Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

Kristoffer Zotchev

High-Resolution 1D ^{1}H Nuclear Magnetic Resonance, Multivariate Analysis and Heteronuclear Single Quantum Coherence Spectroscopy for Comparing Different Sample Preparation Methods and Styles of Beer

Master's thesis in Chemical Engineering and Biotechnology
Supervisor: Eivind Almaas
June 2020

NTNU
Norwegian University of
Science and Technology

Summary

The consumption of beer is deeply embedded in Norwegian history, all the way back to when the vikings celebrated after a day of successful plundering. Worldwide, the variety of different kinds of beers is greatly increasing, with new microbreweries popping up at an impressive rate. Despite the large popularity of beer throughout history, little research has been done on beer using nuclear magnetic resonance (NMR). NMR has previously been used in wines and spirits to gain information on the compounds associated with taste and alcohol content, as well as to ensure product quality. In the present study, we sought to develop a protocol for analysing beer in NMR. The main aim of the study was to see whether there is a difference between the degassing and buffering methods applied to the samples, and which method is most suitable for the creation of a standard NMR protocol. The second aim of this study was to see whether there are any chemical differences between the beer styles that can be detected in NMR. In order to develop such a protocol, we used six different types of beer: stout, IPA, weissbier, lager, sour-stout, and tripel. Each beer was split into twelve samples, of which three were buffered and vortexed, three were buffered and ultrasonicated, three were vortexed and not buffered, and three were ultrasonicated and not buffered. NMR was performed on a Bruker Avance III HD 800 MHz spectrometer, equipped with a 5-mm Z-gradient CP-TCI (H/C/N) cryoprobe at the NV-NMR-Centre/Norwegian NMR Platform at Norwegian University of Science and Technology (NTNU). We performed PCA to compare the different sample preparation methods for each individual beer type. The results showed that the largest difference between buffered and unbuffered samples appears to be their pH, as made evident by the large proportion of variance explained by PC1. PCA did not appear to identify any differences between the degassing methods. The NMR analysis combined with Heteronuclear Single Quantum Coherence (HSQC), revealed that the stout beer had highest intensity peaks related to amino acids, whereas the tripel beer had more than twice the intensity of the other beer styles for the peaks related to lactic acid. For the suggested, but not confirmed, peaks related to histidine, the lager and IPA had the highest intensity, whereas stout had the highest intensity for the peaks related to formic acid. In sum, our results show that buffering beer samples is highly recommended for simplifying the NMR analysis and comparison of the samples. Further, our results demonstrate that NMR analysis can be used

to identify compounds associated with alcohol concentration, vicinal diketones, and esters in beer.

Table of contents

1. Introduction

Beer is strongly embedded in Norwegian culture, and is rich with history. All the way back in the age of the vikings, beer had several mentionings in the book “Poetic Edda”. There was even a god named Ægir, who was dedicated to the art of brewing beer (Poetic Edda, unknown). When Christianity made its advance and crowned itself as the state religion, beer was still as important. It was even enforced by both the state and church upon the citizens to brew a certain amount of beer for each winter. Those who did not abide by these rules were punished by expulsion (Law of Gulating, approx. 1100). These laws may have laid the foundation of the different brewing cultures that can be found scattered across the Western part of Norway today (Preiss et al, 2018).

In spite of the long and copious history of beer, little research has been done on beer using nuclear magnetic resonance (NMR). NMR is a research technique that is commonly used in quality control and research to detect, identify and quantify the content of a certain sample. It has previously been used for research on both expensive wines and spirits in order to ensure a satisfactory quality of the products produced and to establish an understanding of the compounds that are present in these beverages. As the NMR research on wines and spirits has provided interesting and valuable data for its producers, one may ask whether the absence of NMR research on beer is due to the general public considering beer as an overly cheap and uninteresting beverage with little character? Studies that have been performed on beer using NMR have mainly focused on characterizing the composition of the beer for various reasons. In a study by Duarte et al (2002), they were able to identify thirty different compounds in seventeen different beers by using multivariate analysis on ^{1}H NMR spectra. In another study, Almeida et al (2006) also utilized multivariate analysis on ^{1}H NMR spectra gathered from analyzing twenty seven different samples, but to see the effects of brewing site and date of production by obtaining information about composition variability.

In recent years there has been a noticeably sharp increase in the sales of special beer styles (Misje, 2013). It appears that it is more common nowadays to enjoy beer in the same way one might enjoy an expensive wine. This peak in interest may also be a great opening for more

advanced research combined with NMR, to find out what the differences between these special beer styles are, and what the actual content of compounds is.

In this study, we sought to develop a protocol for analysing beer in NMR by utilizing six different beer styles, using different degassing and buffering methods when preparing the samples. **The main aim of this study was to see whether an NMR-based analysis can distinguish between the different degassing and buffering methods applied to the samples, and whether this analysis can help determine the most suitable method for creating a standard protocol for preparing beer samples for NMR analyses.** Due to the Covid-19 pandemic, the laboratories at NTNU were forced to shut down. Therefore, further laboratory work for this study was immediately halted, creating more time for statistical analysis of the already gathered data. This resulted in an additional goal that was added to this study. **The second aim of this study was to investigate distinct chemical profiles of the different beer styles.**

2. Background and theory

2.1 Beer - The Einherjar Beverage

In the old age of norse mythology, where vikings would fight courageously in the battlefield, deathwights would watch over them. These wights are better known as valkyries; female warrior-like beings, riding horses in flocks through the sky. Valkyries are sent out by the wisest and arguably the most powerful aesir (god), Odin, to choose who is to be slaughtered in the battlefield. The fallen men are known as einherjars, and are carried by the valkyries from the battlefield to Asgard, where Odin welcomes the most bloodthirsty warriors to Valhalla, and Frøya welcomes the more honorable to Folkvang. In Valhalla, the valkyries will serve both Odin and the einherjars meat from the hog Særimne and beer. From here on out, during daytime, the einherjars will fight each other in the yard, and later during the evening sit down as friends and enjoy beer together until Ragnarok (illustrated in Figure 2.1.1).

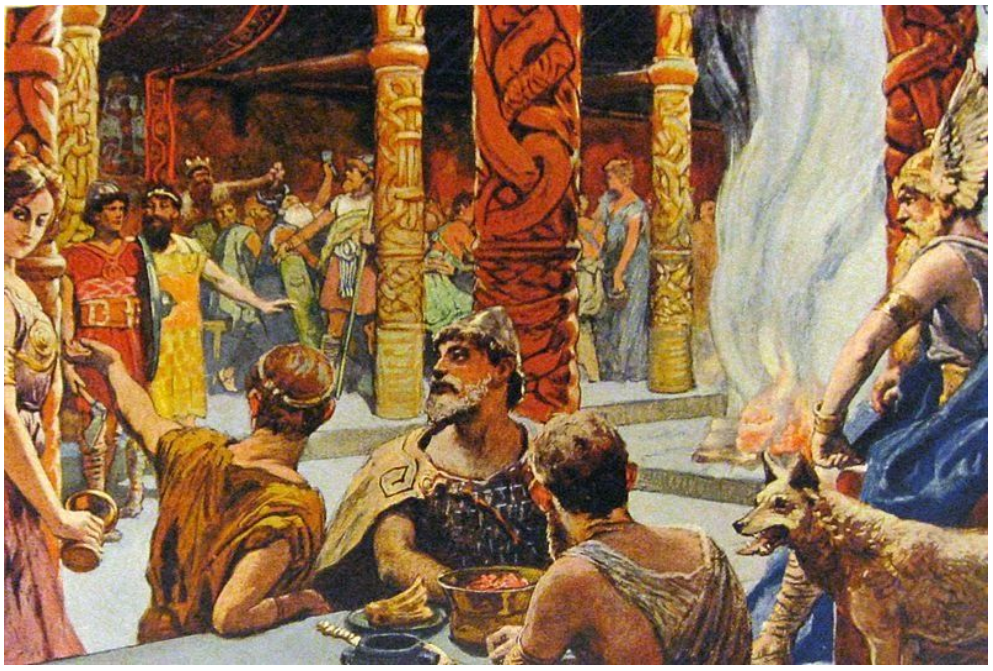


Figure 2.1.1: Fallen warriors, einherjars, being served beer in Valhalla by the valkyries. Odin sits to the right in a gold winged helmet (Illustration: Emil Doepler, approx. 1905).

The tales of valkyries serving einherjars beer in Valhalla is described in the book of the Poetic Edda. In this book, a wisdom poem called *grimnesmål* (grimnes speech), told by Odin himself, goes as follows:

Hrist ok Mist
vil ek at mér horn beri,
Skeggjöld ok Skögul,
Hildr ok Þrúðr,
Hlökk ok Herfjötur,
Göll ok Geirölul,
Randgríðr ok Ráðgríðr
ok Reginleif;
þær bera Einherjum öl.
(Poetic Edda, unknown)

This poem simply says that the valkyrie Hrist and Mist shall bring Odin's drinking horn to him, and Skjeggold, Skogul, Hild, Trud, Hlokk, Hærfjotur, Goll, Geirolul, Randgrid, Rådgrid, and Reginleif shall bring beer to the einherjars. Although feasting and beer consumption among aesir and einherjars in Valhalla is an important and central theme throughout norse mythology, there are also other stories that express the strong importance of beer and how embedded it was in the socio-cultural ideology for this era.

One of these stories is about the jotun Ægir and his yearly feast with the aesir. Jotuns are giant supernatural creatures (illustrated in Figure 2.1.2) and commonly described as evil, dumb, and enemies of the aesir. However, Ægir, the ruler of the seafaring waters, is more of the friendly kind, at least towards the aesir. According to Hymiskviða (Hymeskvadet - story of Hyme), Ægir owned many boilers; enough to cook food for all the aesir for a yearly feast. However, he did not have a big enough boiler to brew beer for them all. Therefore, the feast was to be held on one condition: that the aesir had to fetch him a boiler big enough. The aesir Thor accepted this request, and was told that his father Hyme (another jotun) was in possession of a boiler that was one kilometer wide, and big enough to brew beer for them all. Thus, Thor went to Hyme for the boiler, but was challenged by Hyme to break his drinking

cup for the boiler. The task was thought to be impossible, because Hyme knew that it could only be broken by his own forehead. However, Thor, smart as he was, found out and threw the cup at Hymes' forehead and it was shattered. Therefore, Hyme gave Thor the boiler and told him to leave. Ægir was given the boiler, and he was to be known as the master in the art of brewing beer from here on.

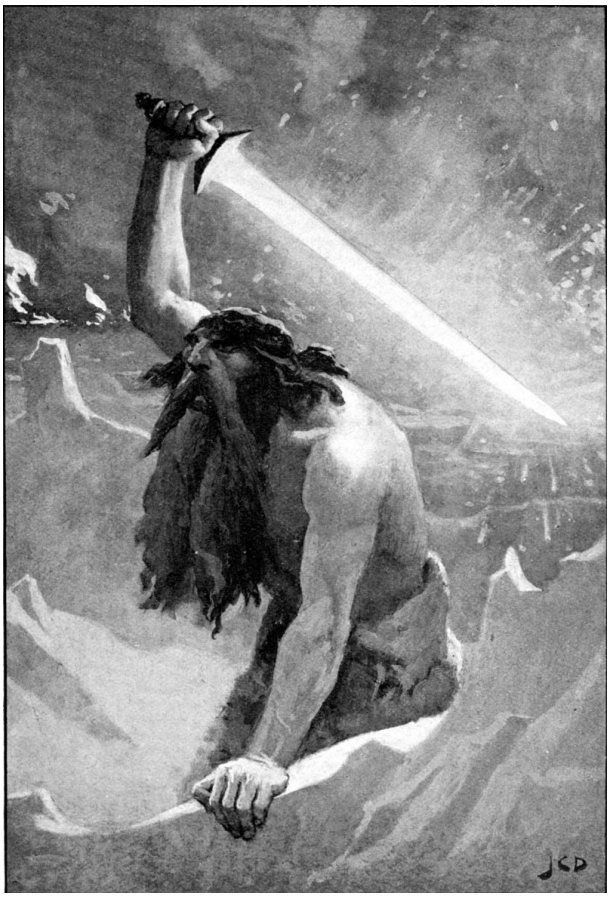


Figure 2.1.2: Illustration of a jotun (Surtr) guarding Muspellsheimen with a sword of fire (Illustration: John Charles Dollman, 1909).

It is not known who wrote the Poetic Edda, but it stems from oral storytelling tradition in the Northern countries probably as early as the 8th century, and was written down during the second half of the 13th century (Store Norske Leksikon, 2019). Even though the book is mainly composed of stories about gods and heroes, it can be interpreted that beer played an important role in social interactions, considering it being mentioned several times by the gods themselves.

2.1.1 Stone beer

When reading the stories about Ægir and his one kilometer wide boiler, one cannot help but ask oneself; how did the vikings make beer? When archaeologist Geir Grønnesby conducted several small excavations on 24 farms in Mid-Norway, he would almost always find a thick layer of stones that were cracked into smaller pieces due to heat treatment. These stones turned out to be from the viking age and earlier and were used for beer crafting. Brewers would heat up the stones until they were glowing red hot and drop them into wooden mashing vessels containing the mash. This sudden change of temperature in the rocks would cause them to crack open, and with enough cracks, they would be replaced by new rocks. Interestingly, in some places, it has been reported that the piles of cracked rocks are so huge, houses have been built on top of them (Bazilchuk, 2017).

2.1.2 The Law of Gulating

From literature and archaeological evidence, we can say with certainty that vikings enjoyed beer just as much as mead. By the great myths, it was told that the aesir Odin gave beer to the humans. Other than that, nobody really knows how beer made its arrival to the Nordic countries (Mortensen and Johnsen, 2009).

When Christianity entered the North in the year 1000 and was declared the official religion in the year 1020 (Pedersen and Sigurdsson, 2019), one would believe, from a modern view on the Christian religion, that people would be forced to abstain from alcohol. Yet, alcohol consumption was only to be sustained. During the Christianization, autumn blot, midwinter blot, and summer blot were changed to Mikkelsmesse, Christmas, and Easter, and the habits of alcohol consumption remained the same throughout this spiritual conversion (Mortensen and Johnsen, 2009).

The importance of continuous beer flow was so fundamental at the time, both in the era of vikings and Christianity, that a Norwegian committee called Gulatinget (in cooperation with the church after the Christianization) imposed the residents in some regions of Norway to

brew a minimum amount of beer each year. This committee was a public assembly that covered the Western counties of Norway, where it exercised judicial and legislative power. The laws of Gulatinget were believed to be passed on orally between the members. Thus, it was each individuals' task to remember the laws without any writing. However, with the Christianization of Norway, the written language became more widespread, and the laws were eventually written down. Today we know these laws by the book of 'Kristendombolk', which consists of several books written at the time of Gulatinget. Although the book mostly consists of Christian injunctions and prohibitions, one can also find rules and regulations securing beer production among the farmers of the land (Law of Gulating, approx. 1100). An excerpt from this book goes as follows:

The next thing is that we have promised beer brewing. That's what people call co-beer. One mæle (16.2 liters) malt to beer from the farmer and another from the housewife. At least three farmers will make it together, except for farmers who live so far out in the islands or in the mountains that he can't carry his beer to other men. Then he alone will brew as much beer as each of the others. Anyone who owns a farm with fewer than six cows or six sown seed (just under 600 liters of grain), he will only brew beer if he wants. The beer must be brewed before Halloween. And the beer must be signed as a thank you to Christ and to St. Mary for the year and to peace. If this is not done by the deadline, they will pay a penny to the bishop and brew beer, even if it is later. If someone does not comply, and is found guilty of sitting for 12 months and does not brew beer, he shall pay three marks for it to the bishop. (...) If someone sits for 3 winters and does not keep beer brewing, and he is found guilty of it, and does not pay the fines that we have imposed for our Christianity, then he has spent every penny of his property. Then the king owns half, and the bishop half. He has the opportunity to go to scripture and remedy for Christ, to be able to stay in Norway. But if he will not, then he shall depart from the land of our king (Law of Gulating, approx. 1100).

The law states that each farmer must brew beer from 16.2 liters of malt, both for the farmer and the wife, and in groups of three farms. Smaller farms may be exempt from the law. However, if those who are required to brew do not follow the law, they will be fined and in the worst case lose their land and be punished by expulsion (Law of Gulating, approx. 1100).

2.2 How beer is made

Figure 2.2.1 illustrates the different steps in the process of beer production on an industrial level (Wunderlich and Back, 2009). The process begins with malt, which is crushed and mashed. The mash is boiled, cooled down and fermented before it is maturated and packed to be ready for consumers. Beer is, as we will see, a product of a long line of microbial activity, and most of these activities are favourable. To avoid unwanted microbial activity, it is of great interest, both for home-brewers and large companies, to control these steps carefully through management and intelligent design.

