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A Chemical and Historical Analysis of Beer: Discovering Brewing Styles and Beer Stages

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ABSTRACT

This interdisciplinary project is designed to explore both the compositional qualities of beer during the brewing process and its impact on society from a cultural, economic, and social viewpoint. Comparing various styles of beer against each other in a historical, societal, and chemical lens allows for a deeper understanding of what creates a beer's identity, and what makes it different from other styles. Here we analyzed two different styles of beer, a bock lager and a saison ale, in order to determine their chemical composition through their developmental stages to their final product. Based on previously published research and extended laboratory testing, methods were designed to run Nuclear Magnetic Resonance Spectroscopy on an array of beer samples in order to identify compounds present. This analysis reveals variations in chemical compositions between saisons and bocks and relates them to stereotypical flavor, body, and aroma profiles in order to get a complete understanding of their impact on the beer. Additionally, differences were seen between a beer's boil and final product, resulting in a better understanding of beer's development. Compounds in each style were identified by reference to established research results, creating evidence-backed assumptions of what each NMR peak represents. Since results from these findings are limited, the compounds possible to identify are therefore limited as well. Carbon NMR baselines were established by use of HSQC, a potential tool in future beer analysis. Humulones were discovered to be a major factor in a beer's identity, a deep historical analysis as well as extraction methods were conducted in order to analyze this. Connecting this chemical analysis to each style's history as well as beer's history as a whole provides a consumer with a deeper knowledge about the product they are consuming and why it is the way it is.

INTRODUCTION

Beer is a broad term for what is a process, a product, and an experience. Beer comes in multiple styles, flavors, colors, aromatics, and so much more. Beyond the actual product, beer has a dense cultural, economic, and social history. With the rise of American microbreweries over the last 30 years it seems as if new styles of beer are emerging every day, and to an extent they are. Chemical analysis of these beers is crucial for customers to understand what they are drinking and how each beer they drink differs from the next. For instance, if a certain style is known for its bitterness, and an intense presence of humulones are recorded in a sample of this style, then we can hypothesize a connection. This will formulate a deeper understanding of how beer styles emerged and diverged over time, as well as giving beer drinkers insight on the social resonance of beer styles.

To date, limited, chemical research has been published that analyzes styles of beer throughout their development. This could be due to major brewing companies not wanting to release their results, microbreweries lacking the funds for the proper equipment needed for research, or a perceived lack of need, since the basic process has worked for approximately 9,000 years. This project aims to refine analytical methods and in part illuminate the question of stylistic development for beer. Analysis of the chemical compounds present in beer intends to reveal why it develops the way it does, how styles compare to each other, and offer methods and analysis procedures for future beer scientific studies. Identifications made are well informed assumptions based on previous research from external groups. Even if the peaks correspond perfectly, without using a standard to confirm the tests, the identifications cannot be completely confirmed.

The modern world of beer consists of steel fermenters and glycol regulated temperature systems. But, beer has a long and complex history, dating perhaps back to ca. 10,000 B.C.[1] The oldest brewery so far discovered is thought to have been between 4000 and 3100 B.C. in Hierakonpolis, Egypt.[2] In this brewery, clay pots facilitated the brewing process: grains submerged in water were heated, and yeast was eventually added in.[3]

Over time brewing culture expanded, and each area developed their own rules and brewing techniques. One of the most influential decisions made in brewing history occurred in 16th century Germany, when it was established that bottom fermented beers should be produced only with the use of barley malt (top-fermented beers were allowed to use wheat malt and beet-cane or invert sugar), hops, yeast, and water. This verdict was known as the Bavarian Purity Law of 1516.[4] This law aimed to keep beer pure, so as not to introduce additional, potentially harmful, additives into beer. The law still serves today as the baseline for many brews, highlighting essential materials for brewing beer.

Brewers make beer for commercial consumption, for social and ritual purposes, to make something they are passionate about, and to enjoy intoxication. While these reasons were also relevant to brewing beer in ancient times, beer also had additional benefits. Historically, beer was a useful, safe, and relatively inexpensive nutritional source, providing vitamin B, riboflavin, and other nutrients.[5] Interestingly, Viking culture used beer's intoxication effects to benefit them in war. They believed that drinking beer would provide them with a courage that would benefit

[1] Smith, Beer: A Global History (Reaktion Books Ltd, 2014), 3
[2] Farag, Revealing the Constituents of Egypt's Oldest Beer Using Infrared and Mass Spectroscopy (Scientific Reports, 2019), 1
[3] Farag, Revealing the Constituents of Egypt's Oldest Beer Using Infrared and Mass Spectroscopy, 1
[4] Narziss, The German Beer Law (Journal of The Institute of Brewing, 1984), 1
[5] Raihofer, Zarnow, Gastl, Hutzler, A Short History of Beer Brewing (Science & Society, 2022), 1

them in battle.[6] Vikings also used the beer as an asset for camaraderie and celebrations after successful battles.[7]

However, a key ingredient of modern beers was not present in these "ancient" styles of beer: hops. Hops, which produce the humulones studied in this research, were not introduced into European beer until the 9th century, when they were implemented in brewing processes in Germany, Hungary, and Austria.[8] It took until the 12th and 13th centuries, however, for hops to become a staple of the brewing process.[9] Hops were introduced into beer not only to provide a bitter flavor profile, but to act as a natural preservative as well.[10] Increasing beer's drinkable lifespan allows for various economic and social applications to occur.

From an economic standpoint, adding preservatives to beer became essential to its commercialization. Originally spreading throughout Germany, hopped beers became a heavily traded item across Europe as early as the 15th century, spreading to Holland and England.[11] Hopped beers were not only limited to Europe, however; their expansion overseas during the 16th century introduced what is now widely considered as the quintessential hopped beer: the IPA (India Pale Ale).[12] George Hodgson, a London-based brewer at Hodgson Brewery, is credited with bringing the IPA to prominence. During the 17th century, Britain had control over India, and Hodgson Brewery was located adjacent to the ports that shipped to India, so he used his close connection to Britain's cargo boat captains to ship his hoppy October Ale to India.[13] While other Hodgson brews were also shipped, his October Ale proved the most popular because

[6] Smith, Beer: A Global History, 6
[7] Smith, Beer: A Global History, 6
[8] De Salle, A Natural History of Beer (Reaktion Books Ltd, 2014), 113
[9] De Salle, A Natural History of Beer
[10] Sethi, Wacky, Wonderful, Wild Hops Could Transform the Watered-Down Beer Industry (2016)
[11] Madsen, Gammelgard, Hobdari, New Developments In the Brewing Industry: The Role of Institution and Ownership (Oxford University Press, 2020), 75
[12] Madsen, Gammelgard, Hobdari, New Developments In the Brewing Industry: The Role of Institution and Ownership, 77
[13] Brown, Hodgson, George (The Oxford Companion to Beer)

its flavor profile was maintained as a result of its added hops. These hops allowed the beer to survive the journey from Britain to India and shaped a new style of beer being created. Other brewing companies noticed its commercial and preservative success and began to brew their own variant of this style, giving rise to the IPA.[14]

Despite the seemingly great economic benefits of hopped beers, major inconveniences resulted. Brewery numbers in both Germany and Holland decreased, as the need for local beer diminished.[15] In response, protectionism led to multiple bans across England, Holland, and Germany, prohibiting the import and export of hopped beers.[16] Doing this aimed to increase the local brewing markets and support local hop farms. During this time, the German Purity Law (stated above) came into effect, strictly controlling what is allowed in beer.

As discussed previously, beer has been around for a very long time. Much before hopped beer, in the ancient city Sumer – located in present day Iraq–, an ancient hymn known as "A Hymn to Ninkasi", written more than 5,000 years ago,. describes how Sumerians used beer to achieve enjoyable intoxication, and describes how beer was a social tool that brought communities together.[17] The Hymn is a recipe for Ninkasi's beer, and since Ninkasi was the Sumerian goddess of beer, the beer held great social and religious significance to the Sumerian people.[18] Ninkasi's beer was commonly shared in communal fermenting jugs which brought people from all social classes together.[19] Being distributed in communal jugs meant that people

[14] Madsen, Gammelgard, Hobdari, New Developments In the Brewing Industry: The Role of Institution and Ownership 77
[15] Madsen, Gammelgard, Hobdari, New Developments In the Brewing Industry: The Role of Institution and Ownership 75-76
[16] Madsen, Gammelgard, Hobdari, New Developments In the Brewing Industry: The Role of Institution and Ownership 76
[17] Smith, Beer: A Global History, 4
[18] De Salle, A Natural History of Beer, 17
[19] De Salle, A Natural History of Beer, 19

who wanted to drink the beer were required to socially interact with others, starting the social nature of beer culture.

Beer-based social patterns seen in Sumer were also present in ancient Egypt. Not only was beer used in religious festivals and for worship, but also helped feed and bond labor crews. In Rob DeSalle and Ian Tattersall's book "A Natural History of Beer", they state that the Egyptian pyramids, specifically the pyramid dedicated to the fourth-dynasty pharaoh Menkaure built ca. 2,500 B.C.E, were built by a group of workers who called themselves "Drunkards of Menkaure".[20] These workers used beer as a method to endure working conditions as well as to come together and work as a team.

When hopped beer began to dominate the beer industry, these communal social patterns started to change. Hopped beer wasn't required to be drunk as quickly, taking away the need to drink the beer promptly in a communal setting. Separately, the popularity of beer itself created opportunities for people to independently brew beer and expand it commercially. Between the 11th and 16th century, northern-Europe began to change its beer patterns into that of commercial brewing. Brewing practices transferred from independent families in the countryside of Europe to towns.[21] Initially, landowners of different countryside estates began to commercialize the breweries on their estates, selling beer for a profit.[22] Then in the 12th century breweries began to expand into urban centers. Specialist brewers, who needed a large brewing premise, became present in different towns across northern-Europe.[23] In addition to its religious and social values, beer now held significant economic weight.

[20] De Salle, A Natural History of Beer, 20
[21] Unger, Beer in the Middle Ages and the Renaissance (University of Pennsylvania Press, 2004), 37-38
[22] Unger, Beer in the Middle Ages and the Renaissance, 39
[23] Unger, Beer in the Middle Ages and the Renaissance, 40

Beer never lost its social or religious aspect, they merely developed over time. It evolved from a beverage people drank due to its cheapness and cleanliness to a commercialized product with intentional flavors and detail-oriented production Even in the modern world, beer still brings people together. Whether at a bar, party, or bonfire, beer remains a dominant social tool in today's world, as it was in ancient times. The major change was, with the development of hopped beer there was no necessity for people to come together to drink beer. Yet its social aspect was retained, even though it could be drunk anytime, anywhere.

For each of these styles, knowing brewing's history and importance is an integral part of this thesis's topic. Understanding how beer is made and how its production methods have varied throughout history will allow for a better understanding of how these methods have changed throughout history, and why they have developed into the way they are today. Since modern day techniques are being studied in this experiment, realizing the similarities and differences it will also be known why the beer is being made the way it is, and what different flavors and aromas were being aimed for in this production.

Present day brewing is a detail-oriented multi step process. These major steps in chronological order are milling, mashing, boiling, fermenting, filtration, and the addition to the bright tank. Both milling and mashing deal with the malts (sugars) of the brewing process. In the milling stage grains are poured into a grain mill where they are crushed in order to expose fermentable sugars[24], whereas the input of the milled grain into boiling water is the mashing phase. Doing so allows for sugar levels to rise in the water. From here the grain infused water is brought to a boil again, this is the beginning of the boil phase. The boil phase acts as an opportunity to sterilize what is now a beer's wort, as well as add flavor enhancements to the beer such as hops, spices, fruit peels, chocolate flakes, and so much more. The product of this, the

[24] Unger, Beer in the Middle Ages and the Renaissance, 4

boil product, is the stage used throughout the study.[25] After the boil is completed, the beer is chilled and moved to a fermentation vessel (in modern breweries this is a steel tank) and yeast is added in order to begin the fermentation process.[26] The fermentation process spans anywhere from a week to over a month depending on the yeast strain being used. In this process yeast converts the sugars from the grain into primarily ethanol, however other byproducts are produced as well. These will be discussed below. After fermentation is complete, hop residues that form a sludge in the fermenter are filtered out of the product and the beer is transferred to a bright tank in order for it to be clarified, carbonated, and ultimately kegged, bottled, or canned.

Beers are generally split into two extremely broad categories, ales and lagers. Both of these subsections of beer vary in their developmental process, yeast, and other brewing components. The main difference between these styles of beer is the species of yeast that is used. Ales use Saccharomyces cerevisiae where lagers use Saccharomyces pastorianus. These different yeast species affect how the beer is fermented, and ultimately how it tastes and its chemical composition.[27] Ales are known for undergoing top fermentation, where the yeast conducts fermentation on the sugars from the mashing process and eventually rises to the top of the fermenter in a foam layer.[28] Using different strains of Saccharomyces cerevisiae is a major component to differentiating between the different sub-styles of ales. They each produce particular flavors, aromas, and colors.

Saisons fall under the category of an ale, whose distinguishable characteristics are determined below. They were traditionally brewed by farms in the countryside.[29] As a result of this, they were known to use local hops in their brewing process, creating varying flavor profiles.

[25] Unger, Beer in the Middle Ages and the Renaissance, 5
[26] Unger, Beer in the Middle Ages and the Renaissance,5-6
[27] SNBC, Ale vs. Lager (SNBC, 2021)
[28] SNBC, Ale vs. Lager
[29] Markowski, Farmhouse Ales: Culture and Craftsmanship in the Belgian Tradition (Brewers Publications, 2004), 1

This style was chosen for investigation due this connection with local hops, which show a rich history of passion and variation in the style. Additionally, analyzing a beer that originated from countryside, independent breweries instead of urban, commercialized breweries provides an opportunity to study a style of beer that represents brewing practices, flavors, and aroma of rural brewing.

When discussing lager fermentation, lagers use a bottom fermenting process. This process is carried out at a lower temperature range of 42-55° F, as opposed to ale's fermenting temperature of 60-75° F.[30] Lager fermentation is a slow process, resulting in the Saccharomyces pastorianus to sink to the bottom of the fermenter (bottom fermentation).[31] Having a slower fermentation process allows for styles –like a bock– to not have all of their sugars consumed during the fermentation process, resulting in more sugars in the final product of the beer and a more malty flavor profile. Additionally, this also creates fewer byproducts such as esters in the final product which usually results in a crisper beer.

Due to lagers brewing and component variation from ales, the lager family was additionally studied. When a person thinks of beer their head most likely goes to Budweiser, Miller Light, or Coors Light. These are all lagers. Modern U.S. society is dominated by this style of beer, so studying it was imperative. Lagers originated on the western edge of Bohemia and Nuremberg, however, when the Bavarian Purity Law of 1516 came into play, it naturally escalated in Bavaria, slowly becoming the traditional beer of that region.[32] In modern day, lagers are usually associated with a lighter more malty taste as opposed to the hop dominant style. Hops still play a major role in preserving the beer and making it what it is, it's just not the

[30] SNBC, Ale vs. Lager
[31] SNBC, Ale vs. Lager
[32] Dredge, A Brief History of Lager: 500 Years of the World's Favourite Beer (Kyle Books, 2019), 15-16

traditional focus piece of the beer. Due to its history, immense popularity, and vast differences from saisons, lagers were a perfect choice to study in this research.

Understanding the historical importance of beer today explains why we drink beer today, why it is important, and its social and cultural value. It shows that people don't drink beer just because it's fun or the popular thing to do, beer has a deep history and important values that relate to why we are drinking beer today. Additionally, understanding chemical differences is crucial in determining what actually makes these beers so different on a physical level. Studying two contrasting styles of beer during this experiment allowed for a wide range of styles to be covered which each have their own history, value, chemical composition, and developmental process.

For the chemical analysis of this project one main analysis technique was used, this is nuclear magnetic resonance spectroscopy (NMR). While different variations of NMR were utilized, and the beer prepared in various ways, NMR was the main analytical tool used in this paper. NMR is presented using an x-axis of parts per million (ppm) and a y-axis of abundance. High performance liquid chromatography (HPLC) was meant to be a critical part of this research paper, and a key factor in humulone analysis. However, after many HPLC runs, proper humulone results for beer were never obtained in a fashion that would prove useful for the analytical argument presented in this research. As a result of this, HPLC experimentation was removed from the research and replaced with a humulone extraction of each beer style. Most of the beer, or early developmental beer products, are products graciously provided by Lost Hollow Beer Company, Greencastle Indiana. Due to unfortunate circumstances, some samples had to be bought from the store. These are mentioned specifically below. Combining all of this, the

proper samples were able to be obtained and an analytical study was able to be conducted of their product in order for them to additionally learn about their product.

