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Biogenic Amines as a Product of the Metabolism of Proteins in Beer

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By

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For many years the only beer that was commercially available in the United States were simple lagers and ales made primarily of barley (and other cereals), water, hops, and yeast. These beer varieties were simple and quick to produce with high levels of consistency. In the last 30 years the rise in craft and microbreweries have dramatically changed the landscape of brewing and beer. As smaller breweries rose in popularity so did the use of unique ingredients such as vegetables, fruits, herbs, meat, etc. along with higher gravity mashes and long barrel aging times. All these ingredients have a profound effect on the chemical makeup of a beer, adding a variety of organic acids, antimicrobials, amino acids, etc. These compounds become important when examining the influences of what may be the most important ingredient in beer (and all alcoholic beverages), yeast.

Yeast, specifically *Saccharomyces* yeast (though many other genera are capable of this), consume simple carbohydrates within the unfermented beer and produce carbon dioxide and alcohol. While these fermentation biproducts are the most well-known compounds produced by yeast, there are many others. During development (log phase) and times of stress/carbohydrate starvation, yeast can often consume proteins and amino acids and produce biogenic amines. Biogenic amines (BA's) are small nitrogenous bases produced through the enzymatic decarboxylation of amino acids by living organisms. Examples of biogenic amines include histamine, serotonin, tyramine, and spermine (though many others

exist). These compounds are important and powerful signaling molecules used by every living creature on Earth. While endogenous biogenic amines produced within the body are critical to survival, exogenous biogenic amines in a consumed food product can be highly dangerous; biogenic amines can cause a variety of symptoms ranging from headaches to hyper/hypotension to pseudo-anaphylaxis. While all living things create these compounds, the levels in most food products are too low to cause any symptoms: The only products with potentially dangerous levels of biogenic amines are fermented foods as bacteria and fungi used to make these products can also produce biogenic amines.

While the biogenic amine content in certain fermented foods like cheese, sausages, and lacto-fermented vegetables has been well studied, formation of biogenic amines in beer remains largely under-examined. The three main objectives of this research were: 1., to perform an extensive review of the literature to ascertain analytical methods, types, levels, and sources of biogenic amines in beer, 2., to develop a quick and effective high performance liquid chromatography (HPLC) method to examine the biogenic amine content in beer, and 3., use this HPLC method to test a variety of commercial beer sample to examine what effect (if any) these new microbrewery recipes had on the biogenic amine profile of the final beverage.

Method development focused primarily on the tagging of BA's using the Waters AccQ Rapid Amine Tag (AccQ Tag Ultra, 2014) and analysis using an Agilent Organic Acids column. In total, more than 17 HPLC methodologies were tested and only one effectively resolved a mixed amine standard solution.

Despite this successful test using a mixed standard, no mobile phase adjustment was able to effectively separate biogenic amines from interfering compounds (free amino acids or other nitrogenous compounds) in a beer sample. It was ultimately decided that moving forward, an effective extraction method, involving sample cleanup and capable of separating biogenic amines from highly soluble peptides and amino acids would be necessary for successful analysis.

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CHAPTER 1

INTRODUCTION

A growing concern for food scientists and regulatory groups is the amount of biogenic amines found in fermented food products (EFSA 2011). Biogenic amines are a diverse group of small, nitrogenous bases that act as signaling molecules in living organisms. Biogenic amines are produced by every living organism on the planet and they monitor and signal a variety of different bodily processes. These amines are perfectly healthy when they are produced within the body (endogenous) but can be extremely dangerous when they are consumed from an outside source (exogenous). Some of these biogenic amines cause a variety of health effects including gastroenteritis, hypertension, hypotension, cell death, and even pseudo-anaphylaxis (Visciano et al. 2014).

While all living organisms produce these compounds, the levels in most food products are so low that no ill effects occur when consumed (Gammone et al. 2019). One exception to this rule is fermented foods. All fermented foods utilize fungi or bacteria to break down various carbohydrates such as glucose, sucrose, amylose, lactose, etc. to produce organic acids, alcohols, carbon dioxide, and a variety of flavor compounds. This process gives rise to all fermented products we readily consume. While bacteria and yeast produce desirable compounds, they can also produce other, less desirable biproducts. When bacteria and yeast run out of digestible carbohydrates, they often start utilizing proteins and amino acids present in that food product as carbon sources (Erdag, Merhan, and Yildiz 2018). When these microbes digest amino acids, they perform various decarboxylating reactions which convert these nitrogenous chemicals into biogenic amines. The major biogenic amines found in fermented foods are histamine, tryptamine, tyramine, spermine, spermidine, putrescine, cadaverine, and gamma-aminobutyric acid (Silla Santos 1996).

While much study has focused on biogenic amines in foods like cheese, sausages, lacto-fermented vegetables, and fish, beer biogenic amines have been largely ignored. Beer is an alcoholic beverage historically produced using barley (although other cereals such as wheat, rye, sorghum, rice, corn, and oats are now commonly used), water, hops, and fermented with *Saccharomyces* yeast. For many years these simple, basic beers (mostly pilsners) were the only brews commercially available in the United States but over the last 30 years the landscape of beer styles has changed drastically(Tucker 2011). As craft breweries have risen in popularity so too have the extent of unique and strange ingredients used in brewing recipes. Herbs, vegetables, fruits, strange microbes, meat, all see use in a variety of breweries, combined with the rediscovery of barrel aging and high gravity recipes the results span a myriad of beer styles and flavor profiles (Tucker 2011). All these ingredients and methods have a profound effect on the chemical makeup of the final beverage - which becomes especially important when considering the types and use of yeast. These eclectic ingredients can introduce compounds like antimicrobials, organic acids, amino acids or environmental conditions like hypertonic solutions (high gravity) and a lack of carbohydrates (barrel aging) to the beer. These various compounds and environmental conditions are known stressors for yeast, which may promote the production of biogenic amines. It isn't currently known what effects these new adjuncts have on yeast or the biogenic amine composition of beer; the increasing concern over the health effects of biogenic amines means discovering the link between beer style and amine profile is even more important than ever.

The work described in this thesis is three fold thesis has two main objectives: 1, to perform an extensive review of the literature to ascertain analytical methods, types, levels, and sources of biogenic amines in beer, 2, to develop a quick and effective high performance liquid chromatography (HPLC) method to examine the biogenic amine content in beer, and 3, use this HPLC method to test a variety of commercial beer samples to examine what effect (if any) these new microbrewery recipes had on the biogenic amine profile of the final beverage.

CHAPTER 2

A REVIEW OF BIOGENIC AMINES ASSOCIATED WITH FOOD AND BEER

2.1. Introduction

Biogenic amines are a large family of diverse chemical compounds. To be considered a biogenic amine the compound must be produced by a living organism ("biogenic") and contain at least one amine group. These various chemicals find use as powerful signaling molecules, precursors to other biologically important chemicals, and as waste products: They can also be found in other organism specific niche roles (Erdag, Merhan, and Yildiz 2018). When considering food science, the most important property shared by members of this family is their ability to act as signaling molecules, especially within the human body (Gammone et al. 2019). Some well-known members include serotonin, histamine, epinephrine, cadaverine, and putrescine (Figure 2.1).

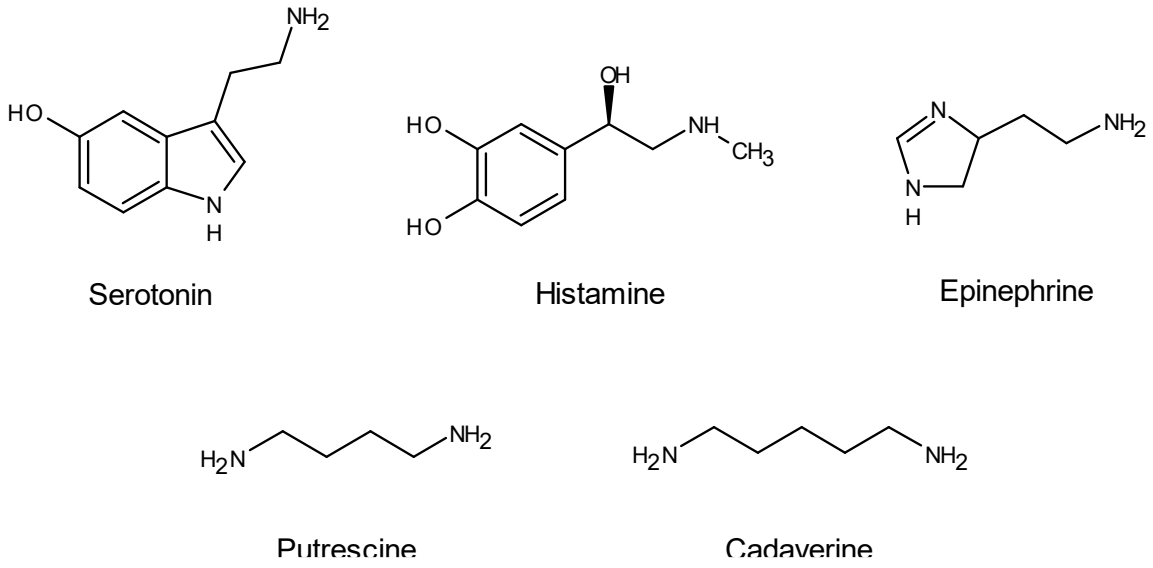

Figure 2.1. Well known biogenic amines

2.1.1. Exogenous vs. Endogenous

An important distinction is made between exogenous and endogenous biogenic amines. Endogenous biogenic amines are produced by various cells within the body and are moved through the circulatory system. These compounds are necessary for human development and survival as they allow organs and cells to communicate. Exogenous biogenic amines are produced by outside organisms (usually present in food) and absorbed through epithelial cells of the gut (Fernández-Reina, Urdiales, and Sánchez-Jiménez 2018). Foreign to the organism, these exogenous chemicals can have healthful or harmful implications.

Once exogenous biogenic amines are introduced to the body, a variety of physiological effects can occur, some of which can be quite severe. Certain biogenic amines such as histamine, tyramine, and putrescine can have powerful vasoconstrictive properties leading to high blood pressure and an increased risk of heart attack and/or stroke (Gammone et al. 2019). Other biogenic amines such as spermine and spermidine appear to have vasodilating effects (Til et al. 1997). Mice fed diets rich in spermine and spermidine have on average lower blood pressure than control mice (Wing, Chan, and Jiang 1993). Tyramine and histamine are commonly believed to be the most dangerous biogenic amines commonly found in foods (Linares et al. 2016). In addition to their blood pressure modulating effects, each have other, unique physiological effects. Ingestion of foods high in histamine can cause a pseudo-anaphylaxis effect with hives, throat swelling, and other allergic-like symptoms occurring; these reactions are most commonly seen in temperature abused seafood (Gammone et al. 2019). Foods with high levels of tyramine can cause intense migraines coupled with nausea and vomiting (Gammone et al. 2019).

2.1.2. Health Effects

The specific pathophysiology of each biogenic amine is unique and not completely understood. The most relevant amine poisoning to the food industry is histamine or so called “scombroid” poisoning, named for the fish family *Scombridae* (Chen et al. 2010). This type of food poisoning is usually caused by temperature abuse of fish like tuna, mackerel, and sardines which allows histidine decarboxylase-producing bacteria to grow. As these bacteria grow, they feed on the large concentrations of histidine in the fish and produce histamine (Visciano et al. 2014). These histamine covered fish are then consumed and poisoning occurs. The symptoms of scombroid poisoning are very similar to that of a severe allergic reaction: Flushing of the skin, inflammation of the tongue and throat, and asphyxiation. This is not considered an allergic reaction because this poisoning occurs in anyone who ingests the food and isn’t triggered by an immune system specific immunoglobin (Comas-Basté et al. 2019).

The mechanism by which histamine causes this poisoning is still unknown, though several theories have been proposed and studied. The most commonly cited theory is that the histamine is simply absorbed through the stomach after which it activates the body’s H receptors (Edigin et al. 2019). H receptors are histamine specific receptor sites located on a variety of cells and tissue types in the body. There are four types of H receptors currently known to medicine, with the H1 receptor being the one capable of triggering allergic responses in the body (Togias 2003). After being activated by increased serum histamine blood vessels are dilated and various inflammatory symptoms begin occurring. There are still unanswered flaws with this theory that cast doubt on the validity of it. The human body and gut are filled with enzymes that decompose histamine, namely diamine oxidase and histamine N-methyltransferase. These enzymes should decompose any histamine present in the stomach before it can be absorbed into the blood stream (Lehane and Olley 2000). For this theory to be correct some other compound in the fish (potentially other amines) must be disabling the enzymes (Comas-Basté et al. 2019).

Another potential biochemical explanation is the “mast cell degranulation” theory. In this model histamine and other molecules present on the fish interact with cells in the stomach (instead of being absorbed) which then signal the body to release histamine from the body’s natural reserves (Lehane and Olley 2000). The release of chemicals stored within cell organelles is known as degranulation.

Cell line studies have shown another, potentially more dangerous side effect: cytotoxicity. Histamine and tyramine have been shown to both directly kill human cells (necrosis) and induce programed cell death (apoptosis) respectively. The mechanism for either form of death is currently not well understood but both have been consistently shown (Linares et al. 2016). Other amines such as cadaverine, spermine, and putrescine have been shown to improve the ability of histamine and tyramine to kill cells (Gammone et al. 2019). This effect occurs through a combination of interactions between these amines and the various detoxifying enzymes present in the body. This cell killing property is still not well characterized but our understanding of it is constantly evolving. Despite long held beliefs that they were not overly damaging spermine and putrescine have recently been shown to destroy cells through necrosis (del Rio et al. 2019). This toxic effect appears to occur at levels present in fermented food products (del Rio et al. 2019). As these amines become better understood researchers are beginning to advocate for stronger legislation regulating the allowable levels of these compounds in food. In the United States the Food and Drug Administration has set a legal limit of 50ppm (5 mg/100 g) of histamine in seafood products, but no specific regulations exist for other biogenic amines or for histamine in beer (FDA 2005)

While most biogenic amines have at best, mixed effects on the human body, there is one that appears beneficial effects; gamma amino-butyric acid (GABA). GABA is an important neurotransmitter in the brain that functions by reducing neuron activity and helping to regulate such bodily processes as appetite, sleep, anxiety, and pain (Kakee et al. 2001). The human body primarily gets GABA by creating it within the brain, but dietary supplementation is becoming a rising trend (Kanehira et al. 2011). The

concept behind dietary supplementation (either through medical supplements or food products with high concentrations of GABA) is to potentially relieve physiological issues as anxiety, insomnia, and seizures. The controversy surrounding these products has historically been between GABA and the blood-brain barrier. It has long been assumed that GABA, much like other neurotransmitters such as serotonin, could not cross the blood brain barrier necessitating the creation of modified compounds like the drug gabapentin to be absorbed via the diet (Cai et al. 2012). This would mean dietary sources of GABA, much like dietary serotonin, would be completely useless but this is not necessarily the case. Recent studies have shown that in actuality GABA may be readily absorbed into the brain through specialized receptors, but that the brain just efflux's GABA out of the brain at a much higher rate (Kakee et al. 2001). Some studies have also shown that we may be able to increase the absorption rate of GABA when exposed to compounds like nitric oxide, though more work needs to be done before this can be completely verified (Shyamaladevi et al. 2002). As our understanding of GABA and the interaction between dietary sources and brain health, knowing the GABA concentrations of food like beer becomes increasingly important. While there has been a lack of studies done on GABA in beer, the few that have been completed show that beer has very high concentrations of this neurotransmitter (Tang et al. 2009). If beer is consistently shown to have a significant concentration of GABA, and if dietary supplementation is deemed effective, the potential for a beer-based functional food becomes very high.

2.2. Biogenic Amine Chemistry and Chemical Properties

When discussing a group of chemicals, it is important to consider the chemistry of those compounds. Biogenic amines are a very diverse group, with individual members having vastly different structures (rings, branched chains, chemical groups, etc.). Despite the variation in structure, members of this chemical family share many properties including formation, volatility, and organoleptic properties.

2.2.1. Formation

Biogenic amines tend to form in two main ways, by transamination of an existing compound or through the decarboxylation of amino acids. While each method is utilized depending on the amine being formed, decarboxylation is the most common. In the decarboxylation reaction the carboxylic acid group is oxidized and removed from the amino acid skeleton producing the amine and a free carbon dioxide (Erdag, Merhan, and Yildiz 2018). This process does not occur spontaneously and requires various enzymes to proceed, usually specific to the amino acid they decompose; histidine decarboxylase exclusively decomposes histidine while tyrosine decarboxylase only decomposes tyrosine (ENZYME entry 4.1.1.25 2012). Living organisms utilize these reactions to either directly create the amines for various purposes or as a waste intermediary for amino acid digestion (in which case an organism may utilize various deamination enzymes to decompose the amine further). Biogenic amines can also be created from other biogenic amines, most famously the putrescine-spermine-spermidine cycle (Erdag, Merhan, and Yildiz 2018).

2.2.2. Chemical Properties

Structurally biogenic amines and amino acids are very similar, usually differing in a single carbonyl group. While the removal of a singular carbonyl is only a small structural change, the effects on the chemical properties of the chemicals is rather large. The amine form of an amino acid is usually less water soluble because the highly water-soluble carbonyl group (which can participate in hydrogen bonding) is replaced with a singular hydrogen. This change also leads to the increased volatility of biogenic amines. The volatility leads to a very recognizable trait most amines share- a strong, unpleasant odor (English, Deore, and Freund 2006). Various biogenic amines, but especially cadaverine and putrescine, make up a large portion of the scent of decaying flesh and rotting meat (Erdag, Merhan, and Yildiz 2018). Amines and amino acids also differ in their relative pH values with amines usually behaving

as bases and amino acids behaving as acids (Beneduce et al. 2010). By decarboxylating the amino acids, organisms are able to control the pH of their environments. When an amine is created from an amino acid, an acidic hydrogen is removed from a solution, leaving behind a basic amine, which can allow microbial protection from highly acidic environments. The structural similarities can be seen in Figure 2. 2.

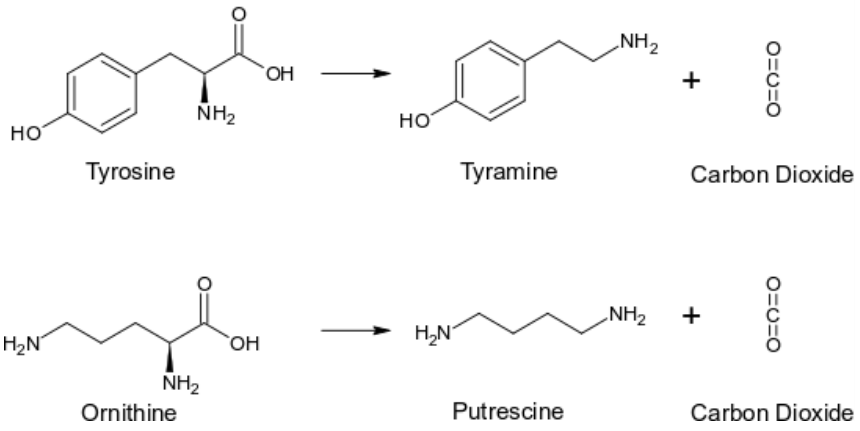

Figure 2.2. Illustrated decarboxylation reaction for Ornithine and Tyrosine

2.2.3. Structural Properties of Biogenic Amines

While amino acids and their conjugate amines have similar chemical structures, significant variation exists between biogenic amines. Amines are broadly characterized by their structural components and/or physical properties and fit into four major categories: aromatic, heterocyclic, aliphatic, and volatile (Silla Santos 1996). The aromatic and heterocyclic amines differ in the structure of their rings with aromatic amines (phenethylamine and tyramine) having a benzene ring while heterocyclic amines (histamine, tryptamine, serotonin) have modified ring structures (double rings, nitrogen substituted rings, etc.). These amines have historically been thought of as the most toxic. Aliphatic and volatile amines are both acyclic molecules, but they differ in the number of amine groups

present on the molecules. Aliphatic amines (putrescine, cadaverine, spermine) have two or more amino groups, while volatile amines (ethylamine, methylamine) contain a single amine, making them much more volatile. These amines have historically been assumed to be less hazardous (though modern studies are offering evidence to the contrary (del Rio et al. 2019)) but more unpleasant due to their intense, repulsive odor and low vapor pressure (Zeisel and DaCosta 1986) . Regardless of the structural differences, biogenic amines all tend to be extremely stable and non-reactive once formed (Veciana-Nogués, Mariné-Font, and Vidal-Carou 1997). Histamine has been shown to withstand boiling, frying, and baking with no evaporative loss or decomposition reactions (Lehane and Olley 2000).

2.2.3.1. Aliphatic Biogenic Amines and Nitrosamines

Evidence indicates that certain aliphatic amines can undergo a series of reactions to become nitrosamines in the presence of nitrites. The mechanism for this reaction is not well understood but it has been hypothesized that amines such as cadaverine polymerize into rings which then combine with nitrites (which can be formed through bacterial interactions or by impure chloride or nitrate salts) to form the nitrosamines(Mitacek et al. 1999). An example of this reaction is shown in Figure 2.3. Nitrosamines are considered powerful carcinogens. Nitrosamines damage DNA by binding to the DNA molecule and causing structural damage. These changes can lead to mutations and eventually cancer (Song, Wu, and Guan 2015). While it is known that these chemicals can cause cancer in animals the effects on humans have not been extensively studied. Meta-analysis of cancer rates in countries with diets high in nitrosamine/nitrite/nitrate content do show a potential link to increased rates of stomach cancers (Song, Wu, and Guan 2015). The lack of direct study makes drawing conclusions difficult, especially as data has begun showing that nitrites and nitrates may be beneficial to the cardiovascular system (Song, Wu, and Guan 2015). It is important to note that while a link appears to exist between biogenic amine content and nitrosamine formation there have been no concrete causal associations.

