Understanding the Colloidal and Aromatic Stability of Dry-Hopped Beer

Margaux Louise Huismann, M.Sc. Brewing & Distilling,

B.S. Microbiology

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The International Centre for Brewing & Distilling

Institute for Biological Chemistry, Biophysics, and Bioengineering

School of Engineering and Physical Sciences

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Abstract

This thesis documents investigations of flavour stability and sporadic haze formation. Historical data was collected to statistically assess process derivations impacting sporadic increases in turbidity. Spearman's rank-order correlation coefficients did not find significant relationships between brewing, conditioning, and filtration/packaging on the increase of turbidity. Therefore, diagnostic studies were used to gradually eliminate potential sources of observed sporadic spikes in turbidity. Concentrations of high molecular weight β-glucans, total protein, and polyphenol were measured in low (≤ 5.0 EBC), high (≥ 5.0 EBC), and control (different brand, always ≤3.0 EBC) samples. Additionally, beer samples were digested with Ultraflo®Max, amyloglucosidase, and pepsin to digest β-glucans, residual starches/dextrins, and protein, respectively. The enzymatic digestion studies saw the greatest differences pre and post digestion by the addition of pepsin. The wet-chemical tests revealed that only β-glucan contents were elevated in high haze samples. Results indicated that mannoproteins were a culprit of turbidity. The use of LC-QTOF-MS and an assay for D-mannose, D-fructose, and D-glucose confirmed this supposition. Flavour-stability studies examined the solubility and extraction rate of hop terpenes into beer, the use of sensory analysis to trace the change in flavour/aroma over time, and an assay-development for the quantification of terpene concentrations in beer. As each hop variety contained different essential oil compositions, a linear extraction rate could not be determined. In addition to this, the chemistry of each hop terpene/terpenoid differs in chemical composition and are more/less soluble in different concentrations of ethanol. Overall, sensory and analytical data analyses did not find any strong relationships. However, the presence of β-myrcene could be linked to fresh beer less than 14 days old. Finally, the Vanillin assay was adapted to develop an assay to determine the concentration of terpenes/terpenoids in beer. Unfortunately, terpene concentrations in beer are too low to be detectable in the assay and the isolation/concentration methods were not successful. However, there is future potential to develop the assay by utilising methanol in place of ethanol and assessing one class of terpenes, such as monoterpene oxides, instead of multiple classes of terpenes. The combined results of this work provide more information to brewers, packaging technologists, and quality laboratories on how raw materials impact the quality of the final product, and therefore increasing the likelihood of consumers experiencing high quality, flavour stable products.

Dedication

I would like to dedicate this thesis to my parents Jim Huismann and Cass Huismann in addition to my siblings Trent Huismann, Marlys Weyandt and Dan Weyandt. I am truly humbled by your support and unconditional love. I have an immense amount of gratitude for your support and compassion, in good times and in bad, throughout this journey. I know none of this was easy, especially given the distance, but I thank all of you from the bottom of my heart for your encouragement and love. I love each of you so much.

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Abbreviations

% ABV- Percent Alcohol by Volume in Solution

°C- Degrees Celsius

A- Absorbance

ADF- Apparent Degree of Fermentation

AIC- Akaike Information Criterion

ANOVA- Analysis of Variance

ATP- Adenosine Triphosphate

CV- Column Volumes

DCM- Dichloromethane

DoE- Design of Experiments

DTT- Dithiothreitol

EBC- European Brewery Convention

FAN- Free Amino Nitrogen

FASTA- Fast-All Nucleotide Comparison

FTU- Formazin Turbidity Unit

FV- Fermentation Vessel

GC- Gas Chromatography

GC/MS-SPME- Gas Chromatography/Mass Spectroscopy paired with Solid Phase Micro-Extraction

GPI-CWP's- Glycosylphosphatidylinositol-Cell Wall Proteins

hL- Hectolitres

HPLC- High Performance Liquid Chromatography

IBU- International Bitterness Units

IPA- India Pale Ale

ISTD- Internal Standard

L- Litres

LC/ESI-QTOF-MS- Liquid Chromatography/Electrosprayionisation-Quadrupole Time of Flight-Mass Spectroscopy

LC/MS- Liquid Chromatography/Mass Spectroscopy

LSD- Least Significant Difference

m- Meters

M- Molar

mg- Milligrams

mM- Millimolar

mol- Moles

mol%- Mole Percent

MW- Molecular weight

NADP- Nicotinomide Adenine Dinucleotide Phosphate

NTU- Nephelometric Turbidity Unit

p- Probability Value

PG- Present Gravity

Pir-CWP's- Proteins with Internal Repeats-Cell Wall Proteins

ppb- Parts per Billion

psig- Pound-Force per Square Inch, gauge pressure

PVPP- Polyvinylpolypyrrolidine

Q-Q- Quantile-Quantile Plot (Probability Plot)

r^2- Coefficient of Determination

RCF- Relative centrifugal force

RPM- Revolutions per Minute

RT- Room Temperature

SDS- PAGE- Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SG- Specific Gravity

SPE- Solid-Phase Extraction

TPO- Total Packaged Oxygen

U- Enzyme Activity Units

V- Volts

v/v- Volume per Volume

VDK- Vicinal diketones

w/v- Weight by Volume

w/w- Weight by Weight (a measure of concentration)

ρ- Spearman's Rank-Order Correlation Coefficient, rho

Chapter 1- Introduction

'You take three compounds, three aromas, different intensities relating to different human threshold levels… You can't quantify that. It would take somebody doing a Ph.D.' He later suggested it would be a particularly ambitious one. -Tom Nielsen of Sierra Nevada in 'For the Love of Hops: the Practical Guide to Aroma, Bitterness, and the Culture of Hops' by Stan Hieronymus

2.1 Introduction

The origin of beer and brewing is a heavily debated topic. Beer was ultimately produced as an evolutionary step with the advancement of civilisation. Gruel, most likely, was produced first, followed by bread and ultimately- beer (Katz *et al.*, 1986). Anthropologists theorise that the discovery of fermentation ultimately arose from human manipulation of grains and that brewing encouraged prehistoric peoples to form and live in settlements (Katz *et al.*, 1986; Joffe *et al.*, 1998). Fermented beverages derived from various carbohydrate sources had been a staple in ancient diets dating back to roughly 3000 BC (Eβlinger, 2009). The earliest written knowledge of brewing dates back to ancient Mesopotamian times. The 'Hymn to Ninkasi', an ode to the goddess of beer, mentions the basic ingredients of beer produced in ancient times (Eβlinger, 2009). Historically, multiple ancient civilisations mention brewing ranging from Israeli and Palestinian peoples to the ancient Celts (Hornsey, 2003).

In medieval times, ales soured rapidly. Prior to hops, herbaceous materials were added to these ales in efforts to prevent the souring and flavour these beverages, known as 'gruit' (Bennett, 1996; Hornsey, 2003; Briggs *et al.*, 2004). Monasteries in the middle ages were some of the first to document the use of hops in the brewing process, which were brought to Northern and Eastern Europe during the migration of the Caucasian people (Hornsey, 2003; Eβlinger, 2009). Monastic breweries were known for producing beer of exceptional quality and the tradition has continued (Hornsey, 2003). Monks brewed with careful consideration and consistency. These early observations were the humble beginnings of brewing research. In modern research, the production of high quality beer relies heavily upon applied principles of biochemistry, microbiology, and organic chemistry. Brewing laboratories utilise these principles to produce high quality beverages with consistency.

2.2 The Brewing Process

2.2.1 Malting/Milling

While beer can be made out of most cereals, malted barley is the most common cereal of use and is considered traditional under the *Reinheitsgebot* Germany Purity Law (Eden, 1993). Barley (*Hordeum vulgare*) is an annual grass plant, planted in late autumn or early spring (Briggs *et al.*, 2004). While the composition and chemistry of a barley kernel is complex, a brewer is primarily concerned about the starchy endosperm, enzyme formation within the aleurone layer, and the husk material (Lewis *et al.*, 2002; Briggs *et al.*, 2004). Barley is harvested by farmers and shipped to maltsters to undergo the malting process.

The malting process consists of three essential processes: steeping, germination, and kilning. During steeping, grain is steeped and aerated in cycles in cool (10-15°C) aerated water to wash away husk components, aerate the grain, and prevent microbial infection. Grain is steeped until the coleorhiza, also known as the root sheath or 'chit', penetrates the micropile (Lewis *et al.*, 2002). This action ensures grain is aerated, microbial growth is kept to a minimum, and that proper modification begins (Briggs *et al.*, 2004). During germination, barley is turned or mixed to maintain even heat and aeration levels. Plant hormones known as giberellins and abscisic acid are produced by the germinating embryo. The production of the giberellins and abscisic acid are key to successful malting as they stimulate the production of hydrolytic enzymes in the aleurone layer which are released into the endosperm. The hydrolytic enzymes modify and begin to soften the starchy endosperm by degrading β-glucans, pentosans, proteins, and starch granules. This enzymatic activity begins the process of converting starch to fermentable sugar (Palmer, 1992; Briggs *et al.*, 2004). When the root sheath penetrates the micropile of barley- a process known as 'chitting'- the necessary enzymes have been formed and germination process is complete (Lewis *et al.*, 2002). Germination is ceased by kilning to halt modification and stabilise the malt while conserving the enzymes within the malt (Priest *et al.*, 2006). Kilning preserves the malted barley by lowering the moisture content from approximately 43% to less than 5% moisture (Priest and Stewart, 2006).

Milling is dependent upon the type of wort separation system used. Roller mills are used to create a coarse grist if a mash or lauter tun is used in the brewing process. Less commonly, hammer mills are employed to create very fine, floury grist and are used a mash filter is employed to separate the finely milled husk material from the

sweet wort. The purpose of milling is to reduce and create uniform particle sizes of malt for even extraction during mashing (Lewis *et al.*, 2002; Priest *et al.*, 2006).

2.2.2 Mashing

The mashing process utilises malt-derived enzymatic activity to gelatinise starches and yield fermentable sugars.

In mashing, a carefully calculated volume of water, at a specific temperature, is combined with the grist to convert malt starches into fermentable sugars, and to break down malt proteins for foam stability and free-amino nitrogen (FAN) to support fermentation. Enzymatic activity is rampant during mashing, the most common enzymes are proteases, endoglucanases, and amylases (Bamforth, 2009). Common mashing techniques include temperature-programmed infusion mashing, decoction mashing, and double mashing.