Results were obtained by use of DePauw University's JEOL 400 MHz NMR spectrometer. An NMR works by inserting a small sample (approximately 700 μL) into the NMR machine. Inside the NMR there is an extremely powerful magnet that creates a magnetic field which induces an energy difference between the spin states of specific atoms inside of the sample. The most commonly studied of these atoms are hydrogen and carbon 13. Once this energy gap is created, radio waves are pulsed into a sample and proton (hydrogen) and carbon NMR results are produced. Understanding how the readings are produced and what the various signals mean allows for the results to be interpreted and to eventually understand what compounds are present in the sample. For this project, previous research from various laboratories will be used as a baseline for results and interpretation of compounds in these results.

Besides the proton and carbon NMR, an additional method was used: HSQC. HSQC is a 2D-NMR experiment where proton and carbon NMRs are plotted against each other in order to identify compounds representing the same molecules. Being 2D means it has two axes representing frequency of the NMR, allowing two different NMR results to be compared against one another. This allows for ^{13}C NMR identifications to be made and utilized in additional research. ^{13}C NMR was not directly utilized in any of the papers found during the course of this study, so providing a baseline of what various peaks represent in ^{13}C NMR will ideally allow this method to be a useful way to analyze beer's chemical composition moving forward.

As for the humulone testing, this was done with the above-mentioned humulone extraction from various beer samples. Humulones are derived from the plant humulus lupulus

(also known as hops). When added to the boil, after the mashing stage in the brewing process a variety of flavors, aromas, and colors can be produced. There are many different variations of humulones that result in different properties in beer. Comparing different beers' humulone levels by extraction will allow for a deeper understanding of a vital flavor factor in beer, and to see how it ranges amongst different beers.

EXPERIMENTAL OUTLINE AND METHODOLOGY

In order to study the beer samples, proper preparation and analysis methods were established. Every sample was degassed using either an ultrasonic bath or manual shaking. The degassing took place until the carbonation left the beer or was nearly eliminated. In addition to the degassing, each beer was filtered using either a 25 mm syringe filter or via gravity filtration. This means that carbon dioxide is present in the final product samples but is intentionally eliminated in order to get clearer results. ^{1}H NMR and ^{13}C NMR were acquired using 128 and 256 scans, respectively, on the JEOL ECZS 400 MHz NMR machine. For proton NMR there were 0 dummy scans, the tip angle 45^{o}, the x offset was 5 ppm, the x sweep was 15 ppm, the proton collection mode was "Acq time", the points or acq time was 2 seconds, and the relaxation delay was 4 seconds. For carbon NMR, there were 4 dummy scans, the tip angle was 30^{o}, the sn ratio was 0, the x offset was 100 ppm, the x sweep was 350 ppm, the collection mode was "Acq time", the points or acq time was 1 seconds, the relaxation delay was 2 seconds, and the temperature was 25 dC. After preliminary tests were completed as described in the results section, 700 µL were added into the NMR. The results were analyzed against multiple different published articles to identify chemical compounds. While their procedures may have been completed with slight variations from ours due to limitations in the laboratory, results of this experiment and of published results appear similar. These research papers provide logical answers when determining NMR results.

In the 2D-NMR HSQC analysis, the ^{1}H NMR and ^{13}C NMR use the same procedure as individual testing except the specifications of the NMR tests. Here the ^{1}H NMR used 128 scans, 0 dummy scans, a tip angle of 45^{o}, x offset of 5 ppm, x sweep of 15ppm, collection mode of "Acq time", points or acq time of 2 seconds, and a relaxation delay of 4 seconds. The ^{13}C NMR

used 256 scans, x offset of 85 ppm, x sweep of 170 ppm, 4 dummy scans, tip angle of 30°, sn ratio of 0, collection mode of "Acq time", points or acq time of 1 seconds, and a relaxation delay of 2 seconds. For the actual HSQC scan, it was linear, with 2 scans, acq time of 0.2 seconds, y points at 128, relaxation delay of 1.5 seconds, temperature of 25 dC, and a temperature state of current. After these tests were run, if better spectra were required then the HSQC spectra would be replaced with a clearer and more revealing spectra.

HPLC methodology consisted of creating a mobile phase based on Sonja Schneider's research with Agilent Technologies.[33] This solution consisted of 0.276 g of NaH_2PO_4 in 100 mL of distilled water in order to get a 2 mM concentration. After this was obtained, concentrated phosphoric acid was added to the solution in order to bring it to a pH of 2.96, the solution was then mixed with 100 mL of methanol and stored until the solution ceased to bubble. The resulting mobile phase was a 50/50 mixture of the above mentioned solutions, this was attached to the machine and used throughout this experiment. Every beer solution was analyzed at a wavelength of 270 nm, and was pushed through a C_{18} HPLC column at a flow rate of 1.0 mL/min. This method proved unsuccessful in generating useful data for analysis.

In order to eliminate the dominating presence of ethanol and water in the NMR results, multiple drying methods were conducted. The first was the air-drying technique. This consists of a 1 mL sample of the filtered boil and final product beer being placed in a test tube and left out for 3 weeks to dry. The method of drying beer samples was shown in previous research experiments as well.[34] After the product is completely dried, 1 mL of D_2O is added in order to dissolve the remnants, and 700 µL of the solution was taken for NMR analysis. NMR needs a solution in order to be able to analyze compounds, so a sample of this residue was placed in

[33] Schneider, Agilent (Agilent Technologies, Waldbronn)
[34] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers (IOP Publishing Ltd, 2022), 7

chloroform in order to test its solubility.[35] Chloroform did not dissolve the substance, so another liquid, D_2O was used and was successful in dissolving the sample. The main concern with doing this method of drying is that something from the laboratory environment will contaminate the sample. Since the beer is left in the open, there is a possibility it could pick up chemicals or molecules form the air, which could then affect the chemical profile of the sample. Air-drying the beer resulted in a completely dried out residue remaining in the test tube.

The second drying technique used was lyophilization. In this, approximately 8 mL of beer sample was placed inside a round bottom flask and attached to the lyophilizer. During the lyophilization process, the beer samples are placed in a beaker and then attached to the lyophilizer, where they are in an airtight environment. The samples are then freeze dried for an extensive amount of time. Samples were taken off when determined completely dry. 1 mL D_2O was used to dissolve the approximately 0.01 grams of dried beer sample The goal of this process is to use a more controlled environment to eliminate water and ethanol from beer samples in order for cleaner and more extensive NMR results.

When analyzing humulones in the beer using NMR a chemical extraction was necessary. Referencing Qiuying Zhang's work at the University of Waterloo, it was determined that combining 3 mL of beer sample with 0.3 mL of 3M HCL, 6 mL of isooctane, and one drop of octanol being added to a falcon tube could properly extract humulones and iso-humulones from a beer sample.[36] Each solution was then centrifuged until there was a clear separation between the beer sample, emulsion, and clear liquid on top.[37] The clear liquid on top was then extracted, put into a small round bottom flask, and attached to a vacuum until as much isooctane as possible

[35] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7
[36] Zhang, Characterizing Humulone Content in Beer Using Differential Mobility Spectrometry (University of Waterloo, 2018), 48
[37] Zhang, Characterizing Humulone Content in Beer Using Differential Mobility Spectrometry, 48

was removed. Leaving -ideally- only humulones and isohumulones in the flask. From here, chloroform-D was added to the flask in order to pull the humulones and isohumulones into solution. This was then run on NMR as described above. In order to prove that these results were actually humulones, they were compared against an ICS - I4 Iso Standard. This is an iso-humulone standard sample, derived from isomerized α-acids of hops, it includes major ISO-α-acids such as trans-isocohumulone, trans-isohumulone, and trans-isoadhumulone.[38] This standard was made into an extremely concentrated solution: .03076 grams of iso-humulone standard in 1 mL of chloroform-D. Once this standard solution was created, a ^{1}H NMR run was performed.

[38] American Society of Brewing Chemists

RESULTS & DISCUSSION

Initial testing was done in order to perfect the experimental methodology explained above. Below are the preliminary testing results. For this reason, the different signals are not identified. Early tests were conducted using 630 µL of Lost Hollow Beer Company's Stone Skipper Saison with 70 µL of D_2O. D_2O is a solvent used to stabilize the magnetic field and lock it into place in the produced results[39]. Additionally, a 700 µL sample of pure beer was analyzed against the previous solution in order to detect if there were differences in their results. These ^{13}C NMR results can be seen below.

[39] Almeida, Duarte, Barros, Rodrigues, Spraul, Gil, Composition of Beer by1H NMR Spectroscopy: Effects of Brewing Site and Date of Production (Food Chem, 2006), 2

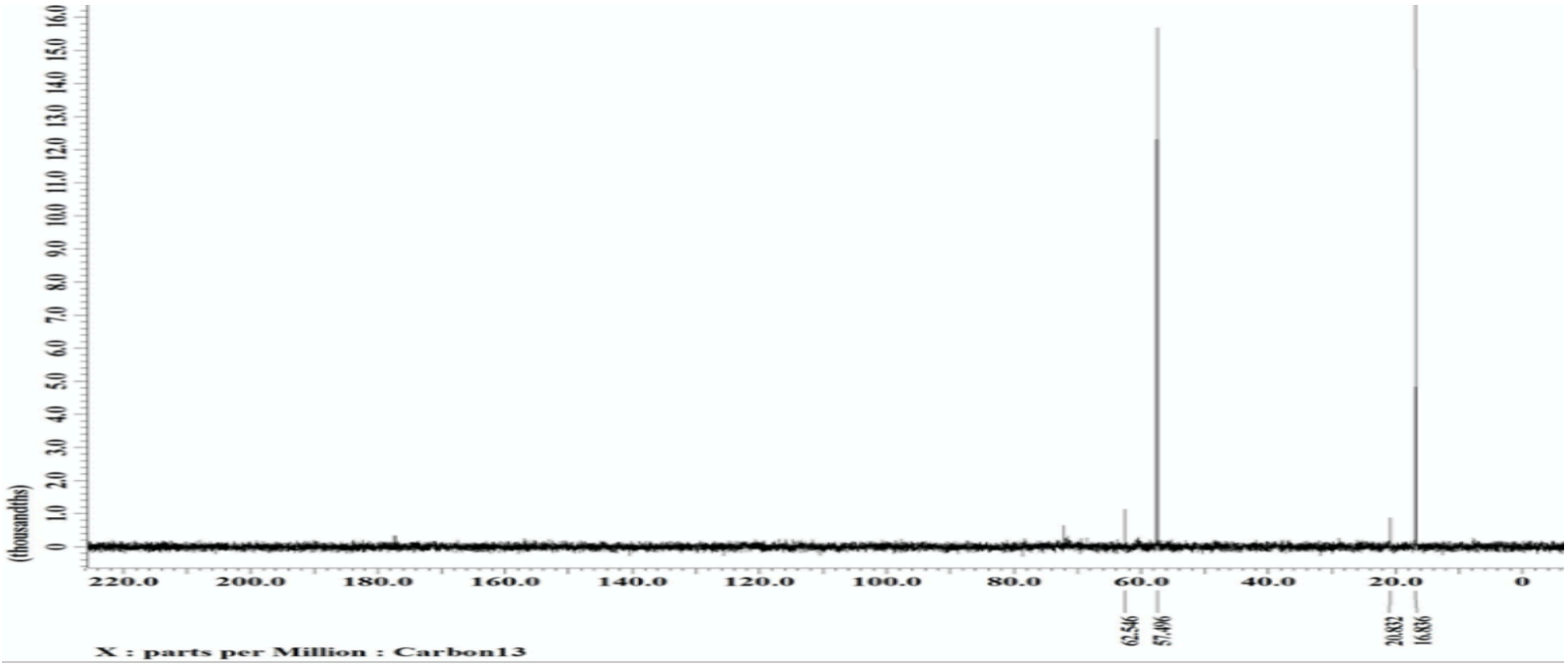

Figure 1: ^{13}C NMR of the Stone Skipper Saison mixed with D_2O in a 630:70 µL ratio. This carbon NMR was completed with 128 scans.

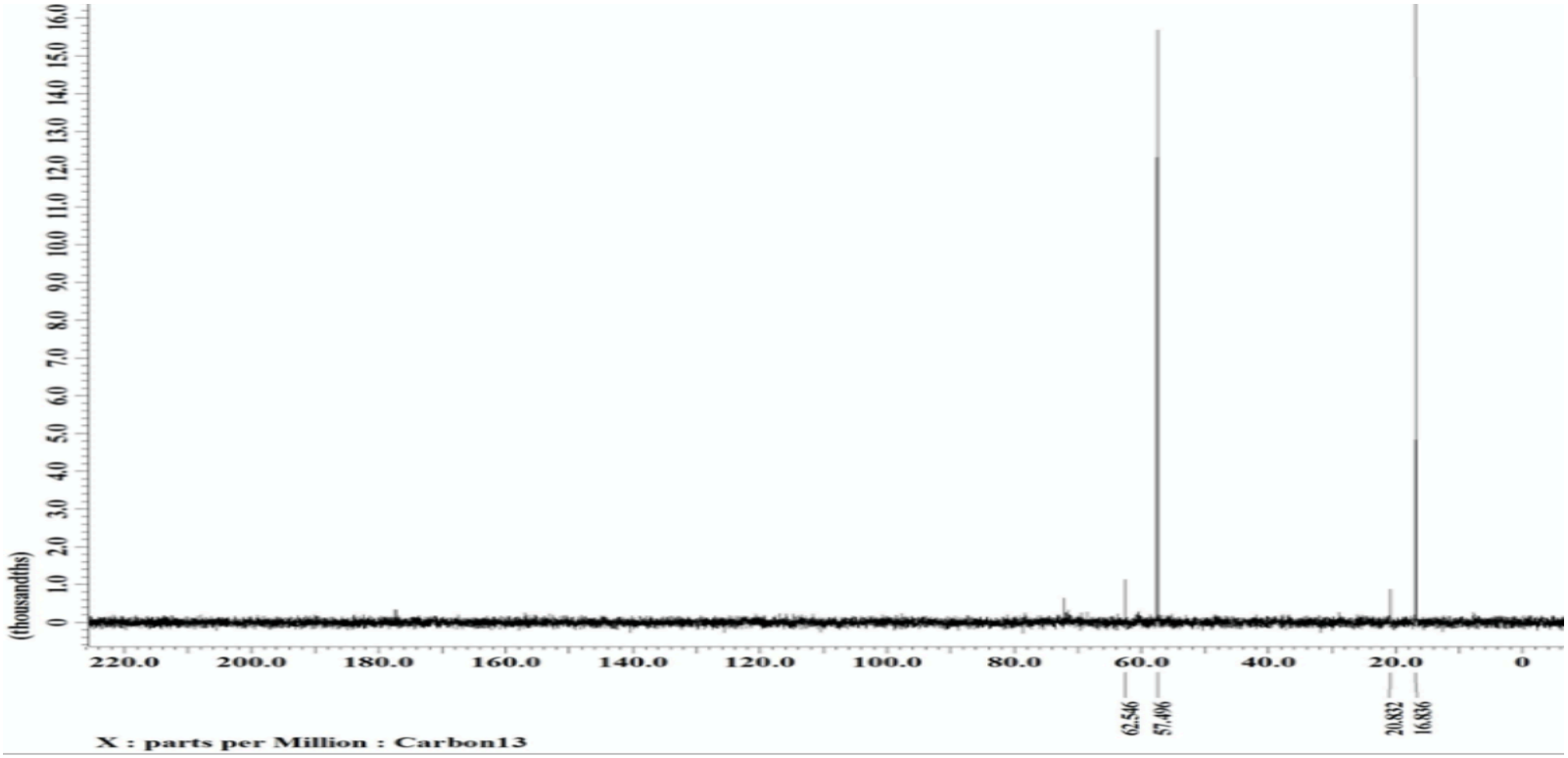

Figure 2: ^{13}C NMR of 700 µL of straight Stone Skipper Saison sample. This carbon NMR was completed with 128 scans.

The results shown in the graph above did not highlight anything significant. The spectra shown are dominated by the ethanol groups in the beer. As a result of these spectra being so similar, the number of scans done of the solution was increased from 128 to 1024. This did not result in any more significant results being shown. Due to a lack of literature research providing ^{13}C NMR, identification using this NMR method proved difficult, however, in the experimental section of each beer, the NMR analysis method of HSQC was used to identify compounds in the ^{13}C NMR spectra produced. ^{1}H NMR results proved to be a useful analytical tool as ^{1}H NMR experiments are more common in published research papers dealing with beer analysis. The preliminary testing of ^{1}H NMR methodology resulted in the figures below.