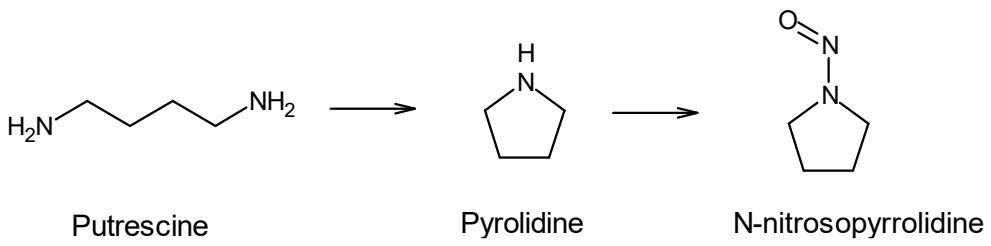

Figure 2.3. The potential reaction chain for the transformation of Putrescine to N-nitropyrrolidine

2.3. Biogenic Amine Analytical Methods

Because of their deleterious health effects, having methods for analyzing biogenic amines in food products is extremely important. A common tool for analyzing these compounds is high-performance liquid chromatography (HPLC) coupled with fluorescence detection. Biogenic amines can't be directly measured through fluorescence detection because they lack fluorophores and must be tagged with a fluorescently active tagging compound (Weremfo et al. 2020).

2.3.1. Dansyl Chloride

The most commonly used technique for measuring the concentration and types of amines in a product utilizes the chemical compound dansyl chloride. Dansyl chloride is a ringed, sulfur containing fluorescent chemical tag (the dye is not fluorescent until it reacts with a primary amine) that binds to the amine groups of amino acids and biogenic amines. The tagged amino acids/amines are run through a fluorescent detector after HPLC separation, and any data is collected and analyzed (Walker 1997). This method is sensitive, effective, and widely used, but several factors make it problematic to utilize. Derivatizing a biogenic amine sample is a complicated and time-consuming task. A given amine sample needs to be mixed with dansyl chloride, solvent (acetone and toluene), sodium carbonate and heated in water bath for 90 minutes (Minocha and Long 2004). After heating is completed the sample needs to evaporated to remove the acetone and centrifuged to break the water-toluene emulsion; the toluene layer contains the amines and is extracted (Minocha and Long 2004). Next the toluene is evaporated,

and the amines are rehydrated using methanol, after which acetic acid must be added to decompose any remaining carbonate (the procedure must occur in this order or the reaction will fail); the reaction will be complete after 4 minutes (Minocha and Long 2004). After these steps are complete the samples can then be run using HPLC with fluorescence detection and any data can be analyzed. So, while the dansyl chloride derivatization method is very effective, it is far from rapid or convenient as it requires 6 different chemicals, several pieces of lab equipment, and more than 90 minutes to complete.

2.3.2. 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Waters AccQ Method)

A promising alternative method is the use of Waters Fluor AccQ tagging system. This method was developed to tag the amine group of amino acids for chemical analysis through HPLC Fluorescence but has been used for biogenic amines in foods like cheese and wine with success (Busto, Guasch, and Borrull 1996). For this method amine groups are tagged with the fluorescent dye 6-aminoquinolyl-n-hydroxysuccinimidyl carbamate which form fluorescent amine complexes (AccQ Tag Ultra, 2014). Derivatizing a sample with this method is quick and relatively simple. A sample of material is loaded into a vial and is mixed with borate buffer and the Fluor AccQ tag chemical; the sample is vortexed or mixed for one minute (AccQ Tag Ultra, 2014). After vortexing, the sample is than heated at 50°C for up to 10 minutes to facilitate the chemical (tagging) reaction; after the heating step the sample is completely derivatized (AccQ Tag Ultra, 2014). After the tagging is completed the sample can be run using HPLC to separate the amine compounds and fluorescence for detection. Compared to the complex and time-consuming dansyl chloride technique, the Fluor AccQ method is quick and easy. One can derivatize a sample and begin the HPLC analysis in only 10 minutes using basic laboratory equipment (a mixing device and a heating device). The Fluor AccQ tagging method hasn't been utilized for beer, but has been used successfully in wine (Vinci, Restuccia, and Antiochia 2011) and was able to successfully separate 11 amines in a single run. There are aspects that may need to be adapted for a beer sample, namely the removal of hop and protein residue, and a number of tag-reactive free amino acids, which aren't as

abundant in wine. It is also important to note that the targeted biogenic amines in beer are different from the biogenic amines targeted in other food products. In seafood histamine is the only targeted amine due to the high content of free histidine (Weremfo et al. 2020) while in fermented cheese tyramine is the primary target, due to the high concentration of tyrosine found in milk (Mayer, Fiechter, and Fischer 2010). Unlike the previously mentioned food products beer has a more diverse variety of biogenic amines. While the most concentrated biogenic amine in beer is gamma aminobutyric acid (due to the high concentration of glutamic acid in barley) several other amines (histamine, tryptamine, tyramine, spermine, spermidine, putrescine, and cadaverine) are also present in anatomically significant concentrations (Bollig 2015).

2.3.2.1 Variation in Waters AccQ Biogenic Amine HPLC Methodologies

The Waters AccQ Fluor system was developed as a rapid tool for the analysis of essential amino acid hydrolysates from food stuffs, and while there is a standardized method for tagging food samples with the Waters AccQ Tag for general amino acid work, there is no standard method for running the HPLC separation with Biogenic Amines. All published methods rely on gradient elution's rather than isocratic separations but the composition of the mobile phase and the gradient itself varies. One methodology published by Thomas Weiss (2005) (testing tumor samples from animals) uses a gradient elution with three mobile phases; solvent A (70 mM acetic acid / 25 mM triethylamine, titrated with phosphoric acid to pH 4.82), solvent B (acetonitrile / water 80:20 (v/v)) and solvent C (methanol) (Weiss 2005). The gradient he utilized can be seen below in Table 2.1.

Table 2.1. HPLC methodology adapted from Thomas Weiss (Weiss 2005)

Elution Time (min)	A %	B %	C %
0	100	0	0
1	78	22	0
15	78	22	0
27	55	39	6
27.5	53	33	14
34	20	10	70
37	0	100	0
57	0	100	0

This methodology was very effective but resulted in poor resolution of similar amines, leading the author to publish a modified method with clear separation of major amines (like histamine) but with reduced clarity for others (i.e., spermine) (Weiss 2005). For this separation three mobile phases were tested, but each was slightly altered from the initial method; solvent A (70 mM acetic acid / 25 mM triethylamine, titrated with phosphoric acid to pH 5.6), solvent B (acetonitrile / water 80:20 (v/v)) and solvent C (methanol) (Weiss 2005). This altered solvent profile was also paired with a modified gradient which can be seen below In Table 2.20.

Table 2.2. Improved HPLC method adapted from Thomas Weiss (Weiss 2005)

Elution Time (min)	A %	B %	C %
0	100	0	0
1	78	22	0
15	78	22	0
27	65	35	0
27.5	60	40	0
34	20	10	70
37	0	100	0
57	0	100	0

Other published articles describe gradient elution's but with only 2 mobile phase solvents, though the solvents vary between publication. The recommended method proposed by Waters in their instruction manual (though designed for amino acids) uses the following parameters, adapted from an article by Nagashima, Yusuke, et al. published in 2012 for analyzing animal tissues. This method uses the following solvents; Solvent A was 4% acetonitrile in 0.1 M ammonium acetate while Solvent B was 60%

acetonitrile in .1 M ammonium acetate both at a pH of 6 (Nagashima et al. 2012). The elution gradient used was as follows “The gradient conditions were: 0% B for 25 min, 0–3.5% B for 20 min, 3.5–6% B for 0.5 min, 6% B for 10 min, 6–7% B for 0.5 min, 7% B for 10 min, 7–8% B for 0.5 min and 8% B for 10 min. Following gradient elution, the column was rinsed with 100% B for 5 min” (Nagashima et al. 2012). Another methodology published by P. Hernández-Orte et al. (2006) for analyzing wine uses a different two solvent system for analysis; Solvent A was a 140 mM solution of sodium acetate trihydrate and 17 mM of Triethylamine adjusted to pH 5.05 and Solvent B was methanol. The elution gradient can be seen in Table 2.3.

Table 2.3. HPLC method adapted from Purificación Hernández-Orte (Hernández-Orte et al. 2006)

Time (min)	A (%)	B (%)
0	75	25
5	75	25
17	60	40
42	35	65
47	20	80
48	0	100

Considering the high level of variability in the published analytical methods there are many possible routes to explore when testing beer samples for biogenic amines. Beer’s unique mix of soluble proteins, peptides, and free amino acids introduces some unique separation and analytical challenges when compared to other food matrices and may necessitate trying different columns and solvent combinations to properly separate the components. Reverse phase columns are the most widely used for amine separation and take advantage of the polarity of the amines, but there is potential for other column chemistries, like ion exchange, to be useful. Ion exchange columns are commonly used to separate amino acids, organic acids, alcohols, simple sugars, and proteins (similar in structure to biogenic amines). It is also necessary to explore the solvent system used for the separation. It would be ideal to use a simple binary separation with acetonitrile and water for ease, but it may be necessary to

use other systems; ternary separations and/or more exotic solvent modifiers, such as ammonium acetate, triethylamine, or acidified acetonitrile. Regardless, more study needs to be done to develop a simplified testing methodology when considering the unique chemical properties of the biogenic amines and beer matrices.

2.4. Microbial Origin of Biogenic Amines

While biogenic amines are found in all living organisms (bacteria to fungi to eukaryotes, etc.), most meat, vegetable, fruit, and grain products don't have high enough concentration on their own to cause any adverse reactions. Additionally, the decarboxylation reaction that converts amino acids to biogenic amines does not occur spontaneously and requires the production of specific enzymes. This means for foods to reach a biogenic amine concentration where health and/or sensory effects occur microbial intervention is required. These enzymes are most commonly produced by a multitude of different bacterial and yeast species. The strongest producers in the bacterial kingdom tend to be found in the *Morganella*, *Enterobacter*, *Lactobacillus*, *Oenococcus*, and *Staphylococcus* genera; of these bacteria *Morganella morganii* is, according to Mastaka Satomi, "particularly well known as a significant histamine producer, owing to its rapid growth and the potent activity of its histamine-producing enzyme" (Satomi 2016). These bacteria are common spoilage microbes for a variety of foods but are sometimes intentionally added to produce fermented products (see *Lactobacillus* and *Oenococcus*). In addition to these bacteria many species of yeast are able digest amino acids into biogenic amines. Of all the yeasts tested by Caruso et al (2002), *Saccharomyces cerevisiae* and *Brettanomyces bruxellensis* were among the most abundant biogenic amine producers. These yeasts were able to produce various amines at a mg/L level (Caruso et al. 2002). Both species of yeasts are commonly used to produce various alcoholic beverages with *S. cerevisiae* being used to produce most beer, all wine, all fruit wine, and all mead at every production scale (amateur to mass production) globally.

2.4.1. Impact of Food Composition on Biogenic Amine Producing Microbes

The production of biogenic amines by bacteria and yeasts is highly dependent on environmental conditions. The first and potentially most important parameter is the chemical makeup of the food product. Amine production depends on amino acid availability, so larger concentrations are usually found in high protein foods. The biogenic amine composition of a food is usually a direct reflection of the types of proteins and amino acids found in the product. One reason the previously mentioned Scombroid fish can have such high levels of histamine is that the muscle tissue of the fish is rich in histidine (Edigin et al. 2019). While the amino acid makeup provides the materials for the reaction to occur, the major trigger to begin producing amines is physiological stress (Beneduce et al. 2010). There are many potential microbial stressors in the food world, including alcohol, dryness, and salinity, but acidity is the most common. The production of biogenic amines allows microbes to modulate the pH of their environment by converting the acidic amino acids to more basic biogenic amines (Satomi 2016). Amine production also increases as glucose and other simple, easily digested sugars are metabolized for energy.

2.4.2. Controlling Biogenic Amine Food Spoilage in Fresh Foods

Controlling food spoilage by amine producing bacteria is a simple process. The best way to prevent contamination of fish (the most commonly impacted food), meat, and poultry by the previously mentioned microbes is by practicing safe meat handling. Foods vulnerable to biogenic amine formation must always be kept at refrigerator or freezer temperatures before cooking. Keeping meat cold can slow or stop the reproduction of amine producing bacteria and yeasts which would rapidly grow on these amino acid and protein rich foods (Nutrition 2020).

It is especially important that fish (due to its high free histidine concentration) is refrigerated at all points from harvest until it is prepared for consumption to prevent bacterial growth. The proper removal of fish offal and gill tissue appears to drastically reduce the level of amine-producing bacteria (Visciano et al. 2014).

The variety of decarboxylating bacteria present in food products makes the changing of various food characteristics like pH, salinity, etc. sparingly effective. It has been shown in various studies that acidic pH and higher salt concentrations may deactivate certain enzymes like histidine decarboxylase while increasing the activity of others, such as tyrosine decarboxylase (Silla Santos 1996). It is important to avoid exposing high protein food products to excess sugar or similar growth-promoting nutrients as they may cause amine-producing bacteria to proliferate. There have been some studies focused on remediation for food thought to be exposed to histamine (Linares et al. 2016)but no officially approved methods currently exist. Because biogenic amines are extremely heat-stable cooking has no useful (reduction) effect on amine levels once they are produced. Some studies even propose that cooking may increase the level of chemicals like histamine in food (potentially due to the increased enzyme activity at higher temperatures) though the mechanism for this is not known at this time (Chung et al. 2017). One promising method of remediation is the use of biocontrol. Certain species of *Bacillus* bacteria such as *B. subtilus* and *B. amyloliquefacines* can produce large quantities of the enzyme diamine oxidase which can deactivate and decompose biogenic amines (Naila et al. 2012). An example of this reaction series is depicted in Figure 2.4. These cultures could hypothetically be used in both a preventative and remediation capacity. While there are promising techniques for fixing amine infected meat no method has been approved by the FDA for commercial use.

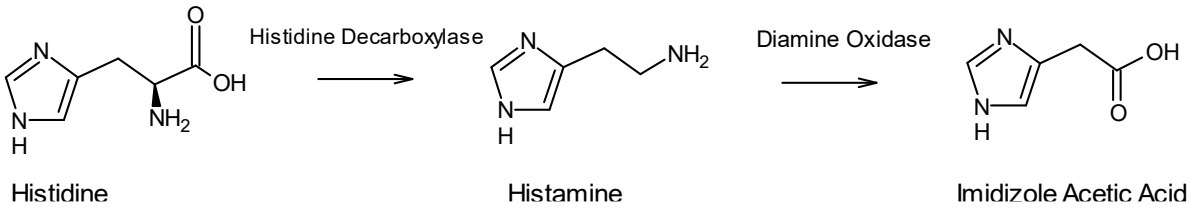

Figure 2.4. Reaction chain for the conversion of histidine to imidazole acetic acid

2.4.3. Biogenic Amines and Lacto-Fermented Foods

While amine contamination of fresh meats can be controlled through proper handling, amine production in fermented foods is more difficult to manage. Food fermentation is an effective method of preserving and creating new food items using microbial cultures. During this process yeast and bacteria consume carbohydrates present in the food and create various by-products such as carbon dioxide, alcohol, and different organic acids. Various bacteria and yeast used to ferment food may also detoxify food components, such as the cyanogen's in cassava root, making foods safer to eat (Padmaja 1995). Some microbes even generate healthful components such as vitamin B12, an essential vitamin that humans can easily become deficient in (Watanabe et al. 2014). For these reasons, humans have produced a variety of fermented meat and vegetable products for thousands of years. It is nearly impossible to find the historic beginnings of fermented foods, but evidence has shown that humans have been utilizing alcoholic fermentation for at least 7000 years (Chambers and Pretorius 2010). During alcoholic fermentation yeasts consume carbohydrates, produce ethanol, carbon dioxide, and a variety of other compounds (organic acids, esters, etc.). This is the process used to produce all drinkable alcohol including beer, wine, cider, mead, and the feed stock for distilled liquor. The major microbes used to make these products include *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* but *Brettanomyces bruxellensis* and *Brettanomyces claussenii* are increasingly used in sour beer production. Lactic acid fermentation utilizes specialized bacteria (lactic acid bacteria) to metabolize carbohydrates and, for some products, produce exclusively lactic acid and in others a mix of lactic acid, carbon dioxide,

and other small molecules. There is a wide variety of lactic acid bacteria utilized but some of the most common include *Lactobacillus casei*, *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, and *Leuconostoc mesenteroides* (Steinkraus 1992). Through lactic acid fermentation we can produce products such as yogurt, cheese, salami, sauerkraut, kimchi, pickles, and many others. Acetic acid fermentation utilizes *Acetobacter* bacteria to convert ethanol to acetic/ethanoic acid, a simple carboxylic acid. This is the process by which all vinegars are produced including rice, malt, and balsamic vinegar. Other types of fermentation exist beyond the previously mentioned, including proteolytic fermentations, but lactic, alcoholic, and acetic fermentation are the most widely used for the production of food.

Most fermented foods take extended periods of time to produce, usually on the order of weeks or months but for products like soy sauce or tamari the fermentation may last years. The primary food source for the yeasts and lactic acid bacteria (LABs) are the various carbohydrates present in the food, though the specific carbohydrates vary with the chemical make-up of the food used. At some point during the fermentation the available carbohydrates will be completely consumed, and the lactic acid/alcoholic fermentations will essentially stop. Even when the sugars and starches have been depleted, the bacteria and yeasts survive and often switch to feeding on other macromolecules such as proteins and peptides. Studies focused on the lactic acid bacteria and *Saccharomyces*/*Brettanomyces* yeasts have consistently shown that they possess the ability to create (among other compounds) biogenic amines from amino acids present in food (Caruso et al. 2002). The chemical makeup of the fermented food has a significant effect on the production of the amines. Foods with low pH, either naturally such as in wine or produced through lactic acid fermentation like yogurt, seem to trigger amine production in bacteria and yeast (Beneduce et al. 2010). The low pH in these foods also makes decarboxylase enzymes more potent and efficient (Linares et al. 2012). Additionally, the production of biogenic amines from amino acids acts as a stress response to the acidic environment the microbes are

living in (Ly et al. 2020). The decomposition of carboxylic acid groups helps adjust the pH of the food to a more livable level by removing an acid from the solution and by also leaving a base behind (the remaining amine group on the molecule). It is important to note that both the quantity and variety of biogenic amines within foods vary greatly based on the chemical makeup of the food.

2.4.4 Biogenic Amine Production in Alcoholic Fermentations

As we have come to better understand the production of biogenic amines, the medical and scientific community has begun to examine their role in alcohol-producing fermentations. Alcoholic beverage fermentation usually utilizes one of two types of yeast: *S. cerevisiae* (ales, wines, mead, cider) or *S. pastorianus* (lager). Studies have shown that *S. cerevisiae* is a prolific producer of biogenic amines being able to produce, according to one study, around 12 mg of amine per liter of mash/must (Caruso et al. 2002). These discoveries have led enologists and food scientists to begin studying the amine content of various wine varietals more closely. Preliminary studies have shown a surprising variety in not only the amine content but the amine character. Based on a study and review done at the University of Calabria in Italy, there appears to be a minor connection between the grape varietal and the amine character of the final wine (Restuccia, Loizzo, and Spizzirri 2018). Minor variations in character is not unexpected given the vast differences between various strains of wine grape. What is interesting about this study is what they found when comparing the fermentative microbes between wine samples. It is not uncommon in Italy for vintners to rely on the ancient practice of spontaneous fermentation instead of yeast pitching. In spontaneous fermentation the brewer/vintner allows wild yeast to inoculate the mash/must instead of adding a pure purchased culture. In winemaking this usually involves allowing the yeasts naturally present on the surface of the grapes to ferment the must. According to the previously mentioned wine study, every batch that utilized spontaneous fermentation methods had significantly higher levels of biogenic amines than batches with pitched yeast, with the concentration being 10x to 50x higher (Restuccia, Loizzo, and Spizzirri 2018). Spontaneous fermentation is becoming more popular

because of the unique flavors produced by wild yeast and a new-found desire to explore traditional wine making techniques. The wild yeasts involved in spontaneous fermentation are incredibly diverse and can vary greatly from location to location and climate to climate. One study isolated more than 10 different yeast strains from a sample of spontaneously fermented wine and found in addition to *Saccharomyces* yeast, yeast from the *Metschnikowia*, *Rhodotorula*, *Pichia*, and *Hanseniaspora* genera and many others (Díaz et al. 2013). Most of these yeast species are very active at the beginning of the fermentation, creating some ethanol and large concentrations of flavor compounds (esters, phenols, alcohols) (Díaz et al. 2013). The *S. cerevisiae* yeast are much more durable and potent alcohol producers, but they reproduce more slowly than the other wild strains, so they aren't active until later in the fermentation. Once the S. cerevisiae have reproduced to a high enough level, they begin generating large amounts of ethanol which proceeds to kill the other less alcohol-tolerant yeasts (Díaz et al. 2013). This process produces wines with very different sensory qualities than standard wines though there is a risk of contamination by spoilage microbes which may ruin a batch. While biogenic amine production has not been specifically studied in wild yeasts the previously mentioned work shows that a relationship potentially exists.

2.4.4.1 Malolactic Fermentation

Beyond yeasts in wine, another microbial source of biogenic amines exists: lactic acid bacteria. Malolactic fermentation is a wine making technique where very sour malic acid is converted to less sour lactic acid. This process is commonly used for a variety of red wines and in a handful of white wines (such as chardonnay) though it is considered a fault in certain varietals. The process is accomplished by adding lactic acid bacteria, usually in the *Pediococus*/*Lactobacillus*/*Oenococcus* genera, and allowing these bacteria to convert the malic acid into lactic acid (Doeun, Davaatseren, and Chung 2017).

This practice is very effective but becomes a problem when the malic acid is depleted. The lactic acid bacteria don't die once they run out of malic acid and will continue to survive by metabolizing amino acids present in the wine and create histamine, tyramine, and other biogenic amines (Restuccia, Loizzo, and Spizzirri 2018).