In a temperature-programmed infusion mash, a mash mixing vessel is utilised to mix and heat the mash until it reaches 62°C. The initial mashing temperature will be lower if malt is poorly modified. Following the first temperature rest, the mash is heated to a 'standing' temperature at 64-68°C followed by a 'mash out' sparge at 75-77°C. Temperature-programmed infusion mashing is a popular method as temperature programmes are specifically designed per beer style and to guarantee sufficient extraction of FAN. Finally, temperature-programmed infusion mashing is more easily-automated and more energy efficient than methods such as decoction mashing (Priest *et al.*, 2006).

Decoction mashing is a commonly utilised method in German breweries. Decoction mashing is a form of step-mashing in which the temperature of the mash is slowly increased by removing a portion of the mash, heating the portion to boiling point, and returning it to the mash tun. Multiple steps may be employed in decoction mashing, depending on the style of beer. Decoction mashing is beneficial as the mash temperature is slowly raised, allowing enzymatic activity to take place in various temperature ranges. Two or three heating steps may be employed for 'enzyme rests' to allow for enzyme degradation in specific temperature ranges. The first 'enzyme rest' sits at a temperature range between 40-50°C for optimal proteolytic action. A portion of the mash is heated and returned to the mash, raising the temperature to approximately 65°C for enzymatic starch hydrolysis. Finally, the mash temperature is raised to approximately 76°C to denature enzymes and decrease wort viscosity for better wort

runoff (Briggs *et al.*, 2004; Priest *et al.*, 2006). While decoction mashing is a very efficient method of mashing, it is cumbersome and requires specialised equipment to pump the mash from the mashing vessel to the external calandria or other vessels.

Finally, double mashing is a technique employed for adjunct brewing. Adjuncts such as rice or maize require pre-cooking to gelatinise starches before adding the adjuncts to the mash. In pre-cooking, the adjunct and a small amount of malt (approximately 10%) are mixed with water in a cereal cooker and heated to approximately 85°C. As the temperature rises, the diastatic power in the malt helps to reduce viscosity. The cereal cooker is held at 85°C for approximately ten minutes to encourage α-amylase activity. The cereal cook is followed by a boil for approximately 10-20 minutes to thin the mash before it is transferred over to the mashing vessel for the second malt-based mash (Lewis *et al.*, 2002; Priest *et al.*, 2006).

2.2.2.1 Wort Carbohydrates

The purpose of mashing is to convert starch into fermentable carbohydrates in the sweet, sugary liquid known as wort. The grist volume and mashing schedule may be tailored to extract specific carbohydrates. However, the wort sugars and dextrins typically observed after mashing are glucose, maltose, maltotriose, maltotetraose, maltopentose and branched dextrins. Yeast commonly used in beer and brewing can easily ferment monosaccharides, utilising glucose and fructose first followed by the disaccharides sucrose and maltose, and finally maltotriose.

As described in Section 2.2.2, mash temperatures dictate enzyme activity and these active enzymes convert starch into dextrins and fermentable sugars. Starch granules are made of amylose and amylopectin with trace levels of protein, ash, and lipids. Amylose is a polysaccharide made of α-(1,4)-linkages of 1600-1900 D-glucopyranose residues. Amylopectin is a highly branched molecule, approximately 15-25 glucose residues (α-(1,4) linkages) long and joined by α-(1,6) branch points **Figure 1.1** (Lewis *et al.*, 2002; Briggs *et al.*, 2004).

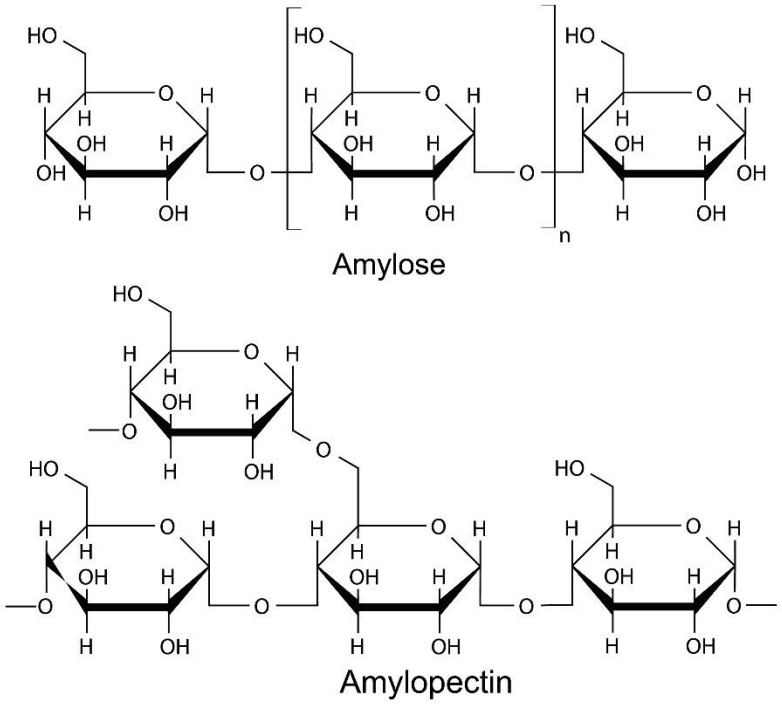


Figure 1.1- Structural differences of amylose (above) and amylopectin (below).

During mashing, enzymes break amylose and amylopectin into smaller, fermentable sugars found in wort. Each enzyme contains differing levels of activity and produce different levels of sugar. There are several subclasses of each enzyme but the enzymes of greatest interest in mashing are α-amylase, β-amylase, and limit dextrinase (Briggs *et al.*, 2004).

Beta-amylase is an exoenzyme, hydrolysing alternating α-1,4-linkages in amylose and amylopectin at non-reducing ends. This cleavage yields maltose, the most abundant malt sugar found in all malt wort. Beta-amylase can only hydrolyse the ends of amylopectin as the enzyme cannot hydrolyse α-(1,6)-bonds (Lewis *et al.*, 2002). Limit dextrinase is able to hydrolyse α-(1,6)-linkages and release straight chain dextrins. The endo-enzyme, α-amylase is similar to β-amylase in the sense that it hydrolyses α-1,4-linkages, except that the hydrolysis is non-sequential, occurring in a random fashion. It is important to note that both enzymes cannot cleave α-1,4-linkages in close proximity to α-(1,6)-linkages. Alpha-amylase is useful in breaking up large starch molecules, creating new non-reducing ends for the β-amylase to cleave. As the enzyme cleaves α-1,4-linkages in a random fashion, it greatly reduces the viscosity of the mash, crucial to wort separation (Lewis *et al.*, 2002; Briggs *et al.*, 2004). The enzymatic action gelatinises and liquefies starches within the malt. With proper gelatinisation and

liquefaction, malt starches are converted to fermentable sugars by the process of saccharification.

The gelatinisation and saccharification enzymes α and β-amylase, function at different temperature ranges. Less thermotolerant β-amylase is less active at 60-65°C. If mash temperatures surpass this range, β-amylase denatures. More thermotolerant α-amylase, has an optimum temperature range between 65-75°C. As α-amylase cleaves α-1,4-linkages in starches in a random fashion, higher mash temperatures produce worts with lower fermentability as dextrins are incompletely saccharified (Lewis *et al.*, 2002; Briggs *et al.*, 2004). Enzyme activity is vital to the concentration of maltose and glucose (Briggs *et al.*, 2004; Schur, Pfenninger, and Narziss, 1973).

The structure of enzymes are also affected by mash pH. Brewery mash pH ranges between 5 and 5.5 but typically, does not exceed 5.7 as proteolytic enzyme activity is affected (Briggs *et al.*, 2004; Bamforth, 2009). Malt enzymes are active at mash pH levels but mash pH levels are not within all malt enzymes range for optimum activity (Briggs *et al.*, 2004). For example, a three-fold increase in limit dextrinase activity occurs when pH levels are reduced to 4.0 (Heisner *et al.*, 2008). Limit dextrinase will be active at mash pH ranges, however, its activity will be limited.

2.2.3 Wort Proteins

Malt proteins and protein breakdown products in brewing are best categorised by their individual properties. For example, LTP1 is not particularly foam-positive when isolated from malt. However, upon boiling, denaturation of LTP1 yields hydrophobic polypeptides. These hydrophobic polypeptides cross-link with hop bitter acids yielding a stable foam (Bamforth, 2011). Additionally, haze-propagating proteins contain high levels of proline residues and easily cross-link with polyphenols. However, these proteins can be easily removed with silica hydrogels or proteolytic enzymes (Asano *et al.*, 1982; Briggs *et al.*, 2004; Bamforth, 2011).

The amount of soluble nitrogen extracted into beer is dependent upon the mashing regime used. Free amino nitrogen (FAN) is an essential nitrogen source to support yeast growth and fermentation. Minimum FAN levels required for a healthy fermentation range from 100-140 mg/L (Briggs *et al.*, 2004). Minimum FAN levels when brewing with unmalted cereals require at least 160 mg/L of FAN (Evans *et al.*, 2012). Mashing temperatures for optimal proteolytic enzyme activity rest between 40-50°C at an optimal pH range of 3.0-6.5 (Briggs *et al.*, 2004).

2.2.4 Wort Separation

The sweet sugary liquid produced during the mashing process, also known as 'wort', must be separated from the spent grains after the mashing process. Two techniques of wort separation exist- mash filtration and lautering.

Mash filtration is utilised with hammer-milled malt. Mash filters feature supported rectangular plates with a central metal grid covered by Kevlar filter cloths (**Figure 1.2**). A deeper frame is found between the two plates for the mash to be pumped into. The frame consists of two sides- one side for the mash/sparge water to be pumped into and one side for the wort/sparge water to be pumped into the kettle. When pressure is applied to the mash filter, the wort filters from the grist through the cloth and flows into a narrow cavity. The wort flows from this cavity to a wort collection pipe and is pumped into the kettle (Lewis *et al.*, 2002).

Figure 1.2- Mash filter and mash filter components- A) Overhead view of mash filter frame covered by filter cloth, B) completely assembled mash filter, C) mash filter plate with mash inlet(indicated by one red circle and a *), wort outlet (indicated by two red circles and **), and deep set frame for mash contents to flow into.