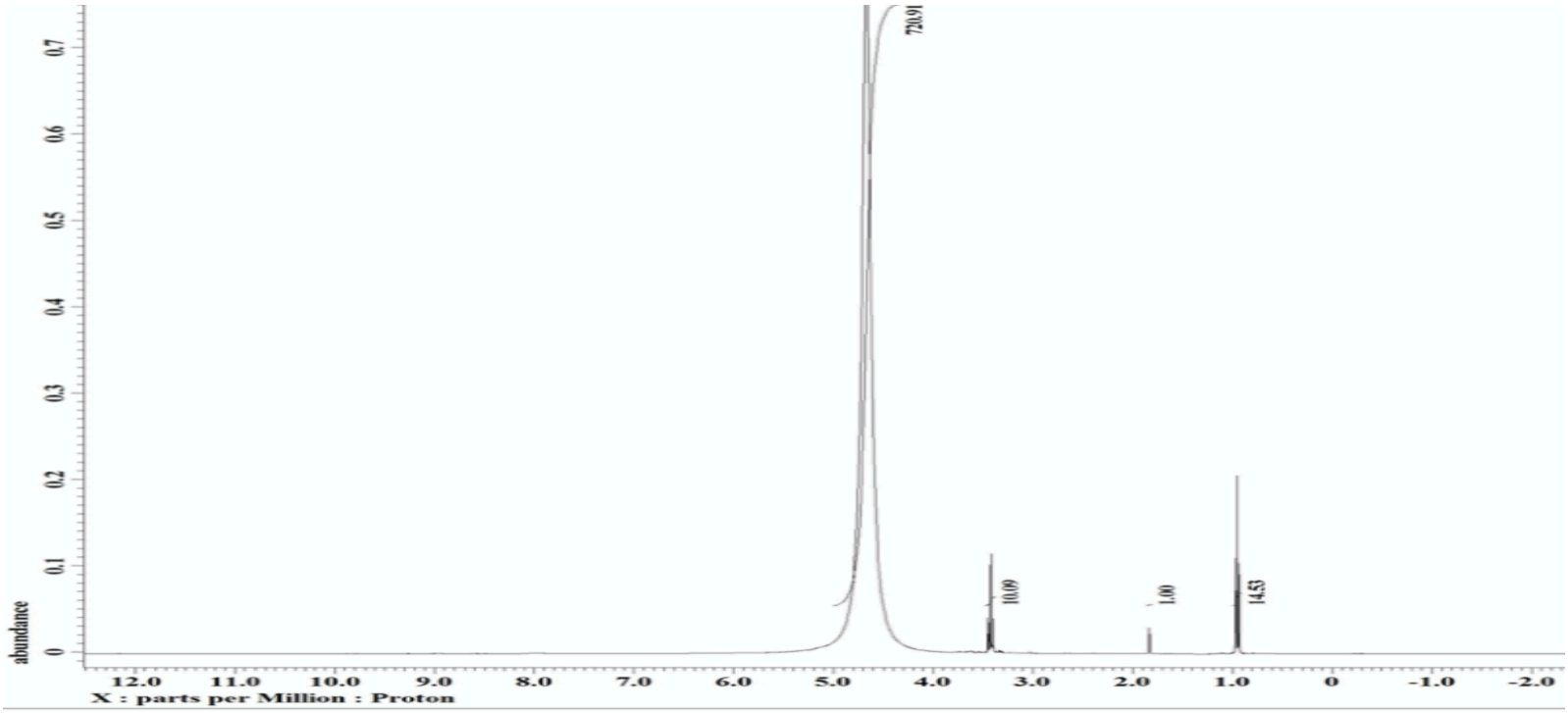

Figure 3: Proton NMR sample of Stone Skipper Saison mixed with D_2O in a 630:70 µL ratio. This proton NMR was conducted with 8 scans.

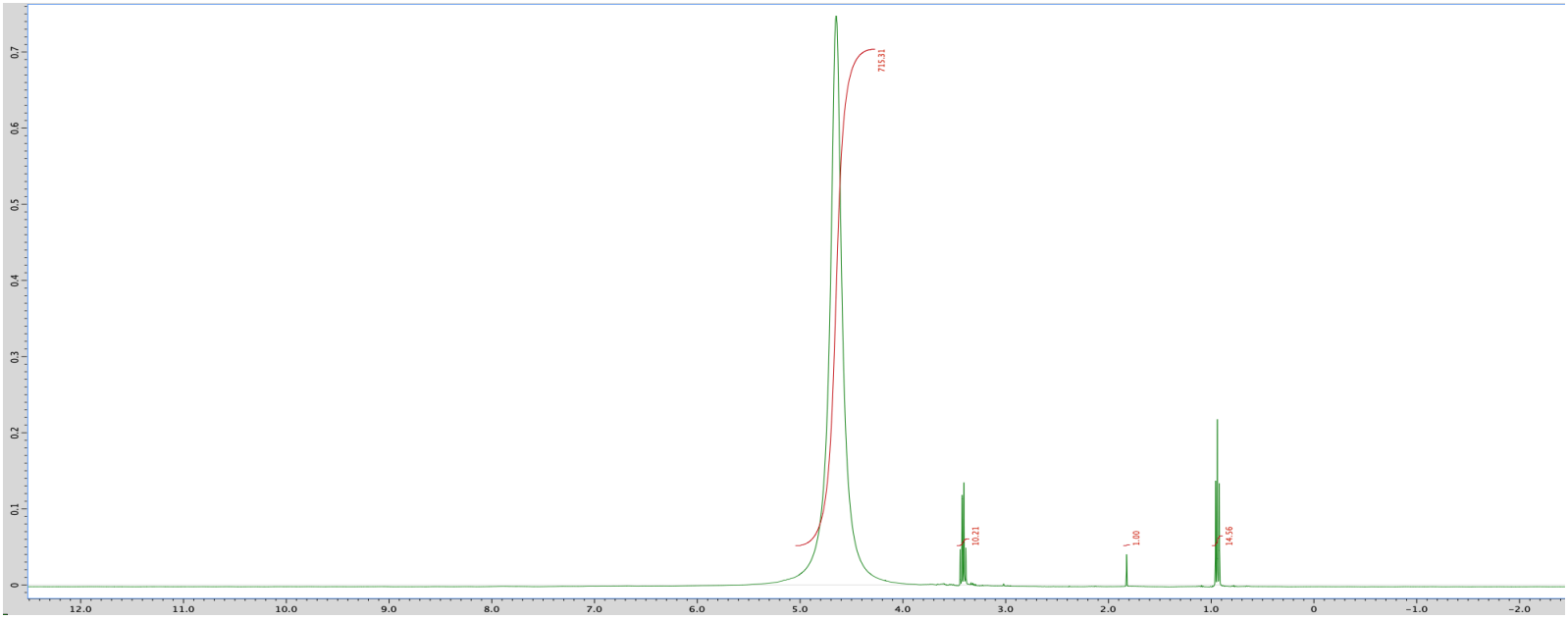

Figure 4: Proton NMR sample of the straight Stone Skipper Saison sample. This proton NMR was conducted with 8 scans.

From Figures 3 and 4 it can be seen that the results are nearly identical to each other. The peaks appear at the same ppm along the x-axis and the peak shape (splitting) looks identical to one another. As a result of this, and the same trend being present in the carbon NMR. It was determined that adding D_2O had no impact on our solution's spectra, as a result of this it was eliminated from use for further testing. However, increasing the number of scans for the proton NMR from 8 to 128 did show improved results.

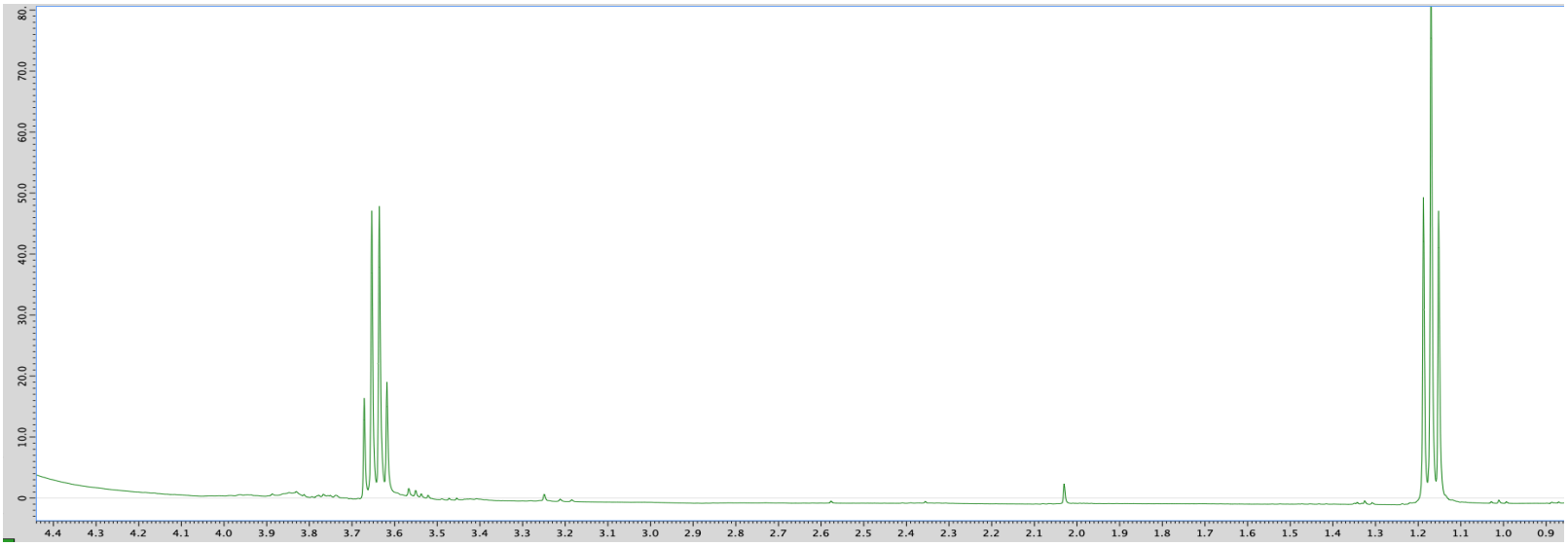

Figure 5: Proton NMR sample of straight sample of Stone Skipper Saison. This figure was produced by 128 scans of proton NMR.

When comparing the ^{1}H NMR with 8 scans to the ^{1}H NMR with 128 scans there does not seem to be a dominant difference. As a result of this, 128 proton scans are the value that will be used throughout this research project. Using 128 scans provides a better signal to noise output and will better indicate what compounds are present in each solution.

SAISON

The first style of beer used in this experiment is an ale. Specifically, the Stone Skipper Saison, a 4.8% ABV (percent alcohol by volume) saison brewed at Lost Hollow Beer Company.[40] This style was chosen for this experiment as it is a traditional style, with a dense history and specialized characteristics. A saison falls under the broad category of farmhouse ales. Saisons originate from Wallonia, Belgium where they were first brewed and the style became quite popular.[41] Typically, saisons were brewed during the winter months, in order to provide farmers with a light, nutritious, and replenishing beer for their work in the fields come spring and summer.[42] What makes saisons such an interesting beer to study is the fact that saisons, and farmhouse ales in general, were typically produced by farmers, as the name suggests. Breweries as we know them today were not a thing throughout history, so sometimes the only way to make beer was for farmers to make it themselves. Farmers used their own grains, yeast, sugars, and spices in order to brew beer for their farm.[43] As a result of this, farmhouse ales were a source of pride for the farm and represented the farm.

In order to produce a beer with a low alcohol percentage and refreshing taste that had the potential to be consumed at a rate of approximately 10.5 pints per day in the Middle Ages[44], specific techniques and ingredients needed to be used. As mentioned above, this style of beer was produced by farmers, using their resources, so the ingredients and exact chemical composition of every saison typically had some variance.

[40] Lost Hollow Beer Company (2024)
[41] Markowski, Farmhouse Ales: Culture and Craftsmanship in the Belgian Tradition, 1
[42] Markowski, Farmhouse Ales: Culture and Craftsmanship in the Belgian Tradition, 1 & approximately 21-23
[43] Garshol, Historical Brewing Techniques: The Lost Art of the Farmhouse Brewing (Brewers Association 2020), 7-8
[44] Markowski, Farmhouse Ales: Culture and Craftsmanship in the Belgian Tradition, 21

The previous information is crucial to understanding the importance of the beer, and its composition. Knowing this provides information about what we should generally expect to see in the chemical results as well as for what purpose was this style of beer chosen. For this saison the boil product and fully fermented beer is analyzed in NMR using the previously outlined techniques.

The first chronological stage in beer's development that we are studying is the boil product. A sample of the Stone Skipper Saison's boil product was taken directly from the boil kettle and stored in a sealed and refrigerated mason jar until analysis. The results of the analysis can be found below.

128 Scan ^{1}H NMR of the Stone Skipper Saison's Boil Product and Final Product.

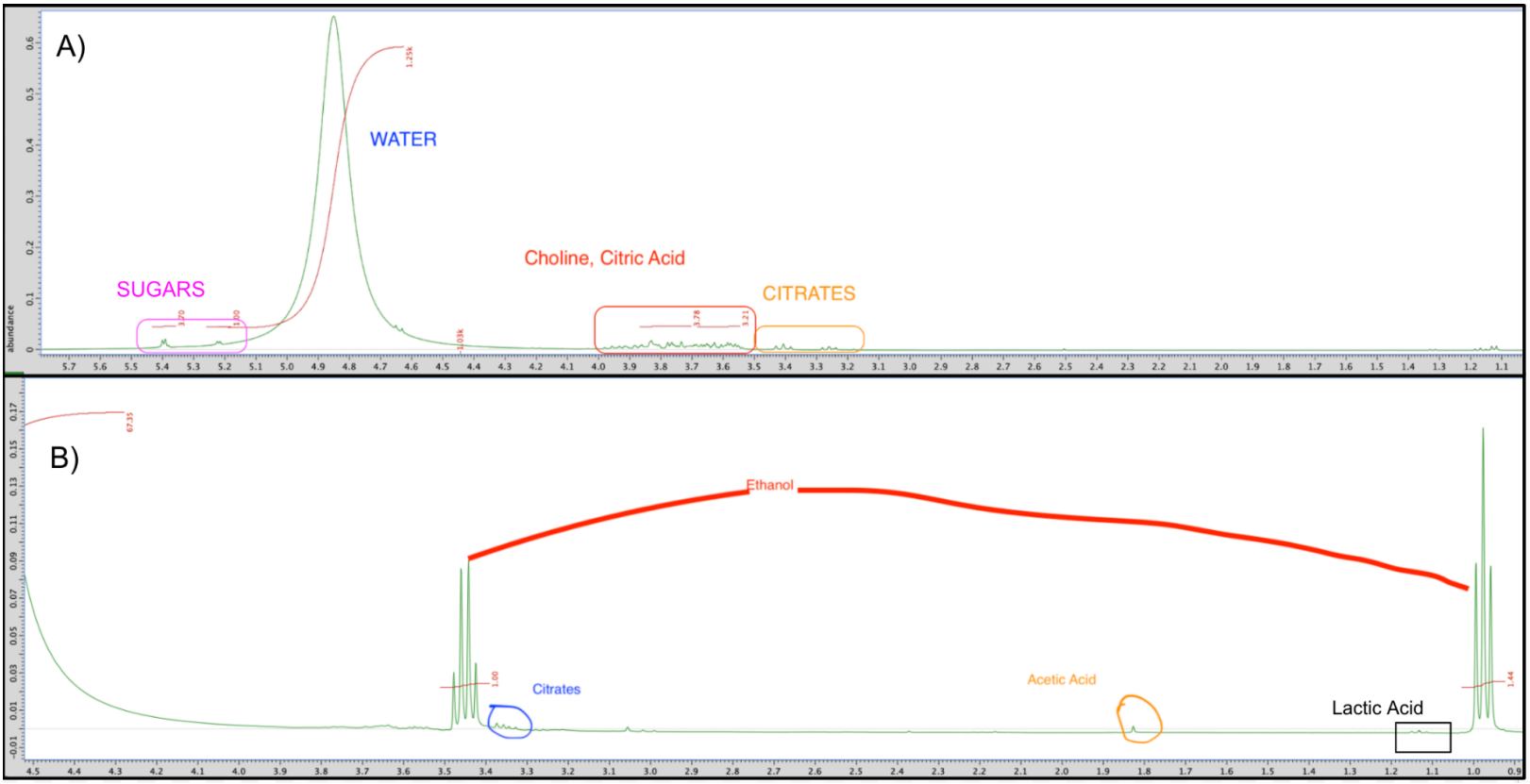

Figure 6: Figure 6A is the boil product, which was filtered through a 25mm syringe filter until approximately 5 mL of the sample was filtered. It can be seen that the dominating peak, at around 4.8 ppm, is water, that maltodextrin can be assumed to be seen from 5.2-5.5 ppm[45], and that citrates at 3.15-3.5 ppm.[46] Additionally, what is assumed to be citric acid is located from 3.5-4.0 ppm.[47] The peaks at 1.1 ppm have the potential to be amino acids.[48] Figure 6B represents the respective final product, filtered by the use of a gravity filtration. The image is zoomed in on differing compounds, which is why water, and the maltodextrins are not present in the figure. In these results citrates are seen again in the 3.1-3.5 ppm range.[49] Additionally two ethanol peaks can be seen at 0.8 and 3.45 ppm.[50] Newly identified compounds, acetic acid, located at approximately 1.8 ppm[51], and lactic acid, located at approximately 1.15 ppm[52] are additionally labeled. Unlabeled peaks were not able to be confidently identified.