2.4.4.2 Ethanol and Biogenic Amine Toxicity

Compared to other fermented foods, alcoholic beverages have lower overall levels of biogenic amines but present a different concern: Ethanol. In the human body ethanol appears to increase both the absorption and damage of biogenic amines (Ladero et al. 2010). The primary method used by the body metabolize biogenic amines is a series of two enzymes known as monoamine oxidase-A and monoamine oxidase-B (Erdag, Merhan, and Yildiz 2018). These enzymes are found throughout the body, but especially large reserves are found in areas where neurotransmitters like serotonin and histamine are abundant like the brain and the gut. Various studies have shown that ethanol appears to behave as a monoamine oxidase Inhibitor, a class of drugs that are able to inhibit the activity of these enzymes (Świder et al. 2020). Inhibiting the MAO enzymes in the gut can allow greater amounts of biogenic amines to pass through the stomach epithelium and enter the blood stream. A study in Europe has shown that when people were fed foods with different levels of histamine, symptoms of histamine intoxication occurred "at levels between 75 mg-300 mg" in both histamine-intolerant and healthy individuals (Comas-Basté et al. 2019). Wine samples spiked with histamine triggered symptoms in histamine-intolerant adults at levels around 4 mg but had no effect on healthy individuals at this level (Comas-Basté et al. 2019). While few studies have been done on the safe dosage of biogenic amines in food in general, there appears to be almost no such work related to toxicity of these compounds in beer and wine.

2.5 Beer Concerns

The beer brewing industry represents an important segment of the U.S. economy. In the U.S. the brewing industry generates upwards of $368 billion annually and in Maine, brewing and beer related industries generate $2 billion dollars, with craft beer alone supplying $268 million (Valigra 2019). Despite the economic impact of this industry very little research has focused on the biogenic amine content of beer. Beer is a unique food when compared to other alcohol-containing beverages such as wine due to the level of innovation in production. Microbreweries have been very innovative with their use of grains, adjuncts, alcohol content, fermentation techniques, and aging conditions.

2.5.1. Beer Protein Level

Protein and amino acid content of grain is a major factor in biogenic amine production as they are the feedstock for the decarboxylation reaction. Beer is usually made from barley but other cereal grains like wheat, oats, rice, and even corn are often added as well. In every 100g of barley there is on average 10.4g of protein (FDA 2019) while grapes contain an average of .72g of protein per 100g (FDA 2020). This large disparity in protein content implies that beer brewing may generate much higher levels of biogenic amines.

2.5.2. Beer Adjuncts

Brewing adjuncts are ingredients other than barley, hops, yeast, and water that are added to beer to create unique properties such as flavors, mouthfeel, foaming, and/or color. Adjuncts range from other grains to fruits, vegetables, spices, to more unique ingredients like wine must, bacon, and chili peppers. Each of the previously mentioned adjuncts could potentially have a major effect on the chemical makeup of the beer and the fermenting behavior of the yeast. Fruits and vegetables often have a variety of indigenous bacteria and yeast on their skins and peels that, once added to beer, may increase biogenic amine production when compared to commercial inoculated brewing yeasts. Adjuncts

like cinnamon, cloves, citrus fruits contain various chemicals like cinnamaldehyde, eugenol, and citric acid that have antimicrobial activity (Lucera et al. 2012). When these products are added to the beer, they likely cause physiological stress to the microbes and trigger them to produce amines to alleviate the damage and stress caused by the antimicrobials (Satomi 2016).

2.5.3. High Gravity Beer

In recent years there has been a rise in popularity of so called “big beers” or beer with high alcohol content (>9% abv). These high gravity beer styles involve creating a wort with a very high sugar content and fermenting with *Saccharomyces* yeast to create beer with a high alcohol content. While no studies have specifically been done to examine the biogenic amine formation in these beers, it is probable that they contain high levels of biogenic amines, as during fermentation they experience a combination of osmotic and alcoholic stress. Osmotic stress may occur because the wort has a much higher alcohol content than other beer styles. The high solute concentration can pull water out of the yeast cell walls through osmosis and is a well-known yeast stressor (Bleoancă and Bahrim 2013). In addition to the high osmotic stress in the beginning of fermentation, the yeast must contend with high alcohol stress towards the end of the ferment and during the conditioning stage. Yeasts are more alcohol tolerant than many microbes, but they are not immune to stress and damage from it. Due to extended log and lag phase, high gravity fermentations take more time to complete than standard beer fermentation resulting in yeast cells being exposed to higher levels of alcohol for long periods of time.

2.5.4. Beer Microbes

Beer most commonly utilizes one of two types of yeast depending on the style being made; *S. cerevisiae* is the yeast of choice for ales while *S. pastorianus* is used to make lagers. While these two *Saccharomyces* yeasts are the most commonly used fermentation microbes, there are several others utilized by brewers. Sour beers, a popular category of beer, utilize either Brettanomyces yeast, wild

yeast, or lactic acid bacteria for their ability to produce a number of organic acids and unique aromatic esters. *Brettanomyces* yeast, a spoilage microbe and scourge of the wine industry, has been studied and found to be a very potent producer of biogenic amines (Caruso et al. 2002). As previously described in the context of wine, beer can also be spontaneously fermented to produce unique flavor components. One method of producing spontaneously fermented beer that is gaining popularity is the coolship technique. In this method hot wort is pumped into open-top metal containers inside specially built buildings with high airflow (usually utilizing intake fans). As the wort cools naturally occurring "wild yeast and bacteria" present in the air inoculate the beer and begin the fermentation process. Allagash Brewery in Portland, Maine is famous for utilizing this technique in their coolship line of beers. Studies examining spontaneous wine fermentation indicate that these wild yeasts are very potent producers of biogenic amines (Díaz et al. 2013). Additionally, some styles of beers (such as gose) utilize lactic acid bacteria in addition to yeasts to produce a mildly acidic fruit beer. As shown in many studies lactic acid bacteria can produce biogenic amines readily (Restuccia, Loizzo, and Spizzirri 2018).

2.5.5. Beer Aging Conditions

One of the big differences between wine and beer production is the length of time the two products require for proper aging. In general, beer is a ready to drink product almost immediately after fermentation is completed and doesn't need time to age to be considered "drinkable" (many styles of beer don't have the necessary chemical make-up to age). Alternatively, most varieties of wine require years of barrel aging to properly develop the flavor components (esters, sulfur derivatives, wood derived compounds) they are known for (Chambers and Pretorius 2010). Due to the combination of alcohol concentration and pH, biogenic amine production is usually quite slow in alcoholic beverages: The alcohol and pH slow the growth of the bacteria and the function of their decarboxylation enzymes (Díaz et al. 2013). Considering beer isn't usually aged like wine, it's been assumed that biogenic amine levels

should be lower. This may no longer be true as we have seen a significant increase of sour beers and aged beers in commercial markets. Many sour beers utilize *Brettanomyces* yeast, a wild strain of fungus that can produce acids and a mix of unique flavor compounds (some are described as horse blanket or musty basement) as well as a variety of biogenic amines. These beers need to be aged for months (sometimes years) so that the *Brettanomyces*-borne flavor compounds can "mellow" and chemically react with other compounds in the beverage. Other barrel aged beers follow wine ageing techniques by filling whiskey, rum, wine, etc. barrels with beer and allowing the product to chemically react and mellow for long stretches of time. Both ageing styles require long stretches of time and may have an extremely profound effect on amine concentration in beer.

2.6 Conclusions

As our understanding of biogenic amines and their health effects become clearer, having accurate data pertaining to specific biogenic amines and their concentration present in foods become increasingly important. While production of these compounds can be controlled in fresh foods by proper storage and refrigeration, fermented foods don't share this luxury. Although biogenic amines in a handful of fermented products, such as cheese and wine, have been well researched and documented in the peer-reviewed literature, biogenic amine identification and concentrations in beer remains largely ignored. Beer is a very popular, and economically important beverage generating more than $118 billion in direct sales every year (Valigra 2019). Every year craft beer is taking an ever-increasing market share from the traditional mass-produced market. Having quick and accurate methods for analyzing the levels of these compounds in beer is an economic and medical necessity.

CHAPTER 3

THE RELATIONSHIP BETWEEN PROTEINS, AMINO ACIDS, AND BIOGENIC AMINES IN BEER

3.1. Introduction

Beer is an alcoholic beverage produced most commonly by fermenting barley, though other cereal grains can be used in combination. Beer brewing has been practiced for thousands of years by various cultures and continues to be an extremely popular and important practice (Tucker 2011). Unlike fruits and honey, which have free fermentable sugars, barley and other cereals require a combination of malting and mashing processes to metabolize their complex starches into maltose and other simple sugars before fermentation can be initiated (Arendt and Zannini 2013). Malting is a complex biochemical process during which barley grains are allowed to germinate and produce a variety of carbohydrase and proteolytic enzymes and other proteins necessary for the brewing process (Celus, Brijs, and Delcour 2006). Mashing occurs when malt is steeped in water and heated to specific temperatures to activate the carbohydrase enzymes with the goal of metabolizing the complex starches into simple mono and disaccharides usable for fermentation (Didier and Bénédicte 2009). This resulting sweet liquid, with high concentrations of maltose, is called wort. The saccharides are eventually converted to alcohol and a myriad of other flavor compounds by yeast fermentation, but the fate of the residual enzymes and proteins is also important.

The proteins and amino acids present in beer are important for a variety of reasons. Proteins in beer can affect mouth feel, color, flavor, and a variety of other properties. These compounds also play an important role in yeast metabolism as many are consumed by yeast for energy, reproduction, and enzyme generations (Lekkas et al. 2005).

Proteins and their amino acids profiles vary widely from food to food. Identification of these nitrogenous compounds present in barley and wort, and which are most likely to be metabolized by yeast is important for predicting the amino acid and biogenic amine makeup of the finished beer.

Biogenic amines are a diverse group of nitrogenous, amine-containing molecules produced through metabolism by living organisms. This group of amines are primarily created through the decarboxylation of amino acids but can also be generated through transamination of various alkanes (Doeun, Davaatseren, and Chung 2017). These compounds, especially when found in food products, are of special interest due to their deleterious health effects. Biogenic amines are potent signaling molecules within the human body; symptoms of biogenic amine consumption can include hypertension, hypotension, migraines, pseudo-anaphylaxis, and gastrointestinal issues (Comas-Basté et al. 2019).

3.2. Storage Proteins of Barley (*Hordeum vulgare*)

Barley (*Hordeum vulgare*) is a cereal grain and one of the first crops domesticated by humans thousands of years ago. Barley continues to be an important crop grown throughout the world for a variety of purposes including food, alcohol, and animal feed. The kernels of this plant contain a variety of organic compounds including lipids, carbohydrates, vitamins, and, for the focus of this review, proteins.

Based on their solubility and structure, storage proteins found in barley can be divided into four general categories. Globulins are the fraction of barley proteins that are very soluble in salt solutions (Osborn 1924). Glutelin's are alkali-soluble proteins that, much like globulins, are insoluble in pure water but soluble in various other solutions including those with high pH (Osborn 1924). Albumins are water-soluble proteins easily dissolved in water (Osborn 1924).

The final family of proteins are Prolamins, or more specifically hordeins. Hordeins are alcohol soluble storage proteins that constitute roughly 50% of the protein content of barley (Osborn 1924). Due largely to their abundance, and the role their catalytic products contribute to beer, hordeins are the most important of these proteins in the brewing process.

3.2.1. Hordeins

Hordeins are storage proteins found only in barley, but several similar analogues exist in other cereals (gliadin in wheat, secalin in rye, etc.) (Thompson 2001). Hordeins are divided into different groups depending on the structure of the specific hordein molecule. The first group are known as “A-Hordeins”. A-Hordeins are relatively small, lightweight proteins roughly 15 to 25 kDa in mass (Görg et al. 1992). Due to their size and structure these proteins aren’t believed to be true storage proteins within the barley seed. The second group of proteins are known as “B-Hordeins”. B-Hordeins are medium size proteins (about 40kDa in mass) and represent more than 70% of the hordein makeup of the barley grain (Görg et al. 1992). These proteins are also known as “sulfur-rich hordeins” as they have high levels of sulfur containing amino acids, especially cysteine (Balakireva and Zamyatnin 2016). These sulfur-rich amino acids allow a high level of crosslinking within the structure of these proteins and help to stabilize dough and bread products made from this grain. The third family of hordeins are the “C-Hordeins”. C-Hordeins are larger than the previously mentioned hordeins, and are roughly 60kDa in mass (Görg et al. 1992). C-Hordeins are also known as “sulfur-poor hordeins” as they lack the high concentration of cysteine found in the B-Hordeins. A lack of sulfur also causes them to exist in a less crosslinked form (Balakireva and Zamyatnin 2016). The final category of hordeins is the “D-Hordeins”. D-Hordeins have very high molecular weight, usually more than 100kDa (Görg et al. 1992). D-Hordeins have been less studied than the previously mentioned barley proteins, but are believed to play an important structural role due to their size and number of cysteine residues (Balakireva and Zamyatnin 2016).

3.2.2. Amino Acid Profile of Barley Protein

To better understand which amino acids and biogenic amines may be present in a finished beer it is important to know what amino acids compose the hordeins. Because barley is a plant, the specific amino acid concentration can vary quite widely depending on the growing conditions (Arendt and Zannini 2013). However, the general amino acid makeup is consistent. It should also be noted that there are a variety of different barley cultivars which may have a significant effect on the amino acid content of the grain; high-lysine barley is a widely grown cultivar but is usually only used as an animal feed. The amino acid profile of barley, like most cereal grains, is comprised primarily of glutamic acid and proline (Singh and Sosulski 1986). The abundance of these two amino acids is shown in Tables 3.1. and 3.2., where they make up roughly 30% of all the amino acids in the plant. The remaining amino acids have variable concentrations with leucine usually being the 3rd most abundant (4.5% of total amino acids), histamine (1.9%), tryptophan (1.6%), and methionine(1.51%) usually the least abundant (Arendt and Zannini 2013). While it was previously mentioned that B-Hordeins are rich in sulfur containing cysteine, the overall concentration of cysteine is low, existing at a level of about 2.1% of the total amino acid content (Arendt and Zannini 2013). With this information, a profile of the potential amino acid/amine content in a final beer product can be constructed as the source of those nitrogenous compounds is ultimately the barley.

Table 3.1. Amino acid Composition of Barley Proteins (Singh and Sosulski 1986)

	Hordein (%)				
Nitrogenous Compound	**Present Work**	**Others (Bishop, 1928)**	**Hordenin (%)**	**Globulin (%)**	**Albumin (%)**
Amide	23	na	10.3	5.1	5.9
Aspartic Acid	1.2	0.81	4.7	5.6	8
Glutamic Acid	23	22.85	11.6	6.8	8.7
Proline	15.3	4.16-9.72	6.6	2.7	4.2
Glycine	1.7	0	5.2	10.7	6.7
Alanine	2.2	0.39-1.22	6.6	0.67	7.2
Valine	3.5	0.09-0.97	4.9	4.1	5.8
Leucine	4.6	3.52	5.8	4.5	5.7
Isoleucine	3.6	4.35	3.5	2.2	4.1
Phenylalanine	3.6	2.48-2.71	2.7	2.1	3
Tyrosine	1.6	0.75-1.80	1.9	1.5	2.7
Tryptophan	0.7	0.36-0.85	1.1	0.65	1.3
Serine	3.2	0.08	4.2	3.9	4.1
Threonine	1.9	na	3.1	2.4	3.4
Cystine	1.5	1	0.9	2.6	1.5
Methionine	0.75	na	1.1	0.9	1.4
Lysine	0.8	0	4.8	6.3	7.9
Histidine	2.2	0.80-2.02	4.3	3.1	4.3
Arginine	6	4.04-5.88	12	22	13
Insoluble Humin	0.85	na	3.9	1.4	1.9
Ammonia	23	20.8-23.3	11.5	8	7

Table 3.2. Total Amino acid content of Hulled and Hull-less Barley (Arendt and Zannini 2013)

Amino Acid Composition (g/Kg) of Barley (% w/w)		
Nitrogenous Compound	**Hulled Barley**	**Hull-less Barley**
Protein	13.2	14
Alanine	0.44	0.47
Arginine	0.6	0.64
Aspartic Acid	0.71	0.75
Cysteine	0.28	0.31
Glutamic Acid	2.98	3.27
Glycine	0.42	0.44
Histidine	0.26	0.28
Isoleucine	0.43	0.46
Leucine	0.79	0.84
Lysine	0.41	0.41
Methionine	0.2	0.28
Phenylalanine	0.68	0.73
Proline	1.32	1.43
Serine	0.54	0.57
Threonine	0.42	0.45
Tryptophan	0.22	0.23
Tyrosine	0.37	0.42
Valine	0.59	0.63

3.3. Brewing Process Effects on Proteins and Amino Acids

Malting, mashing, and kettling (boiling) are three interconnected brewing concepts important for beer production. During the malting process barley grains are allowed to germinate which leads to the creation of a variety of carbohydrase and protease enzymes (Arendt and Zannini 2013). After the germination, the seeds are dried in specialized kilns to drive off moisture and develop various flavor compounds. Lightly kilned grain retains high levels of a wide variety of enzymes, while highly roasted grains sacrifices (denatures) enzymes to develop dark colors and stronger flavors (Baxter and Wainwright 1979). During malting, the Maillard reaction also occurs. During this reaction, reducing sugars react with free amino acids to create a variety of brown pigments (as seen in foods like bread) and a variety of flavor compounds important in the final beer (ACS 2012). The kiln dried product (malt) can be used in a variety of non-beer products including bread, desserts, chocolate products, and beverages. Mashing is the process of steeping malt in hot water to gelatinize and convert long chain starches from the grain into fermentable sugars. The malt and water mixture is than heated to specific temperatures (the temperature is dependent on the specific enzymes to be activated) which promote the activity of various enzymes that cleave amylase and other starches into fermentable sugars, mainly glucose and maltose (Sørensen, Svendsen, and Breddam 1987). The sweet water produced by mashing is known as wort and is analogous to must in wine production.

3.3.1. Malting and Malt

During malting a variety of key steps occur that have lasting impacts on the final product. As previously mentioned, a variety of carbohydrase enzymes such as alpha-amylase and beta-amylase are produced within the seed kernel. These enzymes are extremely important to brewers as they allow the unfermentable barley starch to be broken down into simple sugars. However, the formation of these carbohydrase's is only one of many processes that occur. Proteolytic enzymes are also produced within

the seed kernels. These proteolytic enzymes play an important role in the germination product as they are able to metabolize hordeins. Within the barley seed granules of starch are surrounded by a net of hordein protein which forms a water-tight barrier around the carbohydrate (Baxter and Wainwright 1979). When the germination process begins, the protease enzymes produced by the barley begin cleaving the hordein coat into a variety of smaller water-soluble peptides (Osman 2003). This process isn't well understood, although studies have shown that when the hordein catalysis isn't allowed to fully proceed the resultant malt is almost unusable; the starch molecules appear unable to gelatinize as their protective coating is not degraded (Baxter and Wainwright 1979). While it is known that this process increases the number of water-soluble peptides by removing hydrophobic residues, the specific peptides produced aren't well studied (Silva et al. 2008). In Table 3.3. the production of soluble amino acids and soluble protein is illustrated as a function of this brewing step.

It is clear that the bulk of soluble proteins are produced during the malting step (Jones and Budde 2005). Studies have shown that even after this catalytic process only about 20% of all barley proteins are soluble in the wort (Baxter and Wainwright 1979).

Table 3.3. Soluble Protein Generation by Brewing Step and pH (Jones and Budde 2005)

The Soluble Protein and FAN Levels of Morex and Harrignton Barleys, Malts, and Worts							
	Measured soluble protein % (% of wort protein solubilized)				Measured FAN, ppm (% of Fan released)		
	Barley	**Malt**	**Wort**		**Barley**	**Malt**	**Wort**
Morex							
pH 3.8	1.59 (17)	6.66 (56)	9.14 (27)		35 (12)	220 (64)	289 (24)
pH 6.0	2.32 (35)	5.03 (41)	6.60 (24)		39 (17)	173 (58)	230 (25)
pH 8.0	2.27 (35)	6.19 (60)	6.54 (5)		29 (16)	182 (84)	181 (-1)
Harrington							
pH 3.8	1.56 (21)	5.50 (54)	7.30 (25)		35 (16)	177 (63)	224 (21)
pH 6.0	1.48 (29)	4.13 (52)	5.14 (20)		29 (13)	156 (59)	217 (28)
pH 8.0	2.28 (41)	4.78 (45)	5.55 (14)		40 (27)	149 (73)	146 (-2)
Average							
pH 3.8	(19%)	(55%)	(26%)		(14%)	(64%)	(22%)
pH 6.0	(32%)	(46%)	(22%)		(15%)	(58%)	(26%)
pH 8.0	(38%)	(52%)	(10%)		(20%)	(78%)	(0%)

3.3.2. Mashing and Wort

The malt is converted into wort via the mashing process. During mashing the proteolytic and carbohydrase enzymes continue to cleave proteins (into soluble peptides and amino acids) and complex starches (into simple tri/di/monosaccharides) respectively (Celus, Brijs, and Delcour 2006). Table 3.3. shows that in most cases about 20% of all soluble protein/amino acids are produced during the kettling stage (Jones and Budde 2005). As the wort is heated and boiled a variety of important protein related effects occur. The high temperature and acidic nature of the wort causes up to 70% of the barley proteins to coagulate and precipitate (the first protein removal step) (Jones and Budde 2005). This is an important step for most beer styles as it removes substances that cause haziness, cloudiness, and poor mouthfeel producing a clearer, more pleasant beer. It is also important to note that the goal of this "protein crash" isn't removal of all protein, only insoluble or coagulated protein. All fermentations need some assimilable nitrogen for yeast to reproduce or generate the various enzymes required for survival. Additionally, the soluble proteins provide a variety of important sensory characteristics in beer including mouth feel and foam retention. The overall concentration of amino acids may change but the ratio of abundance for each amino acid is consistent before and after the kettle step. Table 3.4. shows the most abundant amino acids are proline and glutamic acid, while histidine and tryptophan remain at a low overall concentration (Otter and Taylor 1976). It is important to bear in mind that this is a general profile of amino acids in wort and not applicable to every beer style. The actual grain bill of a beer can vary drastically and can include not only a variety of barley malts (each roasted or treated in different ways or for different lengths of time) but a variety of cereal grains (corn, rice, rye, wheat, oats, etc.), which can drastically change the amino acid profile. Additionally, other adjuncts (ingredients) such as fruit, nuts, meat products, etc. commonly used to great effect in beer could significantly affect the available amines.

