        | 4      |            | 1    |
| Flavia            |          | 2      |        | 5      | 9      | 1          | 4    |
| Northstar         |          |        |        | 1      |        | 2          |      |
| Superior Pilsen   |          | 3      | 1      | 1      |        | 2          | 2    |
| Thoroughbred      |          |        |        |        | 1      |            |      |
| Violetta          |          | 1      |        | 4      | 1      | 1          | 2    |

| Sample            | Medium Body | Musty | Neutral | Sour | Sweet | Sweet Aromatic | Vegetal |
|-------------------|-------------|-------|---------|------|-------|----------------|---------|
| VA17M-13DH1720 LX | 1           | 1     | 2       | 1    | 5     | 4              | 5       |
| VA20MFHB-18DH349  | 3           |       | 10      |      | 10    | 1              | 12      |
| VA20MFHB-18DH350  |             | 1     | 1       |      |       |                | 1       |
| VA20BFHB-18DH417  |             | 1     |         |      | 1     |                | 1       |
| VA20MFHB-18DH533  | 1           | 3     | 2       | 1    | 10    |                | 16      |
| VA20MFHB-18DH541  |             |       |         | 2    | 2     | 3              | 2       |
| VA16M-84          |             |       | 3       |      |       |                | 2       |
| Avalon            |             |       | 1       |      | 2     | 1              |         |
| Bestmalz Pilsen   |             |       |         | 1    | 1     |                |         |
| Calypso           |             |       |         |      | 1     |                | 1       |
| Endeavor          |             |       |         |      | 1     |                |         |
| Flavia            |             |       | 1       |      | 5     |                | 4       |
| Northstar         |             | 1     |         |      | 1     | 1              |         |
| Superior Pilsen   |             |       | 1       |      | 4     | 3              | 1       |
| Thoroughbred      |             |       |         |      | 3     |                |         |
| Violetta          |             |       | 3       |      | 3     |                | 3       |

| Sample            | Astringent | Biscuit | Earthy | Floral | Light Body | Stale | Umami |
|-------------------|------------|---------|--------|--------|------------|-------|-------|
| VA17M-13DH1720 LX |            |         |        |        |            |       |       |
| VA20MFHB-18DH349  | 1          | 1       | 7      | 3      | 4          | 1     |       |
| VA20MFHB-18DH350  |            |         |        |        |            |       |       |
| VA20BFHB-18DH417  |            |         |        |        |            |       |       |
| VA20MFHB-18DH533  |            | 2       | 1      |        | 3          | 2     | 3     |
| VA20MFHB-18DH541  | 1          |         | 1      |        | 1          | 1     |       |
| VA16M-84          | 1          |         | 3      | 1      | 1          | 1     | 1     |
| Avalon            |            |         |        |        |            |       |       |
| Bestmalz Pilsen   |            |         |        |        |            |       |       |
| Calypso           |            |         |        |        |            |       | 1     |
| Endeavor          |            |         |        |        | 1          |       |       |
| Flavia            |            | 2       | 2      |        | 1          |       | 2     |
| Northstar         | 1          |         |        |        |            | 2     |       |
| Superior Pilsen   |            | 1       |        |        |            | 1     | 1     |
| Thoroughbred      |            |         |        |        |            |       |       |
| Violetta          | 1          |         | 1      | 2      | 5          |       |       |

These categories were generated in reference to previous malt hot steep research (Bettenhausen et al., 2021), where panelists used terms provided by DraughtLab Pro (Lean Sensory Systems Inc., Webster, New York), an app-based sensory evaluation and data collection tool used in the brewing industry, to evaluate a variety of base malts. Contrary to the free-sorting task performed in the present research, Bettenhausen et al. used descriptors previously identified by DraughtLab to determine what sensory attributes were present in the samples through a check-all-that-apply (CATA) task. Terms in the present research were much more limited, likely due to the choice not to provide a list of descriptors for the panelists, as well as the use of panelists who were familiar with malt and brewing, but not trained specifically to this task.

The most popular flavors identified were, “Sweet,” “Vegetal,” “Grainy,” and “Grassy,” accounting for 43.6% of all descriptors. The top descriptor was “Sweet,” and was used in essentially all iterations of the word – from low, medium, high, and even cloying. This could simply be explained due to the nature of the sample, where the short mashing process called for by the ASBC hot steep malt sensory evaluation method leaves dissolved sugars in the malt, creating a sweet wort. This may also be explained by differences in amino acid concentrations. Amino acids have an impact on flavor profiles and can taste sweet, sour or bitter (Bachmanov et al., 2016). Despite all samples being sweet, some samples were further described by having a thin, medium, or heavy body – indicating that some samples had higher extract or amylase activity.