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[45] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7, & Rodrigues, Quantification of Organic Acids in Beer by NMR-based Methods (Analytica Chimica Acta, 2010), 167
[46] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7
[47] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7
[48] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers,7
[49] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7
[50] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7
[51] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7 & Rodrigues, Quantification of Organic Acids in Beer by NMR-based Methods, 167 & Dicapro and Edwards, From Mash to Bottle: Chemistry of the Brewing Process and NMR-based Quality Control (Process NMR Associates, 2014)
[52] Rodrigues, Quantification of Organic Acids in Beer by NMR-based Methods, 167

While many papers analyze beer's final product, none analyze the boil product, or any developmental stage directly that was found during this project's research. Since there are no previous studies done on these developmental stages, NMR results from experiments done on fully developed beer had to be utilized in order to make assumptions in assigning/identifying spectra features. Though not a direct comparison, compounds are able to be inferred by the analysis of final products NMR results. Of course, deductive reasoning had to be used in this process. Since the boil product is pre-fermentation, if a peak in the boil product's readings correlates to a compound that is developed through beer's fermentation it can be known that this is inaccurate and leaves room for future analysis in this material. Additionally, identifications for all saison spectrums cannot be completely confirmed due to the lack of using a standard for comparison. Molecules can be assumed, and identified with great confidence, but not with complete certainty. This can be seen with the peaks in the 3.5-4.0 ppm range, which are the common range for ethanol to appear.[53] However, since ethanol is produced through fermentation it is known that this cannot be true. Through the use of data with ethanol present, educated assumptions were able to be made about what compounds are represented by the peaks in the 3.5-4.0 ppm range. It was assumed that this cluster of peaks most likely relates to choline, malic acid, citric acid, and succinic acid, however because this data was taken from NMR results which had ethanol present which creates additional uncertainties in identification.[54] Additionally, citrates are suspected to result in peaks in the range 3.15-3.5 ppm[55], but since this same data uses ethanol the exact range may differ, meaning citrate peaks could be seen in the 3.5-4.0 ppm range as well.

[53] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7
[54] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7
[55] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7

While identifying these compounds is an essential part of the process, an equally valuable analytical stage is to understand the impact these compounds have on beer. As stated previously, water is the base of all beer brewing and templates for which the beer is made, and ethanol is the alcohol produced by fermentation which gets people intoxicated. The compounds citrates and citric acid all contribute to the acidity of the beer, which makes sense to have this present since when humulones are boiled, they release acids into the beer.[56] Furthermore, the sugars found in the 5.2-5.5 ppm range[57], are assumed to be comprised of maltodextrins, non-fermentable sugars whose purpose is to increase the body of the beer as well as head retention[58], and glucose, a sugar that is fermented in the brewing process. Having these compounds present in this stage of the brewing process makes sense due to the milling and boiling of the grains occurring directly before the boil product sample is taken.

The next stage that was analyzed of the Stone Skipper Saison was its fully fermented product. This is the finished product, and the stage that is bought in stores. Analysis of this is crucial to knowing why certain flavors, aromas, and colors are present in a beer. The results and experimental developments of this stage's testing are shown in Figure 6B.

Through these initial testing some compounds could be seen, however, these groups do not give much information about the development of the beer. In Figure 6B two ethanol peaks dominant the spectra[59], however, there is still some useful information that can be learned from its results. It appears that both acetic and lactic acid may now be found in the ^{1}H NMR results. Acetic acid is the main acid produced by the fermentation of yeast, and a key component in the generation of ethyl acetate.[60] Ethyl acetate is an aromatic ester that contributes greatly to beer's

[56] Phillisкirk, Citric Acid (The Oxford Companion to Beer)
[57] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7
[58] Brewers Supply Group, Maltodextrins
[59] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7
[60] Spedding, Acetic Acid (The Oxford Companion to Beer)

physical profile. It does so by building a "fruity" flavor and smell profile without requiring the addition of fruit in the beer. Too much however, can create off flavors in the beer.[61] Having acetic acid appear in the final product agrees with the fact that these compounds are produced during fermentation as well as impact the beer's chemical composition. Lactic acid, which is produced by fermentation, is a common product in beer, but an excess of it can result in sourness or a buttery flavor being present in a brew.[62] Something that is interesting about lactic acid, is that it can be mixed into a brew in order to create a style of beer known as a sour. Properly balancing the beer, to fruit, to lactic acid ratio can allow for the creation of variants of certain beer styles.

When comparing the pure beer samples of both the boil product and final product of the Stone Skipper Saison using ^{1}H NMR there are some distinct differences. Specifically, as the beer develops ethanol, lactic acid, and acetic acid form and are suspected to contribute to the beer's flavor and aromatic profile. In Figure 6A's spectra there is a peak that appears similar to that of lactic acid peaks at 1.15 ppm, however, since lactic acid is a product of fermentation it is known that this peak cannot be lactic acid. It is then believed to be an amino acid. Additionally, we see that the sugar groups prevail through the brewing process as well, even though glucose is a fermentable sugar there will still be a smaller concentration left in the beer's final product. Looking in the 3.5 - 4.0 ppm range it appears that compounds are lost, although this is not believed to be the case. The ethanol peaks present dominate this range, in order to eliminate this issue, both the air-drying and lyophilization drying methods were conducted as explained in the

[61] Stewart, Ethyl Acetate (The Oxford Companion to Beer)
[62] Stempfl, Lactic Acid (The Oxford Companion to Beer) & Abedi, Lactic acid production – producing microorganisms and substrates sources-state of art (Heliyon, 2020), 1

experimental section. Both of these results can be seen below, each sample was filtered using gravity filtration.

128 Scan ^{1}H NMR of the Air-Dried Stone Skipper Saison's Boil and Final Product.

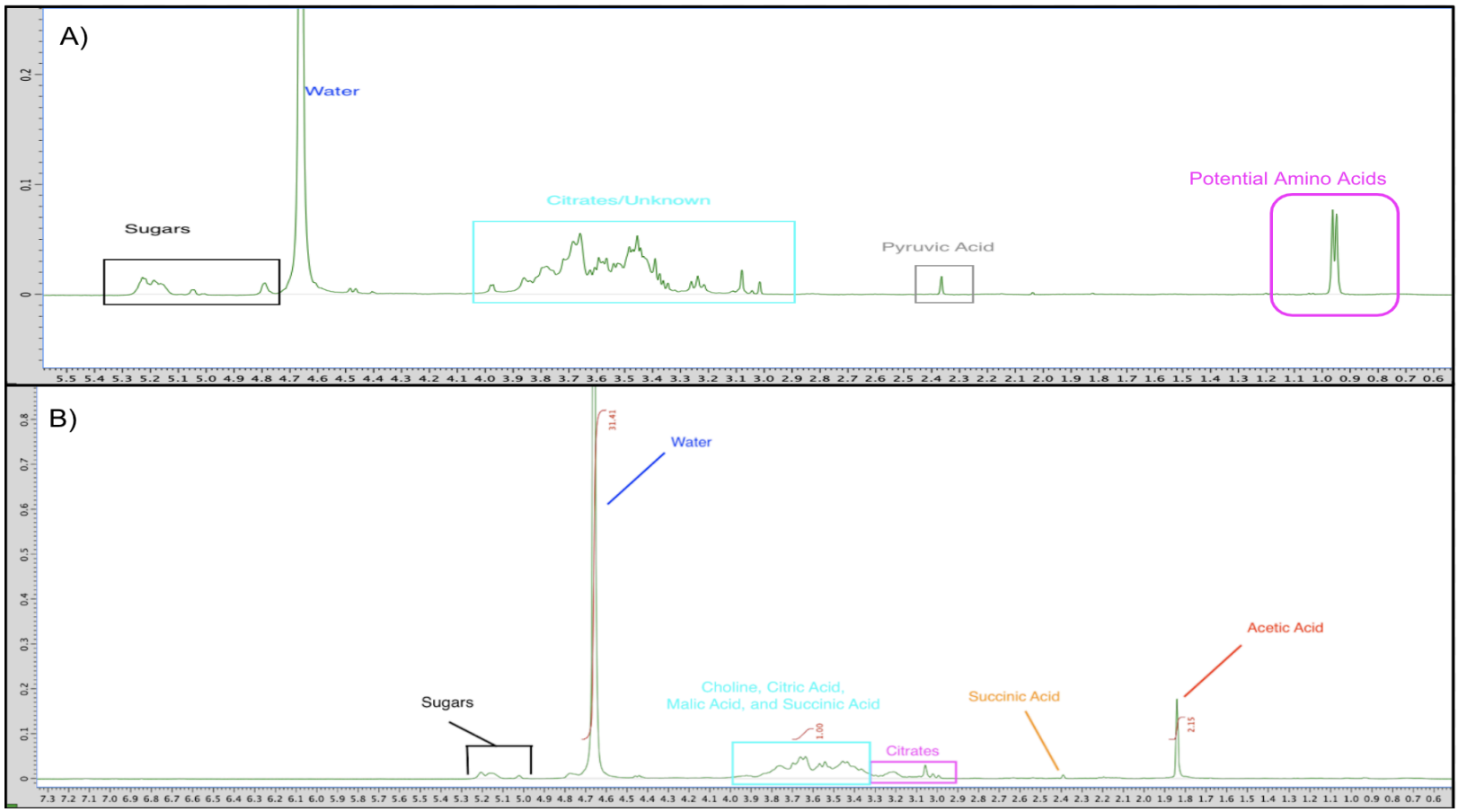

Figure 7: Figure 7A is the boil product. Here sugars are seen in the range of 4.8 - 5.4 ppm, as well as suspected citrates and unidentified compounds in the 2.9 - 4.1 ppm range.[63] Through air-drying a new molecule is believed to be unveiled: pyruvic acid, appearing near 2.35 ppm.[64] The doublet at approximately 0.9 ppm is thought to be an amino acid.[65] Figure 7B is the final product. Once again, sugars can be seen in their common 5.0-5.3 ppm range, in addition to supposed citrates in the 2.9-3.3 ppm range, and acetic acid is once again observed at near 1.8ppm.[66] Besides these compounds, succinic acid is believed to be unveiled, appearing near 2.4 ppm.[67] Additionally, the set of peaks ranging from 3.3 - 4.0 ppm can be associated with choline, citric acid, malic acid, and succinic acid.[68]

[63] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7
[64] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7
[65] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7
[66] Rodrigues, Quantification of Organic Acids in Beer by NMR-based Methods, 167
[67] Rodrigues, Quantification of Organic Acids in Beer by NMR-based Methods, 167
[68] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7

As seen from Figure 7, new peaks arise in the spectra; meaning that drying the solution worked and provided a more detailed chemical profile of the boil and final product. Water concentrations were shown to have greatly decreased as well, indicated by the greatly shrunk water peak around 4.7 ppm. Starting in chronological order with the boil product, it can be seen that a majority of the spectra stayed the same. However, clearer peaks can now be seen of all compounds present in the sample. This increased peak definition is most likely due to the lack of hindrance molecules such as water that the drying process eliminated. From the range of 2.9 - 4.1 ppm it is confidently assumed that the peaks present are representative of citrates and unknown compounds. Citrate's supposed peaks are again seen in the boil product, however, the clumping from other peaks create analytical issues. As stated previously, this range is where ethanol is commonly found, and since the boil product is pre-fermentation, it is known that no alcohol is present. Since there is a lack of research articles relating to boil products NMR analysis there is no ethanol-less spectra for this to be compared to. Additionally, all other identified compounds in previously published final product spectras identify compounds produced by fermentation. This creates uncertainties in what these peaks represent as well as opportunities for future research. The newly identified compound in Figure 7A's spectra is pyruvic acid. Pyruvate, pyruvic acid without a proton, is crucial in beer development and overall taste. In the fermentation stage of the brewing process one of the most important steps is to make sure that the fermentation vessel is tightly sealed so that no O_2 can enter the solution. The lack of O_2 makes it so that yeast conducts alcoholic fermentation. Sugars from the beer's grains are converted to pyruvate through glycolysis, after this alcoholic fermentation converts pyruvate into ethanol and carbon dioxide using pyruvate decarboxylase and alcoholic dehydrogenase.[69] It makes complete sense that this pyruvic acid then appears in the boil stage, due to its important

[69] Morton, Glycolysis and Alcoholic Fermentation (Institute for Creation Research, 1980)

role in the fermentation process. The peak present at approximately 1.0 ppm has the possibility of being an amino acid, although it cannot be said with much certainty. An amino acid seems plausible when looking at Siciliano and Procopio's work, but with no concrete evidence, or exact peaks matching up, it cannot be stated with confidence.

When analyzing Figure 7B, new compounds such as succinic acid, malic acid, citric acid, and choline are believed to be unveiled. These products' appearance makes sense, as they all result from the fermentation process. Succinic acid, supposedly identified at approximately 2.4 ppm, is another acid produced during yeast fermentation that provides a sourness to a beer.[70] In addition to this, in the 2.9 - 4.0 ppm range a similar set of peaks as to what is in Figure 7A appear. However, the compounds, as presented by published articles, that are viable in this range are produced by fermentation, so it can be assumed that the compounds creating the peaks differ from one another. Peaks in this range –in Figure 7B– remain cluttered together, making specific identifications difficult, but the general trend of the peaks give the possibility of the above peaks being present. Additionally, it can be assumed that the peaks in this range were not ethanol, and that air-drying proved a successful method of eliminating ethanol due removal of the 0.9 ppm seen in Figure 6B which resulted from ethanol. Instead, these peaks are assumed to be multiple products. The first being malic acid, malic acid is a yeast fermentation produced molecule that regulates the acidity of a beer. Specifically, it acts in order to lower the pH levels in beer, bringing the beer to the expected acidity and taste of a standard brew.[71] The pH of the boil product of this brew was 4.31, whereas the final product's pH was 4.01. This decrease in measured pH is a direct correlation to malic acid in beer and additionally confirms its presence and role. The next predicted compound was choline. Choline is a vital nutrient which plays a

[70] Micet Craft (2021)
[71] Brew Mart (2024)

role in developing cell membranes as well as regulating mood, memory, muscle control, and more.[72] While fermentation producing this nutrient does not directly affect the taste or aroma of the beer, it relates to the earlier discussed idea that ancient civilizations used beer as a food source, energy boost, and staple in their diet. Beer provides essential nutrients which can aid in someone's health, its content can benefit people despite all of the negative connotations surrounding the beverage today. In the previously specified range, there are still compounds that could be potentially identified, however, the published papers used as the basis of identification all included ethanol peaks. As a result of this, there could be products that the labs were not able to properly identify. Additionally, while not new compounds, citrates, sugars, and water can also be found in Figure 7B.

The air-drying method presented in Figure 7 seemed to unveil useful products, reduce water peaks, eliminate ethanol, and present a cleaner spectra. These products being displayed in the spectra as a result of air-drying supports this preparation technique's validity in future research. Drying methods were taken even further through lyophilization. The results of this are shown below in Figure 8.