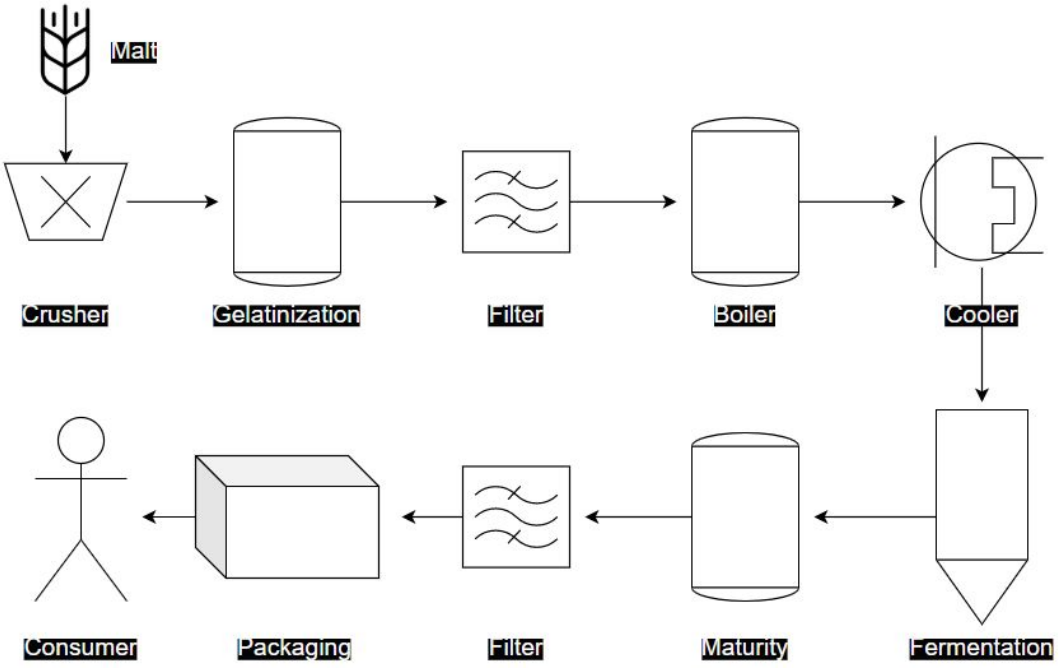


Figure 2.2.1: A simplified flow chart of an industrial beer production. Showing all stages of brewing beer from mashing of malt to packaging and before delivering to consumers.

2.2.1 Malting

For every brewer, independent of size and production quantity, there are three fundamental steps in beer production: malting, mashing and fermentation. Malting is the first step of the production process, and it is here the raw material, cereal, is converted to malt. Malting can

be further divided into three steps called steeping, germination, and kilning. This process is illustrated in Figure 2.2.2, and water is the controlling unit between the stages.

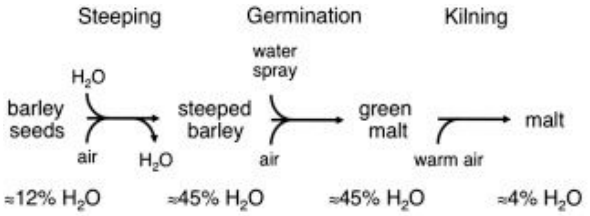


Figure 2.2.2: Illustrates the three steps - steeping, germination, and kilning, of the malting process of barley (Fox, 2018).

Steeping is where cereal is combined with water at a set temperature and this process itself initiates the germination process. In this step, certain enzymes will be synthesized, and the degradation of the protein matrix and cell wall starts. When the cell wall is degraded, starch-degrading enzymes will approach the starch granulates, thus the germination is started. The germination step will require 16-20°C for a period of four to six days. Kilning is the step where the germination is terminated, to stop further degradation, by drying out the barely. This is where the flavour of the malt develops, thus it is a very crucial step (Fox, 2018). The cereal is now considered malt, and it is ready for mashing.

2.2.2 Mashing

Mashing is the process where starch rich malt is turned into mash, which is a dense solution of fermentable sugars. First, the malt is crushed by a mill, to ensure sufficient distribution of starch in the water. Crushed malt is then poured into a mashing tank, containing appropriate volume of water and temperature (usually 64-66°C), depending on the recipe. The temperature of the water is crucial for this operation due to the activation of sugar degrading enzymes, primarily *α-amylase* and *β-amylase* (Ensminger, 1994). The release of these enzymes will start the degradation of starch into smaller sugar molecules, often referred to as disaccharides and oligosaccharides. These carbohydrates are fermentable sugar molecules, meaning they are food for the yeast (yeast metabolomics will be further discussed in section 2.4). Breaking the glycosidic linkages in starch is a time consuming process, thus it is important that the temperature stays the same throughout the process and the distribution of

starch and enzymes is well mixed. Therefore, a mashing tank is often equipped with a heating element combined with a thermostat, ensuring a steady and desired temperature over time. Also, a mixer, usually a rotary blade, is mounted at the bottom of the tank, creating a turbulent flow ensuring maximum contact between the starch and enzymes, thus speeding up the process. Mashing usually takes 1-2 hours, depending on the amount of malt, water volume and temperature. The end product, the highly dense sugar solution, is called the wort, and is boiled for 1-2 hours depending on the recipe. The boiling is to ensure that the solution is disinfected and to stop the enzymatic breakdown of starch (by inactivation of the amylases). It also removes any volatile byproducts of the enzymatic reaction that may cause some off-tastes. It is in the boiling process that hops and other trivial ingredients like vanilla and cocoa are added. After sufficient boiling, a coiled heat exchanger is used to cool the wort down to pitching temperature (usually 20°C), and the wort is ready for fermentation.

2.2.3 Fermentation

During fermentation, the wort is transferred from the mash tank to a fermentation tank. Fermentation tanks come in many shapes and sizes depending on the volume of the wort. Big companies that brew large quantities for the public usually ferment in large industrial cylindroconical vessels, whilst a homebrewer will only require a small bucket. At this point in the process it is essential that all equipment that is to be used is sterile and free from any microbial organisms. This is due to the wort being rich in carbon sources and an easy target for unwanted bacteria that may cause undesirable byproducts in the beer. When the wort is added to a suitable fermentation tank, yeast is added. The type of yeast depends on the beer style and recipe. The wort is now subject to fermentation, and the yeast will start to metabolize the monomeric carbohydrates and, under anaerobic conditions, produce ethanol and carbon dioxide as byproducts (Boulton and Quain, 2013). There are many factors that affect the fermentation time, such as fermentation temperature, type of yeast, original yeast cell concentration, pressure, amount of carbohydrate in the wort, etc. However, yeast fermentation for traditional beer styles usually takes 1.5-2 weeks. When fermentation ends, the wort has become what we call beer, and is ready for packaging.

2.3 *Saccharomyces cerevisiae*

S. cerevisiae (illustrated in Figure 2.3.1) is a yeast species that belongs to the Ascomycota phylum of fungi, and is often called "baker's yeast" or "brewer's yeast" due to its excellent attributes in the craft of wine making, beer brewing and bread baking. Yeast cells are characterized as single cell organisms with favourable diversification in physiological attributes such as carbohydrate metabolism. For the majority of eukaryotic cells, the single monomer of glucose can be considered to be the most important (or at least the most favorable) carbon source for cellular metabolism. Furthermore, these types of cells will under aerobic conditions entirely reduce glucose into carbon dioxide and water. This reaction will yield the maximum amount of energy for the cell, thus causing growth. However, some yeast cells, including *S. cerevisiae*, hold a remarkable ability to metabolize glucose under anaerobic conditions, synthesizing ethanol and carbon dioxide as byproducts instead. This metabolic reaction is often referred to as the Crabtree effect, and is what brewers utilize when brewing beer. The Crabtree effect is a highly sophisticated mechanism that *S. cerevisiae* utilizes to outperform other microorganisms in the race for devouring vital carbon sources in its environment. *S. cerevisiae*'*s* ability to produce high concentrations of extracellular alcohol to eliminate competitors and at the same time be able to tolerate this environment, is not only favourable for the yeast itself, but also essential for brewing beer. Furthermore, yeast cells will to some degree be able to utilize ethanol as a new carbon source after the depletion of glucose (Lin and Li, 2014).

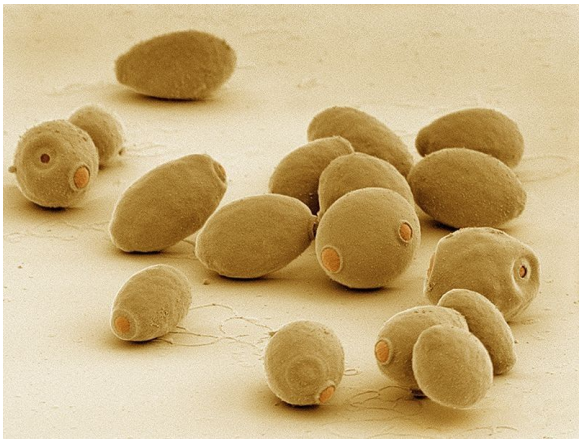


Figure 2.3.1: Microscopic picture of brewer's yeast *S. cerevisiae* (Photo: Thomas Deerinck, NCMIR/Science Photo Library).

2.3.1 Yeast and beer brewing

Perhaps to no surprise (considering the popularity of beer), there is an abundance of different strains in the genus of *Saccharomyces*. However, when it comes to brewer's yeast, one can divide them into two categories: ale yeast, which is a top fermenting yeast, and lager yeast, which is a bottom fermenting yeast (Beer Craftr, 2019). The type of yeast, including other ingredients of course, is what gives beer its character. Thus, generally speaking, one can classify most beers as a lager or an ale.

Top fermenting yeast, such as ale yeast, is fermented at temperatures of 10 to 25°C, yet some of the strains in this category of yeast will not actively ferment at temperatures under 12°C (Beer Advocate, 2019). Due to the higher fermentation temperatures, the ale yeast will produce ethanol and gas at a higher rate than lager yeast. Thus, it will rise and ferment on the top of the wort, hence the name "top fermenting yeast". Also, due to high temperatures, ale yeast produces beer which has higher levels of ester, causing a more distinct character. Ale yeast is used in the production of ales, stouts, porters, and wheat/wit beer (Beer Craftr, 2019).

Lager yeast, on the other hand, ferments at temperatures of 7 to 15°C. This results in a slower metabolization of glucose and a slower rate of carbon dioxide production than for ale yeast. Thus, the yeast will settle at the bottom of the wort, hence the name "bottom fermenting yeast". In addition, cold fermentation causes inhibition of certain chemical compounds that are the bases for some off-tastes in beer (Beer Advocate, 2019). Lager yeast is therefore desired for its capability to produce a cleaner and a purer taste, as can be found in lager, pilsner, and bock beer (Beer Craftr, 2019).

2.3.2 Yeast metabolomics

Although different strains of yeast contribute with different kinds of aromas and sensory attributes to the beer, the basic biochemistry of glucose metabolization follows the same path throughout the genus of *Saccharomyces*. On the surface of a yeast cell, there are two different types of protein-built carbohydrate transporters, as can be seen in figure 2.3.2. It is by these

two transporter molecules a unit of glucose can enter the cell. The first transporter is called HeXose Transporter (HXT), which is a low-affinity glucose transporter. The other transporter is called Galactose Transporter-2 (GAL2) and is mainly used for transporting galactose, however, it is possible for this protein to transport glucose as well. Independent of the path of entry, when a glucose molecule enters a yeast cell, it will undergo a process called glycolysis (Lin and Li, 2014).

Glycolysis of glucose is a 9-step process where glucose will be enzymatically converted to two pyruvate molecules through a series of 8 different intermediates (unless it is galactose, then it will skip the first step). Pyruvate in itself is an intermediate for two different pathways, which is either respiration through the Tricarboxylic Acid Cycle (TAC) or fermentation. The further pathway for pyruvate is determined by the cell's living condition, meaning if it is in supply of oxygen or not. When a yeast cell is metabolizing carbohydrates with the supply of oxygen (aerobic condition), it will utilize the newly synthesized pyruvate in the TAC by enzymatically transforming it to a molecule called Acetyl-CoA. Acetyl-CoA will then be used as an oxidation agent to release energy to the cell for respiration. However, if there is no supply of oxygen (anaerobic condition), the pyruvate molecule will be enzymatically transformed to become acetaldehyde and by the reduction of an energy molecule called NAD^+, into ethanol as a final product (Bailey, 2019).

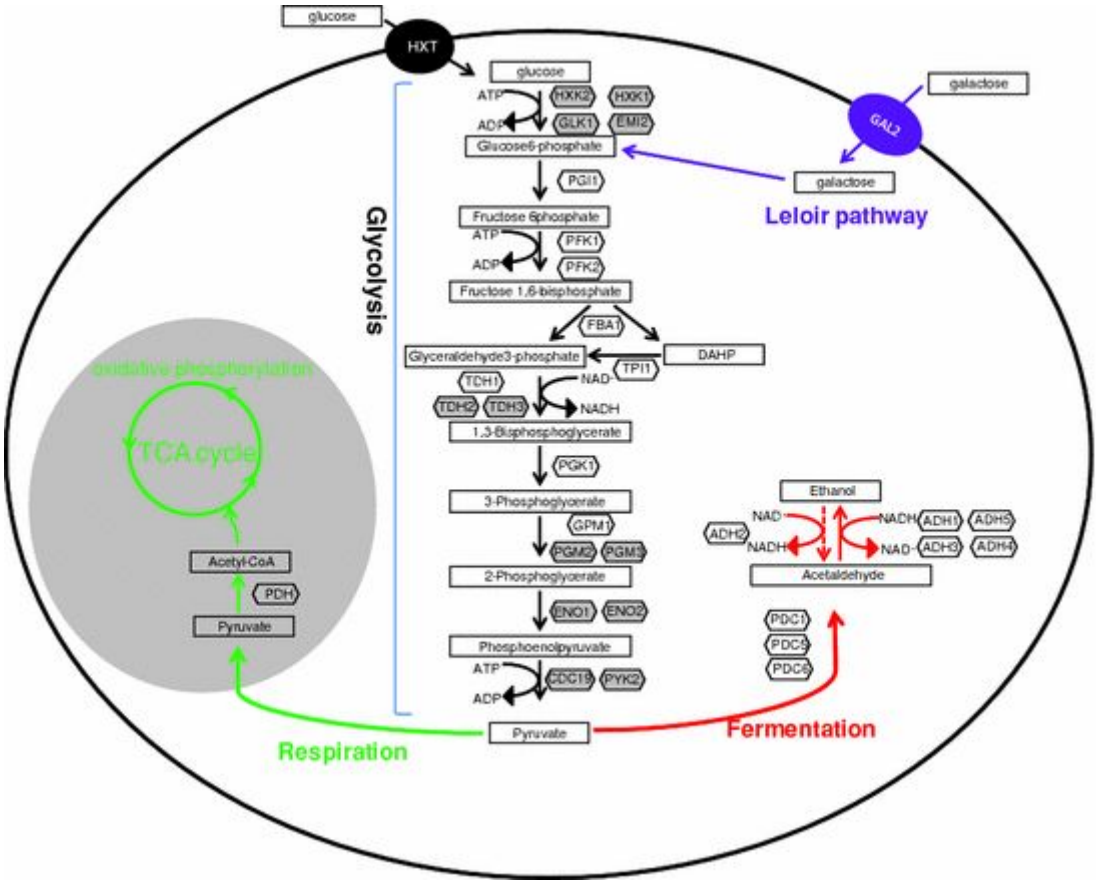


Figure 2.3.2: Schematic illustration of the glycolysis of glucose. The figure illustrates that glucose can enter the cell via two different glucose transporters before entering glycolysis to become pyruvate. Pyruvate can either enter fermentation or respiration cycle, depending on the availability of oxygen in the cell's environment. (Lin & Li, 2014).

2.4 Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) is a form of spectroscopy in the radio-frequency (RF) range of the electromagnetic spectrum. NMR spectroscopy measures the magnetic properties of a certain nucleus that possesses a spin. When placing a sample containing nuclei with half-integer spin (e.g. ½ for 1H and 13C) in an applied magnetic field, the individual spins will populate two energy levels, where the difference in energy levels is ΔE (B_0: strength of the applied magnetic field, : magnetogyric ratio, *h*: Planck constant) (Levitt, 2001).

$$\Delta E = \frac{hB_0}{2\pi}$$

Also, the spin of a nucleus will precess at the Larmor frequency, ω (Beff: effective magnetic field) (Mishra et al, 2017).

$$\omega = -\gamma B_{eff}$$

By exciting nuclei with a radio-frequency that corresponds to the Larmor frequency, an NMR signal can be observed. The electron shell that surrounds the nucleus induces a counter magnetic field (B_i) that interacts with the applied magnetic field (B_0) and partially shields the nucleus (Levitt, 2001). Nuclear spins will therefore experience what is called Effective Magnetic Field (Beff), which determines their spin precession frequency through the previous equation. The interaction of B_0 and B_i is given by the following equation (Levitt, 2001):

$$B_{eff} = B_0 - B_i$$

This electron induced frequency variation is called the chemical shift (δ, expressed in parts per million; ppm), which is defined by the equation below (*v:* frequency in Hz) (Balci, 2005):

$$\delta = \frac{\nu_i - \nu_{TSP}}{\nu_{spectrometer}}$$

The chemical shift is the main component of NMR spectra, and contains information about the electron/chemical environment around the nucleus (i.e. molecular structure). Other parameters that can be extracted from the spectra are: coupling constant (provides information about adjacent atom groups), and signal intensity (which is proportional to the concentration of magnetically equivalent nuclei in the sample) (Levitt, 2001).