The second most popular descriptor was “Vegetal,” which was typically described as corn, asparagus, or by the term itself. This could likely be explained due to the method of sample preparation. The flavor precursor S-methylmethionine (SMM) is naturally found in barley, and is a precursor to the beer off-flavor dimethyl sulfide (DMS) (Yin et al., 2017), which forms in the

presence of heat. Many factors influence the amount of DMS formed in wort, including genotype and malt modification, however previous research suggested that the level of SMM synthetase in raw barley is the largest factor in the development of this off flavor (Yang et al., 1998). Yang et al. (1998) also goes on to explain that the level of SMM in raw and malted barley is dependent on the level of SMM synthetase and the level of free methionine. This flavor compound is easily volatilized during the boiling stage in the brewing process (Yang et al., 1998), however due to the use of sealed, insulated bottles for sample preparation, this compound was not able to be removed. With the method involving steeping the malt at 65°C, this can likely lead to the production and entrapment of DMS in the sample. Flavor effects caused by an increase in methionine may dominate flavor profiles found in the mash when compared the those of other flavor forming amino acids, such as glutamate, which is often described to be savory (Bachmanov et al., 2016). Despite this impacting the flavor profile of the current samples, these flavors would not be expected to be present in beer samples made from these same varieties due to the role the brewing process has in the removal of DMS, in addition to other additive effects that occur during the boiling stage (Morrissey et al., 2021).

Bettenhausen et al. (2021) determined that samples more often termed “grassy,” had lower amounts of Maillard reaction products (MRP) than those that were termed “cereal,” and earlier explains how differences in the amino acid composition of barley varieties can impact this due to their role as in the creation of MRP during the kilning process (Bettenhausen et al., 2021).

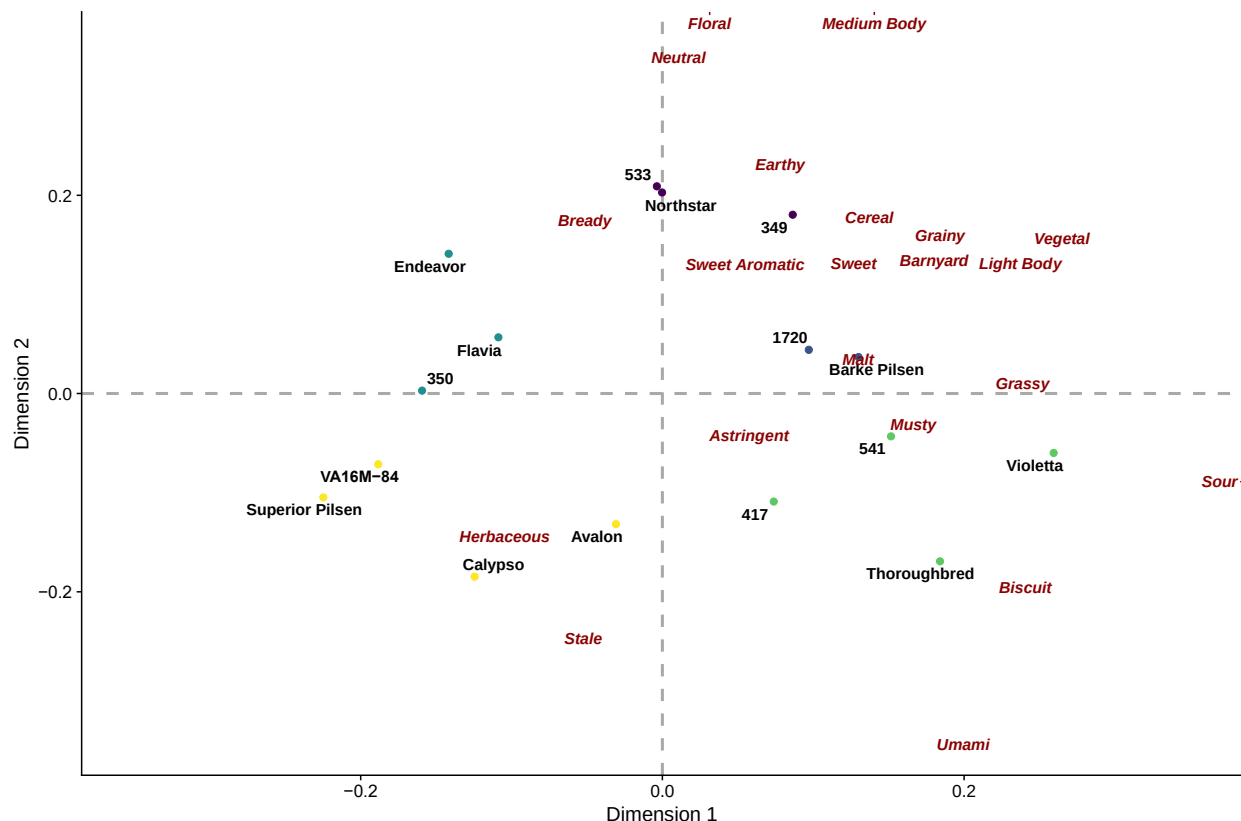


Figure 13: Sorting task results analyzed by DISTATIS. Samples are indicated as points on the plot, and clusters are represented by color. Confidence ellipses (95%) are illustrated with ovals. Descriptors were analyzed via principal component analysis.