[72] National Institute of Health (2022), Choline

128 Scan ^{1}H NMR of the Lyophilized Stone Skipper Saison's Boil and Final Product.

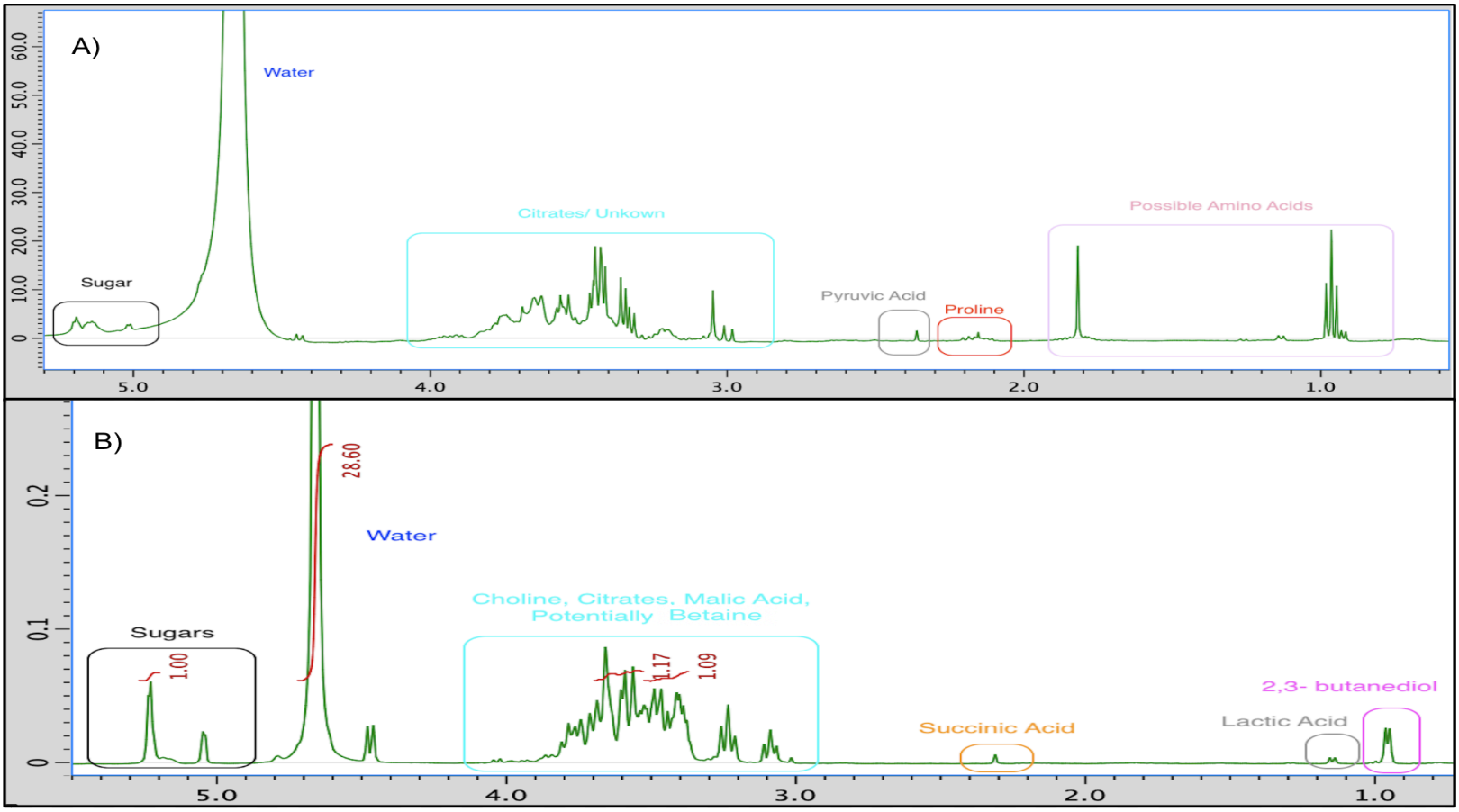

Figure 8: Figure 8A is the boil product. Here sugars are seen in the range of 4.9 - 5.3 ppm, as well as citrates and unidentified compounds in the 2.9 - 4.1 ppm range.[73] In the lower ppm range of the spectra pyruvic acid is suspected to be seen again at 2.4 ppm.[74] Newly unveiled amino acids such as proline are discovered in the 2.2 ppm area, as well as a possibility for more amino acids in the 0.9 - 1.9 ppm range.[75] Figure 8B shows the final product. Here, sugars, choline, citrates, and malic acid are all suspected in their locations as previously described. In this location, there is additionally the potential of betaine.[76] Succinic acid is believed to be present in the 2.4 ppm range as well as lactic acid in the 1.1 ppm range[77], and 2, 3-butanediol.[78]

[73] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7
[74] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7
[75] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7 & Lachenmeier, Frank, Humpfer, Quality control of beer using high-resolution nuclear magnetic resonance spectroscopy and multivariate analysis (European Food Research and Technology, 2005), 218
[76] Palmioli, Alberici, Ciaramelli, & Airoldi, Metabolomic profiling of beers: Combining ^{1}H NMR spectroscopy and chemometric approaches to discriminate craft and industrial products (Food Chemistry, 2020), 7
[77] Lachenmeier, Frank, Humpfer, Quality control of beer using high-resolution nuclear magnetic resonance spectroscopy and multivariate analysis, 218
[78] Palmioli, Alberici, Ciaramelli, & Airoldi, Metabolomic profiling of beers: Combining ^{1}H NMR spectroscopy and chemometric approaches to discriminate craft and industrial products, 7

When analyzing Figure 8A, it can be seen that some peaks remained constant with figures 6A and 7A, in addition to this, new peaks can be seen on the spectra as well. Discovering new peaks establishes lyophilization as a successful preparatory method for ^{1}H NMR beer analysis. For the boil product, the newly discovered products are suspected to be proline and additional amino acids. Proline, seen at 2.15 ppm[79], is an amino acid that is very common in the wort stage of the beer, the stage directly before the boil product is formed. Proline is produced from barley, a common grain used as the sugar basis during the brewing process.[80] Due to these reasons it makes sense why proline is dominant in this stage of the process. Proline's effect in beer is that it, along with other amino acids, can interact with yeast in order to promote fermentation and increase foam quality and stability. However, having a surplus of amino acids can create instability in the beer and result in a haze forming.[81] In the range of 0.7 - 1.9 ppm, it is proposed that these peaks are caused by the CH_3 groups of various amino acids[82], although in the published papers used to analyze these results no specific amino acids results were provided. It would also be assumed that these amino acids would play very similar roles to proline in terms of their impact on beer's flavor, aroma, and color. While this identification cannot be stated with absolute certainty, there is a high probability that it is true. Additionally, it is known that the doublet produced at 1.1 ppm is not lactic acid because lactic acid is produced by fermentation, and since the boil product is pre-fermentation, it would not make sense for lactic acid to be present in this sample.[83]

[79] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7
[80] Koller & Perkins, Brewing and the Chemical Composition of Amine-Containing Compounds in Beer: A Review (Foods, 2022), 2
[81] Fontanna & Buiatti, Amino Acids in Beer (Beer in Health and Disease Prevention, 2009), 1
[82] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7
[83] Abedi, Lactic acid production – producing microorganisms and substrates sources-state of art (Heliyon, 2020), 1

In the analysis of Figure 8B's identifications, there are limited new possible identifications made. Some areas of the spectra, such as the 2.9 - 4.1 ppm range, show different peak patterns but due to the clutter of the peaks it cannot be completely confirmed that identified compounds are present. It can only be assumed that the previously speculated molecules should be in this range, and even with those molecules, the specifics of which peak choline, citrates, and malic acid relate to cannot be identified due to this clutter. A new potential compound is speculated in this range is betaine. The peaks in the above range seem to follow a similar pattern to that of betaine, but with the cluster of peaks and lack similarity to previously published work its identification cannot be confirmed. Betaine is a natural substance produced from microorganisms[84] or plants, so it makes sense that it would only appear after fermentation has occurred (the microorganism yeast was added). While its flavor, aroma, or color impact may be limited, betaine plays a role in preventing alcohol related liver diseases, alcohol-induced hepatic steatosis, apoptosis, and damaged proteins. An additional newly hypothesized molecule that is suspected to be present in Figure 8B's sample is 2,3 - butanediol. The peak at 0.9 ppm, aligns perfectly with Palmiolis group's work, in which they identify the peak to be 2,3-butanediol.[85] 2,3-butanediol is produced via fermentation, so appearing in Figure 8B makes perfect sense; it is associated with a buttery, creamy, or even fruity flavor in beer.[86] A fruity profile is typical for a saison, so it makes sense to see 2,3-butanediol present in this sample.

[84] Arumugam, Beneficial Effects of Betaine: A Comprehensive Review (Biology 2021), 1
[85] Palmioli, Alberici, Ciaramelli, & Airoldi, Metabolomic profiling of beers: Combining ^{1}H NMR spectroscopy and chemometric approaches to discriminate craft and industrial products, 7
[86]Showing Compound 2,3-Butanediol (FDB011934)

On a separate note, an interesting comparison to make between figures 8A and 8B is the fact that the amino acids are not present in the lower ppm section of the spectra in the final product as they are in the boil. Since they play a direct outcome in color it would be assumed that they would be present in the final product. However, it makes sense that there would be low proline levels in the final product of the saison, as saison's are traditionally clear beers that lack haze.

When looking at the saison as a whole, it can be seen that both of the drying methods seemed to prove successful as a way to reduce water, reduce ethanol, create a more defined spectra, and unveil new compounds present at the different stages of the brewing process. Each method provided individual results for at least one spectra and gave us more information to work with.

While the beer's potential compounds and their beer impacts have been identified, it is critical to understand how they develop in a certain style. Stereotypically, saison's have a lighter flavor, with an emphasis on bitterness, fruitiness, and spice driven aroma. Additionally, saisons are less malty with more of an emphasis on their lactic sourness.[87] A majority of these stereotypes greatly align with the research presented and results of the spectra provided in figures 6-8. As seen in nearly every spectrum, beer is mainly composed of water and ethanol, but there is so much more to it. It seems that citrates are non-fermentable, and stay present throughout the entire brewing process affecting the acidity of the beer.[88] Furthermore, sugars are presumed to be present in both stages of the process as well, whether these are maltodextrins, non-fermentable sugars which obviously survive the fermentation process, or glucose, which is fermented but still has some concentration present in the final product, sugars play a crucial role

[87] Markowski, Farmhouse Ales: Culture and Craftsmanship in the Belgian Tradition, approximately 24
[88] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7 & Phillskirk, Citric Acid

in the flavor, fermentation, look, and smell of the beer. During the fermentation process it seems there is a development of choline, malic acid, succinic acid, lactic acid, 2,3-butanediol, and acetic acid.[89] All of these compounds play particular roles in forming the beer into what it tastes, smells, and looks like to a consumer. Their specific roles have already been discussed earlier in this paper, but it is important to understand how these are proven to have developed from one stage to another and show that they are direct products of fermentation.

Learning about what is present in the final product is important, however, it is also essential to know what was lost from the boil product to the final product. This can provide just as much information about the beers creation and development as seeing what compounds developed can. The main compounds that were observed being lost in the saison's development were proline, amino acids as a whole, and pyruvic acid. As discussed before, pyruvate is converted into ethanol and carbon dioxide during the fermentation process, meaning that it is crucial that this compound gets used up if a beer is supposed to develop properly.[90] It can be noticed that in figures 7A and 7B there seems to be similar peaks at the same ppm, however, they are labeled as both pyruvic and succinic acid, respectively. The difference between these two is able to be established due to knowing how these compounds are developed in the brewing process. Since it was established that pyruvic is a crucial part in alcoholic fermentation it is understood that it cannot be present in the final product, furthermore, since succinic acid is produced by fermentation, we know it is in the final product. Some amino acids are expected to be consumed throughout the fermentation process, so it makes sense that these peaks disappear, depending on what amino acid they are. However, proline is not consumed through

[89] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7 & Lachenmeier, Frank, Humpfer, Quality control of beer using high-resolution nuclear magnetic resonance spectroscopy and multivariate analysis, 218
[90] Morton, Glycolysis and Alcoholic Fermentation

fermentation, making its disappearance something to research further.[91] Either they are lost in another pathway in the brewing process, bind with something else, or are present in such small amounts they are not able to create peaks within the NMR spectra obtained. Whether lost or gained, every single compound plays a crucial role in how beer tastes, smells, and looks. Analyzing these compounds tells readers a full story of what is developed as well as provides information about what these compounds do to beer and how they work in the brewing process.

[91] Ferreira and Guido, Impact of Wort Amino Acids on Beer Flavour: A Review (Fermentation, 2018), 5

BOCK

The next style of beer that is being analyzed is a bock. This beer was chosen due to its two very distinct differences from the saison. First, it is a lager, which as discussed previously, means it ferments at different temperatures, for longer, and in different stages. Second, it is a darker beer, which gets its dark color from the use of different malts. It is assumed that these two differences should result in major chemical changes inside of the beer when compared to the saison. This would result in a more definitive answer to what makes a beer taste, smell, and look the way it does, as well as what truly makes beer styles different from one another. It should be noted that due to time constraints Lost Hollow Beer Company's "The Empire Strikes Bock", bock beer was not able to be used in this process but will be used in future research as a comparative point between different beers in the same style. Instead, Michelob's "Amber Bock" was used as a replacement for the final product. While these two beers are of the same style, they were made in different breweries, using different ingredients, and made in different ways. All of these factors could result in some of the chemical compositional differences seen when comparing the boil and the final product. For now, all that is ready to analyze is the final product of Michelob's Amber Bock, its corresponding NMR results are provided below. Due to Michelob being a major brewing company it was not possible to obtain a sample of their boil product. The bock's boil product in this study will be "The Empire Strikes Bock" when it becomes available.

Along with the saison, a bock has a deep and rich history that has created various stereotypical profiles of the beer's characteristics. Bocks are said to have originated from the small German town of Einbeck in the c.13th century. Einbeck was a city located in a prominent hop gardening region.[92] As a result of this Einbeck's business grew and the city became known

[92] Richman, Bock (Brews Publication, 1994), 4 & 7

as a distinguished brewing center, eventually exporting beer to many surrounding countries such as Belgium, the Netherlands, and England.[93] Due to the renowned beer produced by Einbeck –and Munich's lackluster beer industry at the time– it is said that the Einbeck's brewmaster, Elias Pichler, was invited to visit Munich in order to showcase his brewing talents in the year 1612, however, once there his skills were deemed too great and he was forced to stay. In Munich he developed his now coined "bock" style beer to the public. The beer soared in popularity and greatly expanded the brewery production.[94] It began to spread throughout Europe, growing immensely. This, combined with the styles high alcohol content, various flavor, dark color, and rooted maltiness resulted in a slew of sub-styles. These sub-styles include doppelbocks, helles bocks, eisbocks, dunkelbocks, and more. Each one of these subsets provides different standards of body, aroma, and flavor. Making bocks a truly rare style of beer in terms of variety.

As mentioned previously, a main purpose of beer's production and consumption was based around the social and religious aspects of the beverage. Bocks are no exception. While originally created in order to produce an extremely high alcohol content beer for the time (6.5% ABV), bocks eventually grew into a large religious role. This style of beer became extremely popular during lent.[95] It acted as a staple of Catholic's diets and filled their nutritional needs during their religious practices.[96] Specifically, throughout the beer's history monks brewed bocks as a form of liquid bread to aid them through their dietary fast.[97] Bocks playing this role in lent practices reaffirms the importance of beer throughout history as well as the fact that beer was a key social and religious tool used to bring people in a religion together and create a sense of community around the brewing and consumption of this beverage.

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[93] Richman, Bock, 8
[94] Richman, Bock, 8 & Seidl, Bock Beer (The Oxford Companion to Beer)
[95] Seidl, Bock Beer
[96] Seidl, Bock Beer
[97] Smith, Beer: A Global History,

Understanding this historical background is crucial to knowing why this beer is made today, why this research matters, and well as why this beer tastes and looks the way it does. Comprehending this, as well as the flavor profile of the beer, paints a clear and full picture of what a bock truly is. In terms of a bocks physical profile, traditionally they are known for being malty beers. This means they smell of malt, are denser, and provide a deeper sweetness to their flavor. When working with the darker malts that are stereotypical of a bock a caramel, chocolate, or smoke flavor can be introduced to the beer as well.[98] Hops provide a bitterness to bocks that complement the malty profile of the beer, creating a more balanced beer.[99] Having an intense malt-only flavor profile would not be appealing to the general population, it would be too sweet and likely undrinkable. Introducing hops allows the beer to become palatable by creating a proper dynamic between bitter and sweet. As for stereotypical aromas, bocks tend to have a more intense ethanol-based aroma due to the beer's high ABV, but also have notes of sweetness from the malt.[100]

Below the ^{1}H NMR experiments conducted on the final product of the Michelob Amber Bock can be seen. Analyzing these spectra while also keeping in mind the history and flavor profiles of bocks presents a cohesive story about the importance of bocks and what truly makes a bock, a bock. For the Michelob Amber Bock, the NMR results of the undried beer did not provide any useful information for analysis, so they were not included in this research below. Ethanol and water dominated this spectra, creating useless results. Additionally, as with the saison, identifications cannot be made with complete certainty, data can heavily support an identification, but until standards for that compound are tested this identification cannot be said with certainty.