There are several requirements that must be met for the NMR-experiment to be successful. These requirements are listed below:

1. Due to the inherent sensitivity of NMR, high concentration of compounds in the sample is required (usually 2-10mg for an organic molecule with a molecular mass less than 600 (University College London, date unknown)).

2. The magnetic field in a NMR spectrometer will vary spatially and temporally and the samples must experience a homogeneous field, otherwise the signals will broaden and be of poor quality. Therefore, shimming of the magnet is required for the maintenance of the homogeneous field (Petersen, 2007).
3. The sample itself must be homogeneous. Large particles like gas or cell debris will interact with the applied magnetic field and create local field variation. Heterogeneity can not be corrected by shimming, thus the sample must be prepared (degassed/filtered) prior to the experiment (Claridge, 2016).
4. A deuterated solvent is required for its ability to be "invisible" in the spectra due to the different process of frequency for deuterium than ^{1}H. The spectrometer locks on the signal for deuterium to correct for variation in the frequency over time (Mantsch, Saitô and Smith, 1977).
5. For the calibration of chemical shift (ppm), a reference compound needs to be added to the sample. From this reference compound, one can compare our spectra with literature (Hoffman, 2006).

2.5 Analysing Beer using Nuclear Magnetic Resonance

Prior studies have shown that there are several different methods of preparing beer samples for NMR analysis (Lachenmeier et al, 2005; da Silva et al, 2019). A common factor for these studies is that they use ultrasonication baths for degassing the samples. However, the concentration of D_2O and TSP differs, also if a buffer is added or not. In a study by da Silva et al (2019), 126 canned brazilian lager beers from three different manufacturers were analysed and classified. The samples were degassed in an ultrasonic bath for 10 minutes at room temperature. NMR samples were prepared with a total volume of 600 μL of the degassed sample containing 10% D_2O, and 0.5% TMSP-d4 (3-(trimethylsilyl)-2,2,3,3-tetradeutero propionic acid). No buffer was added to the samples in this study, and the pH ranged between 3.7-4.1. This is a relatively narrow range, considering that a sour beer style was included in the study. To ensure correct interpretation of the NMR signals and that the differences between spectra were the result of true sample differences and

not just pH effects, a Recursive Segment-Wise Peak Alignment (RSPA) method was used for spectral alignment (da Silva et al, 2019).

In a study by Lachenmeier et al (2005), high-resolution NMR was used for quality control of 80 different beer samples. In this study, samples were filtered using fluted filter paper (0.2 μm pore size). Filtration, in combination with a 10 min ultrasonic bath, was used as a degassing method. Autosampler vials were used for analysis of the samples. NMR samples containing 2.0 ml of degassed samples containing 10% buffer (pH 5.6 in D_2O), 0.1% TSP, and 0.013% sodium azide (for preservation of the samples) were prepared. The pH of the samples ranged between 4.0 and 4.8 before addition of buffer, and 5.2-5.5 after addition of buffer, resulting in no significant chemical shift differences caused by pH effects (Lachenmeier et al, 2005).

For this study, we aimed to develop a standard protocol for analysing different beer samples by NMR spectroscopy, thus it was important to include different beer types. As seen in figure 2.5.1, sample collection is only one of the first steps in the several steps and considerations required for analysing beer in NMR. There are several strategies one can follow to prepare beer samples, as seen in the studies mentioned in the section above (Lachenmeier et al, 2005; da Silva et al, 2019), yet there is no standardized way of analysing beer by NMR. However, prior research is crucial for future study design and method development. Several different NMR experiments can be used, depending on the study. However, for studying beer metabolomics by NMR, 1D ^1H NMR experiments are preferred due to the high sensitivity to ^1H (Pathmasiri et al, 2020).

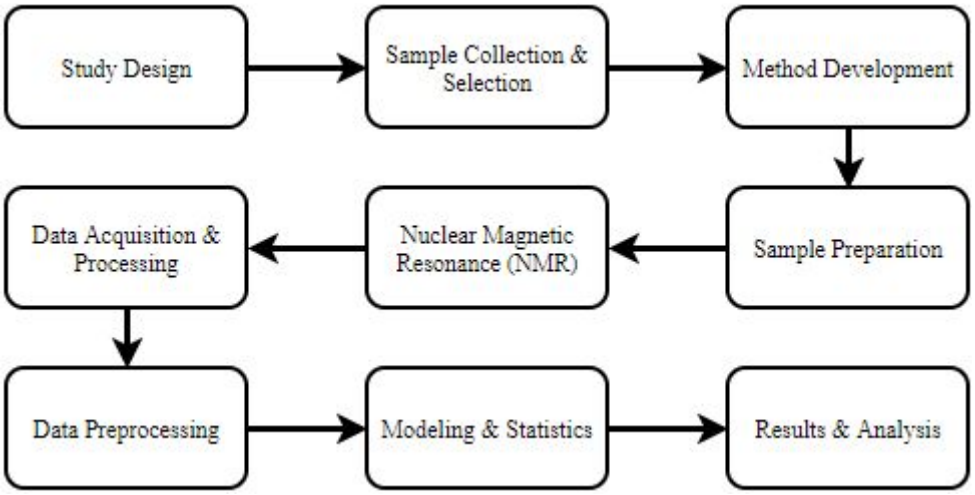


Figure 2.5.1: This flow chart illustrates all of the steps in the metabolomic workflow of the NMR process.

To obtain useful NMR data on beer metabolomics, the data preprocessing step is vital. NMR data can either be analysed using its full resolution, or by binning the data. However, it is important to know that by choosing the latter, some information will be lost with the reduced dimensionality. For further preprocessing of the gathered data, Matlab is, with an appropriate script, a powerful tool. Alignment of the spectra is also possible in Matlab, with the help of a versatile tool called *icoshift*, which provides a rapid alignment of 1D NMR spectra. *Icoshift* is an algorithm designed for clearing up misalignments in metabolomic NMR data analysis. This method of preprocessing the data has proven to be capable of accomplishing full resolution alignment of broad spectra, thus avoiding the issue of binning and reduced dimensionality (Savorani, Tomasi and Engelsen, 2010).

Other meaningful preprocessing steps to consider before moving on to the statistical analysis, are normalization and scaling of the data. Normalization of the data is considered highly essential as it removes any variation in the total amount of material between the samples. There are many methods of normalizing data, yet, there is no single approach on how to normalize any given study. The type of study and samples must be considered and processed before choosing an appropriate method of normalization (Pathmasiri et al, 2020). Scaling is also considered an appropriate pretreatment method of the data. Several methods of scaling are available for metabolomics NMR (Pathmasiri et al, 2020). Mean centering, auto scaling, range scaling, and pareto scaling are all viable methods to be considered, depending on the type of study. For this particular study, pareto scaling was chosen for its ability to keep the

data structure partially intact, and at the same time increase the relative importance of smaller/medium quantities (van der Berg et al, 2006).

There are several different modelling methods and statistics one can apply to multivariate preprocessed data gathered from NMR analysis. Principal Component Analysis (PCA) is an unsupervised method of multivariate analysis that can be fast and resourceful for the analysis of high dimensional datasets. PCA is often used for the analysis of metabolomics, and provides visual information about observed tendencies, arrangements, or outliers in the data set. Partial Least Square Discriminatory Analysis (PLS-DA) is a supervised method that can describe metabolites or features that are discriminatory in a system that is biological. Tendencies or cluster separation that arise from the subjects in PCA are typically depicted in score plots from PLS-DA, and the variables that are subject for these tendencies and cluster separation are given from the loading plot of the PLS-DA (Pathmasiri et al, 2020).

For preprocessing, modeling, and statistical analysis of raw NMR metabolomic data, metaboanalyst (Xia et al, 2009) can be used. Metaboanalyst is a powerful online tool for data analysis and statistical interpretation of metabolomic studies, developed by the Wishart Research Group of the University of Alberta, and first released in 2009. Metaboanalyst supports a wide variety of data input types that are commonly produced in metabolomic studies, and is capable of performing data processing, statistical analysis, functional enrichment analysis, and metabolic pathway analysis (Xia et al, 2009).

3. Materials and methods

3.1 Beer types

In order to develop a method for NMR analysis of beer, a diversity of beer styles were used. It was important to find different styles of beer that could possibly express a wide variety of different compounds in the NMR spectra. Therefore, the selection of different types of beer for this study was done with the knowledge of their extreme attributes, such as high alcohol percentage or a strong acidic/bitter taste.

The beer types chosen were Austmann Lynchburg Latte (Bourbon-Stout, 10.5% alc.), Brewdog Native Son (West Coast IPA, 8.5% alc.), Erdinger (Weissbier, 5.3% alc.), San Miguel Especial (Lager, 5.4% alc.), NTNU-brew (Sour-Stout, unknown % alc.), and Westmalle Trappist (Tripel, 9.5% alc.) (see Figure 3.1.1).

Figure 3.1.1: Beer types used for the development of the NMR protocol. From left: Austmann Lynchburg Latte, Brewdog Native Son, Erdinger Weissbier, San Miguel Especial, NTNU Sour-Stout, and Westmalle Trappist.

3.2 Sample preparation

Figure 3.2.1 explains how each beer style was split into four groups which contained three technical replicates. The first group contained samples that were buffered and vortexed, the second group was buffered and ultrasonicated, the third group was vortexed and not buffered, and the last group was ultrasonicated and not buffered. From six different beers and twelve samples from each beer, a total of seventy two samples were prepared.

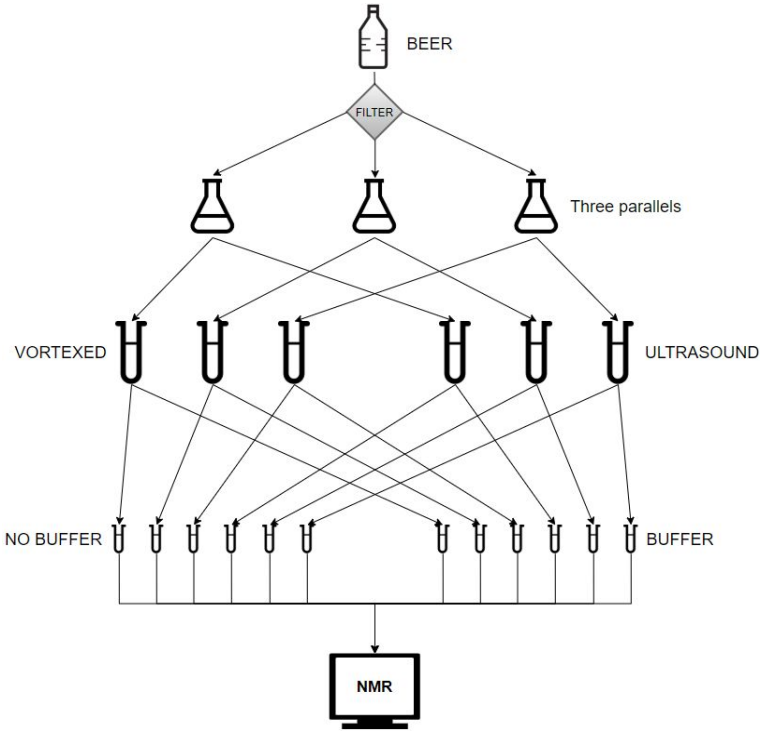


Figure 3.2.1: Flowchart illustration that shows how each beer was split into three replicates (parallels), that were split further into ultrasonicated and vortexed degassed samples, and again split into buffered and unbuffered samples.

The pH of the beer was measured immediately after opening the bottle or can. A small amount of beer (10.0 ml) was then split into three conical Falcon tubes (50.0 ml) and filtered using a syringe filter (WVR, 0.2 µm pore size). pH was measured again after filtration, before splitting each parallel into two smaller samples (5.0 ml), resulting in a total of six samples. Half of the samples were degassed using a vortex mixer, and the other half was degassed by ultrasound using an ultrasonic bath. Vortexing was conducted simply by pressing the sample-tube into the vortex-machine. The other (not vortexed) samples were placed in an ultrasonic water bath for 10 minutes at room temperature. pH was measured once more after

degassing. From each degassed sample, two volumes (540 µl) were pipetted and transferred to NMR tubes (5 mm), where one was added unbuffered stock solution (60 µl) containing D_2O (99%) and TSP (1%), and the other was mixed with 60 µL of a buffered stock solution (500 mM sodium phosphate pH 7.4 buffer) containing D_2O (99%) and TSP (1%). The buffered stock solution was thus diluted 1:10 obtaining a final concentration of 0.1 % TSP, 10% D_2O and, in the case of the buffered samples, 50 mM sodium phosphate buffer. From figure 3.2.1, one can see that each beer yields twelve samples, resulting in seventy-two samples in total.

3.3 NMR data acquisition

All NMR measurements were performed on a Bruker Avance III HD 800 MHz spectrometer (Bruker Biospin) equipped with a 5-mm Z-gradient CP-TCI (H/C/N) cryoprobe at the NV-NMR-Centre/Norwegian NMR Platform at Norwegian University of Science and Technology (NTNU). All spectra were recorded at 300.0 K and a 2 min. waiting period was applied for temperature equilibration after sample insertion. Locking, automated tuning and matching, shimming and calibration of the 90° hard pulse were performed using the standard Bruker routines atma, lock, topshim and pulsecal under the control of home-made scripts and the ICON-NMR software (Bruker Biospin).

The NMR protocol was adapted from Monakhova et al (2009). First, a standard water presaturation pulse program was used to only suppress the water signal. The resulting spectrum was used to measure the exact seven frequencies of ethanol in each sample. These values were used to optimize the shaped pulse necessary for multiple solvent suppression, as previously described (Monakhova et al, 2009). This procedure was automated in ICON-NMR using a home-made script.

The second experiment was a 1D 1H spectrum (NOESYGPPS1D) with only water suppression. The third experiment was a 1D 1H spectrum (NOESYGPPS1D) with suppression of both water and ethanol using the generated shape pulse. In addition, selective

continuous wave irradiation was applied to the center of the ethanol quartet during relaxation delay to remove ^{13}C satellites.

The parameters used for the second and third experiments were:

Presaturation field: 25 Hz
Presaturation pulse: 100 ms
Relaxation delay (d1): 5 seconds
Time domain size (TD): 64k complex points
Acquisition time (AQ): 2.5 seconds
Spectral width (SW): 21.6966 ppm
Dummy scans (DS): 4
Number of scans (NS): 8
Receiver gain (RG): 10

3.4 NMR spectral processing

3.4.1 Topspin processing

Spectral processing was completed in TopSpin software (Bruker, version 4.0.7), with automatic phase correction applied only in the 0th order phase. Free Induction Decay (FID) was multiplied with exponential multiplication, with line broadening of 0.3Hz and fourier transformation. The automatic base correction was deemed satisfactory after a manual inspection. The data was then exported from TopSpin via a python script (Bruker-to-Mat, see attachment 3) to be directly imported into MatLab for alignment, normalization and scaling. A matrix of 35 samples (in rows) and 65536 data points for the chemical shift axis in ppm (in column) was created both for the buffered samples and unbuffered samples from the process.

3.4.2 Alignment (icoshift), normalization and scaling

Alignment of the acquired NMR spectrum was done using an algorithm called *icoshift* (explained in chapter 2.6) in the computer program MatLab. After loading in the matrix data from TopSpin into MatLab, the data was coshifted by using the icoshift command which aligned the whole spectrum using the TSP region as a reference. The TSP ppm region was manually selected from the plot and subtracted from the original ppm variable, creating a new variable for ppm where TSP is corrected for zero. For the buffered samples, the best mode for alignment seemed to be 'average2' with a multiplier of 3. For the unbuffered samples, median seemed best for the alignment (see attachment 4). With the corrected ppm scale, intervals containing peaks of interest were manually defined before the icoshift command was executed.

Normalization and scaling of the acquired NMR spectrum was also executed using MatLab. Both ethanol peaks and the water signal were excluded from the spectra by using the 'excludeAreas' command with defined intervals. The dataset was then normalized and scaled (pareto) using the 'beernormal' command from the script (detailed commands can be found in attachment 5).

4. Results and analysis

In this chapter, results and analysis will be discussed consecutively for the purpose of a more straightforward overview and continuity of the results. The points made here will be summarized to give a full picture in chapter 5, the discussion. In the figures, different abbreviations will be used for the different beer styles with the different degassing methods and whether the beer is buffered or not. These abbreviations are given in table 4.1. For instance, IVB is an abbreviation for IPA (I) that is vortexed (V) and buffered (B), whereas IU is an IPA (I) that is ultrasonicated (U) without the addition of a buffer to the solution. For a more detailed overview of the samples with pH and their corresponding bar-code, see attachment 1.

Table 4.1: Abbreviations used for the different beer types, with different degassing methods, and with or without buffering.

Beer style	Abbreviation
BrewDog - West Coast IPA	*I*
San Miguel - Lager	*L*
NTNU - Sour Stout	*So*
Austmann - Bourbon Stout	*St*
Westmalle - Trappist Tripel	*T*
Erdinger - Weissbier	*W*
Degassing method	
Vortex	*V*
Ultrasonication	*U*
Buffered	
Yes	*B*
No	-

This chapter is split into two parts. Part one assesses sample preparation and method development and consists of results and analyses regarding aim 1: “Is there a difference between the two degassing methods, and is there a difference between the buffered and unbuffered samples?”. The second part assesses the chemical diversity of the beer types and consists of results and analyses regarding aim 2: “What are the chemical differences between the beer styles?”.