Table 3.4. Amino acid Profile of various Worts (Otter and Taylor 1976)

Amino acids in wort-Results expressed as mg amino acid/100 mL wort, 1040 S.G. gravity					
Amino Acid	**All Malt**	**Brewery Wort**	**Malt + 25% Barley Grist**	**Malt + 25% Wheat Grist**	**Proteolytic Malt**
Alanine	15.1	9.9	15.5	16.5	39.4
Valine	13.7	9.2	14	14.7	26
Glycine	7.9	5.2	9.1	10.3	19
Iso-leucine	7.5	5.3	9.4	10.7	18.6
Leucine	18.8	10.9	18.3	19.2	39.7
Proline	44.5	38.9	47.9	47.3	73
Threonine	17.2	2.5	11.2	23.9	1.7
Serine	7.4	4.8	7.5	9.3	28.8
Cysteine	neg.	neg.	neg.	neg.	neg.
Methionine	neg.	2.3	4.7	4.9	12.8
Hydroxyproline	neg.	neg.	neg.	neg.	neg.
Phenylalanine	16.2	11.4	15.8	17.3	34.4
Aspartic Acid	18.9	17.9	23.3	24.8	29
Glutamic Acid	24.8	8	31	40.9	39.6
Tyrosine	8.9	0.8	8.3	8.1	11.5
Ornithine	0.9	neg.	neg.	neg.	13.3
Lysine	10.1	5.6	11.3	8.9	19.2
Tryptophan	trace	neg.	neg.	neg.	neg.
Histidine	trace	0.3	neg.	neg.	0.4
Arginine	15.5	10.8	17.6	13.8	18.3
Cystine	trace	na	neg.	neg.	neg.

3.4. *Saccharomyces* Yeast and Fermentation

After wort is formed and cooled, the yeast is pitched to ferment the sugars into ethanol and other biproducts. The primary yeasts used for alcoholic fermentation are members of the *Saccharomyces* genus, with the *Saccharomyces cerevisiae* being the most common. It is well known that in an anaerobic environment yeasts will consume simple sugars and convert them into ethanol, carbon dioxide, and a variety of fusil alcohols and esters.

Less understood is that yeasts also metabolize nitrogenous compounds, especially amino acids, to reproduce and create more catalytic enzymes. This protein digestion plays an important role in the chemical makeup of the final product as it gives rise to biogenic amines and various other nitrogenous wastes.

3.4.1. Free Amino Nitrogen

"Free amino nitrogen" or "FAN" is an important quality of all alcoholic fermentations. FAN is a measurement of the quantity of available amino acids/digestible peptides in a wort or must. Without a diverse and abundant source of nitrogen yeast are unable to successfully reproduce and ferment (Lekkas et al. 2005). Amino acids vary in the way they are utilized by yeast, which plays an important role in the final makeup of the beer. Yeast digestion of amino acids usually involves the use of decarboxylase enzymes which convert amino acids into biogenic amines. Table 3.5. shows four categories of amino acids (Group A through Group D) which are separated based on how easily and how quickly yeasts can utilize the amino acid. Knowing which amino acids are easily digested during the fermentation process provides a more accurate estimate of the final nitrogenous profile of a beer (Stewart, Hill, and Lekkas 2013). As an example, knowing that glutamic acid is both abundant and easily digested by yeast, it is safe to assume that a finished beer would likely be high in gamma-aminobutyric acid (GABA), the decarboxylated form of glutamic acid. This phenomenon has been observed in other alcoholic beverages, namely grape wine, which has the bulk of its glutamic acid converted into GABA (Pinu et al. 2014). With this information an amino acid profile can potentially be constructed for a beer, even if the grain bill is highly varied. Because Group A and Group B amino acids are easily digested and absorbed by the yeast, there should theoretically be very little of these amino acids in the final beer but their decarboxylated biogenic amines should be present. Group C amino acids are much more likely to go through the brewing process mostly unmetabolized and usually appear during analysis as amino acids, with less of their biogenic amine forms being created. Group D amino acids are a unique case. Proline,

usually the most abundant amino acid in wort, is almost completely unusable to yeast during fermentation. Yeast do have enzymes capable of metabolizing proline (i.e. proline oxidase), but these enzymes require oxygen to function (Smyl 2000). The anaerobic environment that forms during fermentation inhibits the use of these enzymes causing proline to remain almost completely unmetabolized (Smyl 2000). It should be noted that there are some exceptions to this process. First, if a wort is highly oxygenated, enough oxygen can persist in the wort to allow yeast to digest some proline (Salmon and Barre 1998), although this has a minimal effect on the overall proline concentration. A second exception that is more applicable to the future is the rise of genetically modified yeast. While not commercially available, much moderately successful research has been done to create yeast strains that can assimilate proline in low oxygen environments (Long et al. 2018). In the future, it is likely that these yeast species may see wide use as they allow the utilization of the native nitrogen provided by the barley, negating the need to fortify the wort(Long et al. 2018). Several studies have shown that the order yeasts digest amino acids is independent of the amino acid's concentration (Crépin et al. 2012). Even with an abundance of valine and a minimum of glutamic acid, yeast cells will consume glutamic acid before the valine. This preference is coded by a variety of genes in the yeast's DNA and is regulated by several enzymes and the presence of various nitrogen compounds, such as ammonia (Ljungdahl and Daignan-Fornier 2012).

Table 3.5. Amino acids classified by yeast uptake (Stewart, Hill, and Lekkas 2013)

Revised Classification of the Uptake Pattern of Wort Amino Acids and Ammonia			
Group A Fast Uptake	**Group B Intermediate Uptake**	**Group C Slow Uptake**	**Group D Little or No Uptake**
Glutamic Acid	Valine	Alanine	Proline
Aspartic Acid	Histidine	Glycine	
Asparagine	Tryptophan	Ammonia	
Glutamine	Tyrosine		
Serine	Phenylalanine		
Threonine			
Lysine			
Arginine			
Methionine			
Isoleucine			
Leucine			

3.4.2. Yeast-born Amino Acids

In addition to metabolizing amino acids, yeast are capable of producing amino acids from various precursor chemicals. It should be noted that while yeast are able to produce a variety of amino acids through transamination, this usually only occurs in media with extremely limited concentrations of amino acids (Lekkas et al. 2005). While the production of non-proteinogenic amino acids is not well understood, it has been shown that yeast can produce some of these compounds in beer (Watson 1976). One such amino acid that can be produced is ornithine, an intermediary in the production of other amino acids (Qin et al. 2015). Ornithine can also be transformed into another non coded amino acid: citrulline. Citrulline is easily converted to arginine (a coded amino acid) by but also exists in a free form in solutions (Steinle, Bergander, and Steinbüchel 2009). While the full breadth of non-proteinogenic amino acids produced by yeasts isn't well known, these compounds exist in beer and play a role in beer quality and analysis. These amino acids are not usually produced in high concentrations (Watson 1976) but are often present, though they are likely to be over-shadowed by more concentrated amino acids.

3.4.3. Autolysis

Finally, yeasts can interact with the amino acid content in beer in one other important fashion; autolysis. As yeasts age and the alcohol content of the beer increases, yeast cells begin going through the process of autolysis. Autolysis occurs when enzymes within the yeast begin catabolizing the yeast cell, causing the cells to lyse and their contents to be released. This process is very different from normal yeast death as the catabolic cascade causes a variety of proteins to be digested and converted to free amino acids (Kulka 1953). Autolysis is generally avoided in the brewing process as it is a source of off flavors and is considered a flaw. This usually occurs only in beer that has not been properly aged and is allowed to sit on its lees (dead yeast cells) for extended amounts of time (Jacob et al. 2019). Figure 3.1. shows the difference in the protein-bound and free amino acid ratio between mechanically ruptured and autolyzed yeast species (Jacob et al. 2019). This chart clearly shows that when yeasts are autolyzed the concentration of usable, free amino acids is greatly increased. The autolyzed yeast plays a very important role in beer as the released amino acids are both a sensory defect and a spoilage vector. It has been documented that finished beer with autolyzed yeast can become contaminated with lactic acid bacteria, which normally wouldn't survive in the finished product (Kulka 1953). The amino acid profile varies between yeast species and even between members of the same species depending on the environment they are grown in. Table 3.6. shows a relatively average amino acid profile of autolyzed *Saccharomyces cerevisiae* yeasts (Berlowska et al. 2017). This sample was prepared by inducing autolysis in a cultured yeast sample using saponins, so an actual beer yeast autolysate may be different. When examining the data, it appears that the autolyzing process causes a large increase in the levels of certain amino acids like serine, arginine, and asparagine. When analyzing beer that has been left on its yeast for an extended time it is important to keep this information in mind as it will influence the nitrogen/amino acid/biogenic amine profile during analysis.

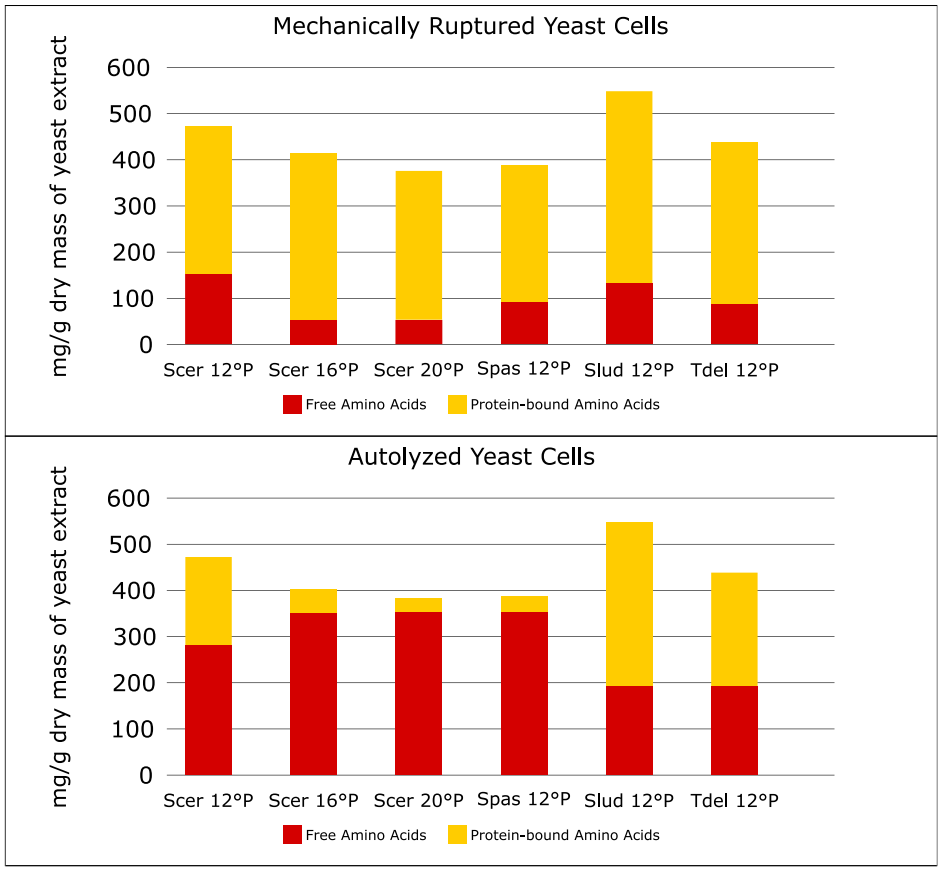

Figure 3.1. Free and bound amino acid concentration in mechanically ruptured and autolyzed yeast species (Jacob et al. 2019) adapted*

Table 3.6. Amino acid profile of autolyzed yeast species (Berlowska et al. 2017)

Amino acid profiles and protein concentration in yeast autolysates			
	Yeast Species		
		S. cerevisiae	*S. stipitis*
Concentration of dry mass (% w/w)		5.62 ± 0.05	6.40 ± 0.06
Protein Content (g per 100 g of dry mass lysate)		0.54 ± 0.05	0.59 ± 0.01
Concentration of free amino acids (g per 100 g of dry mass lysate			
Essential amino acids	Thr	1.09 ± 0.07	2.44 ± 0.23
	Val	1.76 ± 0.14	1.91 ± 0.18
	Leu	2.76 ± 0.15	2.50 ± 0.23
	Ile	1.91 ± 0.15	1.81 ± 0.17
	Met	0.78 ± 0.05	0.74 ± 0.06
	Lys	2.38 ± 0.17	2.35 ± 0.23
	Phe	1.64 ± 0.12	1.55 ± 0.14
	His	0.86 ± 0.08	0.84 ± 0.07
	Sum	**13.18**	**14.14**
Conditionally essential amino acids	Arg	5.03 ± 0.35	1.29 ± 0.11
	Cys	1.39 ± 0.01	1.33 ± 0.08
	Gly	1.69 ± 0.11	1.18 ± 0.11
	Pro	2.06 ± 0.11	1.20 ± 0.10
	Tyr	1.58 ± 0.14	1.55 ± 0.15
	Sum	**11.75**	**6.55**
Non-essential amino acids	Glu	2.46 ± 0.20	3.25 ± 0.32
	Ser	11.28 ± 0.72	9.87 ± 0.96
	Asp	3.59 ± 0.27	3.69 ± 0.33
	Ala	2.71 ± 0.25	2.72 ± 0.25
	Sum	**20.04**	**19.53**
Total sum		**44.97**	**40.22**

3.5. *Brettanomyces* Yeast

While *Saccharomyces* (brewer's yeasts) are the primary alcohol producer in most beer, the *Brettanomyces* or "Brett" genus has regained some of its old-world popularity. This genus of "wild" yeast found throughout the world behaves very differently from traditional brewer's yeast. While *Saccharomyces* yeasts have been cultivated for many years to produce alcohol and minimal amounts of other compounds, Brett yeast is used for the opposite reason. *Brettanomyces* yeast can produce alcohol

but they are well known for their ability to produce a cornucopia of pleasant and unpleasant flavor compounds. Producing flavors that range from “fruity” and “spicy” to “horse blanket” and “urine”, these yeasts are equal parts pest and gift (Joseph, Albino, and Bisson 2017). In some beer styles, like sours and lambics, these yeasts are responsible for the many unique flavors associated with those beverages, but in many others, it is considered a flaw or a contamination (Hersh 1996). The other unique aspect of these fungi is their ability to survive and thrive in inhospitable environments that many other species of yeasts cannot.

3.5.1. *Brettanomyces* Amino Acid Uptake

Of the several strains of *Brettanomyces* yeasts used in beer brewing, *Brettanomyces bruxellensis* is the most commonly utilized and the most commonly studied. *B. bruxellensis* is unique in that it is able to grow easily in beer (including as a brewing spoilage organism), a food product that is acidic, alcoholic, and lacking in usable carbon sources fermentation (Smith and Divol 2016). This yeast genus is able to absorb and metabolize amino acids in a similar fashion to Saccharomyces yeast, shown Table 3.7. (Parente et al. 2017). This chart shows several similarities exist between Brett amino acid uptake and the *Saccharomyces* yeast amino acid uptake; with glutamate, glutamine, and aspartate being the preferred amino acid sources. They differ a bit in the secondary and tertiary tiers primarily with serine and alanine being more easily digested by Brett and hydrophobic amino acids being more readily consumed by *Saccharomyces*. It is important to realize that this is a single test on a single strain of a single species of Brett yeast and is not considered generalizable to all *Brettanomyces* yeast, as assimilation by Brett yeast can vary greatly from strain to strain (Parente et al. 2017). This difference is illustrated in Table 3.8. This study shows the growth potential influenced by various amino acids (AA’s with numbers above 2 are growth-promoting, while AA’s with numbers below 2 have little effect on growth) (Blomqvist et al. 2012).

In this work, amino acids like histidine were shown to have a major effect on cell growth which is completely different from the results from the first study. More work needs to be done to develop a more universal guide to amino acid use by *Brettanomyces* yeasts.

Table 3.7. Classification of amino acids according their potential to promote growth of *Dekkera bruxellensis* GDB 248 in aerobiosis or in anaerobiosis (Parente et al. 2017)

Dekkera bruxellensis Amino Acid Uptake vs *Saccharomyces* Amino Acid Uptake			
Condition	**Preferential**	**Secondary**	**Poorly Usable**
O2	Glutamine	Arginine, Aspartate, Glutamate	Alanine, Asparagine, Cysteine, Glycine, Isoleucine, Lysine, Histidine, Methionine, Phenylalanine, Proline, Serine, Tyrosine, Trptophan, Valine
N2	Aspartate, Glutamate, Glutamic Acid	Alanine, Arginine, Asparagine, Serine, Phenylalanine	Cysteine, Glycine, Isoleucine, Leucine, Lysine, Histidine, Methionine, Proline, Tyrosine, Trptophan, Valine
N2 (*Sachharomyces* Yeast)	Aspartate, Aspartic Acid Asparagine, Glutamate, Glutamic Acid, Serine, Threonine, Lysine, Arginine, Methionine, Isoleucine, Leucine	Valine, Histidine, Tryptophan, Tyrosine, Phenylalanine	Alanine, Glycine, Ammonia, Proline

Table 3.8. Amino acid impact on the anaerobic growth of D. *bruxellensis* CBS 11270 (Blomqvist et al. 2012)

Amino acid impact on the anaerobic growth of *D. bruxellensis* CBS 11270			
Amino Acid	**Number of Divisions**	**Day Sample was Taken**	**NADH Released**
Arginine	3.73	9	4-5
Lysine	3.51	6	6-7
Asparagine	3.34	14	1
Histidine	3.29	9	2
Alanine	3.06	9	1
Glutamic Acid	2.95	14	3-4
Serine	2.68	9	2
Aspartic Acid	2.64	14	1
Methionine	2.5	16	1-4
Threonine	2.46	9	0-1
Glycine	2.42	9	2
Leucine	2.39	16	5
Glutamine	2.28	9	3-4
Phenylalanine	2.26	9	2
Valine	2.11	9	2
Isoleucine	2.03	16	1-2
Tryptophan	1.94	9	3
Proline	1.91	9	1-4
Cysteine	1.22	9	2-5
Yeast Extract	4.2	3	---
w/o Amino Acids	1.4	23	---

3.5.2. *Brettanomyces*, Proline, and GABA

Two other important amine compounds found in Brett-fermented beer are proline and gamma-amino butyric acid (GABA). Proline, as previously discussed, provides an abundant source of nitrogen in beer and wine, but is mostly unusable to *Saccharomyces*. Most microbes use an oxygen-based catalytic enzyme to digest proline, but the fermentation of beer occurs mostly in an anaerobic state. There has been some disagreement on whether Brett yeast can digest proline in a wort/finished beer. A study by Crauwels et al. (2015) implies that proline can be digested as both a carbon and a nitrogen source by *B. bruxellensis* in wine. This information is mostly refuted by other studies, including work by Blomqvist et

al. (2012), which showed proline digestion only if oxygen was present. It is safe to assume that proline is only metabolized in beer that has a high enough concentration of oxygen to allow the oxygenase enzyme to function. GABA is an amine compound formed through the decarboxylation of glutamic acid and is formed in large concentrations during fermentation. GABA is also released in large volumes during the autolysis process (Berlowska et al. 2017). Brett yeasts are generally able to digest this amine, especially when it is the only/most abundant source of nitrogen available. When consumed, GABA is converted into glutamate and succinate, which are both further converted into other various compounds (Smith 2016). The level of digestion varies quite greatly from strain to strain, with some *Brettanomyces* able to digest upward of 60% of the available GABA while others will use effectively none or even produce more. To further complicate matters Table 3.9. shows the uptake/consumption of amino acids by various *B. bruxellensis* strains over 45 days. In this experiment the variable nature of GABA consumption is highlighted with half of strains consuming it, and the other half producing more. In all species proline is initially produced in small amounts, but is further consumed in large quantities, counter intuitive to the assumption that proline catalysis only occurs in the presence of oxygen. One potential explanation of this phenomenon is that Brett yeasts have an alternative catalysis pathway that is not currently known (Smith 2016). The other likely explanation is that proline is not being catabolyzed, but absorbed to repair cellular damage, something commonly seen with microbes exposed to nutrient poor/high ethanol environments (Smith 2016).

Table 3.9. Consumption of nitrogen by several B. *bruxellensis* strains summed over 45 days (Smith 2016)

The nitrogen consumption in N/L by each *B. bruxellensis* strain under all conditions.
Green= strong consumption (above 1 mg N/L).
Orange/Yellow= intermediate/weak consumption (0.2 to 0.9 mg N/L).
Negative Values (red) = a production of that compound. (Day 0-10)= exponential phase,
(Day 10-45)= stationary phase, (Day 0-45)= the overall expirement.