The technique of lautering is used for wort separation when malt has been roller-milled. In this technique, the stirred mash is pumped into the top of the lauter tun and left to settle to form a grist bed. The grist bed has rakes cutting through the bed and sits on top of a false bottom with very thin slits (0.5-1 mm) for wort to flow through. The first runnings of the lautering process are recirculated to the top of the vessel as some starch particles may not have had a chance to be caught by the grist bed from the initial

filling of the mash tun. The wort runoff is recirculated or vourlaufed until the wort runs clear, ensuring that most of the starch particles are trapped in the bed. Rakes in the mash tun are utilised in two different ways to aid in wort separation (Lewis *et al.*, 2002; Briggs *et al.*, 2004).

In one method, the rakes remain on throughout the duration of the lautering process to lift the grist bed and create open channels for wort to percolate through during runoff and sparging. As the bed is compressed, the rakes are lowered and halted once the grist bed has completely compacted. In a similar approach, the same protocol is utilised, however, wort runoff valves are opened to collect wort at a much faster rate. As the runoff rate slows as the bed compacts, wort collection is stopped and sparge water is pumped underneath the false bottom (underletting). The rakes are run faster to resuspend the bed and after the bed settles, wort collection is continued until the lauter tun is required to be underlet again (Lewis *et al.*, 2002).

2.2.5 Wort Boiling and Clarification

Following the separation of wort from the spent grist, wort boiling is an essential step in the production of most beers. Wort boiling serves many purposes. The factors that support a healthy fermentation are found in the European Brewing Convention's (EBC) *Manual of Good Practice- Wort Boiling and Clarification* (Denk *et al.*, 2002). Section 2.1 in the manual states that a successful wort boil yields:

1. "**Colloidal Stability**- by the coagulation of protein/polypeptide chains and subsequent precipitation in hot break (trub) and cold break. The coagulated protein/polypeptide chains often form chains with reactive polyphenols.
2. **Extraction of hop bitterness**- by the consistent isomerisation of the hop bitter compounds (when added to the kettle).
3. **Biological stability of the wort-** by producing a sterile wort, free of beer spoilage microorganisms. Boiling will also destroy any residual enzyme activity carried over from raw materials.
4. **Removal of unwanted volatiles-** by steam distillation of volatile compounds which may originate from the brewing materials or from the addition of hops, but which are not required to be present in the finished beer.
5. **Decrease in wort pH-** wort pH drops during wort boiling owning to the precipitation of proteins, secondary phosphates, the formation of melanoidins and the dissolution of bitter acids. The fall in pH depends on the buffering capacity of the wort and the malt type used. Usually, the higher the original pH

of the wort the greater the pH drop. The fall in pH during boiling is necessary for the flavour and microbial stability of the beer and is a pre-requisite for healthy fermentation.

6. **Formation of colour and reducing compounds-** the increase in colour is dependent on time, temperature, and pH (the higher the pH the greater the colour formation). Some colour increase is obtained through the polymerisation of polyphenols, but it is mainly produced by the formation of Maillard products…
7. **Formation of flavour compounds-** malt flavour components are extracted during mashing. They are not always beneficial to the final beer. Malt also contains compounds that are extracted into the wort and influence beer flavour through their effect on fermentation, for instance lipids."

Reducing power is increased after wort boiling thus protecting the wort from oxidation in other downstream processes (Briggs *et al.*, 2004). Wort boiling is complex as reducing power is increased by the extraction of polyphenols (G. Lermusieau *et al.*, 2001; Jurić *et al.*, 2015). However some hop and malt-derived polyphenols are sensitive to oxidation and may contribute to oxidation as beer ages (Andersen *et al.*, 2000; De Almeida *et al.*, 2015).

2.2.5.1 Wort Boiling

Wort boiling is a critical step in the production of beer with low haze, good flavour development, and substantial reducing power. It is an essential step in the precipitation of nitrogenous compounds from wort. The heat from wort boiling changes the structural organisation of beer proteins as proteins denature and unfold. This action results in a less soluble protein to be present in the wort as it is less hydrated causing the precipitation of proteins (Denk *et al.*, 2002). Proteins coagulate at their isoelectric point. At the isoelectric point, protein becomes less soluble and more hydrophobic due to the molecule's neutral charge, causing the protein to precipitate from solution. Polyphenols assist in the denaturation of wort proteins as polyphenols act as reducing agents (Denk *et al.*, 2002; Briggs *et al.*, 2004).

2.2.5.2 Hop Additions in the Wort Boil

As one of the four essential ingredients in beer, hops provide multiple attributes to beer including bitterness, aroma, and oxidative stability via low molecular weight polyphenols (Almaguer *et al.*, 2014). Hops are discussed in detail in Section 2.3 and Section 1.4.5.2.

2.2.5.3 Wort Clarification

Wort clarification is essential to a healthy fermentation. Inadequate wort clarification results in poor beer filterability due to excess trub and poor fermentation as trub adheres to yeast cells, inhibiting flocculation (Denk *et al.*, 2002). However, it is important to not produce an excessively clarified wort as fermentation is inhibited by a lack of trub material serving as nucleation sites for CO_2 evolution (Siebert *et al.*, 1986; Denk *et al.*, 2002).

Various fining agents are added to the kettle to aid wort clarification. Finings such as carrageenan, also known as Irish moss, encourage protein coagulation and precipitation. Bentonites may be added to ensure complete isomerisation of α-acids in addition to encouraging protein precipitation, which may negatively impact foam (Denk *et al.*, 2002).

2.2.6 Fermentation

Traditionally, fermentation is initiated by the addition of an ale yeast (*Saccharomyces cerevisiae*) or lager yeast (*Saccharomyces pastorianus* formerly *Saccharomyces carlsbergensis*) into cooled wort. *Saccharomyces cerevisiae* are known as top-fermenting ale strains as they flocculate and float to the top of the fermenter at the end of fermentation, presumably due to adhesion to CO_2 bubbles (Speers *et al.*, 1992). *Saccharomyces pastorianus*, is known as a bottom-fermenting lager yeast as these yeast flocculate and sediment to the bottom of the fermentation vessel during fermentation. A brief overview of top-fermenting and bottom-fermenting yeast can be found in **Table 1.1.**

Table 1.1- Comparison of top-fermenting and bottom-fermenting yeasts

Attributes	Bottom-Fermenting	Top-Fermenting
Strain(s)	*Saccharomyces pastorianus*[1] *Saccharomyces eubayanus*[2] *Saccharomyces carlsbergenisis*[1]	*Saccharomyces cerevisiae*[1]
Sugar utilisation	Utilises melibiose[1] Weak respiration in low glucose medium[1]	Unable to utilise melibiose[1] Able to respire in low glucose medium[1]
Flocculation	Precipitates to the bottom of the FV at cessation of fermentation[1]	Floats to the top of the FV at cessation of fermentation[1]
Fermentation Temperature	6-12°C[1]**	18-24°C[4]**
Complex Sugar Fermentation?	Sensitive to catabolic inhibition by maltose[1]	[1]Limited inhibition of the fermentation of maltose[1]
Flavour Profile	Low ester production[3] High sulphur production (SO_2 > 4 mg/L)[1]	High ester production[3] Less sulphur production (SO_2 < 2mg/L) [1]

[1](Denk *et al.*, 2002), [2](Libkind *et al.*, 2011), [3](Pires *et al.*, 2015), [4](Hiralal *et al.*, 2014)
*Adapted from (Denk *et al.*, 2002)
**A wide variation in fermentation temperatures of top and bottom fermenting yeasts exists and can be dependent upon the supplier (White Labs, 2015)

Yeast require nitrogenous compounds and sugars extracted during mashing for a healthy fermentation. Boulton and Quain, (2006) provide a rough mass balance of fermentation (**Equation 1.1**). Micronutrients such as zinc, calcium, and magnesium are also required to support yeast health during fermentation (Dombek *et al.*, 1986; Ciesarová *et al.*, 1996; Briggs *et al.*, 2004).

$$Sugars\left(\frac{150g}{L}\right) + Free\ amino\ nitrogen\left(\frac{150g}{L}\right) + Yeast\left(\frac{1g}{L}\right) + Oxygen\left(\frac{25mg}{L}\right) \rightarrow Ethanol\left(\frac{45g}{L}\right) + CO2\left(\frac{42g}{L}\right) + Yeast(\frac{5g}{L})$$

(1.1)

Besides ethanol and carbon dioxide, yeast by-products of fermentation result in compounds relevant to flavour and aroma including higher alcohols, esters, aldehydes, sulfur-containing compounds and vicinal diketones (Briggs *et al.*, 2004; Pires *et al.*, 2014).

In primary fermentation, most of the fermentable wort carbohydrates are consumed by yeast and converted to ethanol and carbon dioxide. Yeast flocculation indicates the cessation of primary fermentation. Yeast cells begin to aggregate as the wort sugars are exhausted from the medium, through zymolectin receptors that are no longer masked, blocked or inhibited (Stewart, 2018). Low molecular weight ligands exhibit specific binding activity toward high molecular weight molecules, such as proteins. Lectin acts as a receptor for sugars acting as ligands in lectin-sugar interactions and in yeast, zymolectins are proteinaceous lectins anchored to the cell wall, accepting mannose or gluco-mannose residues. Despite their attachment to the yeast cell wall mannan skeleton, it is evident that carbohydrate residues function as ligands and zymolectins function as receptors (Hsu *et al.,* 2001). Research has found that zymolectins are consistently present on yeast cell walls and do not appear upon the event of flocculation (Patelakis *et al.,* 1998; Speers *et al.,* 2006).

The phases and speed of beer fermentations are dependent upon nutrient conditions within the medium. Three stages of brewing fermentations have been documented- the lag phase, the log or exponential growth phase, and finally the stationary phase (Briggs *et al.,* 2004). During the lag phase, yeast cells shift from a dormant state to active cell division. Specific gravity of wort shows little change during the lag phase however, dissolved oxygen levels shift in addition to pH and FAN levels providing evidence of the yeast cell preparation for active growth and nutrient uptake (Anderson *et al.,* 2000; Briggs *et al.,* 2004). Depending on the scale of fermentation and the health of the yeast, the lag phase can last several hours or up to one day. Following the initial lag phase, the yeast cells enter into an exponential growth phase and cell division typically follows a logarithmic scale (Anderson *et al.,* 2000; Briggs *et al.,* 2004). A sharp drop in specific gravity, pH and FAN levels are observed during the logarithmic growth phase as yeast metabolise wort sugars and utilise nitrogenous compounds to support cell growth. During fermentation, yeast consume wort sugars in a preferential manner consuming glucose first, followed by fructose, maltose, and maltotriose, respectively (Briggs *et al.,* 2004; MacIntosh *et al.,* 2016). When wort sugars and micronutrients are depleted and fermentation by-product concentration in solution increases, the log phase slows and cells enter the stationary phase. In stationary phase, cell division ceases and yeast cell biomass maintains constant. If left for a long period of time, yeast cells enter a state of senescence and dormancy (Anderson *et al.,*

2000; Briggs *et al.*, 2004). At this point, the fermentation vessel is set to cool and left to rest for the early stages of conditioning.