Using DISTATIS, a compromise map was created based on panelists' sorting data, and descriptors were later projected based on the DISTATIS plot's projection matrix. Because DISTATIS is a method based on distance matrices, samples are positioned on the compromise map based on their similarity. A total of 5 clusters of similar samples were identified.

Many of these lines share similar lineage with Thoroughbred, Violetta, Flavia, Calypso, Hirondeella, and Endeavor. Avalon (previously named 'VA16M-81,' prior to release) and 'VA16M-84,' are sister lines bred from Thoroughbred as the maternal line and line '2107-2627,' as the paternal line. Despite their genetic similarity, these two lines were not placed in the same cluster as Thoroughbred, indicating differences in flavor profiles. This supports previous research in malt sensory, where flavor profiles of the progeny of the heirloom variety Maris Otter did not match (Morrissy et al., 2021) However, these two (Avalon and 'VA16M-84') were

clustered together indicating that their flavor may have been influenced by genetic factors (Sayre-Chavez et al., 2022). Calypso, Thoroughbred, Violetta, and Endeavor were all placed at the far corners of the compromise map. Due to their role in the breeding process of the experimental barley breeding lines assessed in this study, it appears that despite sharing genetics, the Virginia Tech small grains breeding program has bred lines with a diverse set of flavor profiles. This is favorable to breeding programs, especially for breeding lines early in development.

There was no clear or direct relationship between product placement and agronomic quality, indicating that established European varieties and those adapted to the Eastern Atlantic region share similar quality in terms of flavor and field performance, leaving typical malt analyses such as a COA the determining factor in a malt's success. In general, this is a good indication for the potential success in craft brewing of the lines in the current breeding program that were evaluated in this study. Despite no clear relationship between agronomic quality and flavor, it seems there may have been some role of malting's role and the level of modification. Samples 'VA20MFHB-18DH533,' (represented as "533" on the plot), VA20MFHB-18DH349 ("349"), and North Star Pils<sup>TM</sup> - which is a commercially available undermodified malt produced by Rahr Malting Co. – were all placed into a cluster together. Both samples 'VA20MFHB-18DH533,' and 'VA20MFHB-18DH349,' had very little difference between malted barley and wort FAN, indicating that they were not fully modified. This undermodification may have led to similarities in flavor (Herb et al., 2017), although this is speculative and would not explain other clusters with similarly undermodified malt.