[98] Richman, Bock, 18
[99] Richman, Bock, 18
[100] Richman, Bock, 18

128 Scan ^{1}H NMR of the Air-Dried Bock Final Product.

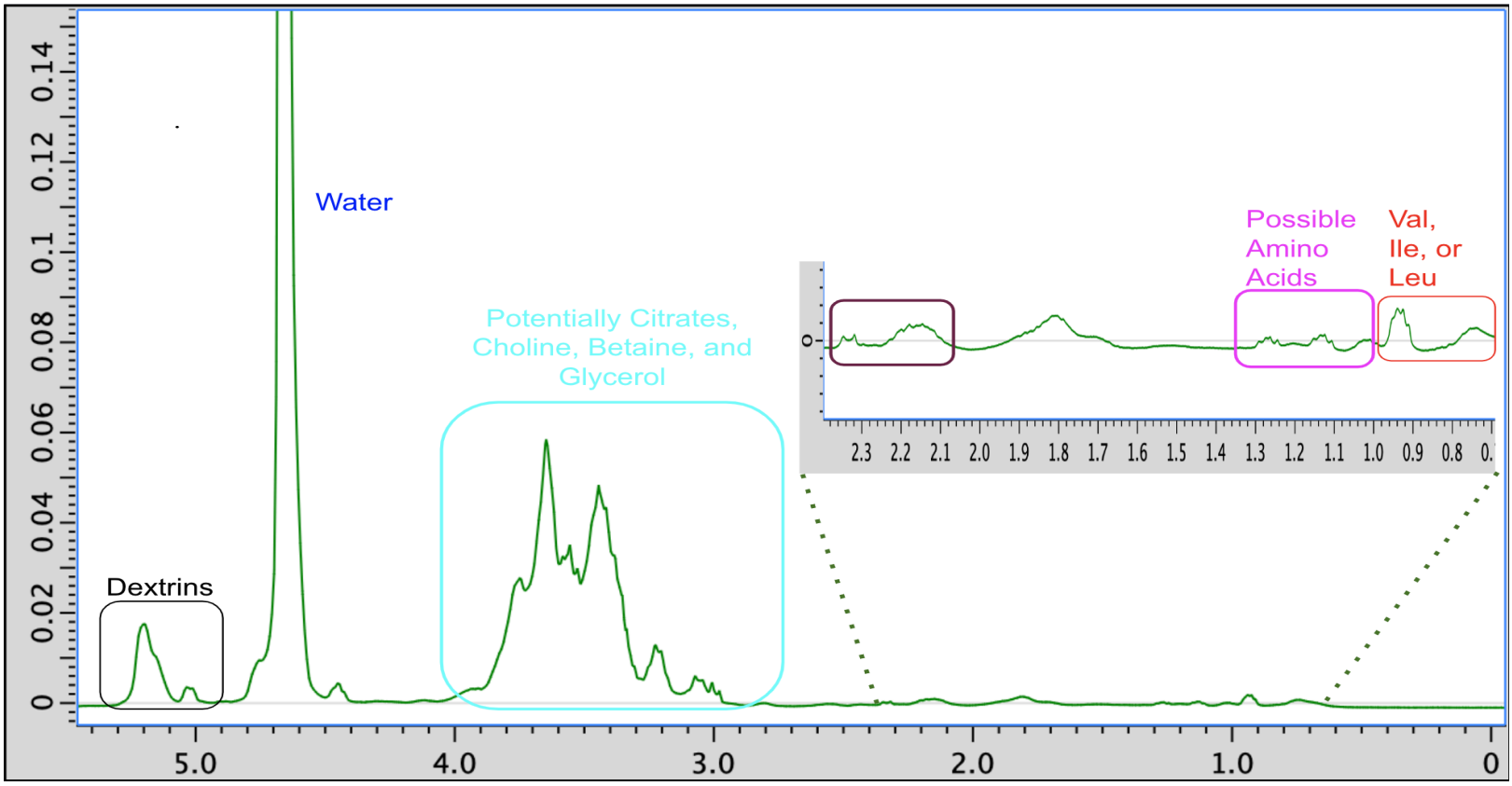

Figure 10: Traditional compounds such as dextrins can be seen here in the 4.9 - 5.3 ppm range, and water in the 4.7 ppm can be found.[101] In the 2.9 - 4.0 ppm, and 1.0 - 1.35 ppm range of the spectra, possibly identifiable molecules are unveiled. These include citrates, choline, betaine, glycerol in the higher range, and amino acids in the lower range. In addition to this, new molecules such as valine isoleucine, and leucine can be potentially identified.[102] Compounds in the 2.1 - 2.3 ppm range remain difficult to identify at this time.

Remaining constant with the Stone Skipper Saison, air-drying the beer allowed for new products to appear in the spectra, as well as some to remain. One thing that is interesting when initially looking at the spectrum is its inclusion of water. This means that the air-drying process wasn't entirely successful in evaporating all the water off of the sample, remaining constant with the saison. Longer time spent on this process could flip this result. When looking at the

[101]Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7, and Rodrigues, Quantification of Organic Acids in Beer by NMR-based Methods, 167
[102]Palmioli, Alberici, Ciaramelli, & Airoldi, Metabolomic profiling of beers: Combining ^{1}H NMR spectroscopy and chemometric approaches to discriminate craft and industrial products, 7

spectrum there is again a conglomerate of peaks in the 2.9- 4.0 ppm range. Due to the merging together of these peaks, it is difficult to confidently state what they represent. However, peaks commonly found in this range, which seem to vaguely match the spectra are citrates, choline, betaine, and glycerol. The first three mentioned compounds have been previously discussed. Glycerol, however, has not. The glycerol peaks presented in Palmiolios group's work seem to match the peaks at 3.4 and 3.6 ppm in Figure 10, better than any previously analyzed figure. Glycerol is produced through fermentation, acting as a carbon competitor with alcohol during fermentation. This means it helps to manage the amount of alcohol in beer, directly impacting the beer's flavor and aroma.[103] Glycerol additionally gives the beer its sense of body and fullness. Not only this, but it is said to increase sweetness and decrease bitterness.[104] In terms of glycerol's effect on aroma, glycerol interacts with 3-methyl butyl acetate and ethyl hexanoate to positively influence the fruity aroma of a beer.[105] All of these patterns comply perfectly with the stereotypical profiles of bocks, a darker beer which is sweeter and full bodied. Bringing reason as to why glycerol is started to become noticed in this beer. There is a small chance that it could be present in the saison, however, its peak patterns were not recognized in the NMR results. In the 1.6 - 2.3 ppm range there are a wide range of peaks present, however, none of the data obtained compares nicely with previously researched work. In the red boxed area, it could be assumed to be some sort of acid, but this cannot be stated with full certainty.[106] When getting into the 1.05 - 1.40 ppm range, possible amino acids are present,[107] but again there is not enough correlation in the peaks to be able to make a firm identification. This theme changes when looking at the 0.7 - 1.0 ppm range of Figure 10. Here the amino acids valine, isoleucine, and

[103]Zhao, Procopio, Becker, Flavor impacts of glycerol in the processing of yeast fermented beverages: a review (Journal of Food Science, 2015), 1
[104]Zhao, Procopio, Becker, Flavor impacts of glycerol in the processing of yeast fermented beverages: a review, 3
[105]Zhao, Procopio, Becker, Flavor impacts of glycerol in the processing of yeast fermented beverages: a review, 3
[106] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7
[107]Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7

leucine can be potentially identified.[108] Their peaks correspond greatly with Siciliano and Procopio's research, but until standard testing can be done with those compounds, no identification can be completely confirmed. These amino acids are produced during fermentation, they are also branched-chain amino acids, which influence the production of higher alcohols isobutanol, isoamyl alcohol, and amyl alcohol.[109] Higher alcohols can add a more alcohol, fruity, or floral taste and aroma to the beer.[110] Since bocks are traditionally higher alcohol beers, especially compared to saisons, it makes sense that these begin to appear in the beer and play a role in its flavor. Amino acids such as valine and leucine, are interestingly formed by yeast, so seeing these amino acids makes sense in the beer's final product.[111] In terms of valine itself, too high of a concentration can result in diacetyl forming. Diacetyl forms a buttery flavor in beer that is avoided by brewers, especially in lagers where temperature changes in fermentation can result in diacetyl forming.[112]

In order to make direct comparisons between the different styles, NMR testing was additionally conducted on a lyophilized sample of Michelob's Amber Bock in order to obtain another spectra of the beer, and ideally find more contrasting peaks from the Stone Skipper Saison. The results of this can be seen below.

[108] Palmioli, Alberici, Ciaramelli, & Airoldi, Metabolomic profiling of beers: Combining ^{1}H NMR spectroscopy and chemometric approaches to discriminate craft and industrial products, 7
[109] Brewing Forward, Amino acids (2024)
[110] Liu, Impact of yeast and bacteria on beer appearance and flavour (Brewing Microbiology, 2015), 359-362
[111] Ferreira and Guido, Impact of Wort Amino Acids on Beer Flavour: A Review, 2
[112] Brewing Forward, Amino acids

128 Scan ^{1}H NMR of the Lyophilized Bock Final Product.

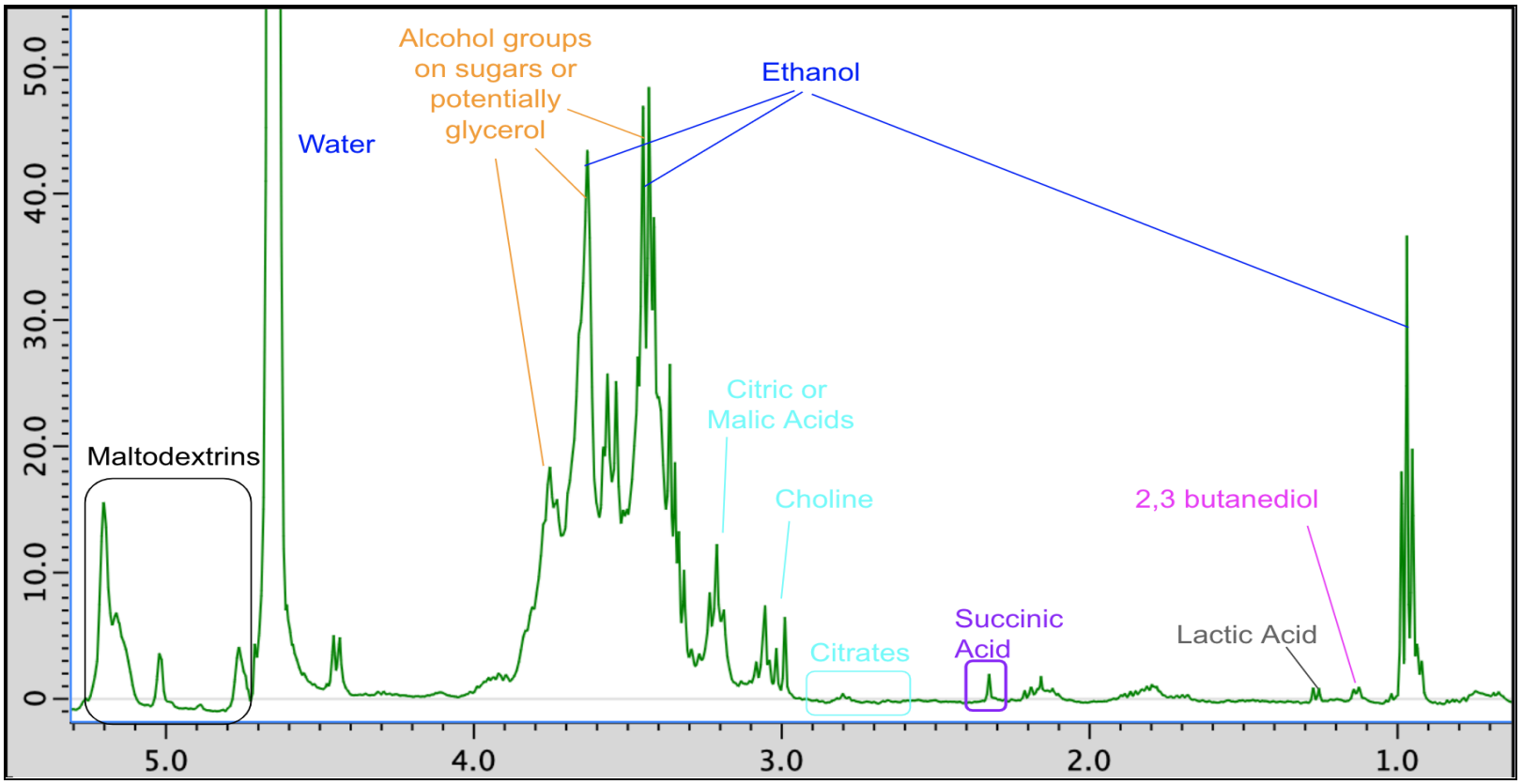

Figure 11: Figure 11 Shows water and maltodextrins in their usual ranges of 4.6 and 4.7 - 5.3 ppm, respectively.[113] In the 2.6 - 4.0 ppm region there is much more defined peaks this time around. Ethanol again shows itself at 3.6, 3.4, and 0.95 ppm.[114] New potential peaks such as alcohol groups, sugars, or glycerol, also seem to be present in this range.[115] The possibility of citric or malic acids can be seen more clearly now by the triplet at 3.2 ppm, choline with the set of peaks at 3.0 - 3.1 ppm, and citrates at the 2.6 - 2.9 ppm range.[116] Succinic acid can once again be seen at the 2.3 ppm mark, lactic acid in the 1.25 ppm range, as well as 2,3, butanediol at the 1.1 ppm mark.[117]

[113] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7
[114] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7
[115] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7 & Rodrigues, Quantification of Organic Acids in Beer by NMR-based Methods, 167
[116] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7
[117] Siciliano & Procopio, High-Resolution NMR and MALDI-MS Molecular Profiling of Craft Beers, 7, and Palmioli, Alberici, Ciaramelli, & Airoldi, Metabolomic profiling of beers: Combining ^{1}H NMR spectroscopy and chemometric approaches to discriminate craft and industrial products, 7

While the lyophilized Amber Bock's ^{1}H NMR results include ethanol and water, the beer's spectra is the best matching one to the previously published papers. The spectra shown in Figure 11, matches the work done by Siciliano and Procopio extremely well and allows for a more specific, peak-by-peak, analysis of the beer to be conducted and for individual identification points to be made. This is extremely useful when discussing the 2.6 - 4.0 ppm range, this range has proven to be difficult to work with in this study, so gaining more clarity within this range is extremely beneficial to this study. These more detailed peaks include the triplet at 3.2 ppm potentially representing citric or malic acid, the clump of peaks at 3.0 - 3.1 ppm possibly being choline, and the peaks from the 2.6 - 2.9 ppm most likely representing citrates. Establishing, and identifying specific peaks allows for a more accurate identification of these molecules and a deeper understanding of what each peak in the spectra actually represents.

While this is by far the most accurate, compared to Siciliano's work, ^{1}H NMR scan in this experiment there are still some uncertainties about what some peaks may truly represent. This comes from the presence of ethanol in this spectra, if ethanol could have been completely eliminated more precise answers would have been given. However, that is not the case, and as a result it can only be said that there is a potential for new compounds appearing in the 2.6 - 4.0 ppm range of this spectra. One alleged new part of a molecule is the CHOH group on sugars. Sugars provide the sweetness and maltiness into a beer's flavor profile. Additionally, since these are seen in the final product it can be assumed that these sugars were either non-fermentable, altered by fermentation, or not fermented, although none of these options can be confirmed with the data provided. An additional compound that can only be assumed to be present is glycerol, in this same range it seems like there is the possibility of it being present in the beer, but due to the clutter and presence of glycerol this cannot be fully confirmed. New compounds for this beer

potentially unveiled in the lyophilization are succinic acid, lactic acid, and 2,3- butanediol. All compounds that play a crucial role in the flavor, aroma, and appearance of the beer. Unlabeled compounds in this spectra were not able to be identified based off of the research presented at this time.