2.2.7 Conditioning/Maturation

During primary fermentation, the rapid consumption of wort nutrients by yeast results in off-flavour formation (including H_2S and vicinal diketones (VDK's), increased turbidity, and astringent flavours. Conditioning (also known as secondary fermentation or maturation), is a phase designed to aid in the reduction and/or removal of undesirable fermentation by-products (Anderson *et al.*, 2000; Briggs *et al.*, 2004).

Valine and isoleucine biosynthesis during fermentation produce VDK's as by-products of the enzymatic oxidative decarboxylation of α-acetolactate (Kobayashi *et al.*, 2005; Krogerus *et al.*, 2013). In valine biosynthesis, pyruvate is converted to α-acetolactate and intermediate transformations result in the production of valine. The conversion step of α-acetolactate to valine is rate-limiting and excess α-acetolactate is pumped through the cell membrane into wort where non-enzymatic decarboxylation and oxidation occurs, forming 2,3-butanedione (Krogerus *et al.*, 2013). Vicinal diketones, 2,3-pentanedione and 2,3-butanedione, provide a buttery and toffee-like flavour that is undesirable in most products (Krogerus *et al.*, 2013). A similar process occurs in the production of 2,3-pentanedione from an α-ketohydroxybutyrate precursor (Briggs *et al.*, 2004). During conditioning, yeast in suspension take up exogenous 2,3-butanedione and 2,3-pentanedione and reduce the compounds to 2,3-butanediol and 2,3-pentanediol, respectively (Anderson *et al.*, 2000; Kobayashi *et al.*, 2005; Krogerus *et al.*, 2013). Good yeast health is imperative to the removal of VDK's. Warm conditioning programmes are scheduled to drive-off other compounds such as hydrogen sulfide (H_2S) and acetaldehyde.

Conditioning regimes vary per beer style and desired flavour profile. Some conditioning programmes hold beer at warmer temperatures (12-16°C) for 1-2 days to allow yeast flavour development and refinement followed by a crash-cool to 0°C to precipitate all yeast cells and colloid particles (Masschelein, 1986; Anderson *et al.*, 2000; Briggs *et al.*, 2004). With modern equipment, knowledge and practice, breweries commonly utilise a conditioning profile of a short rest on yeast, crash cool with yeast cropping, and allow beer to condition at 4°C for several days (Anderson *et al.*, 2000; Briggs *et al.*, 2004). It is important to recognise that conditioning regimes are brewery and beer-style dependent.

2.2.8 Yeast Handling

Yeast handling practices are imperative to the production of high-quality beer. Best practices in yeast handling recommend tightly monitoring propagation, yeast cropping, storage, and serial repitching stages within the brewery.

Yeast must be propagated in an oxygenated, nutritionally sufficient medium at a suitable cell density. Without oxygen, yeast cells are unable to synthesise sterols for cell-wall biosynthesis (Callaerts *et al.*, 1993; Bokulich *et al.*, 2013). Following yeast propagation to a suitable cell density, yeast are pitched into a fermenter at a calculated cell-density (i.e.- cells/mL). If yeast are over-pitched, off-flavours are formed and the cropped yeast result in poor viability. If yeast are under-pitched, slow or stuck fermentations may result (Cahill *et al.*, 2000).

Yeast cropping is the practice of collecting flocculated yeast from a fermenter. At this point, the yeast may be stored or repitched into another beer fermentation. In warm cropping practices, yeast are collected at the end of primary fermentation. Cold cropping is the collection of yeast after-cold crashing the cylindroconical vessel (Powell *et al.*, 2004). Yeast autolysis and petite mutations may be induced by repetitive cold cropping (Alexandre *et al.*, 2006; Lawrence *et al.*, 2012). Overall, cropped yeast are more susceptible to environmental damage, such as shearing from pumps and centrifuges than actively-dividing yeast cells (Stoupis *et al.*, 2002).

If yeast is stored at temperatures of 10°C or above, for extended periods of time, viability and cell glycogen concentrations are drastically reduced, off-flavour production of VDK's is increased, and slow fermentation rates with poor attenuation rates are observed (McCaig *et al.*, 1985). Additionally, acid-washing regimes as part of storage and repitching protocols increases the risk of shear damage. If yeast experience shear stress or damage by pumps or centrifuges, viability, fermentation, and turbidity may be impacted (Lewis and Poerwantaro, 1991; Van Bergen *et al.*, 2004; Chlup, Conery and Stewart, 2007).

Serial repitching refers to the process of recycling brewer's yeast. In serial repitching, yeast is collected from a complete fermentation and reused in subsequent fermentations (Kobayashi *et al.*, 2007). The number of subsequent repitchings (i.e.- generations) is a heavily debated topic and varies depending on the brewery and yeast strain utilised. Some serial repitching studies discuss detrimental impacts to yeast quality with increased generations (Smart *et al.*, 1996; Kobayashi *et al.*, 2007) while

others demonstrate that serial repitching may not have any impact (Speers *et al.*, 2003, 2009; Josey, 2018). Generally, the combination of yeast storage and repitching affect yeast health that ultimately impacts the resulting beer (Lewis *et al.*, 1991; Mochaba *et al.*, 1996; Van Bergen *et al.*, 2004; Speers *et al.*, 2006; Chlup, Conery, *et al.*, 2007).

2.2.9 Dry-Hopping

Dry-hopping has great historical significance as hops were added to casks by the 19th century brewers to enhance microbial stability (Hornsey, 2003). Dry-hopping is a traditional practice in the production of British cask ales but lost popularity with the growing production of various European lagers worldwide (Biendl *et al.*, 2014). The booming American craft brewing market has seen a resurgence of dry-hopping and is a reason why dry-hopping is common practice in the craft brewing industry today (Wolfe and Shellhammer, 2012; Rettberg, Biendl and Garbe, 2018).

Dry-hopping refers to the addition of hops in the cold-side of processing (i.e.-end of fermentation/maturation) to extract highly volatile hop aroma compounds such as β-myrcene, α-humulene and β-caryophyllene (Biendl *et al.*, 2014). Dry-hopping enhances green or 'fresh' hop aromas and various hops are selected to create resinous, spicy, or fruity characters (Biendl *et al.*, 2014). The purpose of dry-hopping is to solubilise flavour and aroma compounds from hops in beer while minimizing oxidation and colloid formation (Wolfe *et al.*, 2012). The volatile hop compounds vary in solubility depending on the alcoholic strength of the beer and the solubility of the individual terpenes. A majority of terpenes are non-polar or weakly polar and do not dissolve easily into water. Ethanol aids in this extraction, but the true effects of ethanol and terpene extraction are still debated (Peltz *et al.*, 2017).

One component of taste extracted during dry-hopping are polyphenols. Hops are rich in polyphenols. Polyphenols provide reducing power and aid in maintaining beer freshness (McMurrough *et al.*, 1996). Polyphenols impact taste, mouthfeel, and increase bitterness in dry-hopped beer (Parkin *et al.*, 2017). The addition of excess polyphenols in beer may increase beer turbidity by the formation of protein-polyphenol complexes (Goiris *et al.*, 2014).

The objective of dry-hopping is to increase hoppy aroma and flavour in the final beer. The essential oils of hops contains over 430 compounds and more recent studies suggest that over 1000 compounds exist in hop oil (Briggs *et al.*, 2004; Roberts *et al.*, 2004; Almaguer *et al.*, 2014). Essential oil content is variety, cultivar and harvest year

dependent. Within the essential oil, terpenes and sesquiterpenes are the most relevant compounds related to hop aroma being that they comprise approximately 80% of the volatile aromatic compounds in hops (Almaguer *et al.*, 2014). In the 1980s, research in aromatic components of hop oil divided hop oil components into three fractions- hydrocarbons, oxygenated compounds, and sulfur-containing groups (Almaguer *et al.*, 2014). These components are discussed in detail in Section 2.3.4.

Dry-hopping is crucial to extracting terpene hydrocarbons to concentrations above their flavour threshold. In non-dry-hopped beer, terpene hydrocarbons will not be present due to their high volatility. The terpene hydrocarbon most relevant and easily traced in dry-hopping is β-myrcene as it is a key terpene in most hop varieties (Rettberg *et al.*, 2018). Hop volatiles, hydrocarbons, oxygenated compounds, and sulfur-containing groups will be discussed in more detail in Section 2.3.4.

2.2.9.1 Dry-Hopping Technology

Dry-hopping is not a prescribed procedure and is entirely dependent upon the hop format utilised, fermentation/conditioning vessels, and separation technology available to brewers. As dry-hopping efforts focus on the extraction of aromatic components, brewers must consider the format of hops utilised and how particles (large or small) affect filtration and packaging, the state of the lupulin glands, the temperature of dry-hopping, if the tank can or will be recirculated, the total contact time, ability to remove dry-hop material, volatile losses by CO_2, and the presence of yeast during dry-hopping (Biendl *et al.*, 2014).

A great risk of dry-hopping is the introduction of oxygen into beer. The use of pelleted hops prevents oxygen ingress as hop pellets are tightly compressed vegetative hop material. In a cylindroconical vessel, hops are typically added to the top of the vessel to precipitate and collect in the cone. Pelleted hops can be milled in low temperature to break apart the vegetative material and increase surface area. The additions may be made to the top of the cylindroconical vessel or hops are placed into a chamber, purged with CO_2 and pumped into the cylindroconical vessel (Biendl *et al.*, 2014).

Another method of dry-hopping is to prepare a slurry of hops with deaerated water or beer. The mixture is contained in an anoxic environment, connected to the cylindroconical vessel, and recirculated through the beer by a hydraulic pump for several hours. This method provides better beer-hop contact and greatly increases hoppy

aroma in beer. A disadvantage of this method is in the addition of the hop slurry. The slurry containing milled pellets introduces a multitude of nucleation points for CO_2 to adhere to, resulting in excessive foaming (Biendl *et al.*, 2014).