# Hot Steeps Clustered By First 2 Dimensions of DISTATIS Consensus

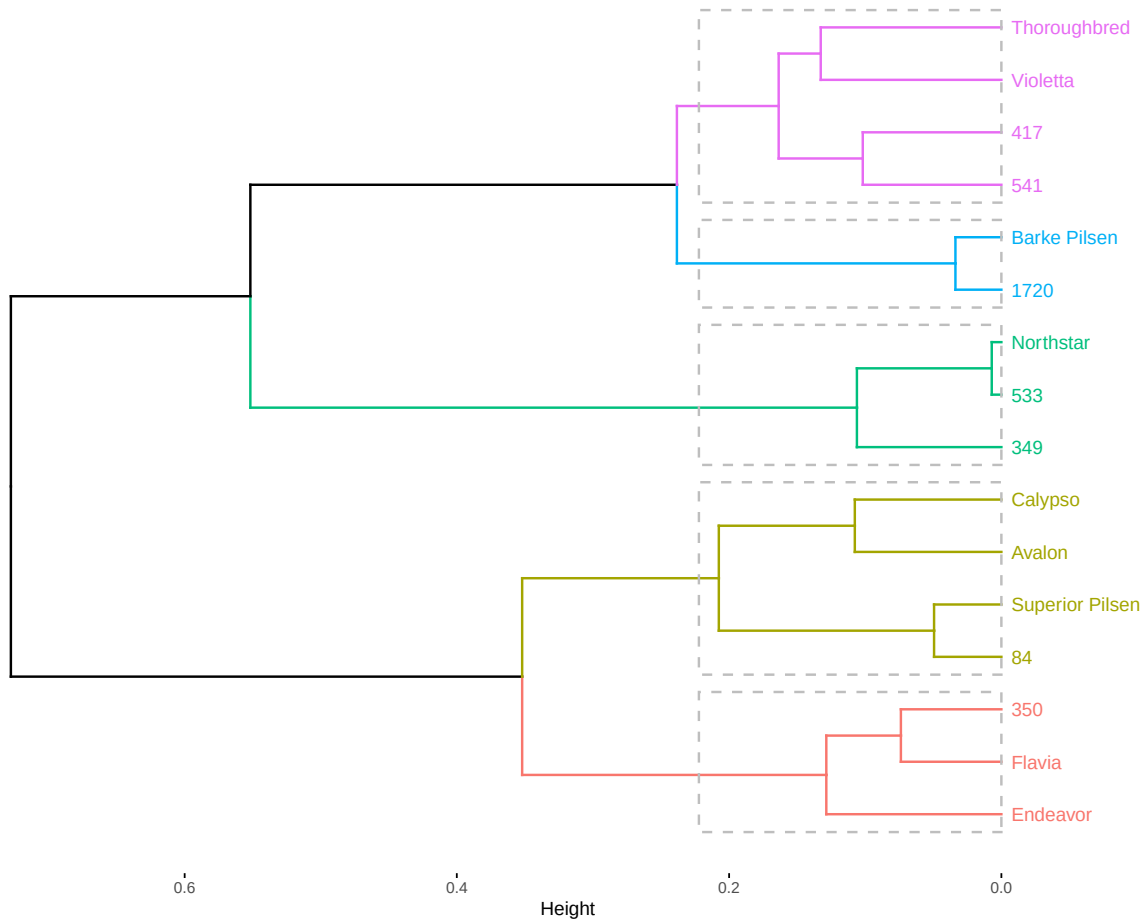


**Figure 14: Dendrogram produced from panelists' sorting task data.**

Hierarchical cluster analysis based on the first two dimensions of the DISTATIS analysis was used to produce a dendrogram (Figure 13) – which can be used to visualize the degree of differences between samples by the length of the branches and vertical lines. Dendrograms with varying amounts of clusters (4 through 6) were created during analysis, and their impact on the clusters shown on the compromise map (Figure 15) was assessed. When clusters were made to represent 4 groups, samples ‘VA17M-13DH1720 LX,’ (“1720”) and Barke® Pilsen (“Barke”) were placed in the same cluster as Violetta, Thoroughbred, ‘VA20BFHB-18DH417,’ (“417”), and ‘VA20MFHB-DH541,’ (“541”). Despite confidence intervals overlapping between the three

samples, VA17M-13DH1720 LX,' and Barke were identified by the dendrogram (Figure 5) to likely belong in their own cluster.

The contingency table of descriptive comments projected onto the compromise map identified malt samples that were labeled with mostly positive descriptors such as “Sweet Aromatic,” “Malt,” and “Biscuit,” from samples that were labeled “Herbaceous,” “Stale,” and “Bready.” The second dimension separated mostly negative attributes such as “Musty,” “Astringent,” “Stale,” “Herbaceous,” “Sour,” from descriptors such as “Sweet Aromatic,” “Malt,” “Biscuit.” The first quadrant of Figure 15 received the greatest number of descriptors, as well as the highest proportion of positive descriptors. Negative descriptors such as “Vegetal,” and “Astringent,” would be expected due to sample preparation, regardless of variety. Husks damaged during milling, particularly over-milling or grinding procedures, will release tannins into the wort causing an astringency (Yin, 2021). Husks may have been damaged during sample preparation due to the use of a burr grinder. The descriptor, “Vegetal,” was placed in close proximity to other descriptors, including, “Grainy,” “Cereal,” and “Sweet,” which is indicative that this characteristic was present regardless of the presence of more positive flavor attributes.

While product maps created by untrained panelists who participate in sorting tasks may be similar to maps created by trained panelists (Faye et al., 2004), the sorting task does not provide a quantitative depiction of sensory results as a Descriptive Analysis task would – and the large number of descriptors used (n=473) can interfere with the precision of terms, especially considering the manual text analysis procedures that must be performed by the researcher (Chollet et al., 2011; Lelievre-Desmas et al., 2008). In the future, depending on the research question, other sensory evaluation approaches such as QDA may prove useful in generating more specific descriptors for a given barley or malt. Another possible source of error was the potential

for panelist fatigue from evaluation of a relatively large sample set (n=16) of samples that were appreciably sweet and astringent. It is possible that panelists would have been better able to describe and distinguish samples if a smaller number were presented to each panelist. However, the literature on sensory sorting tasks indicates that anywhere from 12 to 18 samples can be reasonably evaluated by untrained panelists (Chollet et al., 2014).