4.1 **Part one**: Differences between sample preparation methods

4.1.1 Scaling of spectral data

The full and raw ^{1}H NMR spectrum of all the beer styles is given in figure 4.1.1, where each line is one representative replica from each beer style. The raw dataset is not aligned, scaled, normalized and no peaks are suppressed (ethanol/water). Thus, from the figure below, one can see that the reference TSP-peak is not set to zero, the ethanol peak is extremely dominant (especially for the yellow line - stout), and the peaks are generally varying along the ppm axis, even for the peaks that correspond to the same compound.

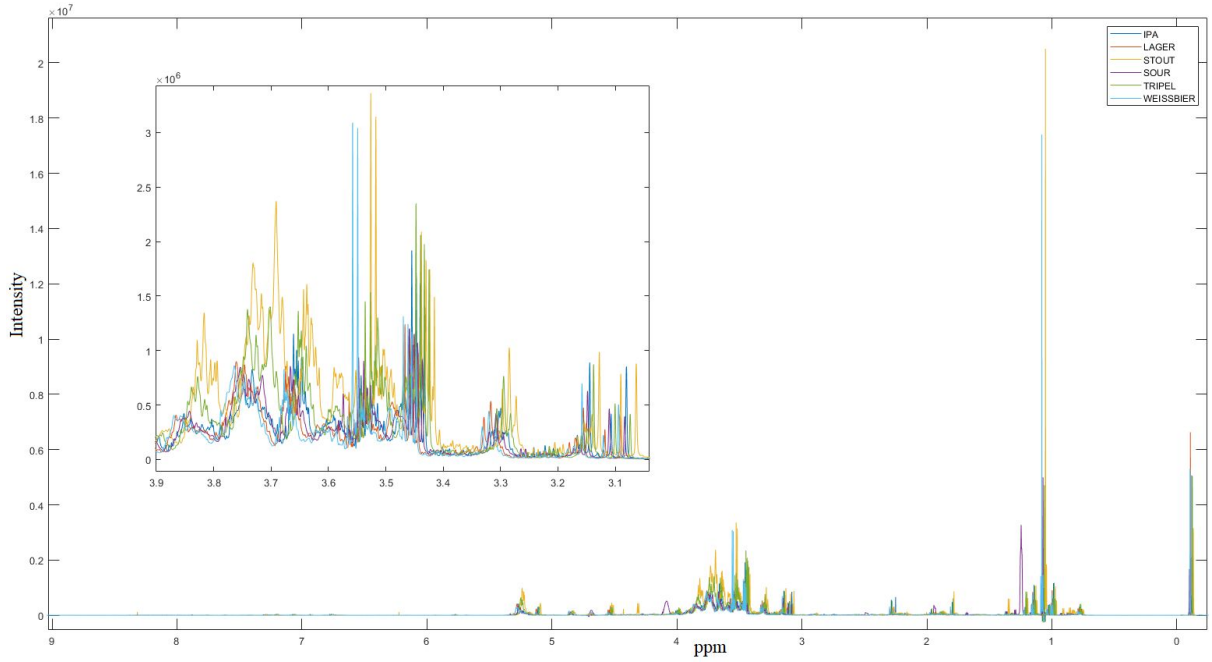


Figure 4.1.1: Full and raw (no alignment, scaling, normalization, or suppression of ethanol and water) ^{1}H NMR spectra with one representative sample (buffered) from each beer style. Chemical shifts of 3.1 to 3.9 ppm are magnified in the figure.

To ensure that prevalent peaks do not dominate others when comparing them, scaling was applied to the spectra. Metaboanalyst offers many different methods for scaling spectral data, as can be seen in figure 4.1.2. Several different scaling methods were evaluated and experimented with by comparing the different outputs, both for normalization boxplots and 2D principal component analysis (PCA) score-plots (full comparison of all the different scaling methods can be found in attachment 2).

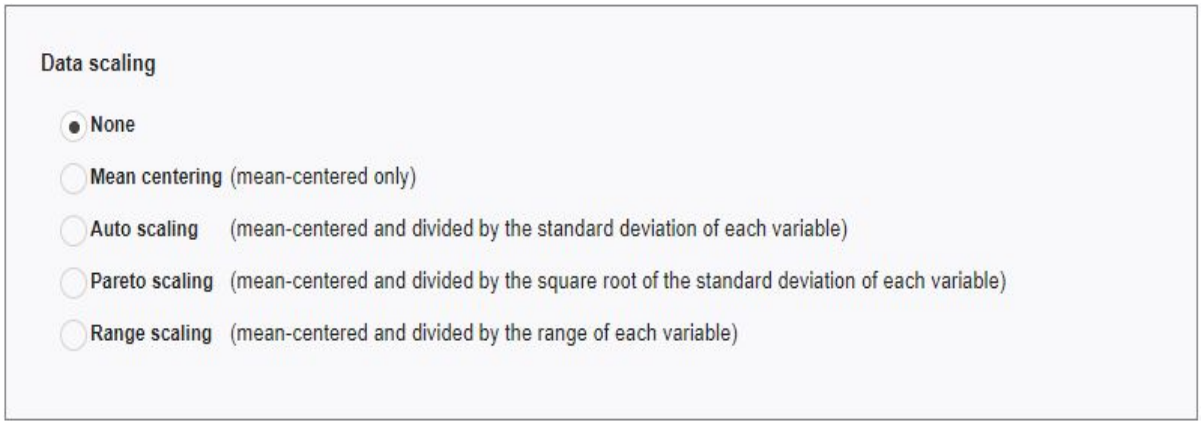


Figure 4.1.2: Different methods of scaling data on the Metaboanalyst website, and the statistical description of what the scaling does (Xia et al., 2009).

Although auto- and pareto scaling are very similar, pareto scaling uses the square root of the standard deviation as scaling factor, rather than just the standard deviation. Thus, the dataset does not become dimensionless (as it would be using autoscaling). In addition, large fold changes will be less dominant to clean data due to a decrease in the large fold changes rather than the small fold changes (van der Berg et al, 2006). PCA was used to compare the effects of scaling, which is illustrated in figure 4.1.3.

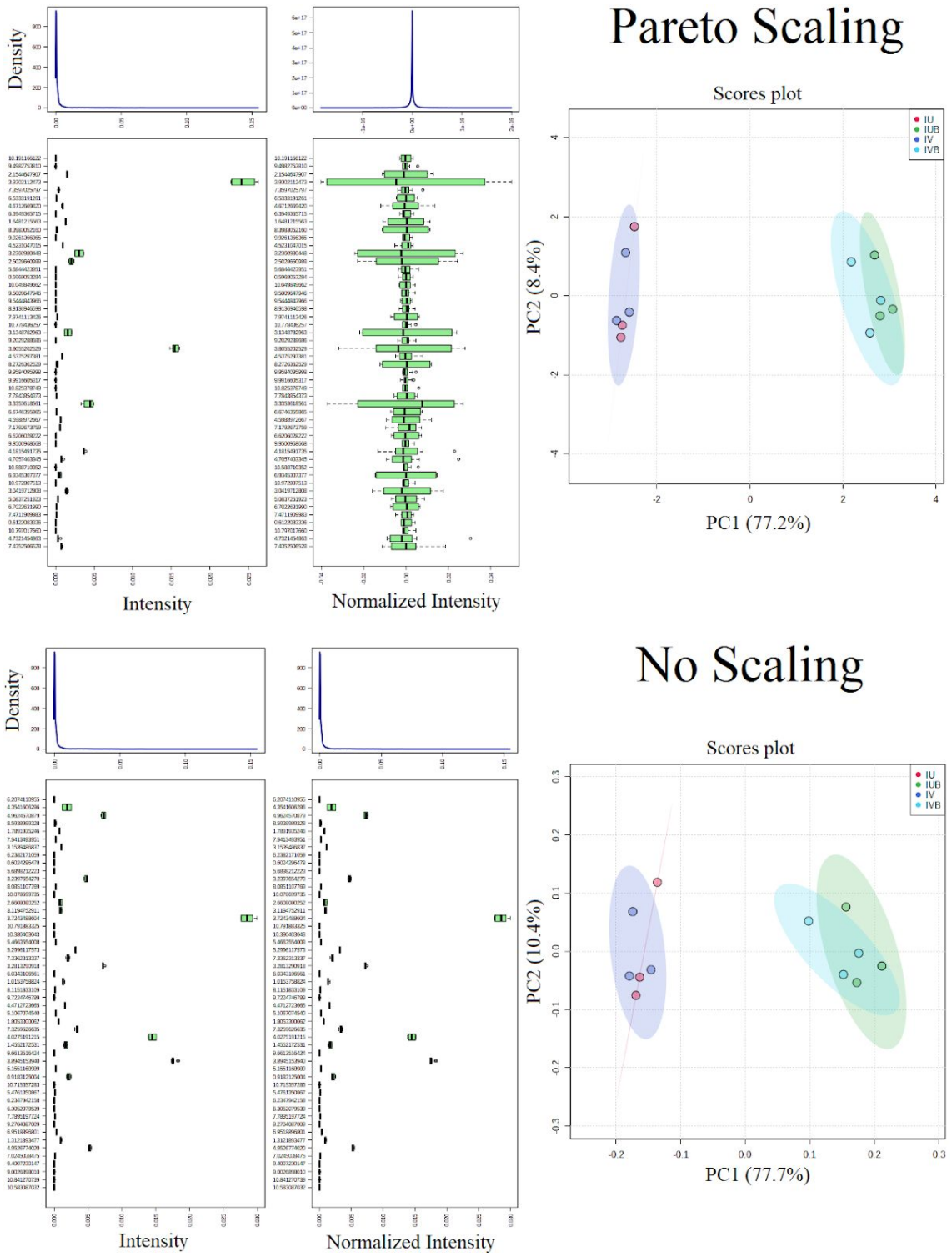


Figure 4.1.3: A comparison of pareto scaling versus no scaling of the IPA-dataset given in 2D-score plots, obtained from the PCA analysis, with corresponding boxplot of the normal distribution of the data. The axis (principal components) in the PCA score-plot represents the combination of the different attributes for each beer style, where PC1 makes up the biggest variation between the data points. The dark blue and red dots in the left cluster represents the non-buffered, vortexed and ultrasonicated IPA beer samples, and the light blue and green dots in the right cluster represents the buffered, vortexed and ultrasonicated IPA beer samples. The 95% confidence interval for the data points are given by the ellipse region surrounding the groups.

In this study, pareto scaling was chosen as the scaling method due to the ability of pareto scaling to keep the data structure partially intact, and at the same time increase the relative importance of smaller/medium quantities (van der Berg et al, 2006). From figure 4.1.3, one can see that the pattern of the data points has been preserved, and the variation has decreased 0.5% for PC1 and 2.0% for PC2.

4.1.2 Comparing the buffering and degassing methods using PCA

To compare the different sample preparation methods for each individual beer type, Metaboanalyst was used to draw a two-dimensional score-plot from the PCA for each beer style with data points for each replicate within each treatment group. The purpose of this plot is to visualize the differences between the two degassing methods and the buffered and unbuffered samples, with respect to the first two principal components (PC1 and PC2), as can be seen in figure 4.1.4.

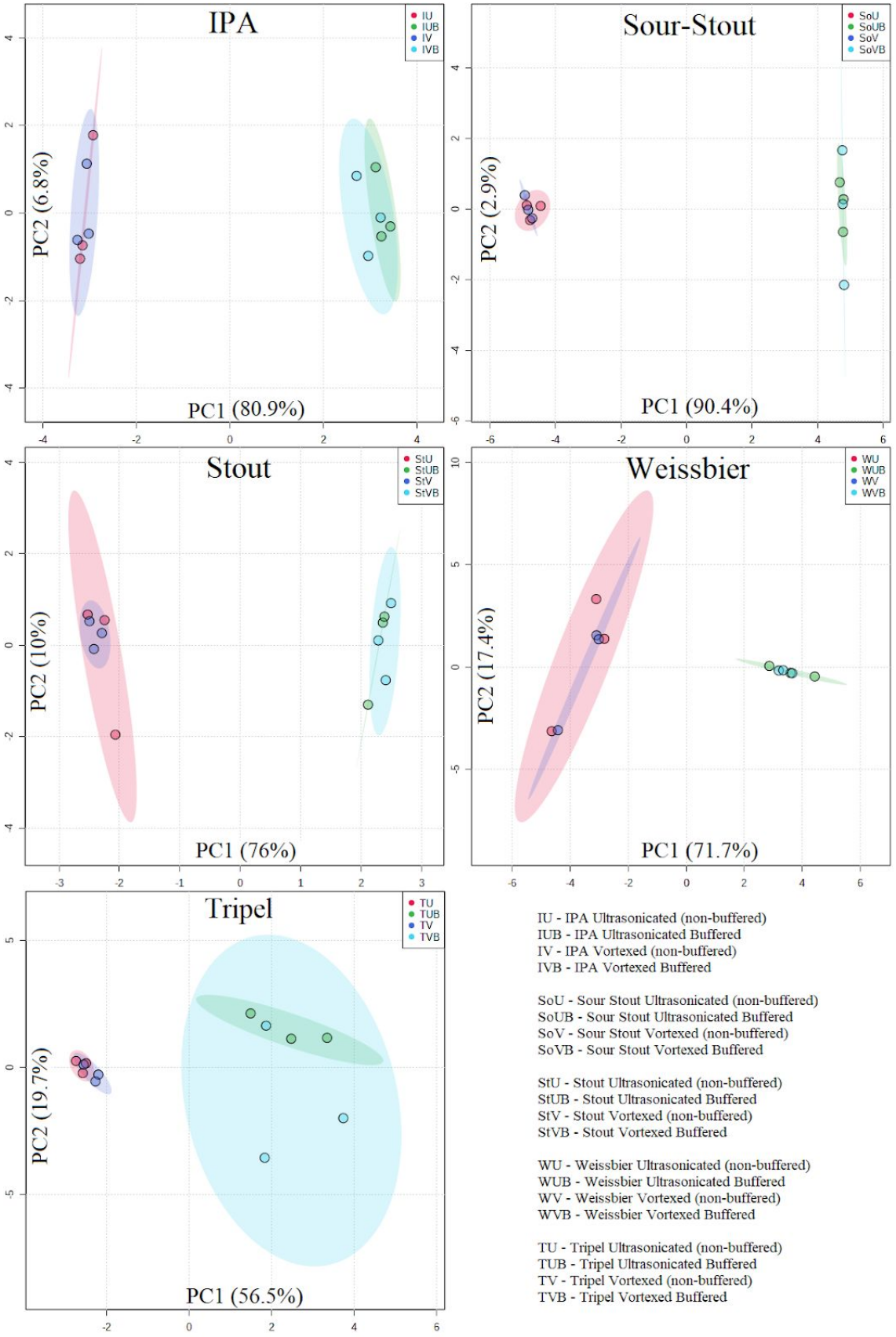


Figure 4.1.4: PCA two-dimensional score-plot for each beer style with data points for all replicates in each group (vortexed with and without buffer, and ultrasonicated with and without buffer). Abbreviations are listed in the figure.

Two samples from the lager beer were discarded due to pipetting error. After excluding these samples there was insufficient replication. Thus the lager beer samples were excluded from the comparison of the different sample preparation methods.

The variation explained by principal component 1 (PC1) ranged from 56.5% to 90.4%. The sour-stout had the highest percentage (90.4%), followed by IPA (80.9%), stout (76.0%), weissbier (71.7%), and finally tripel (56.5%) with the lowest percentage. Further, there is a clear separation between two groups in each plot, where the group on the negative side of the PC1-axis corresponds to the unbuffered samples, and the group on the positive side corresponds to the buffered samples. This indicates that the variation associated with PC1 is most likely explained by differences due to pH. Furthermore, it can be seen that almost all groups are aligning and slightly stretching vertically, thus PC1 does not appear to explain much of the variation between the data points within the two clusters. However, this variation can rather be explained by principal component 2 (PC2).

Although there appears to be an overall vertical stretch to the groups in general, the clusters of the unbuffered samples for the sour-stout and the tripel are quite tight. Thus, their variation appears less explained by PC2, unlike the unbuffered samples for IPA, stout, and weissbier.

From the plots shown in Figure 4.1.4, it is not quite clear what the variation between the replicates PC2 explains. It could be explained by technical errors in the sample preparation method. For example, inaccurate amounts of sample solution in the NMR tubes due to manual pipetting by hand, or that the samples were to some degree heterogeneous, resulting in different concentrations of sample and buffer in the replicates. However, when considering the groupings that can be observed for each score-plot according to PC1, it is clear that two clusters correspond exclusively to the buffered and unbuffered samples. One can therefore assume that the separation in PC1 is explained by the buffering of the samples, and even before looking at the metric values, one can assume that there is a significant difference between buffering and not buffering the samples. For the vortexed and ultrasonicated samples, there is little to no difference within the two first principal components. There is some spread between the clusters of vortexing and ultrasonication for IPA and tripel, though

they are not clearly separated clusters in PCA. This variation may be caused by multiple factors such as pipetting error.