Finally, a classic method of dry-hopping is the addition of whole leaf hops. Whole leaf hops are kiln-dried, vacuum packed, and have not received additional processing. The dry-hop addition of whole leaf hops to beer is similar to the addition of pelleted hops. They may be milled to break up vegetative material however, whole leaf hops are often placed into sterile mesh bags for easy removal post dry-hop (Biendl *et al.*, 2014).

There are several modern technologies utilised to dose hops into fermenters with minimal oxygen uptake, maximum exposure, and minimal risk of microbial infection.

2.2.9.1.1 Hop Torpedo

The hop torpedo is a dry-hopping technology designed by Sierra Nevada Brewing Company in Chico, California. In dry-hopping with the 'torpedo', whole cone hops are added and the vessel is sealed. The vessel is purged with CO_2 and beer from the cylindroconical vessel is pumped through the torpedo for 3-5 days at approximately 20°C (**Figure 1.3**). The method is designed to extract essential oils to boost dry-hop aroma with minimal oxygen ingress (Hieronymus, 2012).

Figure 1.3- Hop torpedo at Sierra Nevada Brewing Company in Chico, US.

2.2.9.1.2 Hop Cannon

The hop cannon is a system designed to 'blast' hops into the top of a cylindroconical vessel. Hop pellets are first added into a holding cylinder of the hop cannon. The hop cannon is then sealed, purged with CO_2 and pressurised with CO_2. The pressure differential between the hop cannon and the tank is designed to force the hops through pipework and into the top or bottom of the cylindroconical vessel, depending the brewery system. The hop cannon system allows hop particulates to be dispersed through the tank (Biendl *et al.*, 2014; Podeszwa, 2016).

2.2.10 Filtration/Separation

The final step before packaging is filtration and/or separation to remove suspended solids from beer prior to packaging. Although filtration is an optional step, many large and small breweries utilise filtration as a technique to enhance beer clarity and quality. In some cases, centrifuges are the sole source of separation and used in conjunction with the addition of finings to precipitate suspended yeast cells and hop-derived particulate matter (Coote *et al.*, 1999). Centrifugation and/or filtration is commonly run at low temperatures (approximately -1°C) to remove protein-polyphenol complexes and chill haze (Briggs *et al.*, 2004). Filtration may also be utilised to sterilise

beer by passing the product through filter sheets with very small pore sizes (<0.2 µm) (Aguilar-Uscanga *et al.*, 2003).

Breweries tend to utilise filter aids to separate particulate matter from beer. Common filter aids are kieselguhr, perlite and cellulose. Kieselguhr is a filtration aid made of diatomaceous earth comprised of approximately 85% silica. The diatomaceous earth particles are calcined, which fuses the particles together to make a robust filter bed. The fine internal pore network has the ability to remove particles with sizes ranging between 7-20 µm in diameter (Coote *et al.*, 1999). Perlite is an inert alumino-silicate rock with lower density than kieselguhr filtration products. Perlite is commonly used for yeast recovery and pre-treatments on filters as perlite does not form a fine internal pore network (Coote *et al.*, 1999). Finally, cellulose filters are derived from wood-pulp and make up a large network of fibres. Many filter sheets are covered with cellulose as this naturally absorbs the shock of changes of filter pressure during the filtration process, ensuring the filtrate is clear (Coote *et al.*, 1999).

It is important to mention polyvinyl polypyrrolidone (PVPP) as it is a specialised stability aid designed to remove polyphenolic material to reduce turbidity and polyphenolic-derived astringency. PVPP is a highly cross-linked polymer and that binds to proteins and polyphenols by hydrogen and ionic bonds. It is added during maturation or pre-filtration and is removed during the filtration process (Coote *et al.*, 1999). Filter sheets impregnated with PVPP are also available for use. The use of PVPP is heavily debated in the brewing industry as it removes reducing power by cross-linking with polyphenolic material (McMurrough *et al.*, 1996; Bamforth, 1999). McMurrough (1996), discovered that reducing power can be significantly decreased from 9-38%, offering less ‘protection’ from free radicals.

2.2.11 Packaging

Packaging is the critical final step in the brewing process. Poor packaging process may cause issues with microbial stability, oxidative stability, and flavour degradation (Section 1.4). Packaging formats differ (keg, cask, can, bottle, etc.) but three facets are key in maintaining robust beer quality.

Oxygen ingress should be avoided at all costs. Oxygen pickup is high in filling vessels that have not been purged or insufficiently purged with nitrogen. Oxygen ingress also occurs in bottle and can formats in the time after the vessels are filled to when they are capped or crimp-sealed. PVC foam liners with oxygen-scavenging

material aid in lowering oxygen levels in package but will not completely remove oxygen (Kuchel *et al.,* 2006). Breweries will typically aim to keep total packaged oxygen (TPO) levels below 50 parts per billion (ppb). High levels of total packaged oxygen in beer will negatively impact beer as it ages (Vanderhaegen *et al.,* 2006).

Monitoring packaging temperature is another critical factor of packaging that should be regularly checked. The bright beer tank (BBT) temperatures are typically held between 0°C and -1°C. As chilled beer passes through filling lines, the beer will warm. Temperature greatly affects CO_2 as it is more soluble in water at colder temperatures. As temperatures rise, kinetic energy is increased and the increase in motion breaks intermolecular bonds, causing the CO_2 in solution to lose solubility. This phenomenon can cause CO_2 losses in packaging leading to product rejection and reprocessing (Briggs *et al.,* 2004).

As with all brewing practices, a critical factor in packaging is brewery cleanliness. All product lines must be clean, free of taints, and oxygen before packaging may begin. General plant cleanliness is important to prevent microbial infection, promote safety, and prevent equipment failure (Coote *et al.*, 1999).

2.3 Hops

The hop plant (*Humulus lupulus)* is the most commonly used species of *Humulus* in the brewing industry. *Humulus lupulus* are native to the Northern Hemisphere can also be cultivated in the Southern Hemisphere between latitudes of 35-55°. Thriving in flood plains, hops require changing seasonal daylight for growth (Briggs *et al.,* 2004; Biendl *et al.,* 2014). During late summer, hops change from slow vegetative growth to active generative growth when day length increases to 16-18 hours per day (Biendl *et al.,* 2014). Hops are dioecious, producing male or female inflorescences on different plants. While male plants are required for pollination, only female plants produce the cones or 'flowers' required for brewing (Biendl *et al.,* 2014).

In the Northern Hemisphere, hops begin to grow in late spring (usually March-April) and staggered depending on variety (Biendl *et al.,* 2014). The staggering allows growers to harvest different varieties at different times during the harvest period.

2.3.1 Structure

The hop plant is a perennial. Each year, buds develop from each plant with shoots extending off of these buds. One shoot is 'trained' to a trellis for the bine to develop and climb as it grows clockwise (Briggs *et al.,* 2004). As the bines grow, leaf

axils develop young flowering shoots at the bracts on the bine (**Figure 1.4**). Female inflorescences develop papillated stigmas which ultimately form the strobiles or hop cones that are harvested (Briggs *et al.*, 2004).

The cones/flowers from hops, provide bitterness, aroma, antioxidative power, enhance microbial stability and foam stability to beer (Wietstock *et al.*, 2010; Schönberger *et al.*, 2011; Almaguer *et al.*, 2014; Biendl *et al.*, 2014). Hops are a fascinating, diverse ingredient due to variances in essential oil content, bitter acids, polyphenol content and growing region (Dresel *et al.*, 2016; Ting *et al.*, 2017).

The hop cone is comprised of one strig with bracts and bracteoles developing from the bracts. Glandular trichomes, also known as lupulin glands, develop at the base of the bracteoles (Champagne *et al.*, 2017). The lupulin glands consist of secondary metabolites- resin, polyphenols and essential oils (**Figure 1.5**) (Wang *et al.*, 2008; Biendl *et al.*, 2014). **Table 1.2** lists the typical constituents that construct the hop cone.

Table 1.2- Components of a hop cone with relative percentages. These values are ranges and may not be representative of all hop varieties.*

Constituents	% (w/w)
Alpha-acids	2-18%
Beta-acids	1-10%
Oils	0.5-3.0%
Polyphenols	2-5%
Waxy components	Trace-25%
Proteins	15%
Water	6-10%
Monosaccharides/Pectin	2%, respectively
Mineral content	10%

*Biendl *et al*., (2014)

Figure 1.4- Hops trained to trellis displaying many flowering shoots (left) and bines growing clockwise around trellis (right).

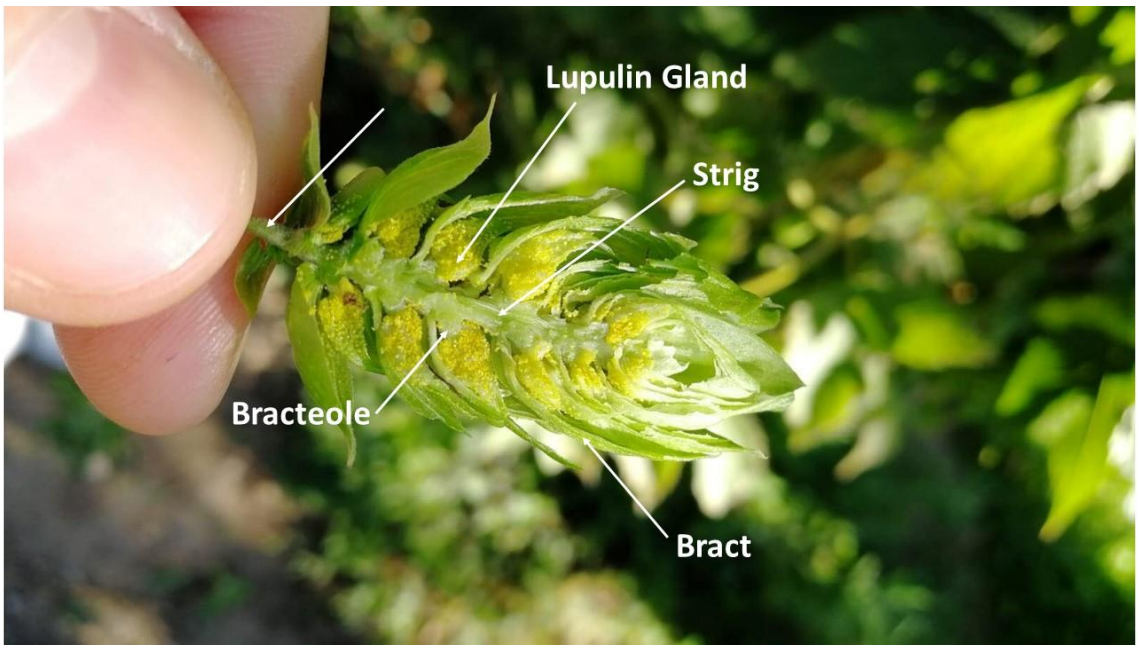


Figure 1.5- Cross-section of a hop cone displaying anatomical structures. Photo provided by Jan Hodel, 2018.