### **Sensory Survey Data**

Panelists (n=46) completed an optional survey following the free-sorting task. Panelists were asked “On a scale of one to ten (ten being the most), what is the importance of using local ingredients to you, and why?” Overall, they reported that purchasing local ingredients would be preferred, but due to pricing, availability, and quality, it is not a driving factor in their purchasing behavior. Many (n=14) reported that flavor is most important when purchasing malt, others valued COA values more (n=9), and others (n=7) reported that they purchase malt based on a balance of COA values and flavor. Almost all (n=26) who responded had reported purchasing malts renowned for their distinct flavor, such as Maris Otter<sup>®</sup>, Golden Promise<sup>®TM</sup>, or Barke<sup>®</sup>, however, it was noted that these were usually used for special releases or certain beer styles. Only a few panelists (n=4) responded that they have not purchased these varieties. Most (n=22) stated that they purchased malt from large maltsters such as Rahr Malting Co., LD Carlson, or Malteurop; although some (n=11) reported using more local suppliers such as Proximity Malt or specialty small scale maltsters.

When asked about supplementation of wort with yeast nutrients prior to fermentation, only (n=5) reported not using any and (n=25) answered yes, however some commented that they only use it for particularly high gravity brewing or seltzers. Many specifically reported using non-nitrogenous sources of yeast nutrient, such as zinc heptahydrate or Yeastex<sup>®</sup> 61 (Brewers

Supply Group, Shakopee, Minnesota). None reported measuring free amino nitrogen (FAN) at any point in their quality control program. This points to an expectation by brewers that commercially available malts will be within an acceptable range of FAN. Fortunately, all of the experimental lines evaluated in this study do fall within the AMBA recommended range, and thus show good potential for brewing with regards to FAN.

Overall, panelists had an average of 10 years of brewing experience, ranging from 0 to 29 (including professional and/or homebrewing experience). Although these responses were gathered from 43 panelists (3 panelists did not respond to any questions), there were only representatives from 13 unique breweries – 4 of which had multiple panelists ranging from 2 to 9. However, differences in responses to the survey questions between those from the same brewery likely reflect their personal experience and may also reflect practices from employment at previous breweries.

### **Conclusions**

In conclusion, it was determined that there are significant differences in the development of free amino nitrogen (FAN) from barley to malt to wort between barley varieties. While FAN did not differ among the raw barley samples evaluated, there were significant differences in the FAN concentration of malt and wort samples. This is indicative that these processes play an important role in determination of quality in a breeding program, and the process of malting barley and creating a wort from those malt samples is necessary to determine differences in malt quality between varieties. In order to determine malt quality, barley breeders must submit their experimental barley line samples to laboratories with micro-malting facilities. This is a hurdle for these programs due to their prohibitive cost. These micro-malting procedures also do not tailor malting procedures to specific breeding lines, which can leave breeders with malt quality

analyses that may not match that produced from a commercial malthouse. This makes breeding for the combined agronomic, malt, and flavor quality much more difficult. Breeding programs require high throughput methods, and based on these results the sensory sorting task applied to malt steep teas may be ideal for these purposes due to their low cost, ability to use untrained panelists, and most importantly – its ability for evaluation of a relatively large sample set. Our findings indicate that experimental winter barley varieties bred for the SE Atlantic region match well with established European varieties that are currently being grown with respect to FAN and malt sensory characteristics, and that there is high potential for successful development of barley varieties for craft brewing applications in Virginia.

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## **Chapter 4: Conclusions and Future Work**

Virginia Tech's small grains breeding program continues to develop barley lines with high potential for craft brewing. These lines' range of sensory characteristics and FAN values are a positive indication that Virginia farmers and craft brewers will be able to establish a local supply chain. Malt hot steep sensory characteristics show that experimental lines differ in terms of sensory characteristics, showing the diversity in the program; but they also align well with a range of established varieties and accepted commercial malt examples – indicative of their potential.

Analysis of free amino nitrogen and the amino acid composition showed that these varieties significantly differ in terms of malting behavior and performance in the mash, however some of this variation may be attributed to the methods involved in micro-malting for breeding programs. Specifically, the fact that different barley varieties may require different malting processes to achieve their highest potential for quality and/or flavor, however by design a standard micro-malting process is just that – standard. The micro-malting process cannot be optimized for the extremely small quantities of grain and extremely high number of samples being evaluated in a breeding program. This leads to fundamental challenges in the breeding process, as barley varieties being developed for craft brewing must be evaluated not only for agronomic performance, but also for their potential for success in brewing, where flavor and other complex parameters play important roles. Different barley varieties have different malting behavior based on a variety of chemical and biological factors, and thus in practice do require optimization of the malting process for each barley variety and intended application in brewing. Through these limitations – breeding for agronomic performance, malt quality, and flavor all at the same time is an uphill battle – and current research revitalizing Maris Otter through sensory