Both figures 10 and 11 provided useful information relating to the bocks chemical profile, however these are not standalone spectra. Comparing them both together provides a full story of what molecules are present in the beer. Both analysis techniques confirm that dextrins and water are present in the beer. As mentioned before Figure 10, has its peaks clumped together in the 2.9 - 4.0 ppm range, whereas in the same range in Figure 11, more defined peaks are presented that allow for more accurate identification of peaks. Combining both Figure 10 and 11's data allows for increased confidence in citrates, choline, citric acid, and malic acid all being present in the beer. Both figures showed the trends of glycerol appearing in the spectra, but neither allowed for a certain identification of the molecule. Having both of these spectra show trends of glycerol being present increases confidence for the likelihood that it is present in the Amber Bock. In Figure 10, betaine is labeled as a potential compound found in the spectra, this is not seen in the lyophilized sample. What is potentially seen in the lyophilized sample is the CHOH group of sugars. Figure 10 also allowed for the alleged discovery and identification of valine, isoleucine, and leucine, amino acids which can greatly alter the taste and aroma of a beer. In addition to this, the potential for more amino acids can be seen in this figure. For the lyophilized spectra, succinic acid, lactic, acid, and 2,3-butanediol may be present. The various similarities and differences between these two different drying methods of the same beer emphasizes why doing multiple drying processes is crucial. Through each method new

compounds can be identified, which adds onto the research completed, but, if no new compounds can be identified there is additional supporting evidence for the already identified peaks.

COMPARISON BETWEEN SAISON AND BOCK

One of the main goals of this research is to showcase the difference between each of the beer styles and illustrate why compounds being present in each style make sense. As discussed previously saisons are traditionally lighter beers, with a more fruity, bitter, and spice driven flavor and aroma. With this, they are less malty and more impacted by lactic sourness.[118] Bocks on the other hand are malty beers with a higher ABV. They typically have a more roasted, chocolate, or smoked flavor to them, along with a balance between bitterness from hops and sweetness from malts. Their aroma is usually more ethanol intense, due to their higher ABV.[119] These beers are completely different on paper, from their yeast, malts, style, origin, and stereotypical flavor/aroma profiles, nothing really seems to be a common ground. When comparing the entire ^{1}H NMR results some clear differences arise, but there are also some fascinating similarities. For this comparison, only the final product's spectras will be compared.

First and foremost, every ^{1}H NMR spectra potentially displayed dextrins, sugars, water, ethanol (only in the final product) in the same regions. Each style also included the potential for malic acid, citric acid, citrates, betaine, and choline. All of these are labeled as potential due to the aforementioned clumping in the spectra and the inability to scientifically validate the labeling for each peak. Malic acid lowers the pH of beer and brings it to proper acidity levels.[120] So this being present in both beers makes sense as it is crucial to the basic taste of any beer. Citric acid and citrates also contribute to the acidity of beer, since they are seen in every sample it can be assumed that they are a general acid involved in the brewing process. Betaine plays a direct role in preventing alcohol related diseases in the body and comes from different microorganisms. It

[118] Markowski, Farmhouse Ales: Culture and Craftsmanship in the Belgian Tradition, 24
[119] Richman, Bock, 17-18
[120] Brew Mart

does not play a major role in the flavor, aroma, or appearance of the beer.[121] It is logical to assume that betaine is standard in most beers since it is labeled as a possible compound in both samples of beer. Something interesting to note about this is that betaine comes from microorganisms, i.e. yeast, and ales and lagers use completely different yeast species in their brewing process. This means that both Saccharomyces cerevisiae and Saccharomyces pastorianus add betaine to the beer. Choline is a nutrient that provides numerous benefits to the body. It does not play a crucial role in the taste, aroma, or look of beer.[122] Two confidently identified components seen in both beers are succinic and lactic acid. Succinic and lactic acid provide a sourness to beer.[123] Their produced sourness makes sense to be present in the saison, since that style is known for its more bitter and sour flavor profile, however, for the bock it is extremely out of character. Bocks are not intended to be sour at all so the presence of these two acids in the bock is either due to an error by Michelob during their brewing process, or they are present in very small amounts to where their impacts on the beer's physical details is not extreme. Another possible reason as to why it is present is because it is a natural product of fermentation and unavoidable to become present. A possibly identified compound found in both the beers is 2,3-butanediol. 2,3-butanediol provides a creamy and/or fruity flavor to the beer.[124] It's reasonable that this would appear in saison, due to its fruit flavor tendencies, however, it is again surprising to see this in the bock. There is the possibility that this compound is not present, and another compound is causing this peak. Additional testing is needed to determine whether it is just present in microscopic amounts which cannot alter the flavor, a mistake in the brewing process, or just a natural part of fermentation which is present in every beer.

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[121] Arumugam, Beneficial Effects of Betaine: A Comprehensive Review, 1
[122] National Institute of Health, Choline
[123] Micet Craft, & Stempfl, Lactic Acid
[124] Showing Compound 2,3-Butanediol (FDB011934)

Despite these commonalities between the beers there were some distinct differences found in the ^{1}H NMR results. For the saison, the only thing that it seemed to have present in the final product that was absent in the bock was acetic acid. Acetic acid is crucial in the production of ethyl acetate, which can give beer its fruity aroma.[125] It makes complete sense that acetic acid is seen in the saison but not the lager, if one of its main contributions is adding a fruity essence to the beer, that fits perfectly with what a saison is. Bocks do not try to portray this, its absence in their spectra makes sense.

For the bock, there were multiple compounds that appeared in its spectra that were not present in the saison's spectra. An example of this is the presence of valine, isoleucine, and leucine in the bock's ^{1}H NMR results. These amino acids aid in the production of higher alcohols such as isobutanol and isoamyl alcohol.[126] Higher alcohols like these can result in a more alcoholic, fruity, or floral taste to a beer.[127] In terms of a bock, it makes sense that these would be present to add the more alcohol-based flavor to the beer, as bocks hold a relatively high ABV. What is interesting is that these higher alcohols also provide fruity and floral flavors, this is not typical of a bock. However, this aspect of the higher alcohols could just not be shining through in the beer or not there in miniscule amounts. Other amino acids are potentially present in this sample as well, but as discussed previously, at this time there is not enough data to confidently confirm that this is true. Amino acids present would have to be ones that can either survive fermentation or are produced by yeast. These could potentially impact the beer in a similar way or provide haziness to the beer.[128] Something that is worth noting is that the Stone Skipper Saison had proline, an amino acid, appear in its boil product but not final product. This

[125] Spedding, Acetic Acid, & Stewart, Ethyl Acetate
[126] Brewing Forward, Amino Acids
[127] Liu, Impact of yeast and bacteria on beer appearance and flavour, 359-362
[128] Fontanna & Buiatti, Amino Acids in Beer, 1

is interesting because there are so many amino acids potentially showing up in the bock's final product, but proline –an amino acid known to prevail through fermentation– does not.. Additionally, saison's are not typically hazy in color, making proline potentially being present out of character. Further research will be conducted into why this difference arises, and what effects that has on the beer. Another interesting thing that appears in only the bocks spectra is the assumed presence of CHOH groups on sugars, and glycerol. The CHOH groups on sugars logically makes sense to only be present in the bock because bocks are more malty, meaning they have a higher sugar profile. So having a presumed sugar peak correlates very well with the bocks style. Additionally, the CHOH is an alcohol group attached to the sugar, bocks are traditionally higher alcohol so having these present makes complete sense in this manner as well. Glycerol is said to make the beer's "body" fuller, increase sweetness, and decrease bitterness.[129] All of these factors play greatly into bocks' traditional darker, more full, and heavier malt profile. Logically confirming that glycerol is most likely present in Figure 11's spectra. Even with the clearer peaks in the lyophilized bock spectra some peaks were still unidentifiable. These peaks range from 1.6 - 2.4 ppm. While there is no certainty as to what these peaks represent, an important thing to notice is that these do not show up in either of the saison final products NMR results. This means that whatever these peaks are, are specific to the bock and influence what makes a bock a bock.

[129] Zhao, Procopio, Becker, Flavor impacts of glycerol in the processing of yeast fermented beverages: a review, 3

HUMULONE EXTRACTION

In this research, humulones have been established as a critical component of what makes beer, beer in today's world. Not only do they hold major implications in the flavor and aroma of beer, but also increase shelf life which, as discussed previously, has various social and economic effects. While the path to studying humulones was long and windy, eventually this method of extraction proved useful in providing data that shows evidence of iso-humulones in beer. Below, the results of an iso-humulone standard can be seen. Identified humulones in this spectra have the possibility to be trans-isocohumulone, trans-isohumulone, or trans-isoadhumulone.[130] Tests were conducted on extracted humulone solutions from multiple beer samples.

128 Scan ^{1}H NMR of Concentrated Iso-humulone Standard

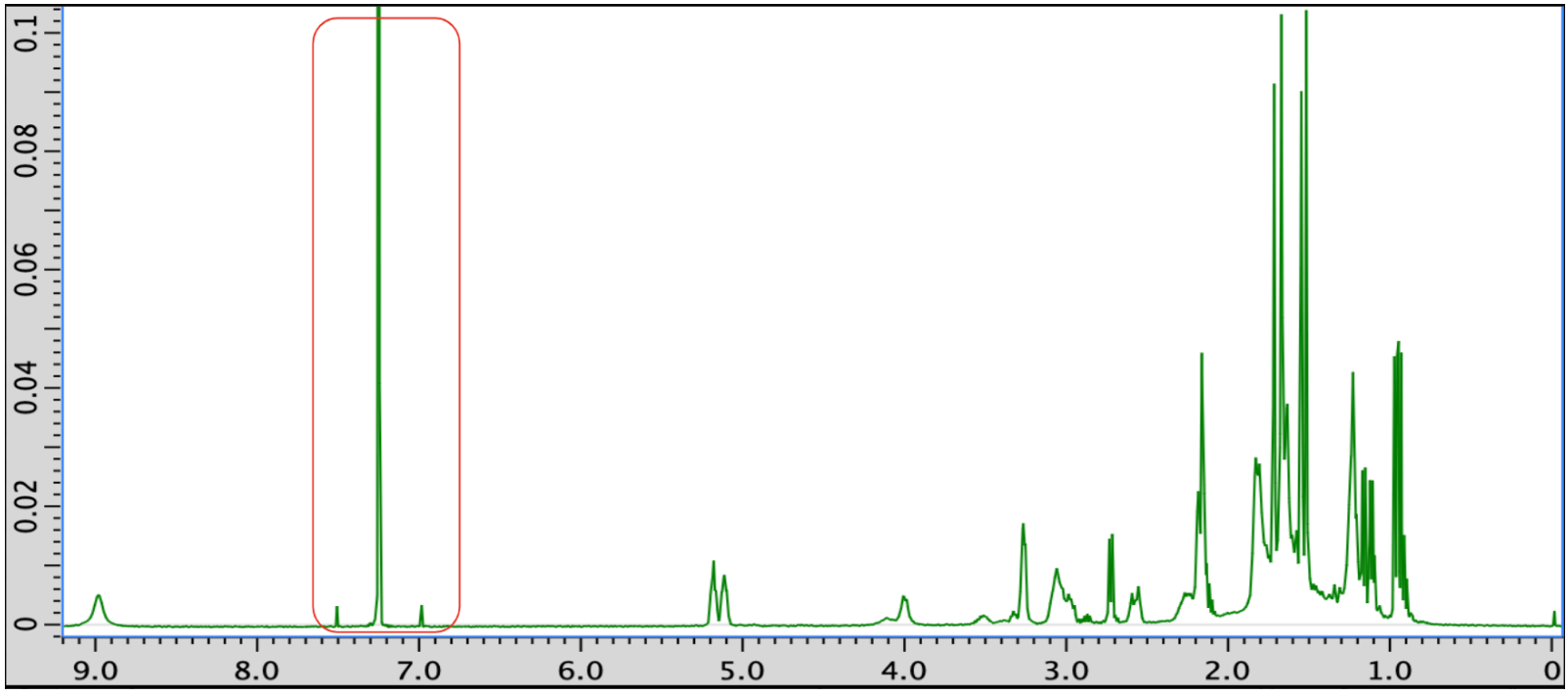

Figure 12: The colored boxes correspond with figures 13 and 14. Matching colored boxes mean it is the same peak.

[130] American Society of Brewing Chemists

Figure 12 shows a wide variety of peaks, ranging from 0.8 -9.0 ppm. These peaks are used as an analytical baseline or reference point for the other beer samples. If the various post-extraction beer samples have peaks that resemble the ones in Figure 12, it confirms that iso-humulones are present in that sample. Confirming that iso-humulones are present is important not only so that it can be confidently stated in research, but also to develop new methods to analyze humulones in beer, and to compare humulone spectras from one beer to another to see if there are any specific differences that occur. Identifications in this section don't have to be assumed, as now test results are being compared to a standard rather than previously published research. This allows for complete confirmation to be displayed. Below are samples from a humulone-extracted saison boil product and bock final product. Due to time constraints these were the only two samples that were able to be extracted and analyzed for this paper, they were chosen because they not only provide information about the boil product and final product of beer, but also information about the differing styles.

128 Scan ^{1}H NMR of Extracted Humulone from the Stone Skipper Saison's Boil Product

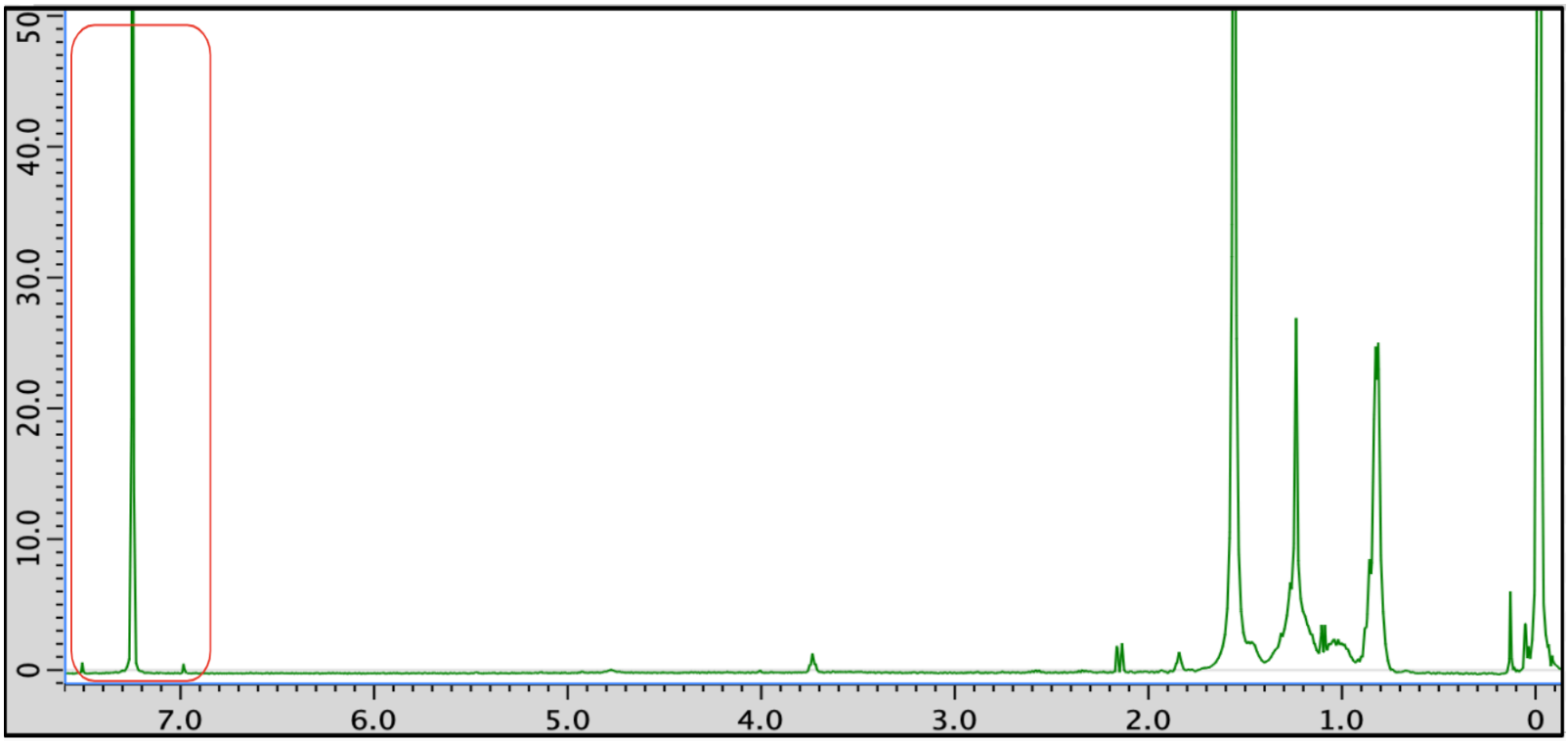

Figure 13: Peaks with boxes around them mean they match with the corresponding-colored box in Figure 12.