Strain	Initial amount (mg N/L)	AWRI1499			AWRI1499			ISA 1649			ISA 1649		
Condition		Anaerobic			Semi-anaerobic			Anaerobic			Semi-anaerobic		
Day		0-10	10-45	0-45	0-10	10-45	0-45	0-10	10-45	0-45	0-10	10-45	0-45
Ammonia	88.05	-0.14	32.42	32.28	17.88	27.58	45.45	28.58	29.84	58.42	20.39	27.5	47.88
Asparagine	2.9	-0.19	-1.9	-2.09	-0.55	-0.49	-1.04	-0.58	-2.49	-3.07	-0.51	-1.69	-2.2
Glycine	1.35	-0.62	0.02	-0.61	-0.6	-1.2	-1.8	-0.74	-0.28	-1.02	-0.74	-0.46	-1.2
Threonine	1.46	-0.03	-0.06	-0.1	-0.09	0.01	-0.07	-0.28	0.26	-0.02	-0.3	-0.07	-0.37
Valine	1.66	-0.17	-0.01	-0.18	-0.2	-0.11	-0.31	-0.12	-0.02	-0.14	-0.13	-0.1	-0.23
Hydroxyproline	0.6	-0.88	0.58	-0.3	-1.24	0.21	-1.03	-0.35	-0.04	-0.39	-0.55	-0.42	-0.97
Alanine	6.15	-0.34	1.03	0.69	-0.78	1.08	0.31	-1.96	1.9	-0.06	-2.58	0.35	-2.23
Isoleucine	1.16	-0.05	0.08	0.03	-0.07	0.17	0.1	0.04	0.29	0.33	-0.04	0.02	-0.02
GABA	2.68	-0.33	1.45	1.12	0.14	1.59	1.73	-0.41	-0.62	-1.03	-0.18	-0.06	-0.24
Phenylalanine	1.39	-0.05	0.45	0.4	-0.02	0.49	0.47	0.27	0.49	0.76	0.1	0.28	0.38
Aspartic Acid	1.9	0.54	0.88	1.41	0.91	0.61	1.52	0.71	0.29	1	0.75	0.2	0.95
Glutamic Acid	2.57	0.19	0.56	0.75	0.39	0.54	0.93	0.09	0.51	0.6	0.02	0.12	0.14
Serine	1.94	0.1	1.36	1.46	0.32	0.79	1.11	0.52	0.46	0.98	0.43	0.25	0.68
Histidine	0.94	-0.2	0.85	0.65	-0.03	0.58	0.55	0.46	0.18	0.64	0.29	0.11	0.4
Arginine	4.56	0.49	3.93	4.41	2.29	1.84	4.14	4.27	-0.01	4.27	4.47	-0.17	4.3
Tyrosine	0.54	-0.03	0.09	0.06	-0.02	0.14	0.13	0.02	-0.1	-0.08	-0.01	0.02	0.01
Cysteine	1.56	-0.48	1.45	0.97	0.19	0.49	0.68	0.79	-0.01	0.79	0.69	-0.01	0.68
Methionine	0.38	0.12	0.02	0.14	0.2	0.01	0.21	0.28	-0.01	0.28	0.29	-0.01	0.27
Tryptophan	0.22	0.06	0.09	0.15	0.1	0.05	0.15	0.1	0.03	0.13	0.07	0.07	0.14
Ornitihine	2.36	-0.09	1.89	1.81	0.14	-0.54	-0.4	-0.46	-0.6	-1.07	-0.39	-3.02	-3.41
Leucine	2.55	-0.03	2.28	2.24	-0.05	2.11	2.05	0.58	1.26	1.83	0.2	1.17	1.37
Lysine	6.2	1.49	4.36	5.86	3.58	2.07	5.65	5.45	0.15	5.6	5.28	31	5.59
Proline	54.08	-4.54	8.43	3.89	-1.98	13.82	11.84	-8.27	23.98	15.72	-8.23	19.74	11.51

3.6. Beer

Creating a generalized view of the amino acids, biogenic amines, and proteins present in beer is challenging. According to the Brewers Association, in 2019 there were well over 120 different recognized styles of beer (Brewers Association, 2020). Each of these beer styles have their own unique grain bill, aging regiment, fermentation style, adjuncts, microbiota, etc. which influence the chemical makeup of the final product. The styles represented on this list include unfiltered and highly hopped New England IPAs, high gravity Russian imperial stouts, rye beers, long aged sour beers, and many more. The effects that these recipes have on a beer's chemical makeup aren't well studied, but the published literature provides a glimpse of some potential patterns.

3.6.1. Amino Acid Profile vs. Beer Style

A study focused on amino acids in several beer styles is shown in Table 3.10. In this study a variety of beer styles were tagged with an amino group specific compound and separated/analyzed using HPLC-MS(Bollig 2015). The three types of alcoholic beer and two types of non-alcoholic beer analyzed in this study were relatively similar, style-wise; Pils and Altbier are both types of lagers (made mostly from barley), while wheat beer is brewed with significant quantities of wheat (all three are "simple" beers without strange adjuncts or long aging methods). The data shows that both broad similarities and stark differences exist between these samples. Some amino compounds like proline and ethanolamine were relatively consistent amongst all five beer styles, but most were vastly different. This highlights the potential influences of beer diversity, as even with three simple, similar beer styles stark differences exist. It may be more helpful to look at beer within a single style.

Table 3.10. HPLC-MS analysis of 5 beer varieties (Bollig 2015)

Results of the determination of amino acids in beer samples (umol/L)					
Amino Acid	**Non-alcoholic pils**	**Pils**	**Alt beer**	**Non-alcoholic wheat beer**	**Wheat beer**
Aspartic Acid	28.6	---	31.4	187.9	43.9
Glutamic Acid	144.2	54.6	43.1	193.8	59.7
Hydroxyproline	6.4	8.5	6.2	4.7	---
Serine	---	9.6	28.1	258.4	39.8
Glycine	208.3	193.2	234.2	316.2	251.3
Histidine	124.1	109.5	63.6	156.7	127.7
Threonine	---	---	20.9	163.3	21.5
Citruline	15.6	8.3	10.7	38	28.1
Alanine	566.1	434.6	242.3	744.4	399.2
Arginine	249.1	223.3	50.7	404.7	50.5
GABA	1315.6	1245.6	1331.5	961.6	890.4
3-Methylhistidine	5.2	---	---	---	---
beta-Aminoisobutryic Acid	---	6.3	7	---	---
Proline	3214.7	3402.3	2987.7	1942.7	1959.7
Ethanolamine	147.2	182.3	167.3	116.7	139.4
alpha-Aminobutryic Acid	15.6	30.5	---	6.1	---
Tyrosine	227.1	283.2	132.5	298.2	216.5
Valine	295.3	252.8	49.3	513.7	290.2
Methionine	---	0.8	12.2	99.4	20.9
Ornithine	14.7	16.5	20.7	68.4	75.1
Lysine	11.2	10.8	37.7	213	30.2
Isoleucine	50.7	27.9	30.1	250.7	55.8
Leucine	121.1	57.9	53.8	570.3	176.6
Phenylalanine	214.1	203.1	35.1	395.9	213.7
Trytophan	142.9	148.2	55.5	178.4	148.1
Total Amino Acid Content	7117.6	6910	5651.7	8074.3	5238.4

While it makes sense that different beer styles have diverse amine profiles, studies have shown variance exists even within a single style of beer. An evaluation of a variety of lager beers from the Czech Republic is shown in Table 3.11. This study revealed that within this family of beer (Lager) amino acid profiles are relatively consistent(Kabelová et al. 2008). The researchers noted that concentrations of amino acids may vary in beer from a single country, but claim that this is largely due to the

concentration of the wort (Kabelová et al. 2008). As can be seen in the charts below, samples from different countries, even if controlled for concentration of wort, vary to a greater or lesser degree. This is relatively normal for plant-derived products because the climate grain is grown in can have a drastic effect on the nutritional and biochemical profile of that plant.

Table 3.11. Analysis of a variety of lager beer from the Czech Republic (Kabelová et al. 2008)

Basic analytical characteristics of beers								
Sample No.	**Land of Origin**	**Alcohol (vol %)**	**Extract of Original Wort (% plato)**	**Real Extract (Wt %)**	**Real Degree of Fermentation (%)**	**pH**	**Color (EBC Units)**	**Density (g/mL)**
1	Czech Republic	4.43	11.76	5.06	58.53	4.73	12.1	1.0116
2	Czech Republic	4.65	11.21	4.11	64.71	4.72	11.4	1.0076
3	Czech Republic	4.76	11.68	4.44	63.44	4.5	27.1	1.0087
4	Czech Republic	4.96	11.47	3.91	67.32	4.62	8.8	1.0075
5	Czech Republic	4.76	11.39	4.13	58.33	4.6	13.5	1.0065

Concentration (mg/L) of amino acids from beer samples																	
Sample No.	**Asp**	**Ser**	**Glu**	**Gly**	**His**	**Arg**	**Thr**	**Ala**	**Pro**	**Tyr**	**Val**	**Met**	**Lys**	**He**	**Leu**	**Phe**	**Total AA**
1	21.3	11.4	17.4	17.5	20.2	32.3	6.3	51.4	241.4	32.2	34.8	5.7	16.1	14.8	25	29.1	576.9
2	9.9	4.6	16.5	11.4	13.9	15.5	2.9	33.1	166.3	26.2	23	3.6	7.3	8.4	16.1	22	380.7
3	9.1	5.7	18.5	7.5	21.1	17.4	3.6	29.1	125.7	37.9	19.4	3.9	9	8.2	13.8	18.4	348.4
4	9.7	6	12.6	14.7	13.8	16	1.5	36.7	200.6	20.4	22.1	2.1	9.9	7.2	9.7	16.5	399.5
5	9.2	4.2	19.4	11.5	30.4	27	3	44.7	232.2	49.5	29.4	4.7	12.8	10.7	19.3	22.9	521.9

These results are consistent with other limited studies focused on amino acids content of beer. Within a given style, different brands of beer are likely to have similar amino acid profiles, but variation can occur when considering the cultivar, growing location, and agricultural practices utilized when growing the grain. Between different styles of beer there can be significant differences in amine content, due to the myriad of ingredients and techniques utilized.

3.6.2. Albumins, Haze Proteins, and Foaming Proteins

As previously discussed, some proteins manage to escape the heating, separating, and fermenting steps and persist in the final beer. These proteins play an important role in the finished beers flavor, mouth feel, and foam formation. The barley proteins most likely to survive in a final product are heat-stable albumins, as they readily dissolve in water and resist coagulation (Osman 2003). These albumins are chemically changed in a variety of ways during the brewing process and are converted into “foaming” proteins. During the brewing process albumins are glycated and acylated using free sugars and acyl groups produced during catalysis of sugars and other compounds (Didier and Bénédicte 2009). As the brewing process continues, disulfide groups are broken and these modified albumins are unfolded, creating foaming proteins (Didier and Bénédicte 2009). A diagram showcasing these steps is shown in Figure 3.2. The other major source of proteins found in beer are the haze-forming proteins. These substances are most commonly formed from the previously discussed hordeins, some of which avoid complete digestion by enzymes/heat coagulation and are soluble in the alcoholic beer(Siebert 1999). These proteins become less soluble and form hazes when they bind to the various polyphenols present in beer. Both haze and foaming proteins are unlikely to cause separation problems during HPLC analysis due to the large disparity in size between the dipeptides/amino acids/biogenic amines and the proteins (~300Da vs. 30,000Da).

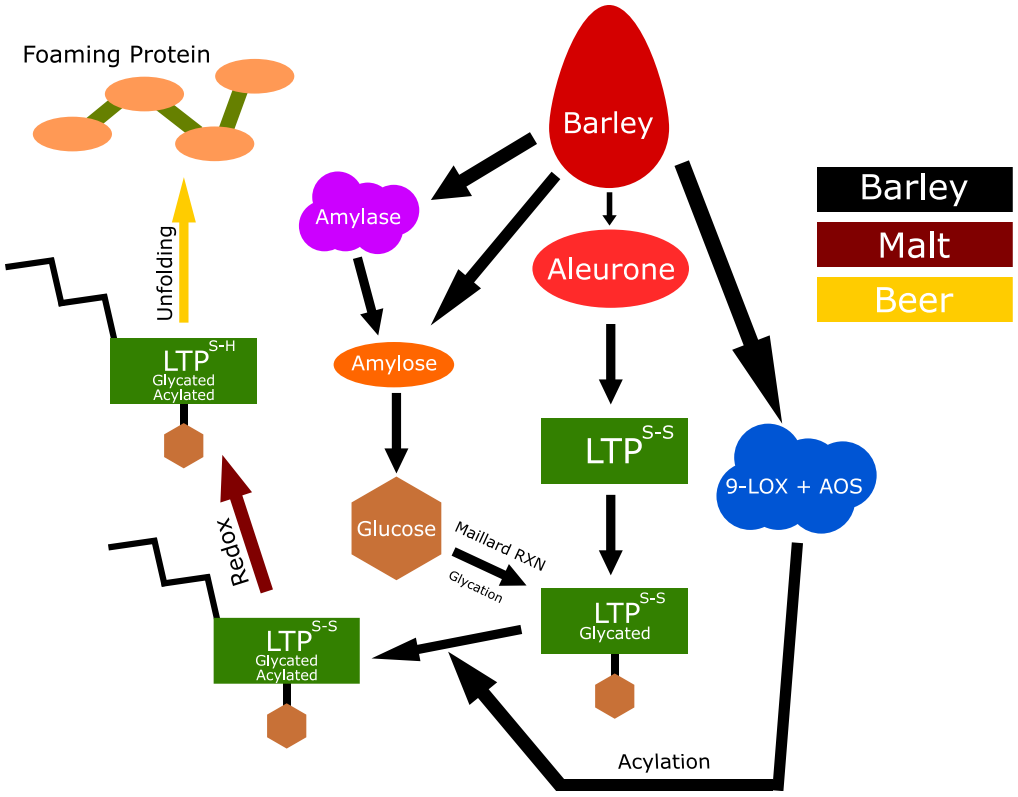

Figure 3.2. The transformation of albumins to foaming proteins (Didier and Bénédicte 2009)

3.6.3 Biogenic Amines in Beer

The final area to consider when examining amine compounds in beer are biogenic amines. Biogenic amines are a class of basic amine molecule formed by the decarboxylation of amino acids or transamination of other compounds and are formed by living beings. Biogenic amines are a family of diverse, powerful signaling molecules and include such chemicals as histamine, serotonin, tyramine, and cadaverine among many others. These compounds are of interest to scientists as they have deleterious effects when consumed in food products. When consumed biogenic amines can cause a plethora of side effects including migraines, high/low blood pressure, pseudo anaphylaxis, and gastroenteritis (Daniel et al. 2015). There is little comprehensive work describing biogenic amine levels in commercial beer products and most of the study done conclude that the concentrations of these compounds are relatively low.

A study in China examined the histamine, tryptamine, phenethylamine, tyramine, putrescine, spermidine, and spermine content of several Chinese beer varieties (the sampler were a mix of different beer styles but were from major breweries) (Tang et al. 2009). These biogenic amines were selected because they are considered to be the most common biogenic amines found in fermented foods. The results of this study (Table 3.12.), showed that all tested beer samples had similar levels (when compared to each other) of each amine. Some of the amines, like tyramine, had a moderately consistent concentration amongst the samples while others, like putrescine varied more significantly (Tang et al. 2009). Considering that all the beers tested were major brands including Heineken, Corona, and Yanjing, made with similar ingredients, the biogenic amine profile should be consistent. The level of processing, simplicity of ingredients, and quick throughput times for these brews would likely limit the production of biogenic amines by the yeasts.

Table 3.12. Biogenic amine content of Chinese beer (adapted) (Tang et al. 2009)

Beer sample Characteristics					
Sample No.	**Varieties**	**Beer-making Areas (Province)**	**Alcoholic Content (v/v, %)**	**Original Gravity (°P)**	**pH**
1	Heineken	Shanghai	≥4.7	11.4	3.98
2	Carlsberg	Guangdong	≥4.0	10.3	4.03
3	Tiger	Shanghai	≥4.7	11.8	4.25
4	Corona Extra	Wuhan	4.6	11.3	4.19
5	Yanjing	Beijing	≥3.6	10	4.13

Concentration of biogenic amines (ug/mL) (mean ± standard deviation)							
Sample No.	**HIST**	**TRYP**	**PHE**	**TYR**	**PUT**	**SPD**	**SPM**
1	1.20±0.04	ND	0.12±0.02	6.35±0.06	4.25±0.08	1.00±0.04	2.64±0.05
2	2.80±0.16	0.45±0.02	ND	5.08±0.12	2.12±0.14	ND	1.06±0.01
3	0.96±0.03	1.62±0.05	ND	5.65±0.03	2.90±0.06	1.35±0.03	ND
4	1.25±0.03	0.36±0.03	ND	3.47±0.05	4.55±0.05	0.71±0.08	2.49±0.08
5	4.62±0.13	ND	ND	3.80±0.03	5.73±0.08	1.41±0.14	3.96±0.11

The levels of amines present in these beer samples vary considerably. The biogenic amine concentrations ranged from 0 ug/mL to more than 7 ug/mL (Tang et al. 2009). Looking at beer sample 5, analysis shows that this beverage had a histamine content of 4.62 ug/mL which is equivalent to 1.64 mg of histamine per 355 mL can of beer. This concentration (equivalent to 4.62 ppm) is far below the FDA's legal limit of 50 ppm for histamine in fish (FDA 2005), but this doesn't mean it is perfectly safe. Concerns about alcohol lowing the intoxicating-threshold for biogenic amines means this could be dangerous in certain individuals (Świder et al. 2020). As discussed previously, histamine-intolerant people experienced biogenic amine poisoning when served a histamine-spiked (4mg) sample of wine which would be matched after just 2.5 cans of this beer (Comas-Basté et al. 2019).

After testing the variation in similar beer styles, it may be more useful to look at the physiological factors that trigger biogenic amine production instead of beer in general. One of the most studied factors that influences biogenic amine production is pH, specifically acidity. Two studies (Pereira et al. 2009; Perez et al. 2015) have shown that when bacteria (in these studies the non-food LAB *Enterococcus faecalis* was examined) produce biogenic amines like tyramine, their ability to survive in acidic environments was greatly improved. In test conducted at pH's from 7 to 1, bacterial strains able to produce tyramine had 50% more surviving cells than those that could not (Perez et al. 2015). Chemically, this may occur because biogenic amines are basic compounds, so the decarboxylation of an amino acid not only removes an acidic compound, but also produces a base. This base production is seen in a variety of lactic acid bacteria, including the genera commonly used in the brewing of sour beer, like *Lactobacillus* and *Pediococcus* (Steinkraus 1992). This implies that in sour beer styles (pH of approximately 3, from lactic acid and small amounts of acetic acid) like gose and lambic. may have higher levels of biogenic amines due to the combination of high acidity, lactic acid bacteria, and yeast.

Metabolizable carbon scarcity, primarily of sugars and carbohydrates, has also been shown to increase biogenic amine production from microbes. Microbes will switch to amino acid digestion when available carbohydrates become scarce producing biogenic amines and carbon dioxide as waste products. This "starvation" doesn't appear to affect biogenic amines formation uniformly (Table 3.13.). While little work has been published specific to beer in this vein, a study focused on sausage fermentation showed that while the concentration of some amines (spermine, spermidine) are identical regardless of sugar content, others amines (tyramine, cadaverine, putrescine, tryptamine, phenethylamine) increased significantly (Bover-Cid, Izquierdo-Pulido, and Carmen Vidal-Carou 2001). Compared to sausages with higher sugar contents, tyramine concentration increased by 159%, cadaverine increased 851%, putrescine increased 700%, tryptamine increase 200%, and phenethylamine increased 450%. The reasoning for this change is not well understood, but it is theorized that the lack of carbohydrates causes the microbes to begin decarboxylating amino acids for energy more quickly than if they had abundant carbohydrates (Bover-Cid, Izquierdo-Pulido, and Carmen Vidal-Carou 2001). While sausages are very different, beer does become a low sugar environment post-fermentation and the only available food source to most bacteria and yeasts are amino acids. There is a potential for this phenomenon to become significant in beer that is aged for long periods of time, such as barrel-aged beers.

Table 3.13. Biogenic amine concentration in fermented sausages (Bover-Cid et al. 2001)

Biogenic amine contents of fermented sausages with and without sugar after 20 days of storage (mg/kg)				
Biogenic Amine	**With Sugar**		**Without Sugar**	
	4°C	**19°C**	**4°C**	**19°C**
Spermidine	6.5±0.1	5.4±0.1	6.7±0.1	5.6±0.1
Spermine	41.8±0.2	37.2±1.6	41.1±0.6	38.2±1.2
Tyramine	113.9±37.9	114.4±21.1	180.4±4.8	210.2±13.9
Cadaverine	23.6±6.2	31.9±1.6	201.0±11.0	254.1±38.2
Putrescine	4.9±0.3	3.8±1.4	34.5±5.9	42.2±4.4
Tryptamine	0.5±0.3	1.4±0.1	5.6±0.2	10.2±1.2
Phenethylamine	0.4±0.3	0.8±0.2	1.8±0.2	4.5±1.7

3.7. Conclusions

The amine component of beer is a complicated mixture of amino acids, proteins, and biogenic amines that develops and changes during the brewing process. Proteins are extracted from the barley seed, which are then catabolyzed, solubilized, denatured, and in some cases coagulated. As the brewing process continues, yeasts utilize amino acids to build peptides and as an energy source, causing amino acids to be removed, created, and digested into biogenic amines. This phenomenon is poorly understood, and more work needs to be done in this area, including identification of the triggers for digestion and uptake by the microbiota associated with beer brewing. The complexity of beer matrices makes analyzing biogenic amines in beer a challenge, especially when fluorescent amine-specific chemical tags are utilized with high performance liquid chromatography (HPLC). HPLC combined with fluorescent detection represents a highly sensitive and analyte-specific approach for identification and quantification of biogenic amines in a number of complex matrices. Unfortunately, there are a number of free (unidentified) amines which co-elute with the targeted analytes. More research, including the development of better analytical methodology, needs to be done to better understand the formation and content of biogenic amines in beer, especially considering the many new exotic styles brewed with unusual microbes, ingredients, and aging regiments, as these factors have a major effect on proteins and protein derived compounds.

CHAPTER 4

HPLC METHOD DEVELOPMENT FOR BIOGENIC AMINES IN BEER

4.1 Introduction

As the deleterious effects of biogenic amines on health become more clearly understood, having accurate data regarding their concentration in beer become increasingly important. Beer is a challenging medium to exam for biogenic amines due to its chemical makeup, specifically the high concentrations of highly soluble free amino acids. Amine-specific fluorescent tagging compounds will react with not only the biogenic amines but every other amine-containing compound present. Without a highly selective HPLC methodology, any data collected will not be usable, as amino acids and their conjugate biogenic amines coelute from the HPLC column. Developing a methodology that can not only remove confounding amino acids but clearly separate the desired biogenic amines from each other is extremely important.

4.2. Preparation of Reagents

4.2.1. Preparation of Amine Standards

Before creating the amine standards all glassware was soaked in a solution of detergent and distilled water to remove any protein or chemical residue. One-hundred mg samples of each amine (histamine, tyramine, tryptamine, gamma-amino butyric acid, spermine, spermidine, putrescine, cadaverine, and phenethylamine; purchased from Fischer Scientific-Pittsburgh, Pennsylvania) were weighed out and added to correspondingly labelled 100 mL volumetric flasks which were then filled with HPLC grade water from a Milli-Q Ultrapure system (purchased from MilliporeSigma- Darmstadt, Germany). Each flask was sonicated for 10 minutes to ensure that each amine standard was totally dissolved. This served as 1000 ug/mL stock solutions for each amine.