evaluation of its better agronomic performing progeny highlights the value that old time-tested varieties still hold in the brewing world (Morrissey et al., 2021). Perhaps with the advent of much faster and less expensive analytical chemistry techniques such as amino acid analysis via UPLC-PDA, we can add value to this challenging process through determination of amino acid profiles in raw barley and gaining a better understanding of the Maillard reactions that occur during the malting process where these free amino acids serve as important flavor precursors. Future research should work towards identifying barley varieties that have similar changes in amino acid profiles that occur during the malting process. Although this is quite complicated and depends on many genetic and environmental factors, it may reveal new and useful information regarding barley varieties with high potential for unique flavor profiles in malt, such as Maris Otter. Studies on beer where classes of flavor compounds were added and evaluated in increments found that the flavor of beer was an all-encompassing and synergistic experience (Yin, 2021); and similar studies on barley varieties with differing amount of amino acids could help to characterize the extent to which this parameter affects beer flavor. Determination of the individual amino acid concentration present in green malt may give indication to potential Maillard reaction products, and in the very least identify barley varieties that may be better suited for darker malts with a higher amount of the MRP flavor notes, such as Vienna or Munich.

Barley varieties that produce malt with a high level of proline should be assessed as to whether they are appropriate in brewing applications. Residual amino acids such as proline (others included) pose a risk to beer's aging potential in as little as one month, and the craft industry which often lacks the means or knowledge to measure free amino nitrogen prior to packaging, as seen in this study, would benefit from these low proline or lower FAN varieties – as suggested by the Brewers Association (Bouckaert et al., 2014).

Taking the current research a step further and producing beer from these barley varieties would be ideal. Although this may not be possible on a large scale due to the small quantities of grain typical available for experimental lines in a breeding program, 5 to 10-gallon batches would allow for sufficient sample for both chemistry and sensory research. The brewing process would alleviate some of the problems that were identified in the current research's sensory study, where samples had a very distinct vegetal odor which may be attributable to dimethylsulfide, and could have distracted panelists from the other similarities between samples. This vegetal character and the sweetness of the samples represented a possible source of error in sensory evaluation due to potential panelist exhaustion. While on one hand the sorting task is ideal for a large number of samples, the effects of sample preparation using the hot steep method may also introduce error due to capturing DMS (vegetal aroma) that could otherwise exit the system during the boil, and other methods of sample preparation or further optimization of the hot steep method for sensory evaluation of malts should be considered. Despite the ability to create distinct clusters of samples showing similarity, the method lacks a robust descriptive nature, as the method of statistical analysis does not allow for group descriptors to be assigned to any projected samples. Descriptive analysis for a smaller set of samples would be a good alternative for targeted research questions regarding effects of individual amino acids or total FAN levels of different barley varieties.

Taken together, our results point to the high potential for development of barley lines suited to Virginia with high quality for craft brewing applications. We have also identified some good questions for future work in the area of malt FAN and the relationship between barley amino acid content and other barley quality parameters on the evolution of FAN and individual free amino acids along the process of malting and mashing. This work should be useful and

informative to barley breeders, maltsters and craft brewers interested in how plant genotypes interact with brewery processing operations to affect the quality of beer.

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## Appendix A

**Table 15: Protein and moisture content of the raw and malted barley used in this study, provided by Montana State University's Barley, Malt & Brewing Quality Lab. Values determined through NIR, and reported as percentages.**