4.2 **Part two**: The Beer Styles

From this point forward, the focus will be on whether there are differences on the NMR spectrum between different beer styles, rather than the different methods of preparing the samples. If so, what are they? Also, given that all the samples will be treated independent of the sample preparation, the lager style beer will be included again for this part.

4.2.1 Analysing the chemical differences

From figure 4.2.1, it is very noticeable that all samples within each beer style cluster to some degree, producing a clear visual difference between the different beer styles. The tripel, however, does not cluster as well as the other beers in this study. PCA cannot by itself describe why there is such a huge variation in the tripel group compared to the others. From a physical examination of the beer itself, it can be seen that it looks unfiltered with small remnants floating through the liquid, making it possible that the solution was highly heterogeneous when a sample was taken from the bottle.

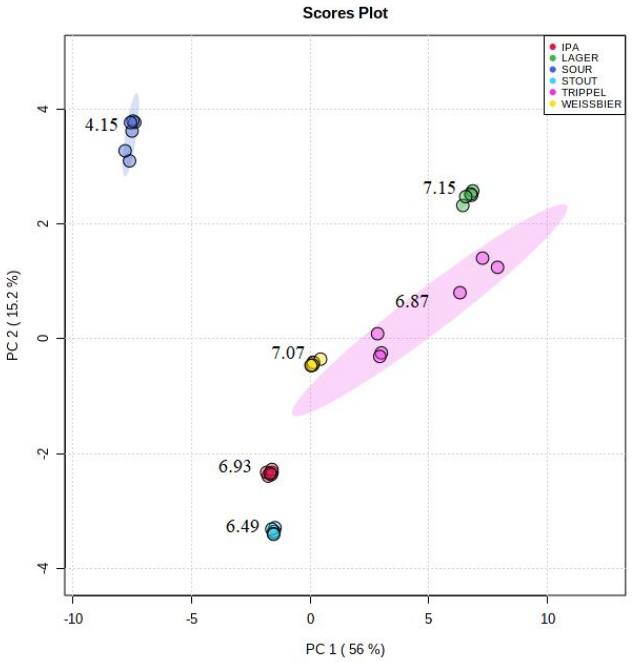


Figure 4.2.1: PCA two-dimensional score-plot for each beer style. Number given to the left for each cluster indicates the average pH (after buffering) for that group of beer samples. The 95% confidence interval is given by the ellipse region surrounding the groups.

Except from the tripel, one can see another group that stands out in the score-plot, not due to the variation within the group itself, but for its outlier position in the plot (see Figure 4.2.1). This grouping is the sour-stout beer with the corresponding average mean pH of 4.15. Obviously, the capacity of the buffer was not sufficient for this beer style, and analysing the differences from the other beers will be difficult at this pH. Therefore, from this score-plot, the differences explained by the principal components will most likely be highly pH dependent. The goal of this part of the study was to find any differentiation between the beer styles by looking for and trying to identify different compounds, and not pH. Therefore, the sour-stout will be excluded for further analysis due to its unsuitable pH.

From the new PCA score-plot given in figure 4.2.2, one can see that all samples within each beer type except the tripel are tightly clustered, indicating that the data measured within each beer style is highly correlated. From the plot, one can see a distinct line that aligns with PC1, with three clusters consisting of IPA, weissbier and lager. This line will from now on be referred to as group one. A second line that aligns with PC1 consists of stout and tripel. This line will be group two. Given that 53.3% of the variation between the clusters is explained by

PC1, and 20.3% by PC2 (see Figure 4.2.2), one can state the following: the variation between the clusters inside group one and two, are greater than the variation between the clusters adjacent to each other along the PC2-axis. For example, IPA is more different from lager, than stout, and stout is more different from tripel, than IPA.

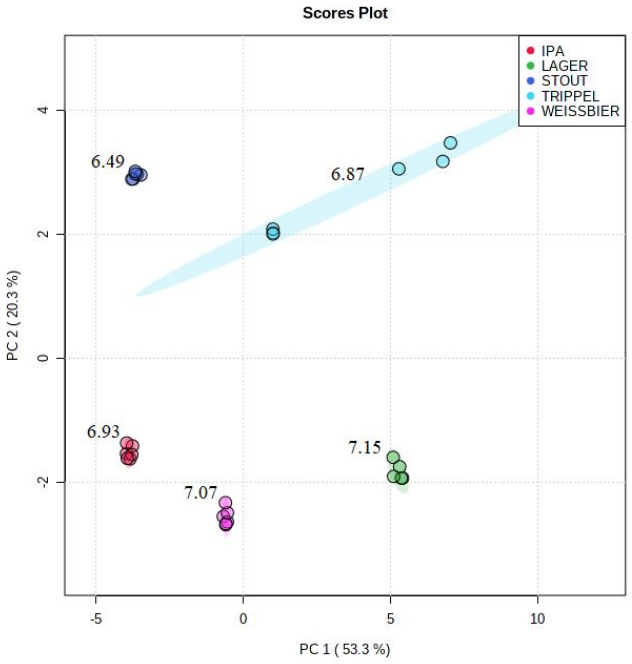


Figure 4.2.2: PCA two-dimensional score-plot of the first two principal components. for each beer style. Number given to the left for each cluster indicates the average pH for that group of beer samples.

Although there is tight clustering and great separation between the clusters in the PCA score-plot already, we can "rotate" the PC axis to maximize the distance between the clusters and view the separation of the six labeled groups in a PLS-DA score plot, as seen in figure 4.2.3.

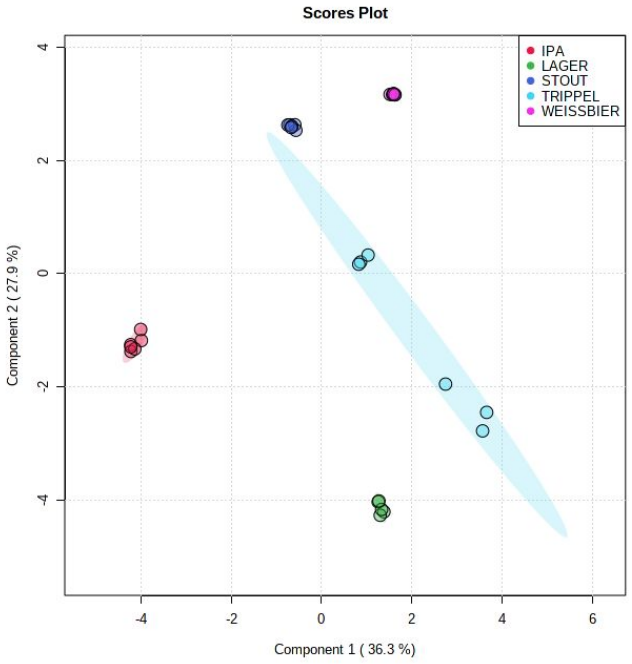


Figure 4.2.3: 2D PLS-DA score plot of the first two components.

A cross validation analysis is used to see if the separation is real and not caused by overtraining. From the cross validation given in figure 4.2.4, one can see that the number of components needed to help drive this separation is six, thus it is a six-component model.

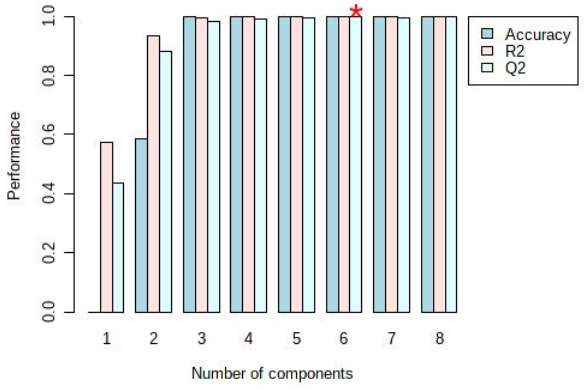


Figure 4.2.4: PLS-DA cross validation results. The red star shows the selected performance value Q2, which indicates that the six-component model is the best.

A permutation test can also be applied to ensure that the results of the PLS-DA score-plot are not a product of random chance. In Metaboanalyst, the option for separation distance was set to B/W-ratio (between-group sum of square/within-group sum of square) and the number of

permutations was set to 1000. The results from this analysis, given in figure 4.2.5, shows that the differences are significant and far from random chance, as all values are below the p-value mark.

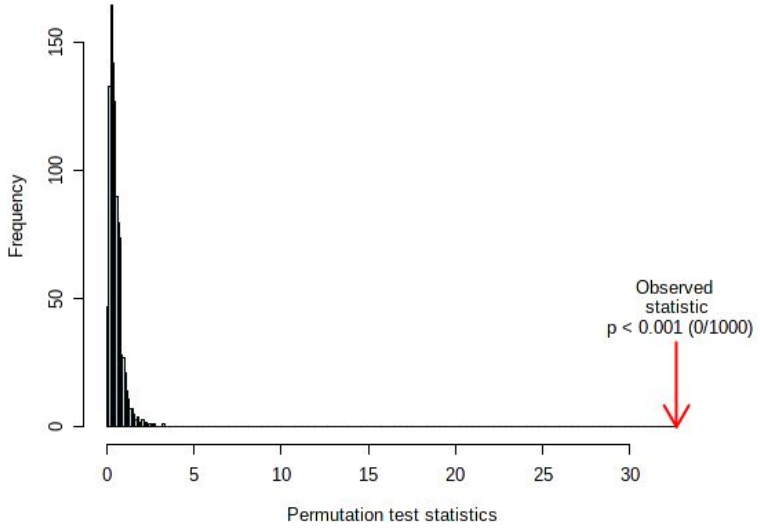


Figure 4.2.5: A histogram of the permutation results, showing that the null distribution composed of permuted data is not a part of the observed original data statistics.

4.2.2 Analyzing differences and content in the different beer styles

The ^{1}H NMR spectra of the five different beer styles (IPA, lager, stout, tripel, and weissbier) given in both figure 4.2.6 and 4.2.8 are consequently conservative due to all of the samples generating the same peaks to a high degree. Therefore, it can be roughly estimated that all of the beers contain the same metabolites, although in different concentrations. To distinguish these differences, a representative sample of different peaks has been selected for further analysis and differentiation. From table 4.2, four different compounds have been identified (valine, isoleucine, lactic acid and alanine) with Heteronuclear Single Quantum Coherence Spectroscopy (HSQC). C-H cross peaks for formic acid and histidine were not visible in the HSQC due to the lack of Cppm for these peaks, therefore these compounds are only suggestions, formed by comparing the peaks to validated formic acid and histidine peaks established in Human Metabolome DataBase (HMDB).

Table 4.2: Overview of the different compounds identified from the NMR spectra illustrated in figure 4.2.6 and 4.2.8, with ID, ppm for both ^{1}H and ^{13}C NMR, assigned carbon, and multiplicity of the peak. The star symbol (*) indicates that the compound is a suggestion and not determined.

ID	δ ^{1}H	Compound	δ ^{13}C	Assignment	Multiplicity
1a	0.99	Valine	19.2	CH3	Doublet
1b	1.04	Valine	20.8	CH3	Doublet
2a	0.93	Isoleucine	13.9	CH3	Doublet
2b	1.01	Isoleucine	17.4	CH3	Doublet
3	1.33	Lactic Acid	22.9	CH3	Doublet
4	1.48	Alanine	18.9	CH3	Doublet
5a	7.06	Histidine C4*	na	CH	Singlet
5b	8.00	Histidine C2*	na	CH	Singlet
6	8.45	Formic Acid*	na	CH	Singlet

Both spectrums given in figure 4.2.6 and 4.2.8 contain five different beer styles whereas each line is a color coded representative replica from each beer. From the first spectrum, one can see that all of the highlighted peaks differ quite well in intensity for almost all of the beer styles. In all the amino acid peaks (1a, 1b, 2a, 2b, and 4), stout makes up the peak with the highest intensity, followed by IPA, and then weissbier, while tripel and lager shows little to no intensity in these areas. For the lactic acid peak, all samples provide roughly the same intensity except for tripel which has more than twice the intensity of the others. At 1.2 ppm one can see that the ethanol peak has been excluded, hence the flat line. Also, the ethanol satellites can be seen as two triplets on each side at 1.11 and 1.27 ppm.

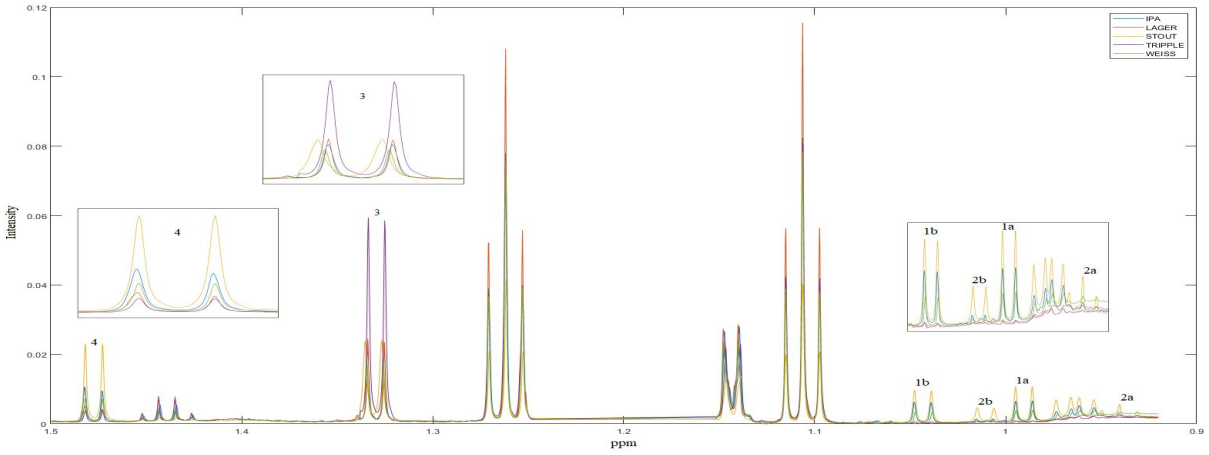


Figure 4.2.6: ^{1}H NMR spectrum for IPA, lager, stout, tripel and weissbier at 0.92 - 1.50 ppm. Whereas the peaks for valine, isoleucine, lactic acid and alanine are magnified for 0.99 - 1.04, 0.93 - 1.01, 1.33, 1.48 ppm respectively. The peaks are identified corresponding to table 4.2. The straight line that can be observed in the middle of the spectra is where the excluded ethanol peak was.

In the loading/ppm plot given in figure 4.2.7, one can see that the peaks that correspond to the amino acid peaks in the main spectrum (figure 4.2.6) are of a relatively high negative value, with no positive values. Thus, the separation between the beers in this region must be due to high concentration of amino acids in the beers that can be seen in the negative side of the component one axis of the PLS-DA plot (stout and IPA, see figure 4.2.3). The lactic acid peak on the other hand has a relatively high positive value with no negative value, which again indicates that the separation between the beers in this region must be due to high concentration of lactic acid in one or more of the beers on the positive side of the component one axis of the PLS-DA plot (tripel).

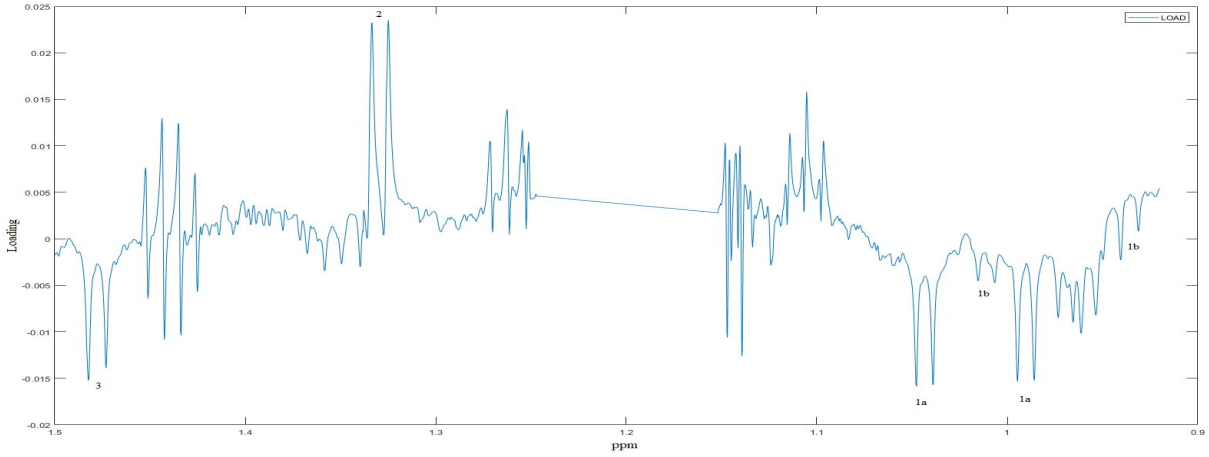


Figure 4.2.7: Loadings values acquired from the PLS-DA plotted on the ppm values at 0.92 - 1.50. The peaks are ID'd corresponding to table 4.2. The straight line (from 1.17 to 1.24 ppm) that can be observed in the middle of the loading-plot is due to no points in this region (excluded ethanol peak).