After allowing each amine to fully dissolve, diluted 50 ug/mL working solutions of each amine were created. To make the diluted working solutions 5 mL of stock solution was pipetted into a 100 mL volumetric flask that was brought to volume with HPLC grade water (Purchased from Fischer Scientific-Pittsburgh, Pennsylvania). Each flask was capped and inverted several times to mix. When not in use all solutions were stored at 4°C.

4.2.2. Preparation of 6-Aminoquinolyl-N-Hydroxy Succinimidyl Carbamate Tagging System

The AccQ Tag Ultra Derivatization Kit was purchased from the Waters Corporation (Milford, Massachusetts). The kit contains three chemical reagents, a borate buffer solution, dry 6-Aminoquinolyl-N-Hydroxy Succinimidyl Carbamate (AccQ tag), and reagent diluent (HPLC grade acetonitrile). The AccQ tag was reconstituted following the instructions provided by Waters. One mL of the reagent diluent was pipetted into the AccQ vial and vortexed for 10 seconds. After vortexing, the AccQ vial was then heated in a sand bath set to 55°C for 15 minutes. When not in use the AccQ tag was stored at room temperature in the dark.

4.2.3. Preparation of 2-Hydroxnaphthaldyhde

An alternative tagging method adapted from Amir Hayat et al. (2015) utilizing 2-Hydroxnaphthaldyhde was also tested. This method required a 2.5% (w/v) solution of 2-Hydroxnaphthaldyhde in methanol made by dissolving 2.5g of 2-Hydroxnaphthaldyhde in 100 mL of HPLC grade methanol in a screw cap bottle covered in tin foil (this solution is very light sensitive). This methodology also requires pH 8.5 boric acid-sodium hydroxide buffer made by dissolving 61.83g of boric acid and 10g of sodium hydroxide into 1L of HPLC grade water(Hayat et al. 2015).

4.2.4. Preparation of Beer Samples

4.2.4.1. Degassing

Before any beer sample can be tagged or used, it required simple preparation. A given beer sample was poured into a clean Erlenmeyer flask and degassed by capping the vial with a vacuum pump and submerging the flask in a sonicating bath. The flask was sonicated and held under vacuum until the beer sample was “flat” and no more carbonation bubbles were observed. This “decarbonation” step took about 10 minutes.

4.2.4.2. Cleaning Samples

Two methods of removing unwanted amino acids and peptides from the beer samples were tested to improve the clarity of the chromatograms. The first was a solid phase extraction method adapted from A Gallego-Pena et al (2009). This method was used to clean wine samples before biogenic amine tagging and offered biogenic amine recovery above 90% (Peña-Gallego et al. 2009). This method utilizes Sep-Pak SPE cartridges (C18), 10mM Phosphoric acid: Methanol [90:10] solution, 10mM Sodium hydroxide: Methanol [70:30] solution, 10mM Calcium chloride: Methanol [70:30] solution, and 100mM Sodium hydroxide: Methanol [65:35] solution. A Sep-Pak cartridge was conditioned using 2 mL of methanol followed by 2 mL of distilled water, after which 0.6 mL of degassed beer was percolated through the filter. After the beer sample was loaded into the filters-Pak, three washing steps were performed to remove interfering amino acids and peptides. The first wash was 2 mL of the phosphoric acid solution followed with 1 mL of distilled water. Next, 2 mL of the 70:30 sodium hydroxide solution was percolated through the Sep-Pak, again followed by 1 mL of distilled water.

Finally, 2 mL of the calcium chloride solution was percolated through the cartridge before rinsing with 1 mL of distilled water. After the three washing steps the filter is eluted using 1.2 mL of the 65% sodium hydroxide solution, tagged and analyzed (Peña-Gallego et al. 2009). An illustration of this technique can be seen in Figure 4.1.

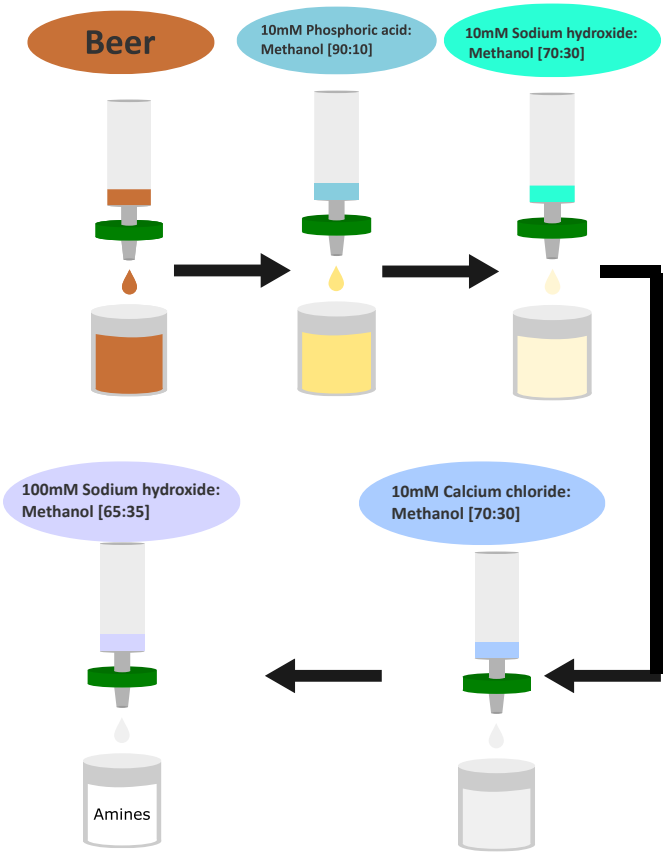

Figure 4.1. Illustration of the SPE procedure adapted from Peña-Gallego et al. (2009)

The second method tested was an acetonitrile-based salting-out assisted liquid-liquid extraction (SALLE) method adapted from Jun Zhang et al (2009). This extraction method utilizes acetonitrile and calcium chloride to completely remove the free amino acids from the biogenic amines. In this method 5 mL of degassed beer is mixed with 5 mL of acetonitrile in a capped vial and inverted a couple times to

ensure proper mixing. After mixing, 1.2g of calcium chloride was added to the vial and allowed to sit until two layers formed. The clear organic layer on top was decanted and stored at 4°C until needed (Jun Zhang et al. 2009). An illustration of this technique can be seen in Figure 4.2.

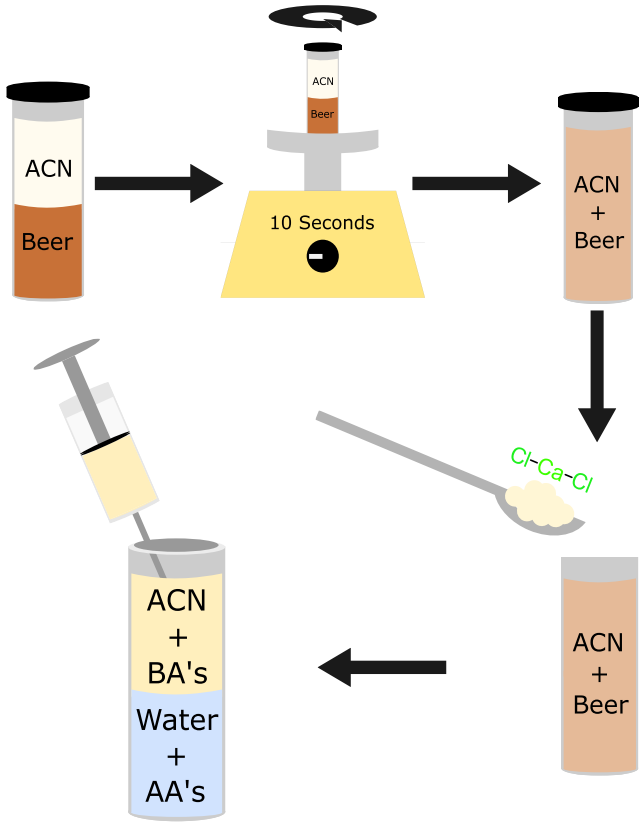

Figure 4.2. Illustration of the ACN based SALLE beer extraction procedure

4.3. Chemical Tagging Procedures

4.3.1. Waters AccQ

The tagging procedure for the AccQ tag was supplied by the Waters Corporation. To tag a sample 70 uL of the borate buffer was pipetted into a 1 mL glass vial, along with 20ul of the AccQ tag, and 10 uL of sample. The glass vial was than vortexed for 10 seconds and heated in a 55°C sand-bath for 10 minutes. After the 10-minute heating the tagged sample was pipetted into an HPLC vial (a vial insert

was used due to the small working volume) the derivatized samples and standard were at room temperature for a maximum of one week (beyond this the derivatized samples would begin to break down) (AccQ Tag Ultra, 2014). An illustration of this procedure can be seen in Figure 4.3.

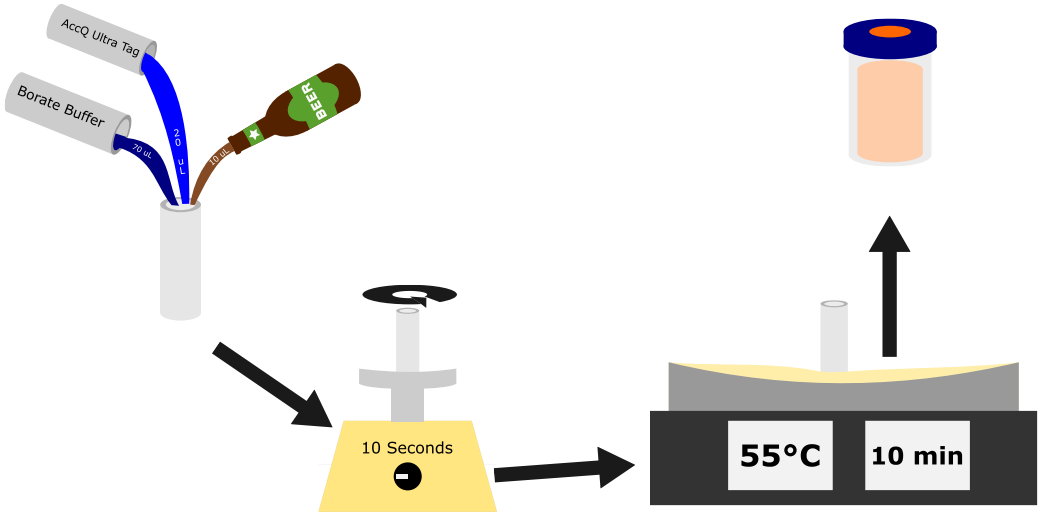

Figure 4.3. Illustration of the AccQ tagging procedure adapted from the Waters Corp (AccQ Tag Ultra, 2014)

4.3.2. 2-Hydroxnaphthaldyhde

The tagging procedure for the 2-Hydroxnaphthaldyhde tag was adapted from Amir Hayat et al. (2015). To chemically tag a sample 1 mL of the sample was added to a 5 mL volumetric flask followed by 1 mL of the 2-Hydroxnaphthaldyhde solution and 0.5 mL of the borate buffer (at this step the mixture turns cloudy yellow). The mix was then gently vortexed for 10 seconds and heated in a sand bath at 85° C for 15 minutes. After heating (at this point the solution should turn a clear fluorescent yellow), the flasks are removed from the sand-bath, allowed to cool to room temperature, and their final volume was adjusted to 5 mL with HPLC grade methanol. The tagged samples and standards can then be added to HPLC vials for analysis(Hayat et al. 2015).

A few minor modifications were made to the process to improve the results. The most important modification was to limit light exposure. Visible light appeared to degrade the tagged samples, so after the heating step was completed the flasks cooled in complete darkness. After cooling and dilution was completed the tagged samples were stored in brown HPLC vials for a maximum of one week at room temperature. An illustration of this procedure can be seen in Figure 4.4.

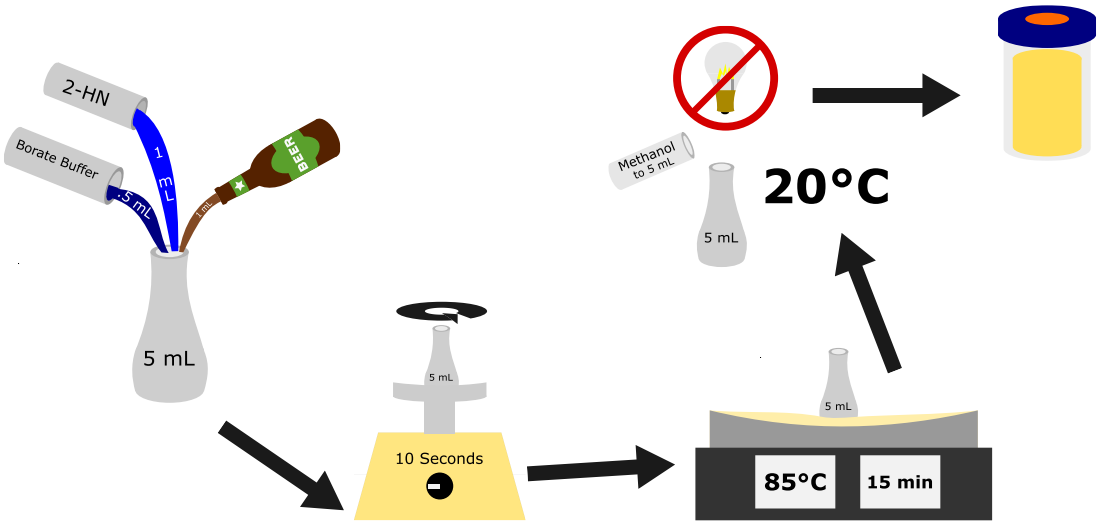

Figure 4.4. Illustration of the 2-HN tagging procedure adapted from Amir Hayat et al. (2015)

4.4. HPLC Method Development

Several variations of HPLC parameters, columns, and mobile phases were tested with the previously mentioned tagging systems. All HPLC runs were accomplished using an Agilent Technologies 1100/1200 High Performance Liquid Chromatography system equipped with an Agilent 1100/1200 quaternary pump, Agilent 1100 autosampler, Agilent 1200 column thermostat, fluorescence detector, and an Agilent 1200 diode array detector. A flow diagram showing all the tested HPLC methodologies is shown in Figure 4.5. while the parameters for each methodology is shown in Table 4.1.

The initial parameters (column and mobile phase) were determined using information provided by Waters Corp. Waters recommends using their Amino Acid Analysis column when analyzing amine compounds tagged with their AccQ tag. This column is a 150 mm long reverse phase C18 column and was ineffective at separating (high coelution) the tested biogenic amine standard solutions. After several unsuccessful runs using the suggested column, the Allure Organic Acids column was tested. This column was selected due to its similar make up to the Amino Acid Analysis column (pore size, C18 makeup, etc.) but longer length (250mm vs. 150mm). The longer length caused the analytes to be retained by the column for a longer period, improving the separation of the targeted analytes. This was the inspiration behind the idea of chaining two Allure Organic Acids columns in series.

The mobile phases tested were primarily Eluent A (a proprietary buffer solution produced by Waters) and either acetonitrile or methanol. Eluent A was needed to keep the tagged biogenic amines stable, as the complex become unstable at high and low pH. Acetonitrile (suggested by Waters) is used for both its ability to dissolve the amine-tag complexes and its miscibility with Eluent A. In the literature, methanol is occasionally substituted for the acetonitrile due to its similar solvent properties but higher polarity.

An iterative design system was utilized to develop the tested elution programs. Each successive method was created by examining the chromatogram and solvent table of the previous one. Solvent percentages and times were adjusted to separate the coeluting peaks. When the initial isocratic methods were unable to resolve the biogenic amines, gradient elutions were developed utilizing the previously described method. This process continued until a successful method was created during iteration 13.

When the Gradient 13 elution was unable to cleanly resolve BA's in a beer sample, extraction methods were tested to remove the interfering free amines from the beer. Polarity-based extraction techniques such as solid phase extraction and salting-out assisted liquid/liquid extraction were chosen. These methods were selected as based on polarity; they are highly effective at separating compounds from complex matrices.

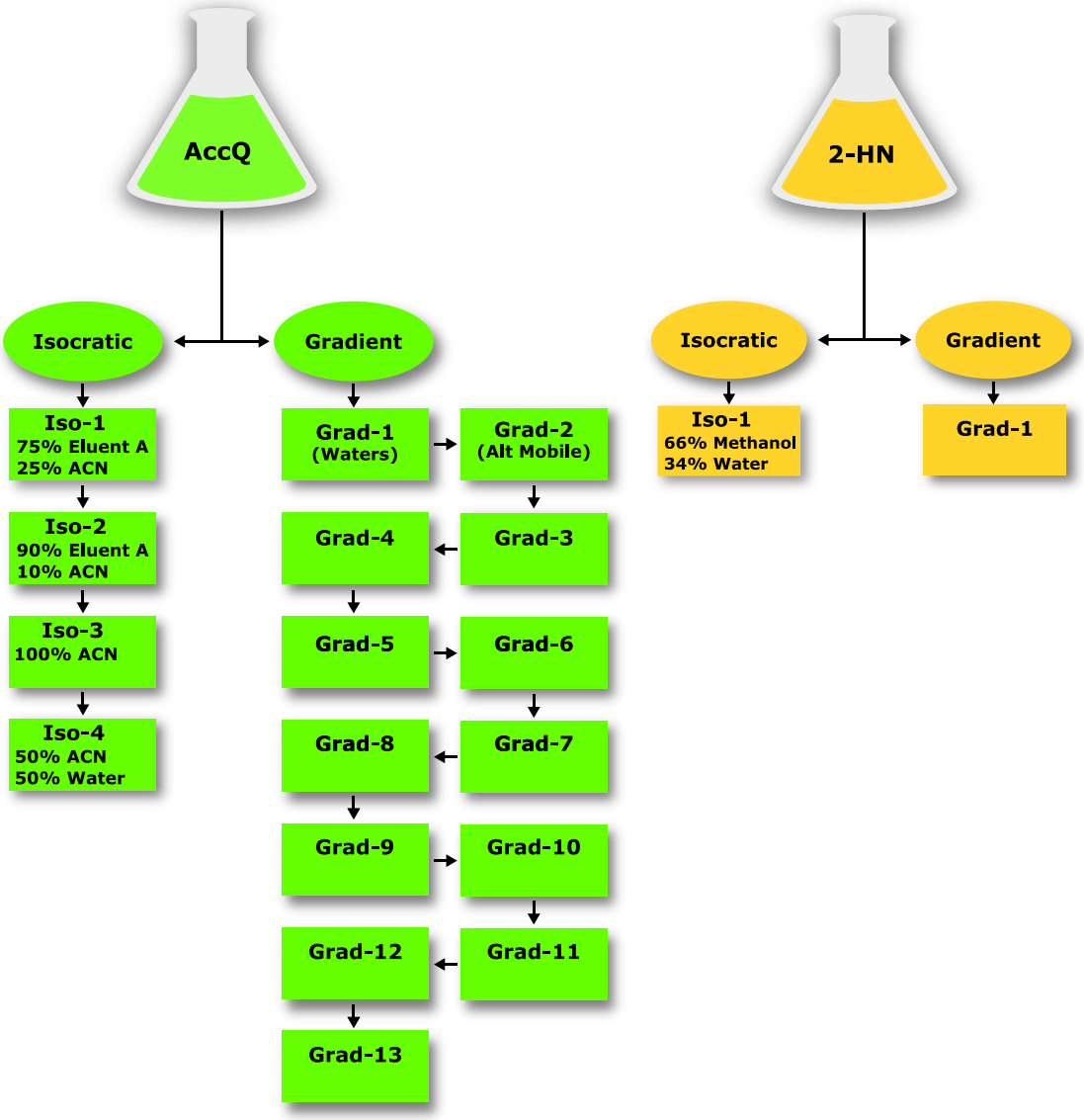

Figure 4.5. Flowchart of chromatographic conditions for the AccQ and 2-HN tags

Table 4.1. HPLC Method Development

Method	Parameters
AccQ Iso-1	**Mobile Phase**: 75% Eluent A & 25% acetonitrile **Run Time**: 25min + 5min post-run **Column**: Allure Organic Acids 250mm & AccQ Ultra Amino Acid C-18 **Flow Rate**: 1 mL/min **Detection**: Fluorescence (EX 250 nm, EM 395 nm)
AccQ Iso-2	**Mobile Phase**: 90% Eluent A & 10% acetonitrile **Run Time**: 30min + 5min post-run **Column**: Allure Organic Acids 250mm **Flow Rate**: 1 mL/min **Detection**: Fluorescence (EX 250 nm, EM 395 nm)
AccQ Iso-3	**Mobile Phase**: 100% acetonitrile **Run Time**: 35min + 5min post-run **Column**: Allure Organic Acids 250mm **Flow Rate**: 1 mL/min **Detection**: Fluorescence (EX 250 nm, EM 395 nm)
AccQ Iso-4	**Mobile Phase**: 50% water & 50% acetonitrile **Run Time**: 15min + 0min post-run **Column**: Allure Organic Acids 250mm **Flow Rate**: 1 mL/min **Detection**: Fluorescence (EX 250 nm, EM 395 nm)
AccQ Grad-1 (Waters)	**Mobile Phase**: Eluent A, acetonitrile, & water **Run Time**: 36min + 5min post-run **Gradient (Eluent A/acetonitrile/water)**: 0min: 100%/0/0, .5min: 99%/1%/0%, 18min: 95%/5%/0, 19min: 91%/9%/0, 29.5min: 83%/17%/0, 36min: 0/60%/40% (AccQ Tag Ultra, 2014) **Column**: Allure Organic Acids 250mm & AccQ Ultra Amino Acid C-18 **Flow Rate**: 1 mL/min **Detection**: Fluorescence (EX 250 nm, EM 395 nm)
AccQ Grad-2 (Alt Mobile Phase)	**Mobile Phase**: Eluent A & methanol **Run Time**: 30min + 5min post-run **Gradient (Eluent A/methanol)**: 0min: 75%/25%, 5.76min: 75%/25%, 7.3min: 75%/25%, 9.30min: 70%/30%, 13.3min: 60%/40%, 17.3min: 40%/60%, 21.3min: 20%/80%, 25min: 0/100% (Berbegal, Pardo, and Ferrer 2016) **Column**: Allure Organic Acids 250mm **Flow Rate**: 1 mL/min **Detection**: Fluorescence (EX 250 nm, EM 395 nm)