2.3.2 Hard Resin

Hard resins are more polar than soft resins and are classified by their insolubility in hexane, and solubility in cold methanol and diethyl ether. Hard resins generally consist of prenylated chalcones and flavanones (Briggs *et al.*, 2004; Taniguchi *et al.*, 2014; Steenackers *et al.*, 2015). Hard resins have been reported to provide a 'pleasant' bitterness as opposed to the astringent bitterness derived from soft resin components (Taniguchi *et al.*, 2014). The content of hard resin components can increase by the oxidation of selected soft-resin components (Palamand *et al.*, 1967; Taniguchi *et al.*, 2014; Almaguer *et al.*, 2015).

An important component of hard resins is the prenylated chalcone, xanthohumol. Xanthohumol is a powerful antioxidant with proven pharmacological benefits for human health and isomerises to, isoxanthohumol, during wort boiling (Biendl *et al.*, 2014; Almaguer *et al.*, 2015). However, evidence of health-giving properties of xanthohumol and iso-xanthohumol in beer is lacking and further studies are required (Biendl *et al.*, 2014). Hard resin components do however, provide foam stability, antioxidative stability, and a pleasant bitterness to beer (Almaguer *et al.*, 2015; Dresel *et al.*, 2016).

2.3.3 Soft Resin

Soft resins account for approximately 10-25% of the total weight of dried hop cones and are soluble in hexane (Almaguer *et al.*, 2014; Steenackers *et al.*, 2015). Soft

resin compounds mainly consist of prenylated phloroglucinol derivatives, also known as the alpha and beta acids (Steenackers *et al.*, 2015). Alpha and beta acids are differentiated by acyl side-chains attached to the main carbon chain (Taniguchi, 2017).

The α-acids are a key component of the hard and soft resins and provide the majority of bitterness to beer in the isomerised form. The heat during the wort boil extracts α-acids and the vigorous, long boil isomerises humulone, cohumulone and adhumulone to their *cis* and *trans* isomerised forms (Almaguer *et al.*, 2014; Dresel *et al.*, 2016). Ratios of *cis* to *trans* isomers generally result in 70% *cis* isomers to 30% *trans* isomers (Taniguchi, 2017).

Beta-acids have similar properties to alpha acids as their analogues isomerise into iso-products upon the application of heat. The beta acids found in hops are lupulone, colupulone, adlupulone, prelupulone, and postlupulone. As β-acids have poor solubility in water, their transformation products are found in low quantities in beer but do provide bitterness attributes in beer (Almaguer *et al.*, 2014).

2.3.4 Essential Oil

The essential oils are secondary metabolites that are produced and contained within the lupulin glands. The essential oil of hops only accounts for 0.5-3% of the hop itself but contains the majority of the compounds responsible for aroma in beer (De Keukeleire, 2000). Compounds that contribute to hop aroma include, but are not limited to, terpenes, terpenoids, esters, aldehydes, and thiols (Rettberg *et al.*, 2018). The aroma-active compounds within essential oil include a multitude of compounds. However, the compounds that are the most pertinent and widely studied in regard to hop aroma are terpenes (terpene hydrocarbons) and terpenoids (terpene oxides, etc) (Wang *et al.*, 2008; Van Opstaele *et al.*, 2012; De Almeida *et al.*, 2015; Praet *et al.*, 2015; Rettberg *et al.*, 2018). Terpenes are hydrocarbon compounds containing at least two interconnected isoprene units and terpenoids contain the same hydrocarbon backbone but contain functional groups attached (Rettberg *et al.*, 2018).

Hop aroma is complex and changes throughout fermentation and maturation, there is no comprehensive list of all hop aroma components. Each hop variety contains different compositions of aroma-active compounds and each of these compounds greatly differ in chemical properties. The extraction of hop aroma is not straightforward. Brewing technology is a limiting factor in hop aroma extraction as some processes and

equipment are more efficient than others. Currently, there is no formula to calculate hop aroma extraction rates in beer (Rettberg *et al.*, 2018).

2.3.4.1 Terpene Hydrocarbons

Hydrocarbon compounds in hop oil are further categorised into three subgroups- monoterpenes, sesquiterpenes, and aliphatic hydrocarbons (Almaguer *et al.*, 2014). The most studied hydrocarbon carbon compounds include the terpene hydrocarbons β-myrcene and sesquiterpenes α-humulene, and β-caryophyllene, as they are the primary components of the essential oils in most hop varieties (**Figure 1.6**). Lacking polar functional groups and a low boiling point, β-myrcene, α-humulene, and β-caryophyllene have limited solubility in wort and beer. Due to this, the compounds are typically, only present in dry-hopped beers (Almaguer *et al.*, 2014; Rettberg *et al.*, 2018).

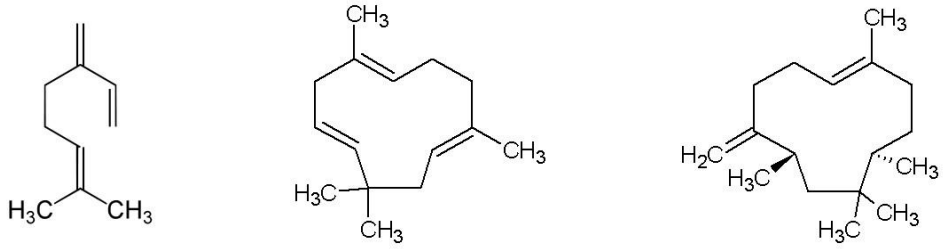


Figure 1.6- Terpene hydrocarbons from left to right- β-myrcene, α-humulene, β-caryophyllene.

It is important to provide special attention to monoterpene hydrocarbon, β-myrcene as, in most hop varieties, it is the aroma compound in the greatest abundance. The compound is prone to oxidation and evaporation during storage and has been found to oxidse to form the terpenoids linalool, nerol, geraniol, citral, α-terpineol, or carvone. Additionally, myrcene has been observed to form cyclic products such as α-pinene, β-pinene, or camphene (Dieckmann *et al.*, 1974; Rettberg *et al.*, 2018). The flavour threshold (30-100 µg/L) of the monoterpene hydrocarbon is only surpassed in dry-hopped beer, thus, is a crucial monoterpene hydrocarbon to measure in dry-hopped beer (Rettberg *et al.*, 2018).

2.3.4.2 Terpene Oxides

Oxygenated terpene compounds are classed as terpenoids due to the functional group(s) attached to the hydrocarbon backbone (Rettberg *et al.*, 2018). Monoterpene oxides such as linalool are key components of ‘green’ or ‘fresh’ hop aroma in beer. Fresh, fruity, and citrusy sensory descriptors have are used to describe the aroma of

linalool in beer. The monoterpene oxide has also been recorded above sensory threshold levels (10µg/L) pre-dry-hopping and is pertinent to beer aroma (Almaguer *et al.*, 2014). Other important monoterpene oxides contributing to hop aroma in beer are geraniol, citronellol, nerol and α-terpineol (Almaguer *et al.*, 2014). In beer, the aromas of various monoterpene oxides have been found to synergistically impact the aroma of one another. This phenomena is observed with the combination of linalool, geraniol, and β-citronellol providing a new, distinctive lime-like aroma to beer (Takoi *et al.*, 2014). It is difficult to measure the true concentration of monoterpene oxides as many of the compounds undergo yeast-mediated biotransformation into other monoterpene oxides (Section 2.3.4.4).

The importance of sesquiterpene oxides has been debated as they typically exist below flavour threshold in beer (Goiris *et al.*, 2002; Van Opstaele *et al.*, 2013; Biendl *et al.*, 2014). Research suggests that some sesquiterpene oxides are relevant aroma active compounds. Humulene epoxide III, humulenol II, several caryophyllene epoxide enantiomers have been found to be aroma-active by Gas Chromatography-Olfactometry analysis (Van Opstaele *et al.*, 2013; Praet *et al.*, 2016a; Praet *et al.*, 2016b). Sesquiterpene oxides provide 'spicy' 'woody' and 'earthy' sensory attributes to beer, are formed during the wort boil, and are commonly referred to as a 'spicy' hop flavour (Goiris *et al.*, 2002; Van Opstaele *et al.*, 2013; Praet *et al.*, 2016b).

2.3.4.3 Non-Terpene Aroma Compounds

Hop essential oil also contains a substantial amount of other, non-terpenic compounds. These compounds include but are not limited to- aldehydes, ketones, thiols (mercaptans), carboxylic acid compounds (acids and free esters) (Biendl *et al.*, 2014).

Aldehydes and ketones are of very little concern in regard to dry-hopping as the respective sensory thresholds are very high. Hexenal is an aldehyde that is abundant in fresh hops, imparting a grassy aroma. However, upon kilning, hexenal is lost as epoxydecenal isomers form by the degradation of linoleic acid during hop kilning. Expoxydecenal compounds increase upon beer storage by the degradation of hop carboxylic acids (Biendl *et al.*, 2014; Rettberg *et al.*, 2018).

Hop-derived thiols have exceptionally low sensory threshold levels (ng/L). Some hop varieties grown in New Zealand and the United States contain polyfunctional thiols that are key aroma compounds to hop varieties- providing melon and 'muscat' grape flavour/aromas (Almaguer *et al.*, 2014; Biendl *et al.*, 2014; Rettberg *et al.*, 2018).

Carboxylic acid compounds are the third most abundant group of compounds within hop essential oil following terpenes and terpenoids (Biendl *et al.*, 2014). Carboxylic acid compounds provide positive and negative sensory characteristics to beer as carboxylic acid esters provide 'fruity' aromatic qualities (ethyl isobutyrate) and free carboxylic aids provide 'cheesy' (butanoic acid) sensory qualities (Biendl *et al.*, 2014).