From the second spectra (figure 4.2.8), there are three singlets (5a, 5b, and 6 from table 4.2) that have been highlighted at 7.06, 8.00, and 8.45 ppm. These peaks have been suggested to be histidine (5a and 5b) and formic acid (6). From prior analysis, one can see that there is a seemingly large amount of the amino acids valine, isoleucine, and alanine in the stout-beer, though this is not the case for histidine. From reading the graph, one can see that the lager and IPA have the highest intensity in the 5a and 5b peak. On the other hand, for peak 6, the intensity for the stout is much stronger than the other beer types.

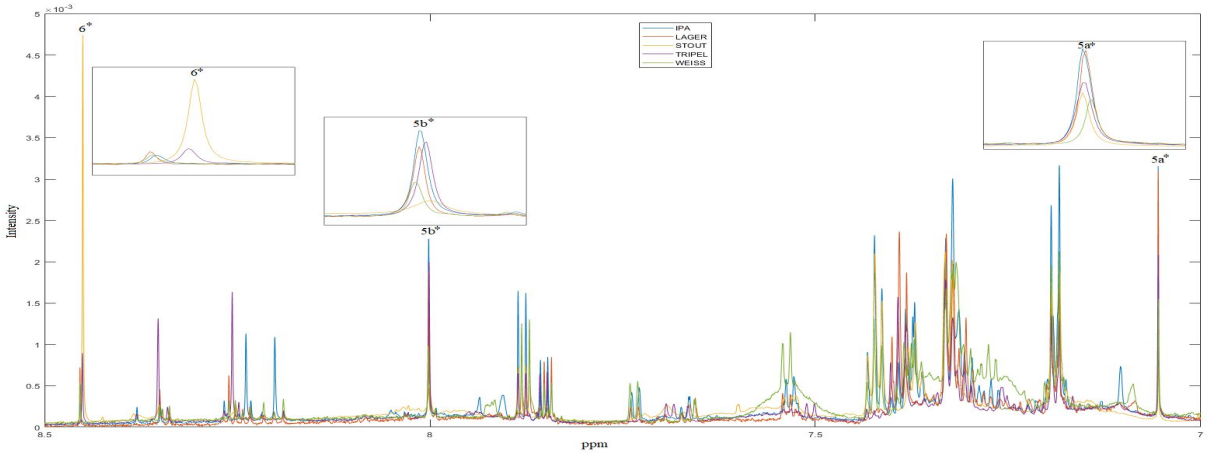


Figure 4.2.8: ^{1}H NMR spectrum for IPA, lager, stout, tripel and weissbier at 7.00 - 8.50 ppm. Whereas the peaks for formic acid* and histidine* are magnified for 8.45 and 7.06 - 8.00 ppm respectively. The peaks are ID'd corresponding to table 4.2. The star symbol (*) indicates that the compound is a suggestion and not determined.

Common for all of the highlighted peaks in figure 4.2.8 is that they do not contribute much to the separation between the beer styles. In figure 4.2.9, one can see that the 5a and 5b peak are relatively small in the negative direction. This correlates well with the spectra, given that they are very much alike. Also, peak 6 is even smaller, which is expected given that only the stout had a high value for this ppm, and from the PLS-DA plot one can see that the stout is not far out on the negative axis for component one.

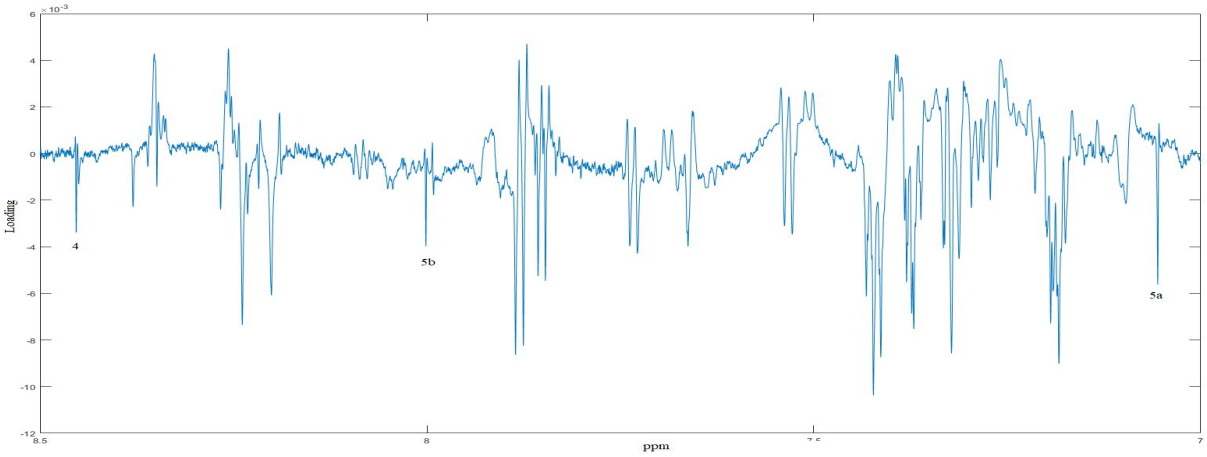


Figure 4.2.9: Loadings values acquired from the PLS-DA plotted on the ppm values at 7.00 - 8.50. The peaks are ID'd corresponding to table 4.2.

5. Discussion

In this section, the results of the present study will be discussed in light of the aims of the study and prior research. The present study had two aims. First, we sought to identify differences between two degassing methods of beer, as well as differences between buffered and unbuffered samples of beer in NMR. The second aim was to identify chemical differences between the different beer styles using NMR. The main findings of this study were that the largest difference between buffered and unbuffered samples appeared to be their pH, as made evident by the large proportion of variance explained by PC1. However, the PCA did not appear to identify any evident differences between the different degassing methods. Further, the NMR analysis revealed that the stout beer had highest intensity peaks related to amino acids, whereas the tripel and lager beer had very low to no intensity peaks related to amino acids. For the peaks related to lactic acid, tripel had more than twice the intensity of the other beer styles, which did not differ significantly from each other. For the peaks that were suggested, but not confirmed, to be histidine, the lager and IPA had the highest intensity, whereas for the peaks that were suggested to be formic acid, the stout had a much higher intensity.

Results of the PCA showed that the first principal component appeared to explain a large proportion of the variation between the buffered and unbuffered samples. This difference is most likely related to pH, as the buffered signals appeared simultaneously on the NMR spectrum, whereas the unbuffered signals did not. Thus, this suggests that the buffer has succeeded in changing the pH of the beer samples, making them more similar. This most likely makes it easier to analyze the samples using NMR, as where the signal for a compound appears can be affected by pH (Lachenmeier et al, 2005; da Silva et al, 2019). Further, there were no noticeable differences between the vortexed and ultrasonicated beer samples on the PCA plot.

We found several differences between the different beer styles on the NMR spectrum. One of the differences was related to the concentrations of the amino acids histidine, valine, isoleucine, and alanine. Prior studies have found that differences in amino acid concentration

in beer wort can significantly affect beer taste through their effect on metabolic products of fermentation, including higher alcohols, esters, carbonyls, and sulfur-containing compounds (Ferreira and Guido, 2018). Although the results of this study only illustrate the intensity of the NMR signal, these differences in signal intensity may reflect the differences in amino acid concentration, explaining some of the sensory differences between the different beer styles. For instance, the stout beer had the highest peak intensity of all the beer styles for valine, isoleucine, and alanine. Prior studies have shown that the addition of valine and isoleucine increases formation of higher alcohols (He et al, 2014). As stout is a beer style which typically has a high alcohol percentage, our results illustrate that NMR can be used to identify the amino acids possibly contributing to the alcohol concentration. Further, the amino acids isoleucine and valine also affect the production of vicinal diketones, which are characterized by strong "butterscotch" and "toffee" aromas and tastes. Too high concentrations of vicinal diketones in lagers may result in an unpleasant taste (Ferreira and Guido, 2018). In addition, the lager beer sample had the highest peak intensity related to histidine, which has been found to have a large impact on the flavour of lager beer by increasing the formation of higher alcohol and esters (Lei et al, 2013). This indicates that NMR can be utilized to identify compounds related to vicinal diketones, alcohol concentration, and esters in lager beer. However, it is worth noting that the histidine compound only was a suggestion formed by comparing the peaks to validated histidine peaks established in HMDB. Thus, the results related to histidine must be interpreted cautiously. The same goes for the results regarding formic acid, which will not be further discussed in this chapter.

Lactic acid produces wanted (and unwanted) sour taste in beer (Li and Liu, 2015). In our study, the tripel beer sample had the highest peak intensity related to lactic acid. In fact, the peak intensity related to lactic acid was more than twice as high for tripel than for the other beer types. This is somewhat unexpected, as lactic acid is typically used in brewing of most beer types, and one would not typically expect tripel to be more loaded with lactic acid than other styles. It is of course worth remembering that the results of the NMR analyses must not be interpreted as compound concentrations, and the high peak intensity of lactic acid for the tripel sample may thus be the result of an unknown factor. Unfortunately, we were not able to utilize the data from the sour-stout beer due to unsuitable pH, as the capacity of the buffer

was not sufficient for this beer style. Analysis of the sour-stout could have produced interesting results which would have further improved our understanding of the use of NMR in beer research.

The present study has several strengths and limitations. Some uncertainty was associated with the pipetting when preparing the samples, due to possible pipetting errors that may have been caused by an inexperienced master's student that was pipetting the samples by hand, which may have caused some variation in the samples. To test this hypothesis, we planned to repeat the experiment in the same manner, but with different people involved in the process of pipetting the samples, in order to investigate whether there was any significant difference between the PCA score-plot clusters. Unfortunately, due to the covid-19 pandemic, this task could not be accomplished.

Although further testing for pipetting accuracy was never executed, one can see from both PCA and PLS-DA score plots that there is a clear separation between all beer styles and sufficient clustering within them. However, for the beer style tripel, the clustering was not as tight as for the other beer styles. If there were more beer styles included in this study, overlapping may have been a problem. This variation may have been caused by pipetting error, however it is likely that this beer style is more heterogeneous considering that the beer is unfiltered.

One of the strengths of this study is the wide variety of beer styles that have been selected for NMR analysis. This shows that the protocol developed in this study is versatile, yet at the same time one can see where it lacks. For example when the buffering capacity for the sour stout was not sufficient, making exclusion of the beer type an easy choice.

6. Conclusion

In this study, we compared different methods of preparing beer samples for NMR analysis for the purpose of developing a standard protocol. Degassing by vortexing or ultrasonication and the addition or no addition of buffer was compared for six beer samples. Our results indicate that there is no considerable difference between the two degassing methods. However, the PCA score analysis showed great variation between the buffered and unbuffered samples. Our results suggest that buffering the samples is highly recommended for simplifying the process of spectral alignment and ease of comparison between the beer styles. Comparison of the different beer styles revealed that the stout beer had the highest intensity peaks related to amino acids which are associated with higher alcohol concentration and vicinal diketones, whereas the tripel beer had the highest intensity peak related to lactic acid. Our results indicate that NMR analysis can successfully be used in order to identify compounds associated with alcohol concentration, vicinal diketones, and esters in beer. Future studies should investigate the difference between hand pipetting and auto pipetting (machine pipetting), and its effects on the results. Also, the protocol developed in this study should be applied to several unfiltered beer types, to examine if there is an extra variable to take into consideration for this protocol (e.g. more complex filtration).

Acknowledgments

I am very grateful for being given the opportunity of carrying out my master's research at the Almaas Lab at the Norwegian University of Science and Technology (NTNU). I would like to express my sincere gratitude to my supervisor professor Eivind Almaas, and my co-supervisors Christian Schulz and Leesa J. Klau for providing me with their invaluable guidance, comments, and suggestions throughout the project, securing the accomplishment of this study. And I would like to thank everybody at the Almass Lab Group for taking the time to evaluate my findings and present useful feedback along the way.

I am sincerely thankful to Gaston Courtade, Finn L. Aachmann, and Trygve Andreassen for providing me with the idea and aim of this thesis, but also for great guidance in the field of NMR.

Finally, I would like to thank my lovely wife Ekaterina Sergeevna Zotcheva, for being the sole reason and inspiration for even thinking of higher education, and providing unconditional support in all aspects, til the end. Last, but not least, I am thankful to my son Nikola Zotchev, for being such a good little boy during the writing of my thesis.

References

Almeida, C., Duarte I. F., Barros, A., Rodrigues, J., Spraul, M., and Gil, A. M. (2006) Composition of Beer by 1H NMR Spectroscopy: Effects of Brewing Site and Date of Production. *Journal of Agricultural and Food Chemistry*, 54(3), pp. 700-706. doi: 10.1021/jf0526947

Bailey, R. (2019) Glycolysis: The First Stage in Cellular Respiration. Available at: https://www.thoughtco.com/steps-of-glycolysis-373394 (Accessed December 12th 2019)

Balci, M. (2005) *Basic 1H- and 13C-NMR Spectroscopy.* Amsterdam: Elsevier Science.

Bazilchuk, N. (2017) Vikinger brygget øl med steiner. *Forskning.no*, October 21st 2017. Available at: https://forskning.no/ntnu-historie-partner/vikinger-brygget-ol-med-steiner/315183 (Accessed April 1st 2020)

Beer Craftr (2019) The Ultimate Beer Yeast List. Available at: https://www.beercraftr.com/beer-yeast-list/ (Accessed December 5th 2019)

Beer Advocate (2019). Yeast Guide. Available at: https://www.beeradvocate.com/beer/101/yeast/ (Accessed December 5th 2019)

Boulton, C. & D. Quain. (2013) *Brewing Yeast and Fermentation*. Hoboken, NJ: John Wiley & Sons. p. 294.

Claridge, T. D. W. (2016) Practical Aspects of High-Resolution NMR, in Claridge T. D. W. *High-Resolution NMR Techniques in Organic Chemistry (Third Edition)*. Amsterdam: Elsevier Science. pp. 80

da Silva, L.A., Flumignan, D.L., Pezza, H.R. and Pezza, L. (2019) ^{1}H NMR spectroscopy combined with multivariate data analysis for differentiation of Brazilian lager beer according

to brewery. *European Food Research and Technology,* 245, pp. 2365–2372. doi: 10.1007/s00217-019-03354-5

Duarte, I., Barros, A., Belton, P. S., Righelato, R., Spraul, M., Humpfer, E., and Gil, A. M. (2002) High-Resolution Nuclear Magnetic Resonance Spectroscopy and Multivariate Analysis for the Characterization of Beer. *Journal of Agricultural and Food Chemistry*, 50(9), pp. 2475-2481. doi: 10.1021/jf011345j

Ensminger, A. (1994). *Foods & Nutrition Encyclopedia, 2nd Edition, Volume 1*. Boca Raton, FL: CRC Press. p. 188.

Ferreira, I. M. and Guido, L.F. (2018) Impact of Wort Amino Acids on Beer Flavour: A Review. *Fermentation*, 4(2), pp. 23. doi: 10.3390/fermentation4020023

Fox, G. (2018) Starch in Brewing Applications, in Sjöö, M. & Nilsson, L. (eds). *Woodhead Publishing Series in Food Science, Technology and Nutrition, Starch in Food (Second Edition).* Cambridge: Woodhead Publishing, pp. 633-659.

He, Y., Dong, J., Yin, H., Zhao, Y., Chen, R., Wan, X., Chen, P., Hou, X., Liu, J. and Chen, L. (2014) Wort composition and its impact on the flavour-active higher alcohol and ester formation of beer—A review. *Journal of The Institute of Brewing,* 120(3), pp. 157–163. doi: 10.1002/jib.145

Hoffman, R.E. (2006) Standardization of chemical shifts of TMS and solvent signals in NMR solvents. Magnetic Resonance in Chemistry, 44, pp. 606-616. doi:10.1002/mrc.1801

Lachenmeier, D.W., Frank, W., Humpfer, E., Schäfer, H., Keller, S., Mörtter, M., and Spraul, M. (2005). Quality control of beer using high-resolution nuclear magnetic resonance spectroscopy and multivariate analysis. *European Food Research and Technology,* 220, pp. 215–221. doi: 10.1007/s00217-004-1070-7

Law of Gulating, approx. 1100. Available at: https://www.norgeshistorie.no/hvordan-blir-historie-til/hoymiddelalder/K0901-Gulatingsloven-om-%C3%B8lbrygging.html (Accessed December 9th 2019)

Lei, H., Li, H., Mo, F., Zheng, L., Zhao, H. and Zhao, M. (2013) Effects of Lys and His supplementations on the regulation of nitrogen metabolism in lager yeast. *Applied Microbiology and Biotechnology*, 97(20), pp. 8913–8921. doi: 10.1007/s00253-013-5137-x

Levitt, M. H. (2001) *Spin Dynamics: Basics of Nuclear Magnetic Resonance*. Hoboken, NJ: Wiley.

Li, H. and Liu, F. (2015) The chemistry of sour taste and the strategy to reduce the sour taste of beer. *Food Chemistry*, 185, pp. 200‑204. doi: 10.1016/j.foodchem.2015.03.135

Lin, Z. and Li., W.-H. (2014) Comparative Genomics and Evolutionary Genetics of Yeast Carbon Metabolism, in Piskur, J. & Compagno, C. (eds), *Molecular Mechanisms in Yeast Carbon Metabolism,* Berlin: Springer, pp. 97-120.