Table 4.1. Continued

Method	Parameters
AccQ Grad-3	**Mobile Phase**: Eluent A & acetonitrile **Run Time**: 30min + 5min post-run **Gradient (Eluent A/acetonitrile)**: 0min: 100%/0, 20min: 20%/80%, 20.01min: 20%/80% **Column**: Allure Organic Acids 250mm **Flow Rate**: 1 mL/min **Detection**: Fluorescence (EX 250 nm, EM 395 nm)
AccQ Grad-4	**Mobile Phase**: Eluent A & acetonitrile **Run Time**: 30min + 5min post-run **Gradient (Eluent A/acetonitrile)**: 0min: 100%/0, 10min: 90%/10%, 20min: 20%/80%, 20.01min: 20%/80% **Column**: Allure Organic Acids 250mm **Flow Rate**: 1 mL/min **Detection**: Fluorescence (EX 250 nm, EM 395 nm)
AccQ Grad-5	**Mobile Phase**: Eluent A & acetonitrile **Run Time**: 30min + 5min post-run **Gradient (Eluent A/acetonitrile)**: 0min: 50%/50%, 19min: 10%/90%, 19.01min: 10%/90% **Column**: Allure Organic Acids 250mm **Flow Rate**: 1 mL/min **Detection**: Fluorescence (EX 250 nm, EM 395 nm)
AccQ Grad-6	**Mobile Phase**: Eluent A & acetonitrile **Run Time**: 30min + 5min post-run **Gradient (Eluent A/acetonitrile)**: 0min: 80%/20%, 20min: 20%/80%, 20.01min: 20%/80% **Column**: Allure Organic Acids 250mm **Flow Rate**: 1 mL/min **Detection**: Fluorescence (EX 250 nm, EM 395 nm)
AccQ Grad-7	**Mobile Phase**: Eluent A & acetonitrile **Run Time**: 30min + 5min post-run **Gradient (Eluent A/acetonitrile)**: 0min: 30%/70%, 20min: 20%/80%, 20.01min: 20%/80% **Column**: Allure Organic Acids 250mm **Flow Rate**: 1 mL/min **Detection**: Fluorescence (EX 250 nm, EM 395 nm)

Table 4.1. Continued

Method	Parameters
AccQ Grad-8	**Mobile Phase**: Eluent A, acetonitrile, & water **Run Time**: 30min + 5min post-run **Gradient (Eluent A/acetonitrile/water)**: 0min: 80%/20%/0, 20min: 20%/80%/0, 20.01min: 20%/80%/0, 24min: 20%/80%, 26.01min: 0/10%/90% **Column**: Allure Organic Acids 250mm **Flow Rate**: 1 mL/min **Detection**: Fluorescence (EX 250 nm, EM 395 nm)
AccQ Grad-9	**Mobile Phase**: Eluent A, acetonitrile, & water **Run Time**: 30min + 5min post-run **Gradient (Eluent A/acetonitrile/water)**: 0min: 80%/20%/0, 20min: 20%/80%/0, 20.01min: 20%/80%/0, 24min: 20%/80%/0, 26.01min: 0/10%/90% **Column**: Allure Organic Acids 250mm **Flow Rate**: 1 mL/min **Detection**: Fluorescence (EX 250 nm, EM 395 nm)
AccQ Grad-10	**Mobile Phase**: Eluent A & acetonitrile **Run Time**: 30min + 5min post-run **Gradient (Eluent A/acetonitrile)**: 0min: 80%/20%, 10min: 50%/50%/, 10.01min: 50%/50%, 12min: 50%/50% 15min: 25/75%, 16min: 20%/80% **Column**: Allure Organic Acids 250mm **Flow Rate**: 1 mL/min **Detection**: Fluorescence (EX 250 nm, EM 395 nm)
AccQ Grad-11	**Mobile Phase**: Eluent A, acetonitrile, & water **Run Time**: 35min + 5min post-run **Gradient (Eluent A/acetonitrile/water)**: 0min: 100%/0/0, 2min: 55%/45%/0, 10min: 55%/45%/0, 20.01min: 20%/80%/0, 29min: 20%/80%/0, 31min: 0/10%/90% **Column**: Allure Organic Acid, s 250mm **Flow Rate**: 1 mL/min **Detection**: Fluorescence (EX 250 nm, EM 395 nm)

Table 4.1. Continued

Method	Parameters
AccQ Grad-12	**Mobile Phase**: Eluent A, acetonitrile, & water **Run Time**: 35min + 5min post-run **Gradient (Eluent A/acetonitrile/water)**: 0min: 80%/20%/0, 6min: 65%/35%/0, 7min: 65%/35%/0, 8min: 50%/50%/0, 9min: 50%/50%/0, 10min: 45%/55%/0, 11min: 40%/60%/0, 12min: 40%/60%/0 13min: 35%/65%/0, 14min: 35%/65%/0, 15min: 30%/70%/0, 16min: 30%/70%/0, 20.01min: 20%/80%/0, 29min: 20%/80%/0, 31min: 0/10%/90 **Column**: Allure Organic Acid, s 250mm **Flow Rate**: 1 mL/min **Detection**: Fluorescence (EX 250 nm, EM 395 nm)
AccQ Grad-13	**Mobile Phase**: Eluent A, acetonitrile, & water **Run Time**: 35min + 5min post-run **Gradient (Eluent A/acetonitrile/water)**: 0min: 80%/20%/0, 20min: 20%/80%/0, 20.01min: 20%/80%/0, 31min: 0/10%/90% **Column**: Allure Organic Acids 250mm **Flow Rate**: 1 mL/min **Detection**: Fluorescence (EX 250 nm, EM 395 nm)
2-HN Iso-1	**Mobile Phase**: 66% methanol & 34% water **Run Time**: 20min + 2min post-run **Column**: Allure Organic Acids 250mm & AccQ Ultra Amino Acid C-18 **Flow Rate**: .8 mL/min **Detection**: Diode Array with the detection wavelength set to 254nm, 230nm, and 330nm (Panrod, Tansirikongkol, and Panapisal 2016)
2-HN Grad-1	**Mobile Phase**: methanol & water **Run Time**: 20min + 2min post-run **Gradient (methanol/water)**: 0min: 40%/60%, 2min: 50%/50%, 5min: 60%/40%, 10min: 70%/30%, 12min: 75%/25%, 15min: 80%/20% **Column**: Hypersil GOLD C8 HPLC column 100mm **Flow Rate**: .2 mL/min **Detection**: Diode Array with the detection wavelength set to 254nm, 230nm, and 330nm (Hayat et al. 2015)

4.6. HPLC Methodology Results

4.6.1. Waters AccQ Tag HPLC Methods

4.6.1.1. Tag Consistency

The Waters derivatization system was a very efficient and consistent tagging system. Between all runs and trials the tagging procedure worked without issue, always tagging any given sample (beer or analytical standard) within 10 minutes. The major advantage of this system seems to be the simplicity and efficiency. The tagging procedure is rapid and solution preparation is easy. Including all reagents in a single kit with just a single preparation step (rehydrating the AccQ powder) affords a low level of variance.

4.6.1.2. Isocratic Elution

An example chromatogram for a Waters AccQ Tag isocratic HPLC run (AccQ Iso-2) is shown in Figure 4.6. The injected sample was a 50 ug/mL mixture of histamine, gamma-aminobutyric acid, tyramine, and spermine. Ideally there should be six resolved peaks present (one for each amine, one for unreacted tagging dye, and an impurity peak of unknown origin) but only three highly rounded and overlapped peaks eluted. None of these isocratic elution methods resolved biogenic amines consistently. All HPLC runs eluted as an overlapping mass of unresolved target analytes toward the middle of the run time. Despite the varying conditions and columns used these results were typical for all AccQ-tagged isocratic runs and were ultimately unsuccessful for biogenic amine separation.

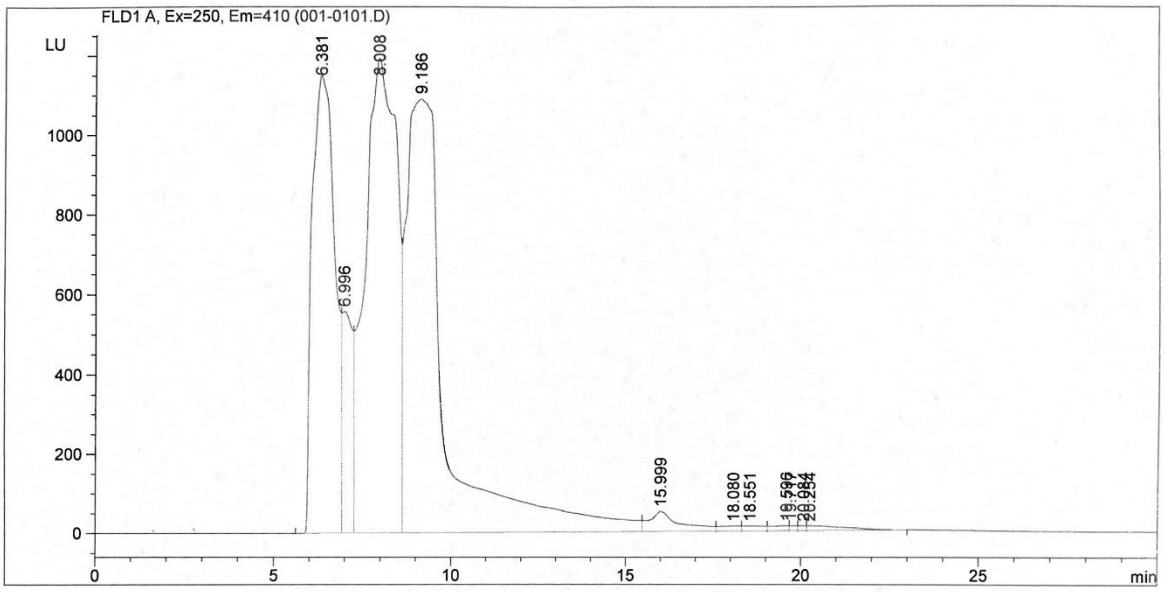

Figure 4.6. Chromatogram for AccQ Iso-2 method used on a mixed standard solution of histamine, GABA, tyramine, and spermine

4.6.1.3. Gradient Elution's 1-12

As simple HPLC isocratic separation of biogenic amine standards using a number of column chemistries was unsuccessful, several mobile phase gradient elution schemes were tried. An example chromatogram for this set of methods is shown in Figure 4.7. The injected sample for this run was a 50 ug/mL mixture of histamine, gamma-aminobutyric acid, tyramine, and spermine eluted using the AccQ Grad-10 protocol. Ideally, there should be six distinct, sharp peaks (four biogenic amines, the tagging dye, and an impurity peak) present on the chromatogram but this did not occur. This chromatogram shows three large peaks (9.692, 11.429, 14.432 min) and one small peak (9.076 min) clearly visible, but with several issues. The only clearly resolved peak eluted at 9.692 min and typically correlates to an unknown impurity (glass residue, ammonia, breakdown products of the amines or dye, impurities in the standards are all possible causes) while the peaks at 11.429 min and 14.432 min are both rounded and clearly overlapped with secondary peaks. In addition to the highly overlapped peaks

there is considerable trailing and noise at the base of each peak. These results were better in comparison to the isocratic elution attempts. While this is the result of just a single gradient elution protocol, all methods 1-12 had similar results despite differences in the conditions or use of different columns. While these results were better than the isocratic methods, they were ultimately not effective at separating a standard mixture of amines and were not used for beer beyond a single test shown in Figure 4.8.

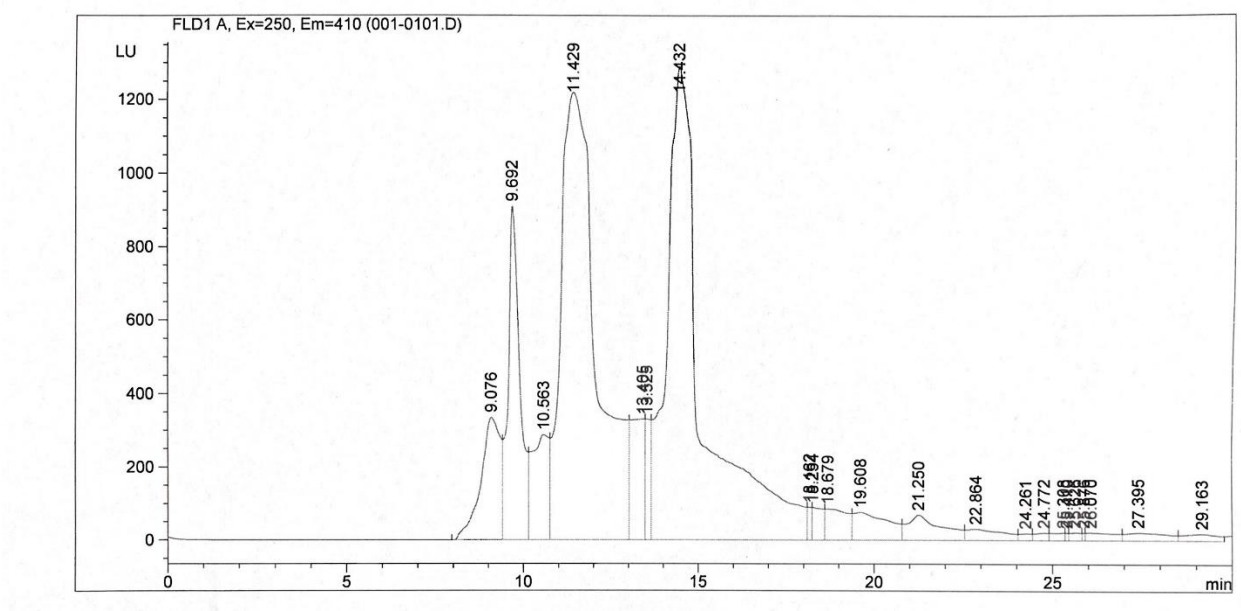

Figure 4.7. Chromatogram for AccQ Grad-10 method used on a mixed standard solution of histamine, GABA, tyramine, and spermine

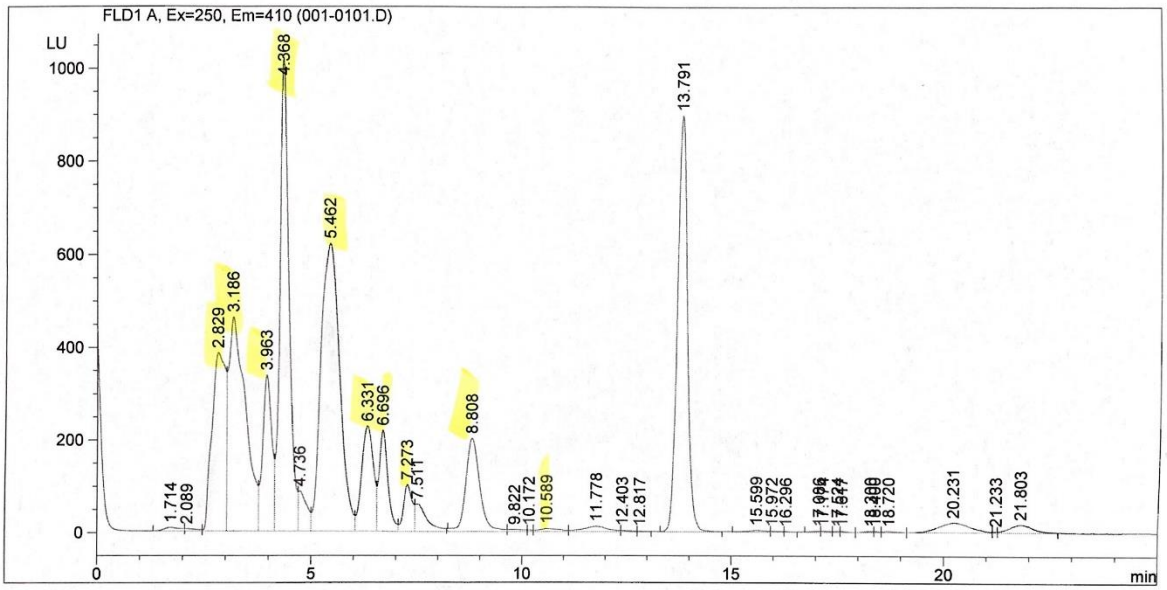

Figure 4.8. Chromatogram for AccQ Grad-10 method used on a sample of Oak Pond Brewing's Storyteller Dopplebock

4.6.1.4 Dual Columns

In addition to the single column elution, a dual column run of the AccQ Grad-10 run was also tested. Two Allure organic acids columns were connected in series and injected with a 1000 ug/mL sample of histamine. Figure 4.9. shows the chromatogram of the single column vs. the double column run. The overlapped peak at 9.896 min (single column) and 14.491 min (double column) is histamine. It appears that this method may be useful for resolving similar compounds as the second run had a much cleaner peak. This method wasn't tested further as excessive back pressure caused system failure.

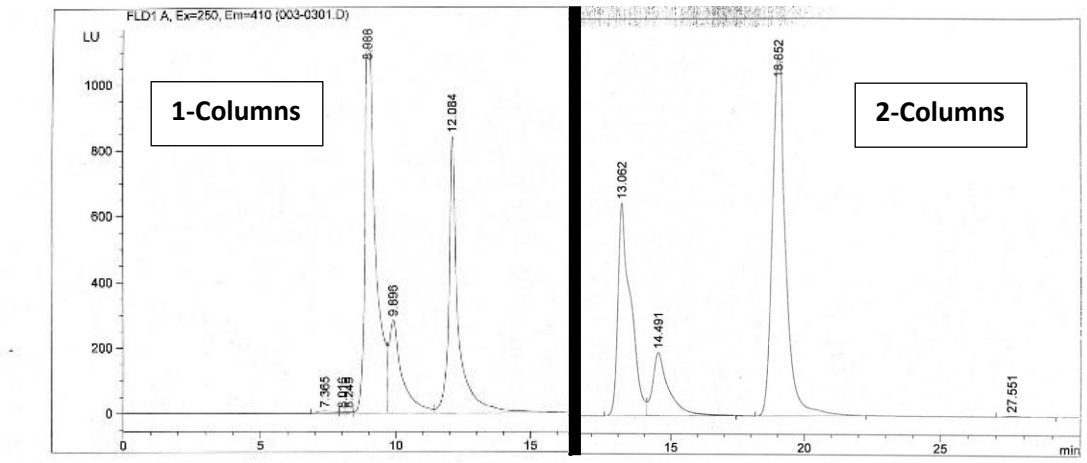

Figure 4.9. Single vs. Double column elution of tyramine using the AccQ Grad-10 method

4.6.1.4. Gradient Elution 13

An example chromatogram for this method is shown in Figure 4.10. The injected sample for this run was a 50 ug/mL injection of gamma-aminobutyric acid, histamine, tyramine, phenethylamine, putrescine, and cadaverine eluted with the AccQ Grad-13 protocol. Ideally there should be eight peaks present (six biogenic amines, the tagging dye, and an impurity peak) on this chromatogram and all expected peaks are resolved. At 8.652 min a GABA peak appears, followed by a small impurity peak at 9.378 min, histamine at 10.548 min, unreacted AccQ dye at 12.320 min, tyramine at 13.041 min, putrescine at 15.741 min cadaverine at 17.531 min, and phenethylamine 19.071 min. While there is some minor overlapping between tyramine and unreacted AccQ tag (though this is the best separation for those two compounds) and some peak tailing (especially between cadaverine and phenethylamine) this is by far the clearest and most successful separation of all tested methods. When this method was tested on BA-fortified beer samples the results were much less promising (Figure 4.11.). This run was poorly resolved, and the biogenic amines were not clearly resolved from small peptides and free amino acids found in the beer matrices.

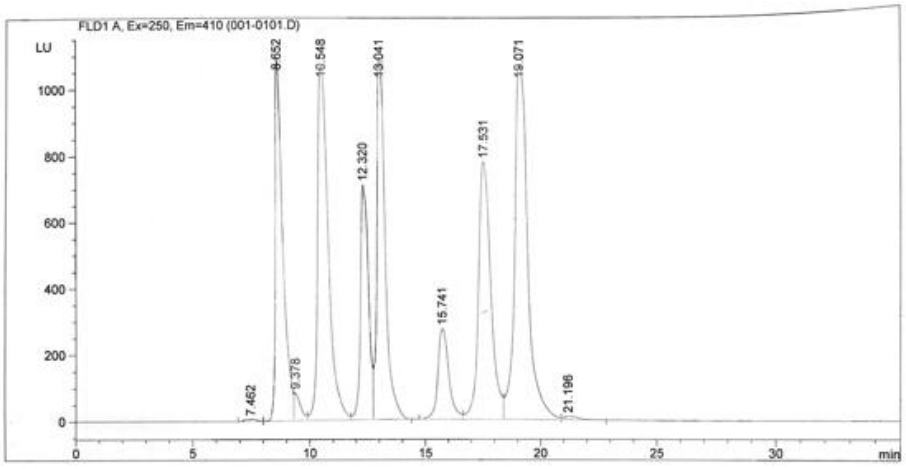

Figure 4.10. Chromatogram for AccQ Grad-13 method used on a mixed standard solution of histamine, GABA, tyramine, phenethylamine, putrescine, and cadaverine

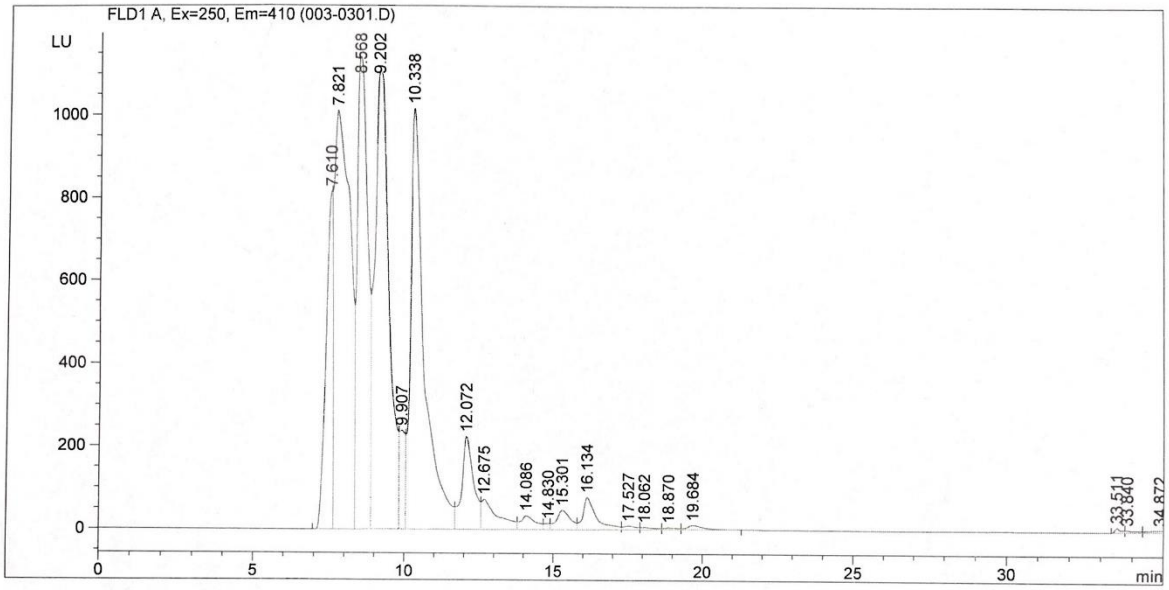

Figure 4.11. Chromatogram for a beer sample analyzed using the AccQ Grad-13 method

4.6.2. 2-Hydroxnaphthaldyhde HPLC Methods

4.6.2.1. Tag Consistency

The 2-HN derivatization was much less consistent and considerably more difficult to use compared to the Waters AccQ method. The tagging procedure often failed leaving samples completely unreacted. Another issue that occurred during the process of tagging samples was precipitate formation. After the heating step a white solid would often form in the vials. None of the published methods using this procedure reported this problem, but it is likely the result of the presence of an impurity.