2.3.4.4 Biotransformation of Flavonol Glycosides by Yeast

Several of the monoterpene oxides exist in free and glycosidically bound forms, primarily bound to β-D-glucose (Biendl *et al.*, 2014). Geraniol is a monoterpene oxide reported to undergo glycosidic cleavage of β-D-glucose by yeast during fermentation, yielding β-citronellol (Takoi *et al.*, 2010, 2012, 2014; Biendl *et al.*, 2014). Similar reactions have been observed in the two-step conversion of geraniol or nerol to linalool and finally, α-terpineol (King *et al.*, 2003; Biendl *et al.*, 2014). Biotransformation of hop compounds is commonly observed during brewing and fermentation processes, but is also observed post-packaging with fluctuations in pH (Biendl *et al.*, 2014). Finally, it is important to note that yeast activity during fermentation has also been found to produce acetate esters of monoterpene oxides, geraniol and citronellol (King *et al.*, 2003).

2.4 Beer Quality

2.4.1 Introduction

Beer quality is a measurement of consistency and stability. Stability encompasses several subcategories- microbial, colloidal, foam and aroma/flavour stability (Stewart, 2004; Bamforth, 2011). Poor quality raw materials, mashing, wort boiling, fermentation conditioning, and inefficient brewery equipment are all of detriment to beer quality.

Modern brewing techniques and systems have been designed for high brewing efficiency. A vast amount of research has been dedicated to malting, hop-production, and yeast physiology in the production of high quality raw materials. These topics are comprehensively covered by Briggs, (1998); Briggs *et al.*, (2004); Boulton and Quain, (2006); Biendl *et al.*, (2014). High quality raw materials produce beer with stable flavours and enhanced reducing power.

The use of analytical chemistry techniques are crucial in beer quality research and multiple tools are utilised to assess beer quality. With the use of electron

paramagnetic resonance techniques, flavour stability and reducing power of various beer styles can be assessed (Skibsted *et al.*, 1998; Foster *et al.*, 2001; Kocherginsky *et al.*, 2005; Marques *et al.*, 2017). Gas chromatographic techniques are utilised to measure various constituents that compose beer aroma (Andres-Iglesias *et al.*, 2014). Proteomic, metabolomic, and genomic techniques are used to measure nutrient compositions in beer and wort and their impact on colloidal and aromatic stability (Colgrave *et al.*, 2013; Spevacek *et al.*, 2015; Ye *et al.*, 2015; Heuberger *et al.*, 2016; Hughey, McMinn and Phung, 2016; Schulz *et al.*, 2018).

2.4.2 Colloidal Stability

Beer haze occurs when colloids or suspended particles in solution cause light scattering (Cejnar *et al.*, 2016). Biological and non-biological hazes occur during beer production and during storage (Bamforth, 1999, 2011; Suzuki, 2011). Biological hazes arise from microbial contaminations in fermentation, tank transfers, and/or packaging (Suzuki, 2011; Macintosh *et al.*, 2014).

Non-biological beer hazes may arise from β-glucan, starch, calcium oxalate in calcium deficient wort, protein-polyphenol complexes, hop derived resin hazes, excessive copper or iron content in wort, insoluble starch fines, excessive dextrin or carryover of diatomaceous earth. Any of these can contribute to increased beer turbidity (Bamforth, 1999; Steiner *et al.*, 2010; Kotlikova *et al.*, 2013).

2.4.2.1 Haze Active Protein

Beer contains substantial proportions of protein and protein breakdown products. Protein break-down products survive the brewing process and are the protein constituents observed in finished beer (Hejgaard *et al.*, 1983). Haze active beer proteins are protein break-down products derived from the alcohol-soluble prolamin, hordein (Asano *et al.*, 1982). Hordeins, present in the starchy endosperm of barley, contain low lysine, high proline, and high glutamine levels (Steiner *et al.*, 2011). These proline-rich proteins are known to induce haze formation as they are strongly attracted to free binding sites -polyphenols containing vicinal hydroxyl groups attached to an aromatic ring (McManus *et al.*, 1985; Mulkay and Jerumanis, 1983) on polyphenols (Aron *et al.*, 2010). Proteins rich in proline range in the size of 15-35 kDa. In addition, glutamic acid hordeins have also been found to initiate haze formation in the size range between 10-30 kDa (Asano *et al.*, 1982).

Non-haze active protein concentrations range between 3-100 mg/L (McMurrough *et al.,* 1983, 1992). Haze active proteins in beer are reported to be between 65-130 mg/L (Ishibashi *et al.*, 1996; Kakui *et al.*, 1998). The respective protein contents are dependent on beer style and grist bill.

2.4.2.2 Haze Active Polyphenol

There are many polyphenols in beer- each identifiable by their respective chemical properties. Beer polyphenols may be divided into two groups- hydrolysable tannins and flavonoid elagitannins *or* phenylpropanoids (flavones, flavonols, flavanonals, flavanones, flavan-3-ols, and condensed flavan-3-ols or proanthocyanidins) (Marais *et al.,* 2006; Aron *et al.,* 2010). Not all polyphenols are responsible for haze formation. In fact, condensed flavan-3-ols or proanthocyanidins are polyphenols strictly involved with beer haze formation (McMurrough *et al.*, 1992; Siebert, Troukhanova, *et al.*, 1996; Ye *et al.*, 2016). Haze active proanthocyanidins are mainly derived from the testa of barley compared to hop-derived proanthocyanidins in which only 20-30% survive the brewing process (Steiner *et al.*, 2011; Biendl *et al.*, 2014). Levels of hop-derived proanthocyanidins (procyanidin B3) will ultimately increase with dry-hop additions (Biendl *et al.*, 2014).

Polyphenol-protein binding affinity increases with the presence of hydroxyl groups attached to an aromatic ring. Binding is stereochemically favoured when the hydroxyl groups are vicinally located to the protein (McManus *et al.,* 1985; Mulkay and Jerumanis, 1983). The most haze active polyphenols are dimeric proanthocyanidins (anthocyanogens), as very few tetramers and trimers of proanthocyanidin survive the brewing process (McMurrough *et al.*, 1992). Proanthocyanidins are higher polymers of gallocatechin, epicatechin, and catechin (Siebert, 1999; Cejnar *et al.*, 2016). Two catechin dimers are mainly present in beer. Procyanidin B3, responsible for catechin-catechin bonding and prodelphinidin B3, responsible for catechin-gallocatechin binding are speculated to be contained in colloidal haze (McMurrough *et al.*, 1994; Bamforth, 1999; Cejnar *et al.*, 2016).

Dimeric polyphenols contain two or more free binding sites on the same molecule. This enables cross-linking with two or more haze active proteins (Siebert, Troukhanova, *et al.*, 1996). Polyphenols with one free binding site are able to bind with proteins but lack the ability to cross-link (Siebert *et al.*, 1998). When beer is chilled to 0°C, dimeric polyphenols cross-link, forming ionic bonds with proteins. However, when beer is warmed to 20°C these bonds break, causing the haze to disappear. This

phenomenon is otherwise known as ‘chill haze’ (Bamforth, 2011). However, as haze flocs polymerise and covalently bond, increasing in bond number and size, the haze particles are no longer soluble when beer is warmed to 20°C and form what is otherwise known as ‘permanent haze’ (Siebert and Siebert, 2005).

2.4.2.3 Protein-polyphenol haze

Protein-polyphenol interactions are also known to produce increased turbidity in beer (McManus *et al.*, 1985; Siebert, Carrasaco, *et al.*, 1996; Siebert, Troukhanova, *et al.*, 1996; Siebert *et al.*, 2000). Gliadin, a wheat prolamin, is used as a model protein for barley hordein due to its similar chemical composition, activity, and wide commercial availability in turbidity studies (Siebert *et al.*, 2000; Siebert and Lynn, 2005; Li *et al.*, 2008). Tannic acid is utilised as a haze active polyphenol in research to model the activity of haze-active dimeric polyphenols (Siebert, Carrasaco, *et al.*, 1996; Siebert, Troukhanova, *et al.*, 1996; Siebert *et al.*, 2000; Miedl *et al.*, 2005; Li *et al.*, 2008). Due to the nature of haze active compounds and available binding sites, ratios of 2:1 and 5:1 of gliadin and tannic acid, respectively, are reported to induce the highest levels of haze formation (Siebert *et al.*, 2000).

The majority of polyphenols in beer tend to be non-haze active polyphenols (McMurrough *et al.*, 1983, 1996; Li *et al.*, 2008). Haze active polyphenols, however, are challenging to measure in beer as large proportions of polyphenol are complexed with haze active proteins in beer and are difficult to isolate for analysis (Li *et al.*, 2008).

Protein-polyphenol complexes are frequently discussed in the formation of chill-haze or permanent haze. Chill hazes occur, as per their nomenclature, when beer is chilled to 5°C or lower. In chill haze, proteins and polyphenols complex in hydrogen and hydrophobic bonding to form a visible haze (Siebert, 2006, 2009; Cejnar *et al.*, 2016). The non-covalent bonds are easily reversible and broken when beer is warmed above 5°C explaining the phenomena of ‘chill’ or ‘reversible’ haze (Schulte *et al.*, 2016). However after packaged beer has aged, the protein-polyphenolic bond polymerise and form irreversible covalent bonds. Due to ionic bonding strength, the complexes are not soluble and unable to dissolve as the beverage warms. Polymerisation will increase over time, increasing turbidity levels in beer (Schulte *et al.*, 2016). Finally, it is important to note that the formation of protein-polyphenol complexes are reliant on pH. Bonding activity is stable at low pH values with a majority of hazes formed just above pH 4, right in the range of a typical ale pH (Bamforth, 1999; Siebert, 2006, 2009; Cejnar *et al.*, 2016).

2.4.2.4 Yeast Haze

Yeast derived hazes are not typically considered a probable cause for increased turbidity. However, yeast have an impact on beer quality, including a potential impact on turbidity levels (Kupetz *et al.*, 2015; Lewis and Poerwantaro, 1991; Omura and Nakao, 2009). These hazes are speculated to arise from yeast cell wall β-glucans (Kupetz *et al.*, 2015), α-glucans/glycogen (Stewart, 2018), changes in cell-membrane protein expression due to variances in oxygen during propagation (Omura *et al.*, 2009), and from the presence of excess metal ions (Mochaba *et al.*, 1996). Beta-glucans affect filtering speed are known to cause unfilterable beer hazes (Jin *et al.*, 2004; Chlup, Conery, *et al.*, 2007; Kupetz *et al.*, 2015).