In Figure 13, there can be one exact match seen. This comes with the three singlets at 7.0 ppm, 7.3 ppm, and 7.6 ppm. This exact match confirms that the saison's boil product contains some form of iso-humulone. No other peaks could be confidently connected back to the standard. The peak near 3.75 ppm, and the peaks near 2.0 ppm have vague similarities to the corresponding peak ranges in Figure 12, however, none of these similarities came with enough confidence to make the connection. The peaks appearing to the left of 2.0 ppm should all be humulone peaks, as the extraction was designed to eliminate all other compounds, they just may not be isohumulones. In order to discover this, more standards are needed for comparison. Peaks in the 1.0 - 2.0 ppm range cannot be confidently studied at all due to that being the range where octanol produces peaks.[131] Although the solution was placed in a vacuum with the intent

[131] 1-Octanol(111-87-5) ^{1}H NMR (2017)

of removing the octanol, there is more than likely still some remaining. This remaining octanol would produce peaks in this range and make there be no certainty that any of these peaks are iso-humulone peaks. Below the humulone extraction from Michelob's Amber Bock final product can be seen.

128 Scan ^{1}H NMR of Extracted Humulone from Michelob's Amber Bock

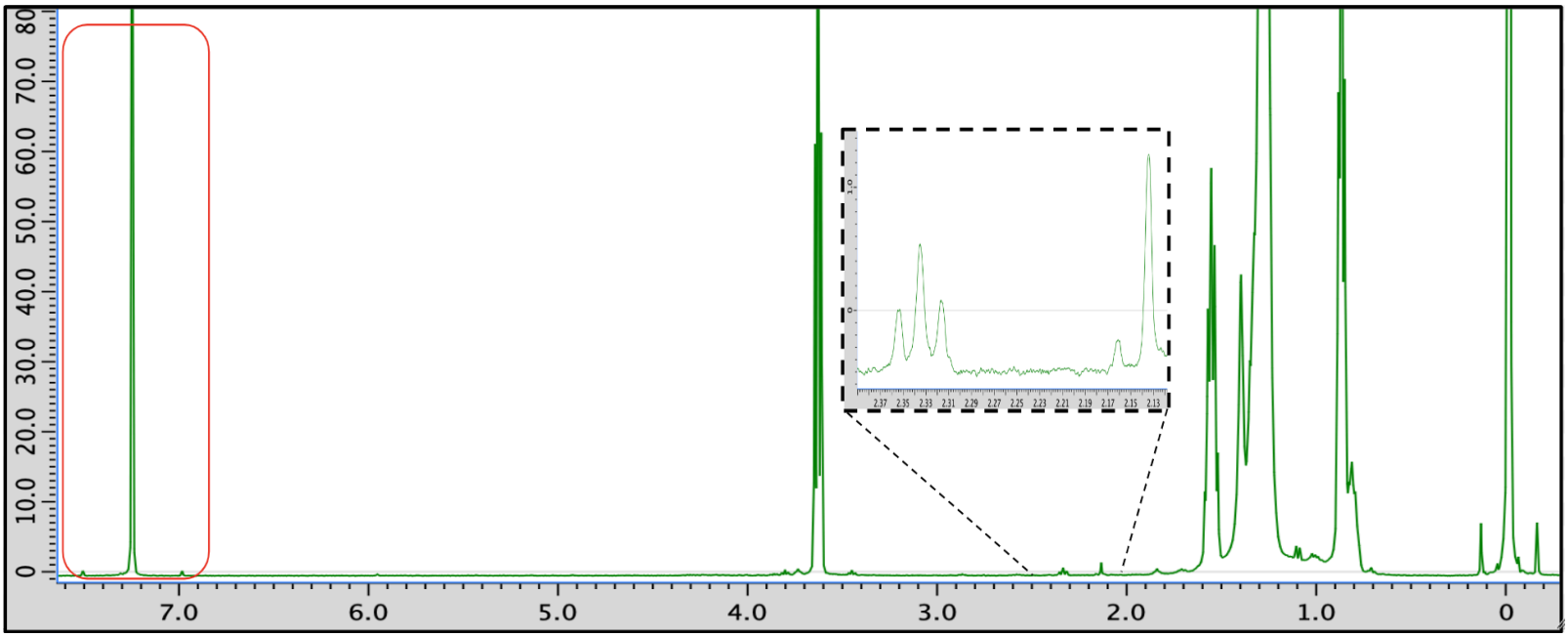

Figure 14: Peaks with boxes around them mean they match with the corresponding-colored box in Figure 12.

In Figure 14, the same set of peaks in the 7.0 - 7.6 ppm range that were present in figures 12 and 13, is also present in the bock's final product. Unfortunately, this sample follows the previous trend of not showing any more correlating peaks. Some are close such as the ones near 2.0 ppm, but again, there is not enough correlation to confidently make the connection. Additionally, near 4.65 ppm there is a massive set of peaks, these do not correlate with any peaks in Figure 12, but theoretically should be some variant of humulone. The same concerns in the 1.0 - 2.0 ppm range that arose in Figure 13 also appear here. Even though the sample was

intentionally attached to the vacuum longer, it appears that some octanol remains. There is a possibility that using a vacuum to remove every bit of octanol may not be possible and a new method for removal may be necessary.

While not many connections were able to be made for either the saison's boil product, or bocks final product, there are some interesting differences between the two spectra that are worth mentioning. After the octanol interference, in the saison's boil product a singlet and doublet are seen near 2.0 ppm, whereas for the bock's final product there is a triplet and what appears to be two singlets. These are all at different ppm locations. In addition to this, at 3.7 ppm the saison expresses a singlet, whereas with the bock there is a large triplet at 3.7 ppm, and many smaller peaks surrounding it. These are clear differences between the different styles and stages, each having the potential to represent an array of humulones. Having these variations does show that these two spectrums are different and humulones inside of them act specifically to make that style of beer its own. Since these are taken from two different brewing stages it is difficult to make definite claims and direct comparisons, however, with additional experiments a clearer similarity test will be able to be conducted.

HSQC

Making the initial identification of compounds in beer is a crucial step in discovering more about the brewing process, beers development, and the chemical profile of beers. However, establishing future methods for identification is equally important for exterior studies to continue. HSQC was used to do this. Below the results can be seen of the HSQC testing on the lyophilized boil and final product of the saison. Due to water and ethanol dominating the straight sample's spectra, those results were not included. Additionally, proper data for the air-dried samples could not be conducted and every Amber Bock version did not result in a successful ^{13}C NMR result, so those were not able to be analyzed in HSQC at this point in time.

HSQC Results of Fully Lyophilized Saison Boil Product

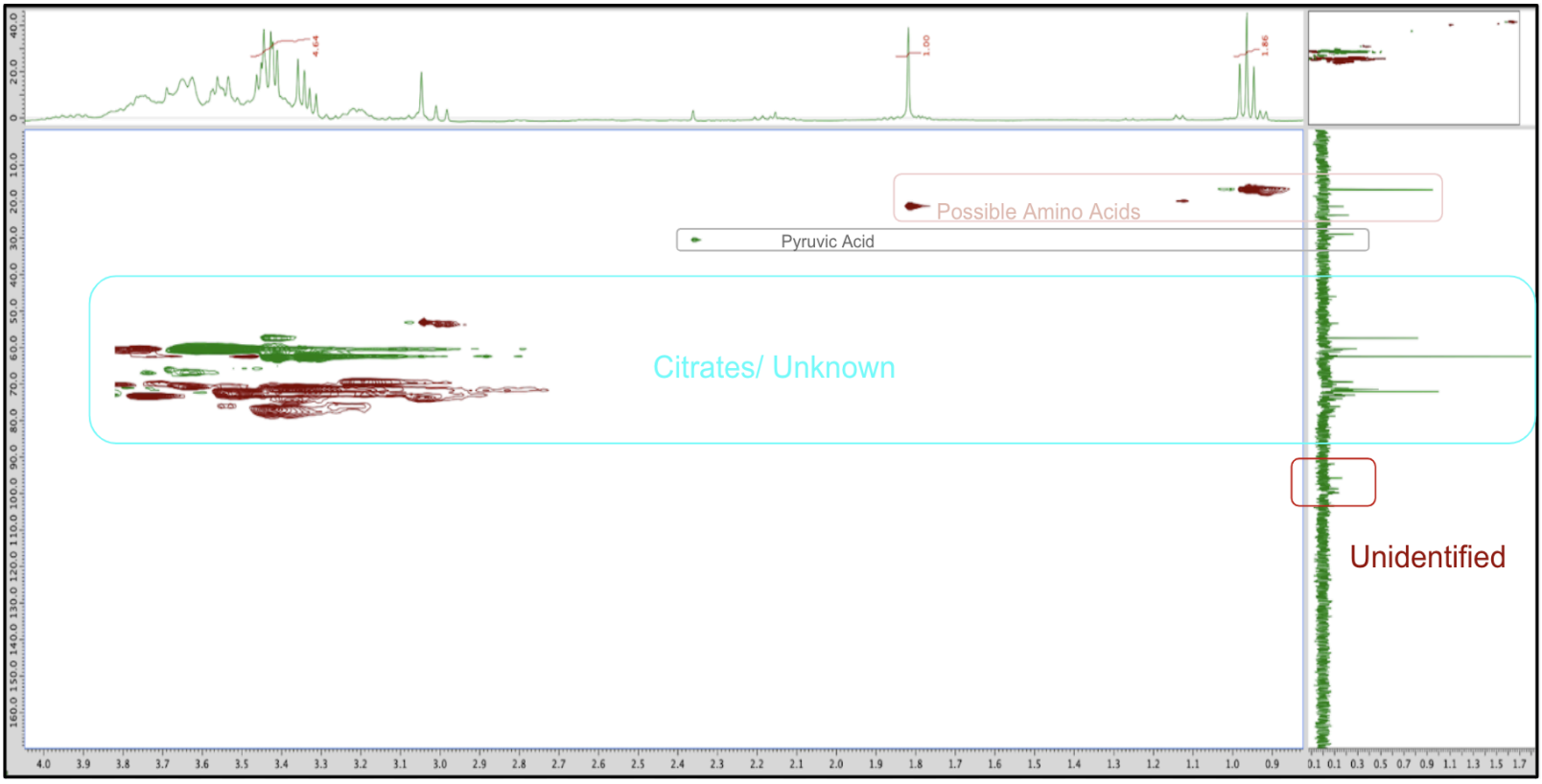

Figure 13: HSQC Result of a 128 scan ^{1}H NMR (top) and 256 ^{13}C NMR (right). The proton NMR's x-axis is in ppm, whereas the y-axis is abundance. The carbon NMR's x-axis is abundance, and y-axis is ppm. All possible products are labeled as accurate as can be.

When looking at Figure 13, useful ^{13}C NMR data is provided in this experiment, as there are matches between the proton and carbon NMR. This allows for potential use of ^{13}C NMR as an analytical tool moving forwards. While in the saison's boil product there was a lot of uncertainty as to what some peaks represented it seems that the potential amino acid triplet at 0.9 - 1.0 ppm for the proton NMR matches with a peak in the range of 14.0 - 18.0 ppm in the carbon NMR. As well as the potential amino acid singlet peak at 1.8 ppm on the proton NMR matches with the ^{13}C NMR peak at 20.0 - 22.5 ppm. What these correlations show is that when peaks appear in those ^{13}C NMR ranges, they have the potential to be amino acid peaks. Continuing off of this, a peak at 30.0 ppm on the ^{13}C NMR corresponds to the singlet at 2.35

ppm on the proton NMR that represents pyruvic acid. The conglomerate of peaks from 2.7 - 4.0 ppm on the proton spectra was largely unidentifiable in the above research, however, it is worth noting that peaks in this range correlate to the following peaks on the carbon NMR spectra: single peaks at approximately 52.0 ppm, 55.0 ppm, and 62.0 ppm, as well as a group of peaks at approximately 60.0 ppm, and 68.0 - 82.0 ppm. Additionally, in the ^{13}C NMR, a set of peaks arise that do not coincide with any ^{1}H NMR data. These peaks are present at 90.0 - 105.0 ppm and will be analyzed further in order to determine what they are produced by.

The HSQC results from the lyophilized final product of the saison can be seen below, here some of the data matches what is mentioned above, but new identifications can be made as well.

HSQC Results of the Fully Lyophilized Saison Final Product

Figure 15: HSQC Result of a 128 scan ^{1}H NMR (top) and 256 ^{13}C NMR (right). The proton NMR's x-axis is in ppm, whereas the y-axis is abundance. The carbon NMR's x-axis is abundance, and y-axis is ppm. All possible products are labeled as accurate as can be.

Figure 14 establishes that the 2,3 - butanediol –usually found at 0.9 - 1.0 ppm– correlates to a ^{13}C NMR peak at approximately 14.0 ppm. Additionally, the cluster which potentially represents choline, citrates, malic acid, and betaine at the 2.9 - 4.2 ppm range on the ^{1}H NMR again represents a cluster of unidentifiable peaks in the 55.0 - 80.0 ppm range on the ^{13}C NMR. As a result of the proton NMR and HSQC spectra being cluttered and having indistinguishable peaks no individual peak identifications can be made or related to the carbon NMR. However, it once again confirms that these compounds do appear in the 55.0 - 80.0 ppm range of carbon NMR, specific locations just cannot be confirmed. Sugars and maltodextrins can also be connected to carbon NMR peaks at approximately 92.0 ppm, 100.0 ppm, and 103.0 ppm. There

is an unidentified proton NMR doublet peak that appears around 4.45 ppm, which correlates with a ^{13}C NMR peak at 95.0 ppm. The contents of what this peak represents is not yet identified.

Through the use of HSQC, suspected compounds such as sugars, maltodextrins, 2,3 - butanediol, and pyruvic acid can all be confidently identified on a carbon NMR spectra. Compounds such as amino acids, citrates, choline, malic acid, and betaine's general ppm range have been discovered for ^{13}C NMR, however, specifics of where exactly they appeared were not able to be determined. This allows for carbon NMR to become a more viable analysis tool moving forward in analytical beer studies. HSQC thus proves useful in creating new carbon NMR identifications and allows for additional research to be conducted on this topic using carbon NMR as a base for research.

CONCLUSIONS

In this work the historical and societal significance of beer was established. Throughout time, beer has played a crucial role in how societies function. Whether it be bringing different social classes together, experiencing religion through beer, or changing how countries' economies operate, beer has had a major impact in the world's development. The styles of the saison and bock were historically covered in-depth in order to paint a wider picture of why that individual style matters, as well as to showcase its impact on the world. Chemically, NMR tests showed that beer's chemical profiles do differ throughout their developmental stages as well as between styles. While identifications were educated assumptions based on previous research, they still indicated clear differences between the beer styles. Through these identifications, correlations were able to be made between the beer's stereotypical flavor, aroma, and appearance and the results seen in the NMR. Discussions on the logic of different identifications were also discussed so a clearer understanding of what the beer typically is, and what it chemically appears to can be made. Using an iso-humulone standard, humulones were proven to be in each sample that was analyzed. Building off of this, the importance of humulones and hops in beer was showcased.

Although not directly related to information gained about the beer's history or chemical profile, a major discovery of this project was the methodology and procedures that were used. A large amount of time in this research was spent on perfecting analysis methods so that the beer's NMR results lacked interference from water and ethanol, were clear and easy to interpret, and unveiled everything that could be unveiled in the beer sample. In these experiments we demonstrated that air-drying and lyophilizing beer samples was effective in producing more detailed spectras that also lacked interference. Not only this, but through the use of HSQC.

Carbon NMR spectras with assumed identification were established. Doing so allows for carbon NMR to be utilized in future analytical beer research and provides another analysis viewpoint when studying beer.

FUTURE PLANS

While the work for this thesis is complete, the scientific research will continue on. In future research a main focus will be to shift from using previously published research as a baseline of analysis, to using chemical standards for identifications. This will eliminate a vast amount of the speculation that came with compound identification. On the other hand, more literature research is needed in order for additional compounds to be initially identified in NMR. While extensive research has been conducted, there is a small chance more could be unveiled about the compounds in the beer. However, what we have now is a very good spectra with great identifications.

Testing will continue using the methods described in this paper. For NMR, the main focus will be conducting experiments on Lost Hollow's "The Empire Strikes Bock". Once this is completed, a bocks development from boil product to final product will be able to be studied as well as two different bock's final products will be compared to see if there are noticeable chemical differences between two beers of the same style, made from two different breweries. In addition to this, more humulone extractions will be performed on every sample mentioned in this paper in hopes to learn more about humulone profiles in beers. More HSQC runs will be completed on these samples as well so that additional ^{13}C NMR peak assignments can be made.

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