4.6.2.2. Isocratic Elution

An example chromatogram for isocratic HPLC elution is shown in Figure 4.12. The injected sample for this run was a 50 ug/mL solution of cadaverine which was analyzed using a diode array detector set at 254nm, 230nm, and 330nm. Ideally, there should be two peaks present in the chromatograms (one biogenic amine, one for unreacted 2-HN) but in each there is only a single peak at 7.5 minutes. This single peak appears in every run and is likely the unreacted 2-Hydroxnaphthaldyhde. Due to time constraints, only a few runs were completed but combined with the tagging/derivatization problems, separation of biogenic amines in beer using 2-HN derivatization combined with various isocratic HPLC methodologies was unsuccessful.

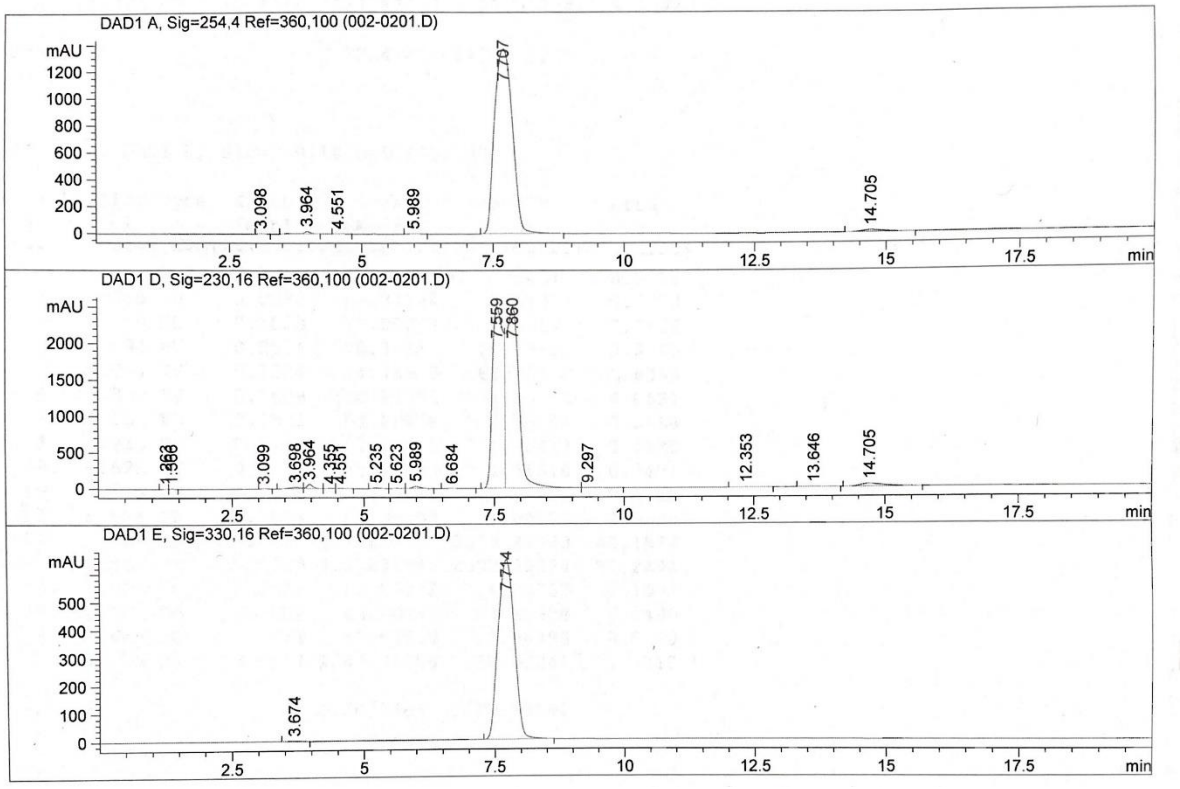

Figure 4.12. Chromatogram for the 2-HN Iso-1 method used on a sample of cadaverine

4.6.2.3. Gradient Elution

An example chromatogram for 2-HN derivatization with gradient HPLC elution scheme is shown in Figure 4.13. The injected analytical standard for this run was a 50 ug/mL solution of tyramine, which was analyzed using a diode array detector set at 254nm, 230nm, and 330nm. Ideally, there should be two peaks present in the chromatograms (one biogenic amine, one for unreacted 2-HN) which did absorb two of the three wavelengths examined. The peak at 7.079 min is the tyramine peak, while the larger eluting at approximately 10 min is likely the unreacted 2-Hydroxnaphthaldyhde. Due to time constraints, only a few runs were completed but combined with the tagging issues the results were very inconsistent (though seemingly more consistent than in the isocratic method). A beer sample fortified with tyramine was analyzed using this method (Figure 4.14.) was also unsuccessful.

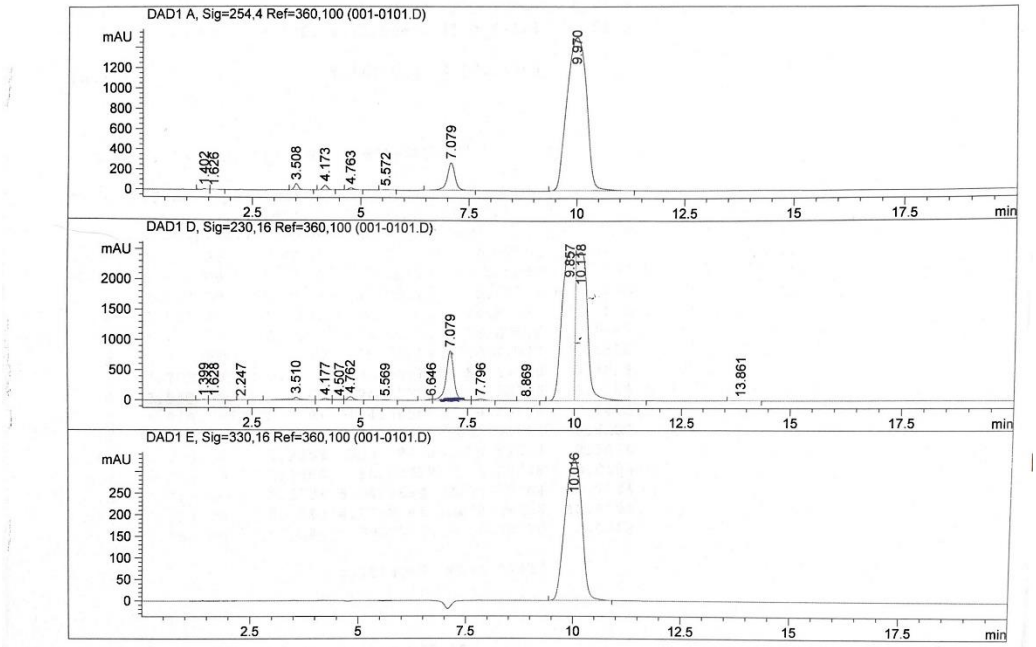

Figure 4.13. Chromatogram for the 2-HN Grad-1 method used on a sample of Tyramine

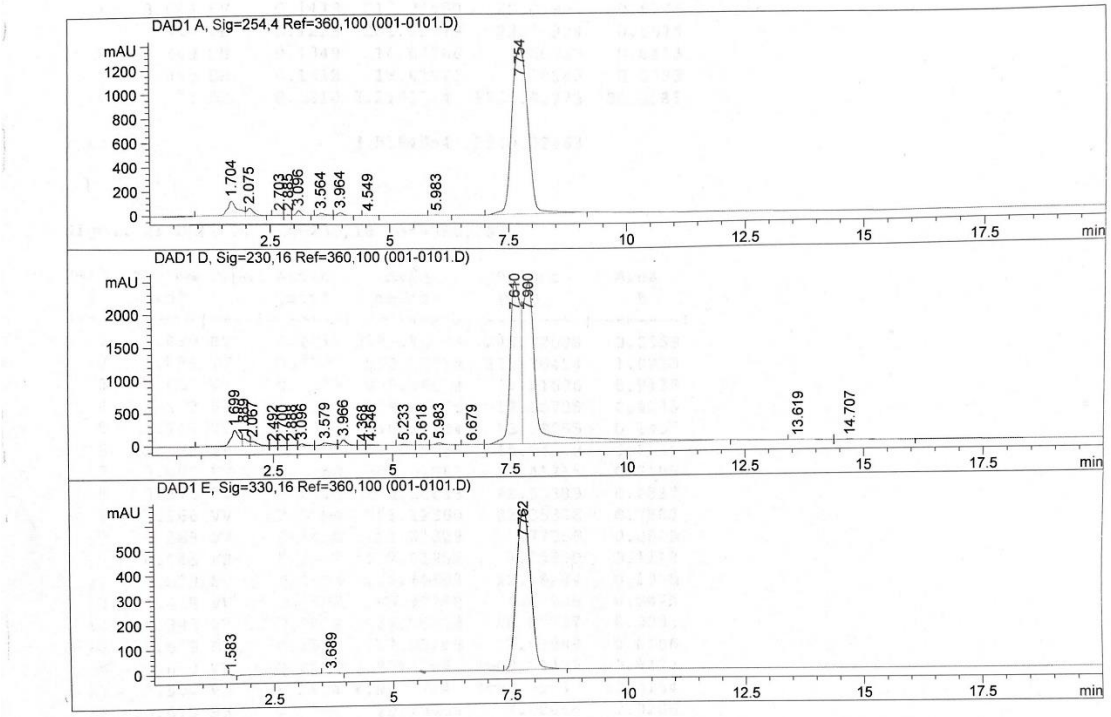

Figure 4.14. Chromatogram for the 2-HN Grad-1 method used on a sample of beer

4.6.3. Beer Extraction

4.6.3.1. Solid Phase Extraction

An example chromatogram for this solid phase extraction is shown in Figure 4.15. This chromatogram is for a beer sample (Oak Pond Brewing's Storyteller Dopplebock) that was treated with the previously discussed solid phase extraction method. Looking at this run it is quickly apparent that this extraction procedure is not effective for biogenic amine in beer. The three consecutive washes appear to remove every amine compound in the sample, including the amines that are being examined. The large peak at 12.839 min is the Waters AccQ tag which is applied after the extraction and thus unaffected by the extraction. The second peak at 14.990 min is not currently known as it doesn't directly correspond to a known peak and could be any peptide, amino acid, or amine present in the drink.

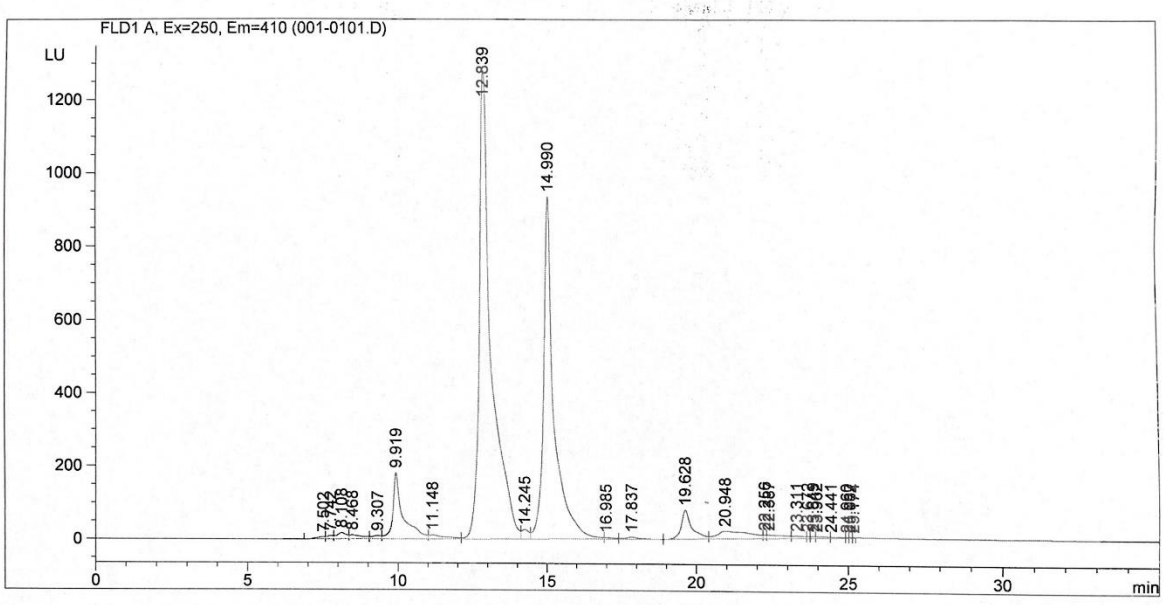

Figure 4.15. Chromatogram for a sample of beer after solid phase extraction and analyzed using the AccQ Grad-13 procedure

4.6.3.2. Salting-out Assisted Liquid/Liquid Extraction (SALLE)

An example chromatogram for the SALLE extraction method is shown in Figure 4.16. This chromatogram is generated from a mixed biogenic amine standard solution consisting of gamma-aminobutyric acid, histamine, tyramine, putrescine, cadaverine, and phenethylamine. This sample (pre-extraction) is identical to the one used in Figure 4.6. Looking at this method, it appears as that is could be used for beer as each biogenic amine is still present after extraction. Unfortunately, this method clearly doesn't extract all biogenic amines equally, due to variations in the polarity of each amine. More polar, water soluble amines like histamine and GABA aren't extracted well as they are soluble within the aqueous layer while the more nonpolar amines like phenethylamine are almost completely extracted into the acetonitrile phase. Another problem associated with this method relates to the acetonitrile used in the extraction. In the non-SALLE runs the injected sample is either an aqueous solution (standards) or a mixture of ethanol and water (beer) which have peaks in consistent locations, but the SALLE samples are dissolved in less-polar acetonitrile. Acetonitrile has a different compatibility with the mobile phase and the acetonitrile-dissolved samples elute at different times, making peak identification difficult.

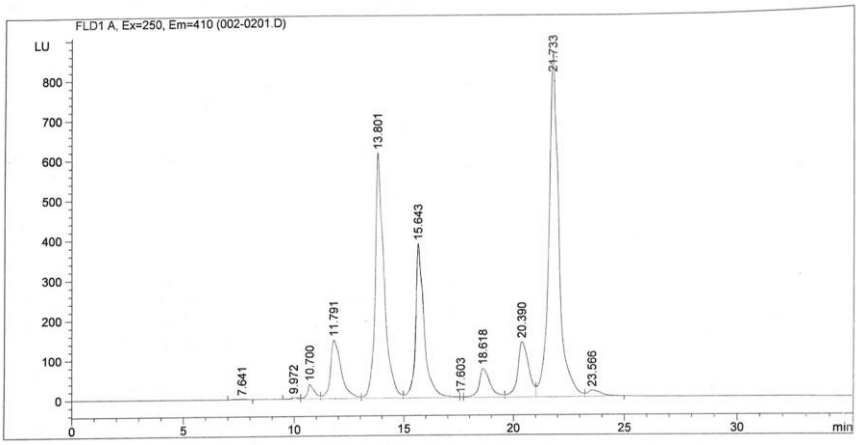

Figure 4.16. Chromatogram for a sample of beer after salting-out assisted liquid/liquid extraction and analyzed using the AccQ Grad-13 procedure

4.7. Conclusions

While the development of an effective method for separating and analyzing biogenic amine in beer was not successfully completed, of the tested methods, the simple sample preparation technique using the Waters AccQ tag kit, combined with the "Gradient Elution 13" method appeared to be the best option. The Waters AccQ tagging procedure was by far the most consistent and effective method for tagging amine-containing compounds. Additionally, the only preparation method tested that resolved a standard solution mixture of the tested biogenic amines was the Waters AccQ tag. The 2-HN derivatization procedure results were disappointing and inconsistent, deemed ineffective when compared to the Waters AccQ tag.

Moving forward, while expensive, the amine-specific concept of the Waters AccQ has great potential. Waters AccQ is used regularly for amino acid analysis and once combined with the proper HPLC separation technique, should be useful for biogenic amine analysis in beer and other complex food matrices. While 2-HN derivatization does have the advantage of being considerable cheaper (about 1/20 the cost of the Waters tag) it is much more time-consuming and challenging to work with. Only a handful of papers have been published using this method so there is very little practical advice or information to draw from. Coupled with the challenges mentioned previously, this is a much more difficult method to utilize successfully.

The Allure Organic Acids column was the only tested column resulting in any reasonable separation of the derivatized amines. Using a number of mobile phases combinations and conditions, the Waters Amino Acid Analysis column was unable to successfully resolve the targeted derivatized amines, and other columns with unique phases, such as the Hypersil Gold, were also unsuccessfully trialed.

The Allure Organic Acids column was effective and consistent as a single column but also showed some promise when used in series. Chaining two Allure columns in sequence resulted in better resolution for certain biogenic amines, but this option was ultimately discarded as impractical.

While the Allure Organic Acid column was superior to the other columns tested, there are a number of other column chemistries that were not tried. There are many different column chemistries, brands, and sizes with potential for successful resolution for the biogenic amines. Typical of most published HPLC methods, there is little consensus among researchers on the "correct" column to use for biogenic amine analysis, with nearly every paper describing a different reverse-phase column. Columns described in literature include Nova-Pak C18 (Jinjie Zhang et al. 2011), GL Sciences Alkyl columns (Yeh and Chen 2011), and C18 Waters Reverse columns (Arrieta and Prats-Moya 2012).

While there are many different extractions and separation techniques for BA-rich food matrices described in the literature, of the two tested (salting-out assisted liquid/liquid extraction and solid phase extraction) for our work with beer, only salting-out assisted liquid/liquid extraction (SALLE) was effective. The SALLE method enabled the extraction of most amines far better than the solid phase extraction method tested. The major advantage to SALLE was its effectiveness for extracting some nonpolar or hydrophobic biogenic amines (tryptamine, phenethylamine, etc.) with very little loss. A major drawback with this method was the low recovery of hydrophilic biogenic amines (GABA, histamine, etc.) from beer matrices. The solid phase extraction methods trialed were not effective as most targeted compounds did not bind to the solid phase and eluted from the SPE cartridge while loading the sample.

4.8. Future Work

Pursing the further development of an HPLC method, sample extraction may be the most important area for further research. At first look beer seems like a simple food product to analyze for biogenic amines, as it is a (usually) highly filtered liquid with extremely low levels of fats, but beer presents some difficult challenges. Most analytical-focused biogenic amine research food has been done with solid foods like cheese, sausages, fish, and vegetables. These extraction techniques won't work on a liquid food product and the only usable published methods to investigate and modify were performed with wine. Wine methodologies can be useful, but the chemical makeup of beer presents a unique problem: higher levels of proteins and free amino acids. During the brewing process there are several stages (boiling, whirlpool/centrifuge, cold crashing, filtering) that coagulate and separate protein from the final product, but these processing steps do not remove all potentially interfering compounds. Remaining after these processing stages are highly soluble foaming proteins, tri and di peptides, and amino acids (along with the biogenic amine). Normal coagulation and separation methods (acidifying/basifying, cold crashing, salting, heating, centrifuging, etc.) won't remove these proteins from a finished beer sample. Finding an effective extraction method is key for analyzing beer samples. SALLE appears to be a relatively effective extraction method, but only works for a few hydrophobic amines. Due to the highly varied chemical properties of biogenic amines it may be necessary to utilize multiple extractions in parallel or in series to extract the hydrophobic and hydrophilic amines

In addition to the suggested HPLC method development and modification, there are gas chromatographic – mass spectrometer (GC/MS) techniques that may also be employed to successfully detected and quantify biogenic amines in a variety of beer styles. A GC/MS method developed by the Phenomenex company called the "EZfaast" system shows a lot of promise for analyzing biogenic amines in beer. This technique was developed to analyze amino acid hydrolysates extracted from complex matrices (body fluids, animal feed, wine, etc.) that would have poor resolution when analyzed with

HPLC. Phenomenex claims that this system can give “full resolution of 50 amino acids and related compounds in a 7-minute GC run” (Badawy, Morgan, and Turner 2008) . GC capillary columns offers far greater resolution compared to HPLC packed columns, which was a consistent problem experienced during this HPLC-BA research project. The EZfaast method can resolve large numbers of amine compounds from complex matrices without the need for extraction or sample cleaning and full sample prep for analysis is achieved in 8 minutes. While there is little published literature regarding the use of the EZfaast system and beer, it could be a solution to quickly resolve, identify, and quantify biogenic amines and other amino acids in a number of finished beer styles.

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