| Variety             | Grain Protein       | Grain Moisture      | Malt Protein        | Malt Moisture      |
|---------------------|---------------------|---------------------|---------------------|--------------------|
| Hirondella          | 8.29 ( $\pm$ 0.29)  | 11.70 ( $\pm$ 0.50) | 9.19 ( $\pm$ 0.25)  | 3.00 ( $\pm$ 0.16) |
| Violetta            | 9.58 ( $\pm$ 0.12)  | 11.82 ( $\pm$ 0.74) | 10.16 ( $\pm$ 0.30) | 3.21 ( $\pm$ 0.13) |
| Avalon              | 9.76 ( $\pm$ 0.86)  | 11.86 ( $\pm$ 0.61) | 10.23 ( $\pm$ 0.58) | 3.27 ( $\pm$ 0.33) |
| Flavia              | 8.54 ( $\pm$ 0.75)  | 11.58 ( $\pm$ 0.53) | 9.89 ( $\pm$ 0.53)  | 3.31 ( $\pm$ 0.22) |
| Calypso             | 8.56 ( $\pm$ 0.42)  | 11.89 ( $\pm$ 0.46) | 9.54 ( $\pm$ 0.24)  | 3.18 ( $\pm$ 0.21) |
| Thoroughbred        | 8.81 ( $\pm$ 0.55)  | 11.59 ( $\pm$ 0.32) | 9.27 ( $\pm$ 0.31)  | 3.18 ( $\pm$ 0.22) |
| Endeavor            | 9.92 ( $\pm$ 0.12)  | 11.56 ( $\pm$ 0.42) | 10.46 ( $\pm$ 0.30) | 3.24 ( $\pm$ 0.15) |
| VA16M-84            | 11.02 ( $\pm$ 0.77) | 11.76 ( $\pm$ 0.38) | 11.33 ( $\pm$ 0.71) | 3.60 ( $\pm$ 0.31) |
| VA17M-13DH1720 LX   | 8.33 ( $\pm$ 0.59)  | 11.70 ( $\pm$ 0.55) | 8.91 ( $\pm$ 0.18)  | 2.97 ( $\pm$ 0.28) |
| VA20MFHB-18DH349    | 9.55 ( $\pm$ 0.31)  | 11.53 ( $\pm$ 0.41) | 10.15 ( $\pm$ 0.32) | 3.51 ( $\pm$ 0.18) |
| VA20MFHB-18DH350    | 8.77 ( $\pm$ 0.79)  | 11.41 ( $\pm$ 0.24) | 9.98 ( $\pm$ 0.57)  | 3.39 ( $\pm$ 0.17) |
| VA20MFHB-18DH533    | 9.31 ( $\pm$ 0.70)  | 11.44 ( $\pm$ 0.20) | 10.20 ( $\pm$ 0.50) | 3.57 ( $\pm$ 0.15) |
| VA20MFHB-18DH541    | 9.31 ( $\pm$ 0.58)  | 11.32 ( $\pm$ 0.17) | 10.35 ( $\pm$ 0.54) | 3.48 ( $\pm$ 0.19) |
| VA20BFHB-18DH352    | 8.60 ( $\pm$ 0.36)  | 11.59 ( $\pm$ 0.09) | 9.83 ( $\pm$ 0.51)  | 3.33 ( $\pm$ 0.14) |
| VA20BFHB-18DH417    | 8.81 ( $\pm$ 0.45)  | 11.55 ( $\pm$ 0.31) | 9.81 ( $\pm$ 0.42)  | 3.27 ( $\pm$ 0.11) |
| VA20BFHB-18DH534 2R | 9.98 ( $\pm$ 0.08)  | 11.20 ( $\pm$ 0.25) | 10.37 ( $\pm$ 0.36) | 3.22 ( $\pm$ 0.20) |

**Table 16: Standard micro-malting protocol used by MSU's Barley, Malt & Brewing Quality Lab.**

| 3 Steep Malting Protocol |              |                 |
|--------------------------|--------------|-----------------|
| Phase                    | Temperature  | Duration        |
| Steep 1                  | 15 °C        | 10 Hours        |
| Air Rest 1               | 15 °C        | 18 Hours        |
| Steep 2                  | 15 °C        | 6 Hours         |
| Air Rest 2               | 15 °C        | 10 Hours        |
| Steep 3                  | 15 °C        | 4 Hours         |
| <b>Germination</b>       | <b>15 °C</b> | <b>96 Hours</b> |
| Kiln 1                   | 60 °C        | 12 Hours        |
| Kiln 2                   | 65 °C        | 6 Hours         |
| Kiln 3                   | 75 °C        | 2 Hours         |
| Kiln 4                   | 85 °C        | 3 Hours         |

**Table 17: Malt specifications of the ASBC recommended sample versus the sample used in this research for grist calibration. CM-SPIL is a commercially available Pilsen malt.**

| Malt Specification | ASBC Recommended Value | CM-SPIL Lot 7245                     |
|--------------------|------------------------|--------------------------------------|
| Variety            | AMBA 6-Row             | 2-Row                                |
| Moisture           | 4.0%                   | 4.4%                                 |
| Color              | $\leq$ 2.0 °L          | 1.5 °L                               |
| S/T                | 41%                    | 41.2%                                |
| FG:CG Difference   | $\leq$ 2.0%            | 1.1%                                 |
| Viscosity          | $\leq$ 1.50 cP         | 1.5 cP                               |
| Assortment         | 100% 6/64"             | 79.5% 7/64", 15.5% 6/64", 4.1% 5/64" |

Table 18: Results from grist analysis using CM-SPIL Lot 7245 as the malt used in calibration.

| Sieve Type           | Percentage Grist Remaining |
|----------------------|----------------------------|
| U.S. Standard No. 10 | 0%                         |
| U.S. Standard No. 30 | 71%                        |
| U.S. Standard No. 60 | 15%                        |
| Receiving Pan        | 14%                        |

Table 19: Malt analyses performed by USDA-ARS for Virginia Tech's Barley Breeding Program for the Warsaw, VA 2022 crop year. This data is not directly comparable to samples used in sensory analysis due to being malted by separate laboratories.