Mantsch, H. H., Saitô, H. and Smith, I. C. P. (1977) Deuterium magnetic resonance, applications in chemistry, physics and biology. *Progress in Nuclear Magnetic Resonance Spectroscopy,* 11(4), pp. 211-272. doi: 10.1016/0079-6565(77)80010-1

Mishra, R. K., Cherusseri, J., Bishnoi, A. and Thomas, S. (2017) Nuclear Magnetic Resonance Spectroscopy, in Thomas, S., Thomas, R., Zachariah, A. K. and Mishra, R. K. (eds.) *Micro and Nano Technologies, Spectroscopic Methods for Nanomaterials Characterization*. Amsterdam: Elsevier Science. pp. 369-415

Misje, H. K. (2013) Kraftig økning i spesialøl-salget. Available at: https://e24.no/naeringsliv/i/RR4j4x/kraftig-oekning-i-spesialoel-salget (Accessed April 6th 2020)

Monakhova, Y. B., Schäfer, H., Humpfer, E., Spraul, M., Kuballa, T. and Lachenmeier, D. W. (2011) Application of automated eightfold suppression of water and ethanol signals in 1H NMR to provide sensitivity for analyzing alcoholic beverages. *Magnetic Resonance in Chemistry*. 49(11), pp. 734‑739. doi: 10.1002/mrc.2823

Mortensen, H. and Johnsen, V. (2009) *Den komplette guiden til norsk øl.* Oslo: Tun.

Pathmasiri, W., Kay, K., McRitchie, S. and Sumner, S. (2020) Analysis of NMR Metabolomics Data, in Li S. (ed.) *Computational Methods and Data Analysis for Metabolomics. Methods in Molecular Biology*. Humana, New York, NY, pp. 61-97.

Pedersen, U. and Sigurdsson, V. (2019) Landet blir kristnet. Available from: https://www.norgeshistorie.no/vikingtid/religion-og-verdensbilder/0813-landet-blir-kristnet.html (Accessed December 10th 2019)

Petersen, D. (2007) Shimming: Bruker NMR instruments. Available from: https://www.mn.uio.no/kjemi/english/research/about/infrastructure/nmr/manuals/miscellaneous/Shimming.pdf (Accessed April 15th 2020)

Poetic Edda. (2014) translated with an introduction and notes by Carolyne Larrington. Oxford: Oxford University Press

Preiss, R., Tyrawa, C., Krogerus, K., Garshol, L. M., and van der Merwe, G. (2018) Traditional Norwegian Kveik Are a Genetically Distinct Group of Domesticated Saccharomyces cerevisiae Brewing Yeasts. *Frontiers in Microbiology*, 9, pp. 2137. doi: 10.3389/fmicb.2018.02137

Savorani, F., Tomasi, G. and Engelsen, S.B. (2010) icoshift: A versatile tool for the rapid alignment of 1D NMR spectra. *Journal of Magnetic Resonance*, 202(2), pp. 190-202. doi: 10.1016/j.jmr.2009.11.012

Store Norske Leksikon (2019) Den eldre Edda. Available at: https://snl.no/Den_eldre_Edda (Retrieved March 6th 2020)

University College London. (unknown) Sample Preparation. Available from: https://www.ucl.ac.uk/nmr/sample-preparation (Accessed June 4th 2020)

van den Berg, R. A., Hoefsloot, H. C., Westerhuis, J. A., Smilde, A. K., and van der Werf, M. J. (2006) Centering, scaling, and transformations: improving the biological information content of metabolomics data. *BMC genomics*, 7, pp. 142. doi: 10.1186/1471-2164-7-142

Wunderlich, S. & Back, W. (2009) Overview of Manufacturing Beer: Ingredients, Processes, and Quality Criteria, in Preedy, V. R. (ed.), *Beer in Health and Disease Prevention*, Cambridge, MA: Academic Press, pp. 3-16

Xia, J., Psychogios, N., Young, N., and Wishart, D. S. (2009) MetaboAnalyst: a web server for metabolomic data analysis and interpretation. *Nucleic acids research*, 37(Web Server issue), W652–W660. doi: 10.1093/nar/gkp356

Attachments

Attachment 1: Overview of beer types, pH, sample method, and barcode.

BrewDog - Native Son, West Coast DIPA

Original pH	Parallell	pH_1	Degas	pH after degas	Buffersys.	Barcode	pH_2
4.567	1	4.585	Vortex	4.640	Y	755779	6.99
					N	575500	5.07
			Ultra	4.596	Y	573508	6.93
					N	561213	4.88
	2	4.565	Vortex	4.624	Y	918196	6.95
					N	746465	4.98
			Ultra	4.599	Y	906777	6.93
					N	653709	4.84
	3	4.550	Vortex	4.598	Y	891916	6.86
					N	545990	4.89
			Ultra	4.594	Y	673611	6.93
					N	542054	4.95

San Miguel - Lager Beer

Original pH	Parallell	pH_1	Degas	pH after degas	Buffersys.	Barcode	pH_2
4.155	1	4.160	Vortex	4.240	Y	986371	7.12
					N	865940	4.3
			Ultra	4.185	Y	771137	nan
					N	675141	4.88
	2	4.155	Vortex	4.239	Y	741538	7.21
					N	806259	4.9
			Ultra	4.199	Y	968674	7.08
					N	578198	4.87
	3	4.157	Vortex	4.220	Y	537564	7.17
					N	865591	4.76
			Ultra	4.190	Y	549163	7.19
					N	666499	4.69

Austmann - Lynchburg Latte, Bourbon Breakfast Stout

Original pH	Parallell	pH_1	Degas	pH after degas	Buffersys.	Barcode	pH_2
4.616	1	4.630	Vortex	4.709	Y	672463	6.46
					N	620972	4.84
			Ultra	4.657	Y	549635	6.46
					N	756084	4.9
	2	4.646	Vortex	4.653	Y	785985	6.5
					N	814865	4.86
			Ultra	4.634	Y	735575	6.5
					N	750695	4.81
	3	4.626	Vortex	4.634	Y	671305	6.49
					N	911302	4.73
			Ultra	4.631	Y	881702	6.5
					N	683982	4.77

NTNU - Sour-Stout

Original pH	Parallell	pH_1	Degas	pH after degas	Buffersys.	Barcode	pH_2
3.460	1	3.404	Vortex	3.400	Y	801704	4.1
					N	642437	3.42
			Ultra	3.415	Y	623874	4.2
					N	548500	3.47
	2	3.395	Vortex	3.380	Y	588379	4.15
					N	813684	3.48
			Ultra	3.434	Y	778910	4.07
					N	895505	3.49
	3	3.392	Vortex	3.387	Y	789181	4.26
					N	649445	3.5
			Ultra	3.399	Y	872162	4.13
					N	604884	3.42

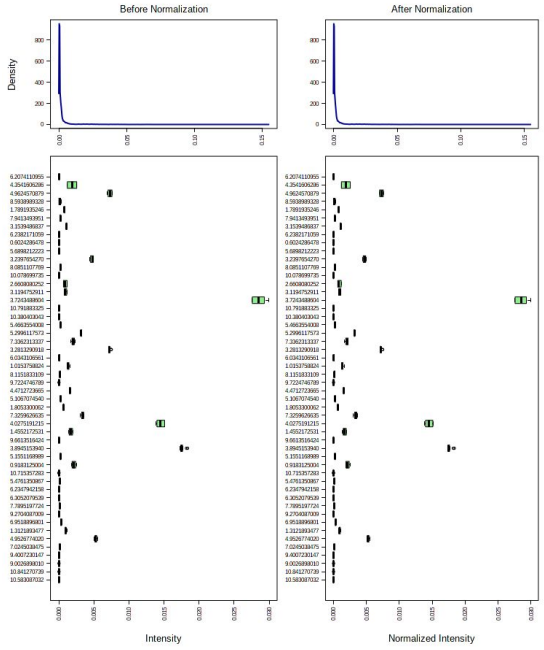
Westmalle - Trappist Tripel

Original pH	Parallell	pH_1	Degas	pH after degas	Buffersys.	Barcode	pH_2
4.358	1	4.379	Vortex	4.462	Y	658940	6.84
					N	671114	4.7
			Ultra	4.376	Y	812650	6.87
					N	559210	4.72
	2	4.381	Vortex	4.408	Y	631749	6.89
					N	628802	4.87
			Ultra	4.376	Y	929895	6.86
					N	564834	4.72
	3	4.419	Vortex	4.393	Y	652607	6.86
					N	738006	4.75
			Ultra	4.383	Y	895303	6.87
					N	797012	4.8

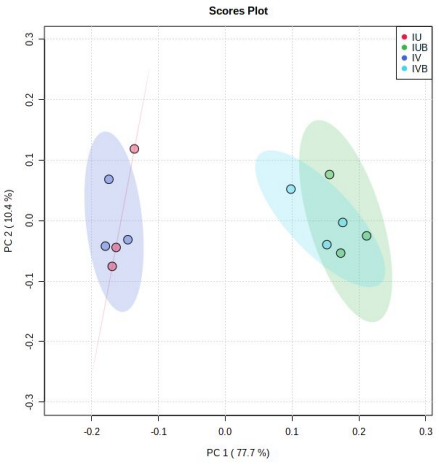
Erdinger - Weissbier

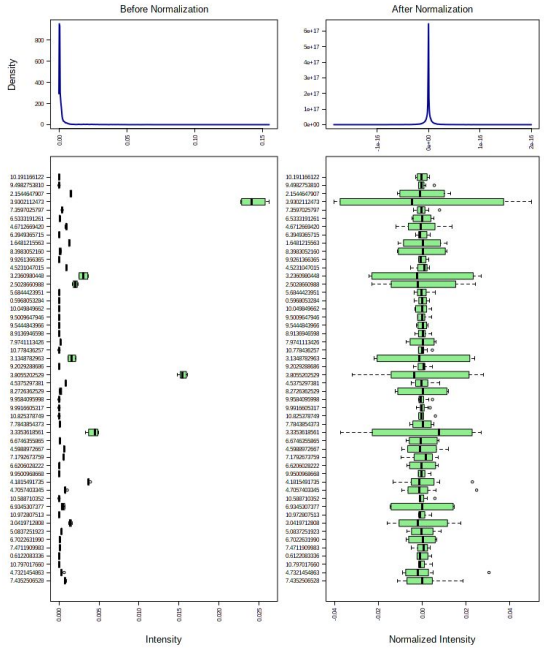
Original pH	Parallell	pH_1	Degas	pH after degas	Buffersys.	Barcode	pH_2
4.250	1	4.301	Vortex	4.361	Y	828859	7.06
					N	975335	4.89
			Ultra	4.293	Y	801254	7.09
					N	746634	4.93
	2	4.296	Vortex	4.326	Y	650896	7.05
					N	676366	4.85
			Ultra	4.303	Y	986483	7.09
					N	528890	4.85
	3	4.293	Vortex	4.300	Y	925116	7.07
					N	690227	4.84
			Ultra	4.297	Y	796493	7.05
					N	587592	4.97

Attachment 2: Score-plots and norm. dist. boxplots comparing the scaling methods (for IPA).

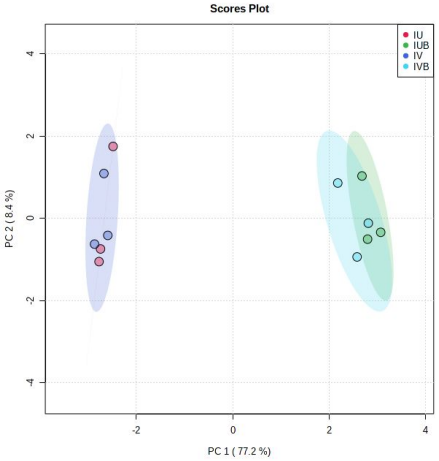


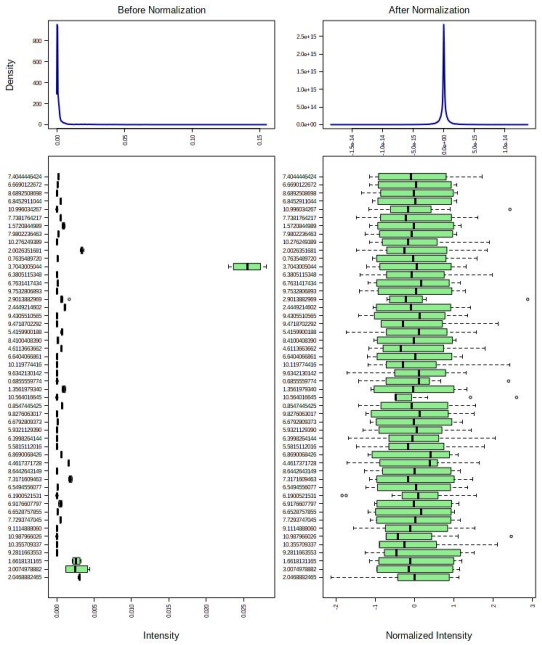
No Scaling



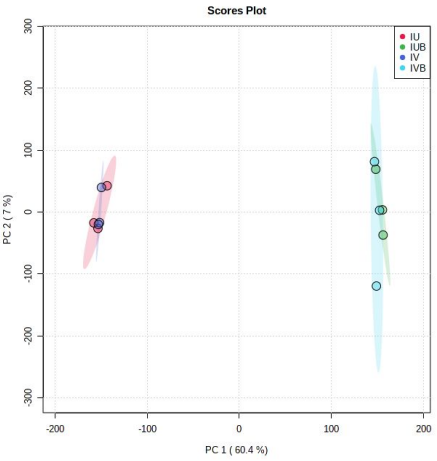


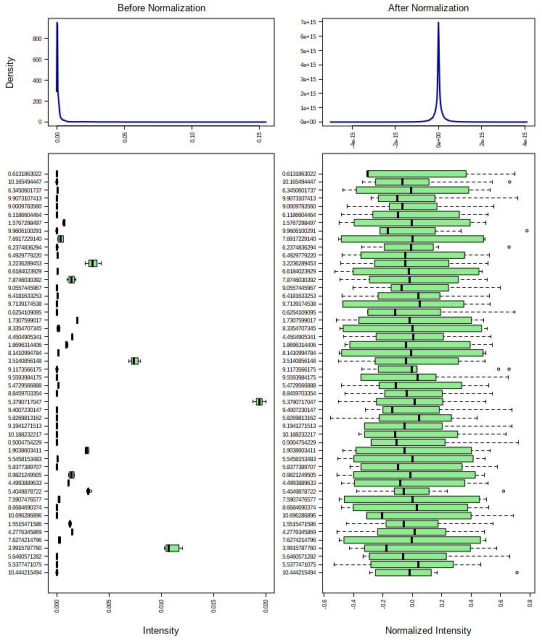
Pareto



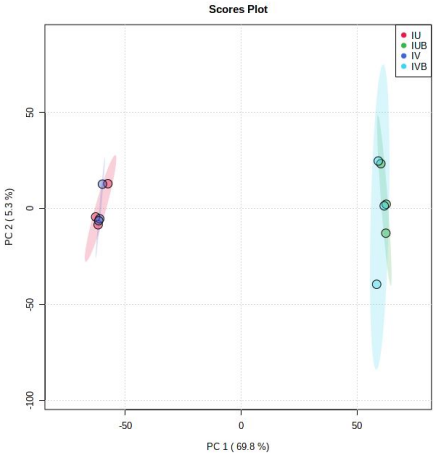

Before Normalization
After Normalization
Density
Intensity
Normalized Intensity

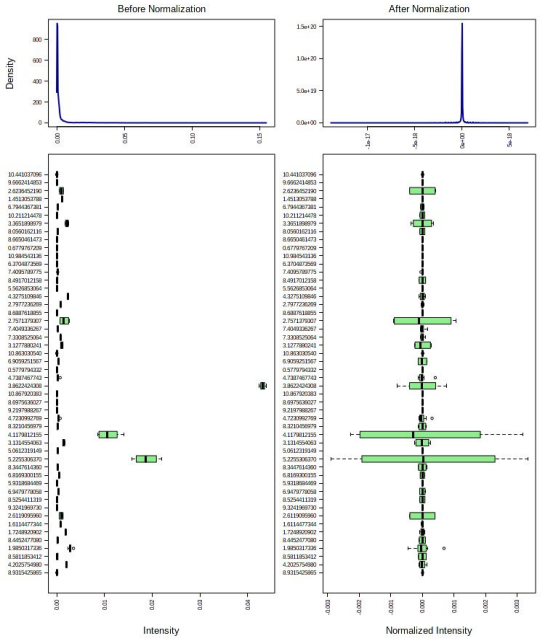
Auto


Scores Plot
IU
IUB
IV
IVB
PC 2 (7 %)
PC 1 (60.4 %)