| Line              | Rowed | Malt Extract (%) | Barley Protein (%) | Kolbach Index | Diastatic Power (°L) | β-Glucan (mg/L) | FAN (mg/L) |
|-------------------|-------|------------------|--------------------|---------------|----------------------|-----------------|------------|
| Calypso           | 2     | 80.24            | 11.27              | 34.31         | 141.15               | 139.89          | 105.18     |
| Violetta          | 2     | 79.85            | 11.49              | 36.91         | 156.95               | 205.25          | 133.05     |
| Flavia            | 2     | 78.37            | 10.87              | 35.62         | 88.86                | 395.42          | 114.01     |
| Avalon            | 2     | 80.17            | 11.63              | 43.42         | 156.37               | 141.79          | 160.91     |
| VA16M-84          | 2     | 81.43            | 11.76              | 34.65         | 98.03                | 431.07          | 120.54     |
| VA20MFHB-18DH349  | 2     | 80.65            | 11.51              | 31.72         | 108.33               | 504.9           | 123.68     |
| VA20MFHB-18DH350  | 2     | 81.16            | 10.01              | 36.78         | 121.91               | 242.86          | 123.9      |
| VA20MFHB-18DH533  | 2     | 80.87            | 11.12              | 31.89         | 135.73               | 493.3           | 95.75      |
| VA20MFHB-18DH541  | 2     | 82.22            | 9.68               | 38.06         | 123.34               | 251.27          | 129.72     |
| Thoroughbred      | 6     | 79.78            | 10.23              | 37.45         | 113.72               | 505.96          | 113.31     |
| Hirondella        | 6     | 80.2             | 9.28               | 35.97         | 101.09               | 547.58          | 97.63      |
| VA17M-13DH1720 LX | 6     | 78.7             | 9.93               | 34.88         | 109.6                | 577.96          | 101.37     |

Table 20: Certificate of analysis (COA) data for Superior Pilsen (CM-SPIL), a commercially available malt that was used in the sensory analysis portion of this study.

| Item #  | Lot # | Sizing 7/64" | 6/64" | 5/64" | THRUS | FRI. | Homo | WK  | MOIST | F.G. AS IS | F.G. DRY | C.G. AS IS | C.G. DRY | F/C DIFF. | COLOR | VISC. | pH  | b Gluc | DP    | ALPHA | TTL PROT | SOL PROT | S/T  | FAN   | (Clarity) TURB. | PHENOL p.p.m. |
|---------|-------|--------------|-------|-------|-------|------|------|-----|-------|------------|----------|------------|----------|-----------|-------|-------|-----|--------|-------|-------|----------|----------|------|-------|-----------------|---------------|
| CM-SPIL | 7245  | 79.5         | 15.5  | 4.1   | 0.9   | 89.4 | 99.2 | 0.2 | 4.4   | 77.8       | 81.4     | 76.7       | 80.3     | 1.1       | 1.5   | 1.5   | 6.0 | 113.0  | 122.0 | 47.5  | 10.8     | 4.5      | 41.2 | 170.0 |                 |               |

Name: \_\_\_\_\_

Age: \_\_\_\_\_

Sex: \_\_\_\_\_

**Malt Sensory Evaluation – Sorting Task**

There are **16** malt tea samples in front of you in labeled cups. Please smell and taste these samples, rinsing your mouth with water between each. If you would like to spit out the samples, the red cup in front of you can be used for that.

Please group the samples based on similarities. Similar samples should be placed in the same group. You may create any number of groups between two (2) and fifteen (15). Please record the groupings below, using the three-digit numeric codes as the name for the samples. Do not place samples in more than one group.

**In the space below, please record:**

- ☐ Your groupings (using the sample's respective three-digit code).
- ☐ A few words describing the defining characteristics of each group. In other words, what descriptors could you use for these samples that make them similar to each other, but different than other samples? You may add frequency terms as well, such as "low," "medium," or "high."

Thank you for your participation. Once completed, please turn the page to complete a brief survey.

Figure 15: Sorting task instructions provided to panelists.

**Malt Sensory Evaluation – Survey**

Please answer these questions to the best of your ability. If you are uncertain or the question does not apply to you, please write “N/A.”

1. What is your current base malt for your flagship brand, and how/who do you source it from?
2. On a scale of one to ten (ten being the most), what is the importance of using local ingredients to you, and why?
3. In your opinion, what makes an ingredient local? At what point, or distance, does an ingredient become no longer local?
4. What is the barley variety of the base malt you purchase?
5. Have you ever sourced malt based on the barley variety (i.e. Maris Otter, Golden Promise, Barke)? If so, how often do you do this?
6. Do you purchase base malt based on its contribution to flavor, or are certificate of analysis (COA) targets valued more?
7. Do you currently use yeast nutrient? If so, what factors motivate you to do so? If you use nutrients, do you adjust the amount used based on the free amino nitrogen content (FAN) of your malts?
8. Do you currently have a quality lab that measures FAN? If so, is this measured at multiple points in the brewing process?
9. How many years of brewing experience (or malt sensory experience) do you have?

**Figure 16: Survey questions for panelists to respond to following the sorting task.**