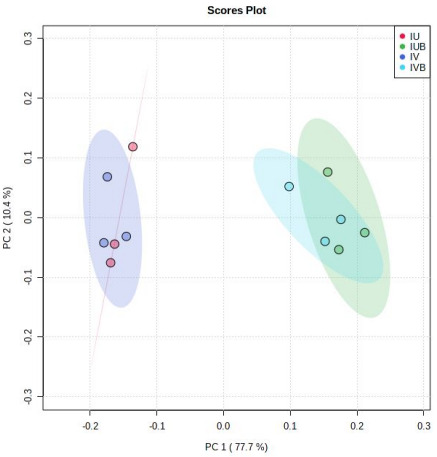

Before Normalization
After Normalization
Density
Intensity
Normalized Intensity

Range


Scores Plot
IU
IUB
IV
IVB
PC 2 (5.3 %)
PC 1 (69.8 %)


Before Normalization
After Normalization
Density
Intensity
Normalized Intensity

Mean Center


Scores Plot
IU
IUB
IV
IVB
PC 2 (10.4 %)
PC 1 (77.7 %)

Attachment 3: Bruker-to-Mat python script

```
# Read and plot processed 1H bruker spectra
# Also save them as .mat files
# Gaston Courtade - 06.03.20

import nmrglue as ng
import numpy as np
import matplotlib.pyplot as plt
import seaborn as sns
import scipy.io as sio


# edit for experiment number required from topspin
start = 76
end = 181
skip = [96,97,183,184,204,205]
filename = 'filename.mat'


# conversion of spectra to data matrix
spectra_list = []
i = start
while i < end+1:
    if i not in skip:
        spectra_list.append(i)
    i+=3


data_matrix = []
ppm_list = []
SF = 800.13
for expno in spectra_list:

    # nmrglue magic
    dic, data = ng.fileio.bruker.read_pdata(f'{expno}/pdata/1')
    udic = ng.bruker.guess_udic(dic, data, strip_fake=True)
    udic[0]['car'] = 3760.61 # 4.7 ppm
    uc_1h = ng.fileiobase.uc_from_udic(udic, dim=0)
    ppm_1h = uc_1h.ppm_scale()
    print(ppm_1h)
    #add this data to the matrix
    data_matrix.append(data)


#save to matlab
sio.savemat(filename, {'ppm':ppm_1h, 'X':data_matrix})
```

Attachment 4: MatLab *Icoshift* script for spectral alignment

```
% Notes for processing beer 1H NMR spectra using icoshift
% buffered samples, with double suppression (H2O and EtOH) used as examples
%
%
% SUMMARY OF DATASETS
% --------------------
% filenames:
%   noesygpps1d_13Cdec_buff.mat
%   noesygpps1d_13Cdec_unbuff.mat
%   noesygpps1d_buff.mat
%   noesygpps1d_unbuff.mat
%
% experiment type
%   noesygpps1d_13Cdec:    double suppression (H2O and EtOH)
%   noesygpps1d:          single suppression (H2O)
% sample type
%   buff:                 buffered samples
%   unbuff:               unbuffered samples
%
% contents of each dataset:
%--------------------------
% barcode:      barcode ID number for each sample
% buff_ints:    defined intervals used for alignment for the buffered samples
% ppm:          x-axis scale for [] in ppm
% ppm_corr:     ppm scale corrected using TSP reference peak
% TSPppm:       co-ordinates of TSP peak in [XcoshiftTSP] (used to correct ppm
scale)
% unbuff_ints:  defined intervals used for alignment for the buffered samples
% X:            raw data, samples x ppm
% Xaligned:     aligned data, samples x ppm (use with ppm_corr)
% Xcoshift:     coshifted data, samples x ppm
% XcoshiftTSP:  coshifted and referenced to TSP, samples x ppm (use with
ppm_corr)
%
%
%
% ANLIGNMENT STEPS
%-----------------
%
% 1. load the data file - add filename
% ------------------------------------
% datafile should consist of:
% ppm : row vector of the ppm scale
% X : matrix of raw data, samples x ppm
%
load('noesygpps1d_13Cdec_buff.mat')
%
%
% to generate plot of raw data:
% -----------------------------
%
plot (ppm, X'); set(gca, 'XDir', 'reverse')
%
%
% 2. create a matrix of COshifted data
% ------------------------------------
% this is an alignment based on the whole spectrum. the best mode for the
```

```
% buffered samples seems to be 'average2' with a multiplyer of 3. for the
% unbuffered samples the best seems to be using 'median'. also using the
% 'b' setting: algorithm search for the best 'n' for each interval
%
% buffered samples
[Xcoshift] = icoshift ('average2',X,'whole','b',[2 1 0],ppm);
%
% unbuffered samples
[Xcoshift] = icoshift ('median',X,'whole','b',[2 1 0],ppm);
%
%
% 3. align coshifted data using TSP reference region
% ----------------------------------------------
% define the TSP region from the coshifted matrix.
% Note:
%   in buffered samples, seems there are a couple that affect the TSP
% peak? Therefore only used the main peak (on the right) for the TSP
% alignment.
%   in unbuffered samples the TSP peak seems more widely affected by the
% the individual sample?
%
%
% buffered samples
[XcoshiftTSP] = icoshift ('average2',Xcoshift,(52390:52610),'b',[2 1 0],ppm);
%
% unbuffered samples
[XcoshiftTSP] = icoshift ('average2',Xcoshift,(52280:52480),'b',[2 1 0],ppm);
%
%
%
% 4. compute a TSP corrected ppm axis
% -----------------------------
%
% plot the TSP corrected matrix data
plot (ppm, XcoshiftTSP'); set(gca, 'XDir', 'reverse')
%
% select the TSP peak using the "data tips" tool
% right-click, select "export curser data to workspace"
% name "TSPppm"
% compute corrected ppm scale:
ppm_corr = ppm - TSPppm.Position(1);
%
% plot with corrected scale
plot (ppm_corr, XcoshiftTSP'); set(gca, 'XDir', 'reverse')
%
%
% 5. define intervals and align coshifted data
% ----------------------------------------
% use the coshifted and TSP aligned matrix (XcoshiftTSP) generated from
% previous steps as a starting point. use corrected ppm scale (ppm_corr).
% best mode for alignment seems to be 'average2' with multiplyer of 3.
% select missaligned regions and manually define intervals
% (buff_ints/unbuff_ints). this usees the column numbers from the
% ppm/ppm_corr vectors and therefore is the same for both scales. the
% buffered and unbuffered intervals differ slightly. They have the same
% number of intervals and same approximate positions but have been moved
% to accomodate approximately the same signals between both datasets
```

```
%
%
% buffered samples
[Xaligned] = icoshift ('average2',XcoshiftTSP,buff_ints,'b',[2 1 0],ppm_corr);
%
% unbuffered samples
[Xaligned] = icoshift ('average2',XcoshiftTSP,unbuff_ints,'b',[2 1
0],ppm_corr);
%
%
%
```

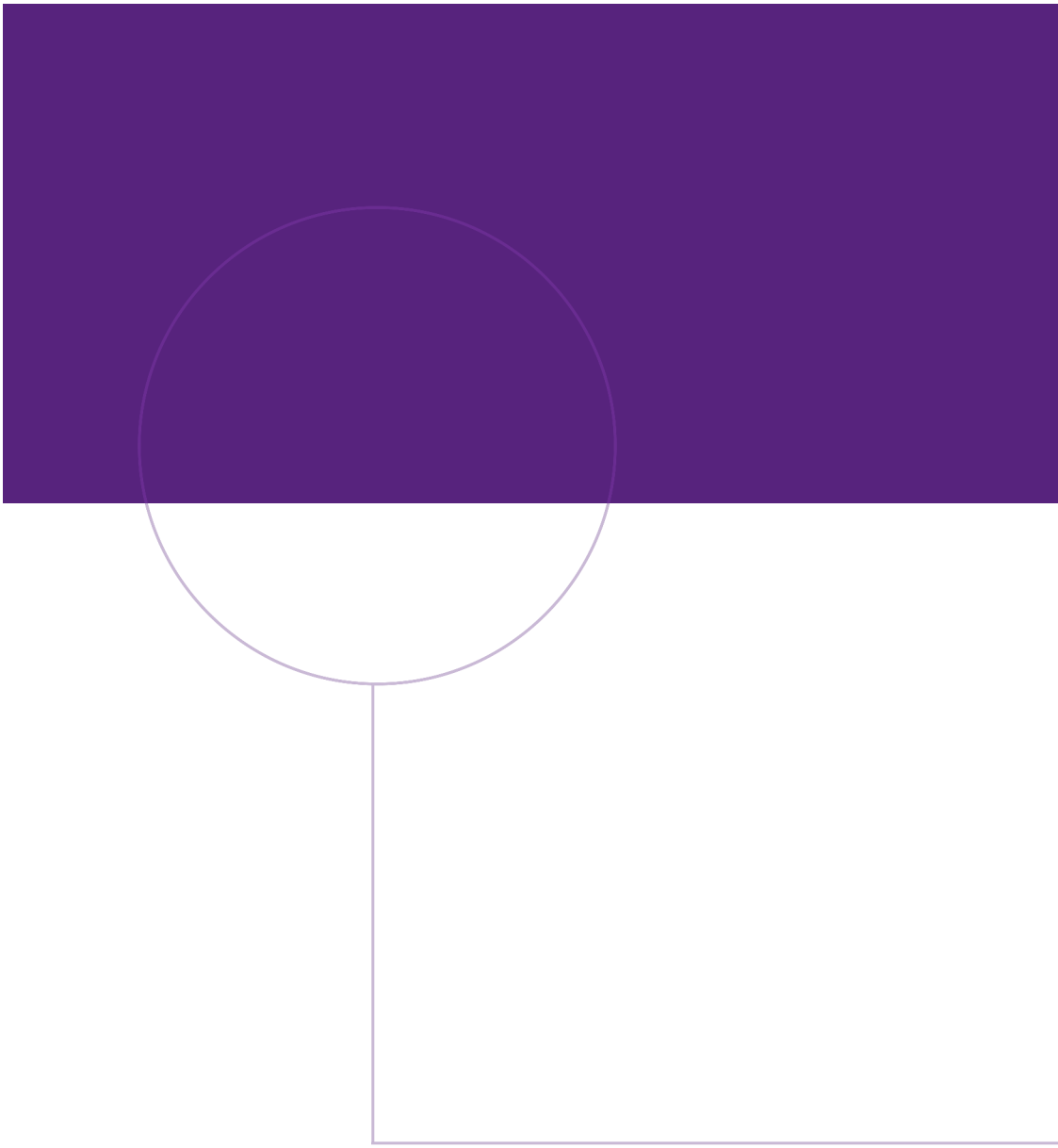
Attachment 5: MatLab beernormal script for normalization and scaling of the spectra

```
function [normalised, ppmNew] = beernormal(inputdata, ppm, excludeAreas,
varargin)
% This function normalises data using pqn based on
% [1] Dieterle, F et al., Analytical Chemistry 78(13), 4281-4290
% Additionally, it excludes areas from the data, such as ethanol and water
% areas before the normalisation
% The function truncates the spectrum automaitcally: Typically only from -0.5
% to 11 will be used.
% Furthermore, negative values will be eliminated.
%
% Scaling will be performed based on
% [2] R. A. van den Berg, H. C. Hoefsloot, J. A. Westerhuis, A. K. Smilde, M.
J. van der Werf, BMC Genomics. 7, 142 (2006).
% The options are:
%    'auto', 'pareto', 'vast', 'level' or 'range'
% which should be given as variable arguments.
%
%
% Example usage:
% excludeAreas = {{1.152,1.247},{3.608,3.681},{4.751,4.928}};
%
%
% [normalised, ppmNew] = beernormal(Xaligned, ppm_corr, excludeAreas, 'scale',
'pareto');
% This removes the unwanted areas, truncates the data, normalises it and
% scales it according to pareto scaling.
%
% [normalised, ppmNew] = beernormal(Xaligned, ppm_corr, excludeAreas);
% This normalises data, truncating it, without removing further areas nor
% scaling the data.
%
%
% HOWEVER: Scaling and removal of water/ethanol peaks is recommended!
%
%
% Christian 22.04.2020
%


% ppm truncate values:
ppmscaleEnd = 11;
ppmscaleStart = -0.5;



 %% check inputs and add the truncates to possible excluded areas.

% Check ppm scale
if numel(ppm)~=size(inputdata,2)
    error('The ppm scale and input data do not match in length.')
end

% generating and checking the ppm truncats
startPPM = ppm(end);
```

```
endPPM = ppm(1);
if startPPM > endPPM
    error('Something wrong with the ppm scale (maybe inverted?).')
end
if ppmscaleEnd > endPPM
    error('The ppm scale is shorter than 11. Fix this script in the start for
the truncation.')
end
if ppmscaleStart < startPPM
    error('The ppm scale is shorter than -0.5. Fix this script in the start
for the truncation.')
end
addlisting = {{endPPM,ppmscaleEnd},{ppmscaleStart,startPPM}};

% Adding the ppm truncates to the list
if exist('excludeAreas', 'var')
    excludeAreas{end+1} = addlisting{1};
    excludeAreas{end+1} = addlisting{2};
else
    excludeAreas = addlisting;
end

% actually checking the excluded areas
for ii = 1:numel(excludeAreas)
    if numel(excludeAreas{ii}) ~= 2
        fprintf(2,"Error in excluded Areas: Area %i has not two ppm values
defined.\n", ii);
        error('Returning.')
    end
end
inpLoc = excludeAreas;
for ii = 1:numel(excludeAreas)
    for jj = 1:numel(excludeAreas{ii})
        inpLoc{ii}{jj} = find(ismembertol(ppm,excludeAreas{ii}{jj}, 0.00001));
% TO adjust for non exactly values in the ppm scale, since they have more
digits
        if numel(inpLoc{ii}{jj}) ~=1
            fprintf("ppm couple:%i value: %i\n", ii, jj);
            error('Given ppm values not in ppm list. Maybe there is an error
with the accuracy (NMR ppm are NOT EXACTLY)')
        end
    end
end

% Checking input for rhe scaling
scaleType = [];
% Valid options for scaling
options = {'auto','pareto','vast','level','range'};

% Check input type
for j = 1:length(varargin)
    if isequal(varargin{j},'scale')
       for n = 1:length(options)
           if isequal(varargin{j+1},options{n})
              scaleType = n;
           end
```

```
        end
    end
end

if isempty(scaleType)
   scaling = false;
   fprintf(2,"No scaling will be performed!\n");
else
    scaling = true;
end


%% remove the exctration areas from the matrix

RemovedMatrix = inputdata;
ppmNew = ppm;
removeline = [];
for ii = 1:numel(excludeAreas)
    num = [inpLoc{ii}{1} inpLoc{ii}{2}];
    minnum = min(num);
    maxnum = max(num);
    numbers = [minnum:maxnum];
    removeline = [removeline,numbers];
end
RemovedMatrix(:,removeline) = [];
ppmNew(:,removeline) = [];

%% Adjusting for negative values

RemovedMatrix(RemovedMatrix<0) = realmin;
% This uses the smales value in matlab (2.225073858507201e-308) instead of
% a negative value.

%% normalistion using pqn

[row, col]=size(RemovedMatrix);
% Perform an integral normalization (typically a constant integral of 100 is
used).
integral=sum(RemovedMatrix,2);
integral_normed = 100*RemovedMatrix./repmat(integral,1,col); %now each spectra
sum to 100
%choose a reference or template spectrum, am selecting the median
ref=median(integral_normed);


% Addition here
% Apparently, a 0 in the ref is a problem...
% Therefore, we will manualy check it
if sum(ismember(ref, 0))>0
    posnill = find(ismember(ref, 0));
    for ii = 1:numel(posnill)
        med = median(integral_normed(posnill(ii)));
        if med ~=0
            ref(posnill(ii)) = med;
        else
```

```
            ref(posnill(ii)) = median([ref(posnill(ii)-
1),ref(posnill(ii)+1)]);
        end
    end
end


% Calculate the quotients of all variables of interest of the test spectrum
with those of the reference spectrum.
quotients = integral_normed./repmat(ref,row,1);
% Calculate the median of these quotients.
median_q = median(quotients,2);
% Divide all variables of the test spectrum by this median.
data = integral_normed./repmat(median_q,1,col);

%% scale data

% Handle scale type
if scaling
    if scaleType == 1 % auto scaling
        scaledData = zscore(data);
    elseif scaleType == 2 % pareto scaling
        for j = 1:size(data,2)
           scaledData(:,j) = (data(:,j)-
mean(data(:,j)))./sqrt(std(data(:,j)));
        end
    elseif scaleType == 3 % level scaling
        for j = 1:size(data,2)
           scaledData(:,j) = (data(:,j)-mean(data(:,j)))./mean(data(:,j));
        end
    elseif scaleType == 4 % vast scaling
        for j = 1:size(data,2)
           scaledData(:,j) = ((data(:,j)-
mean(data(:,j)))./std(data(:,j))).*(mean(data(:,j))./std(data(:,j)));
        end
    elseif scaleType == 5 % range scaling
        for j = 1:size(data,2)
           scaledData(:,j) = (data(:,j)-mean(data(:,j)))./(max(data(:,j))-
min(data(:,j)));
        end
    end
    normalised = scaledData;
else
    normalised = data;
end



%% plott the new graph

% plot (ppmNew, normalised); set(gca, 'XDir', 'reverse')
```



NTNU

Norwegian University of
Science and Technology