2.4.2.5 Beta-glucans

Beta-glucans ((1,3)(1,4)-β-D-glucans) are derived from the malting, milling and mashing process. Beta-glucans are structured from linear chains of units of β-D-glucopyranose and are a major component (70%) of the endosperm cell walls (Palmer, 1992). Malting processes should be sufficient in degrading β-glucans, however, undermodified malts contain high levels of β-glucans. Sufficient levels of β-glucans can impede wort separation by raising the viscosity of the liquid (Jin *et al.*, 2003). Beta-glucans are broken down in the mashing process by β-glucan solubilase and β-glucanase (Bamforth *et al.*, 1983). Paradoxically, β-glucans can also be extracted into the wort in increasing concentrations after the mash is heated above 45°C, as β-glucans are easily solubilised at higher temperatures. Beta-glucan solubilase survives at warmer temperatures, contributing to a higher concentration of β-glucan with warmer mash temperatures (Briggs *et al.*, 2004).

2.4.3 Polyphenols and Beer Quality

The influence of polyphenols on haze stability is discussed in Sections 2.4.2.2 and 2.4.2.3. However, further elucidation is required as polyphenols contribute a multitude of attributes to beer. It is important to discuss the chemical structure of polyphenols and the other roles of polyphenols in beer stability.

Polyphenols are defined as chemical compounds containing at least two (usually multiple) linked structural phenol units (Aron *et al.*, 2010). Plant polyphenols consist of two broad classes: phenolic acids and flavonoids. Relevant to beer, flavonoid egalitannins and phenylpropanoids are two plant phenols derived from flavonoids and phenolic acids, respectively (Marais, J., 2006). The flavonoid egalitannins and phenylpropanoids consist of flavan-3-ols, condensed flavan-3-ols, flavanones,

flavanoals, flavones, and flavonols (Aron *et al.,* 2010). The polyphenols of interest to beer quality are that of (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, and (-)-epicatechin-3-O-gallate as they are present in beer, malt, and hops (**Figure 1.7**) (Aron *et al.*, 2010). Polyphenols provide up to 60% of the endogenous reducing power in beer (Vanderhaegen *et al.*, 2006). However, research has reported that polyphenols such as Xanthohumol greater than 5 mg/L can exhibit pro-oxidative effects in pilsner-type beers (Carvalho, *et al*., 2016).

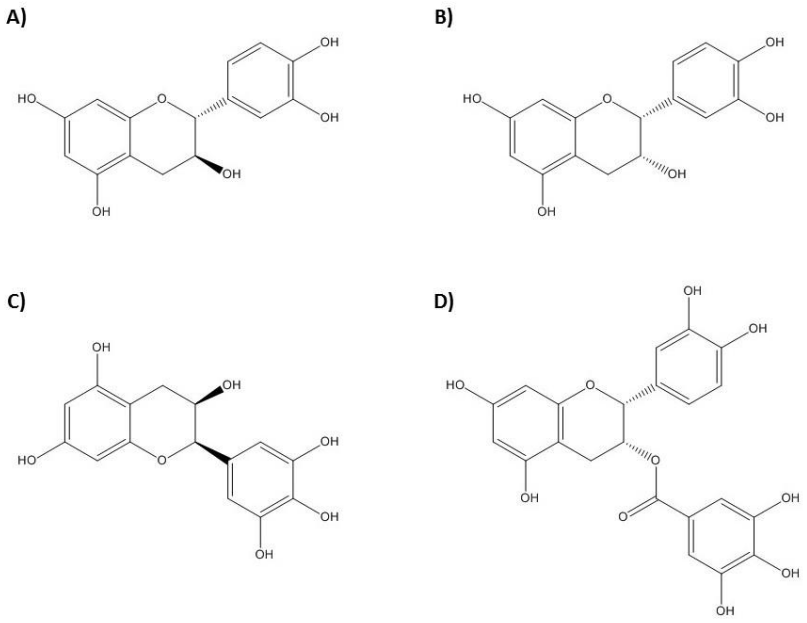


Figure 1.7- Common Polyphenols- A) (+)-catechin, B) (-)-epicatechin, C) (-)-epigallocatechin, and D) (-)-epicatechin-3-O-gallate.

Flavan-3-ols (**Figure 1.7**) and proanthocyanidin are beer polyphenols proven to support antioxidative activity (Aron *et al.*, 2008). Flavan-3-ols act as antioxidants scavenging free radicals, chelating transition metals, and inhibiting enzyme activity (Aron *et al.*, 2010; D. Carvalho *et al.*, 2016). The ease of electron transfer causes flavan-3-ols to serve as antioxidants. Flavan-3-ols oxidise to form semiquinone radicals that form oligomers by nucleophilic addition. The remaining structures positive-scavenging catechol and pyrogallol structures, scavenge free radicals (Bors *et al.*, 2000; Aron *et al.*, 2010). The accessibility of these compounds to act as scavengers again, is reliant upon stereochemistry. Less crowded (+)-catechin C4-C8 linked dimers oxidise much more readily than C4-C6 linked dimers (de Freitas *et al.*, 1998; Aron *et al.*, 2010). Galloyl groups and hydroxyl groups enhance antioxidative activity while

methoxylations and glycosylations act to inhibit antioxidative activity (Nanjo *et al.*, 1996; Aron *et al.*, 2010).

Polyphenols have the ability to chelate transition metals in an ionic state or as a free radical (Aron *et al.*, 2010). Chelators bind transition metals and feed into oxidative mechanisms of ageing. Aron and Shellhammer, (2010) provide a thorough description of the chelating properties of various polyphenols on transition metals and more recent research discusses the biological activity and antioxidative capacity of hop-derived flavonoids (Karabin *et al.*, 2015).

Flavan-3-ols are lower in molecular weight than proanthocyanidins. Typically, polyphenols of a high molecular weight have limited reducing power against oxidative reactions and cannot quench free radicals as effectively. High molecular weight polyphenols are polymerised via oxidation or acid catalysis during a long boiling time (De Schutter *et al.*, 2009). High molecular weight polyphenols act as pro-oxidants, stabilizing transition metal ions by electron transfer which later catalyse Fenton/Haber-Weiss reactions (Vanderhaegen *et al.*, 2006).

Tannoids (polymers consisting of many phenol groups) and oxidised phenol monomers form insoluble complexes in beer. These complexes covalently bond with beer protein and form insoluble beer hazes (McManus *et al.*, 1985; Bamforth, 1999, 2011; De Schutter *et al.*, 2009).

Finally, Walters, Heaseman and Hughes (1997) found that the presence of (+)-catechin and ferulic acid hinder oxidation by quenching the superoxide anion and hydroxyl radicals, respectively. In high oxygen environments, these compounds demonstrate antioxidative behaviour but are not as effective in low oxygen environments demonstrating pro-oxidative behaviour in chemiluminescence studies (Walters *et al.*, 1997; Vanderhaegen *et al.*, 2006). Ferulic acid might also be detrimental in regard to flavour shifts as 4-vinylguiacol is derived from ferulate following decarboxylation via ferulic acid decarboxylase. Some wild yeast strain contaminants contain ferulic acid decarboxylase, ultimately changing beer flavour with age (Walters *et al.*, 1997; De Schutter *et al.*, 2009).

2.4.4 Flavour/Aroma Instability of Beer

Beer flavour/aroma is continuously dynamic in that components contributing to beer flavour/aroma are in a non-stable state upon extraction. Hop-derived and malt aromatic compounds are dynamic and are altered throughout the brewing process,

undergoing various oxidation and biologically-mediated transformations (Section 2.3.4) (Takoi *et al.*, 2010; Biendl *et al.*, 2014).

Aromatic stability or instability rather, is dependent upon the compounds which are extracted in the brewing process, and research tends to be directed toward understanding changes in flavour over time.

Aroma-active compounds in beer are chemically diverse and concentrations of the compounds greatly depend upon beer style and raw materials used. Components of some raw materials are more prone to oxidation than others while other raw materials contain more endogenous reducing power. These factors ultimately affect the development of beer flavour over time in addition to factors such as total packaged oxygen, storage temperature, and pH (Madigan and Clements, 1998; Vanderhaegen *et al.*, 2005; Kuchel, Brody and Wicker, 2006; Liu, Li and Gu, 2008; Heuberger *et al.*, 2012; Taniguchi *et al.*, 2013; Heuberger *et al.*, 2016).

Many aromatic stability studies draw attention to aromatic stability in beer fermented with lager yeast (McMurrough, Madigan and Kelly, 1996; Vanderhaegen, Delvaux, Daenen, Verachtert and Delvaux, 2007; Saison *et al.*, 2009; Rodrigues *et al.*, 2011; Suarez *et al.*, 2011; Blanco, Nimubona and Caballero, 2014). As lager yeast fermentations produce beers with a low-ester profile and a mellow hop character, flavour development over time can be more easily assessed (Vanderhaegen *et al.*, 2007; Hiralal *et al.*, 2013).

Some of the most commonly discussed beer flavour compounds are esters, aldehydes, terpenes/terpenoids, lactones and sulphur-containing compounds to name but a few (Hiralal, Olaniran and Pillay, 2014; Pires *et al.*, 2014; Cantrell and Griggs, 1996; Kunz, Frenzel, Wietstock, and Methner, 2014; Kunz and Kroh, 2013; Mizuno, 2013). To provide a compendious summary of flavour/aromatic stability in beer is impossible. Thousands of compounds derived from malt, hops, and yeast contribute to beer flavour/aroma, often with synergistic or antagonistic interactions (King *et al.*, 2003; Briggs *et al.*, 2004; Vanderhaegen *et al.*, 2006, 2007; Almaguer *et al.*, 2014; Stewart, 2016; Rettberg *et al.*, 2018). **Table 1.3** lists some of the compounds most commonly associated with aged beer taste and aromas.

Table 1.3- Compounds associated with aged beer flavour/aroma*

Class	Compound	Sensory Perception	Sensory Threshold (µg/L)
Linear aldehydes	Acetaldehyde	Green apple	1114- 20000[a], 10000[b]
	(E)-2-octenal	Waxy, fatty, green	0.3[b]
	(E)-2-nonenal	Papery, wet-cardboard[a,b,d]	0.03-0.11[a]
	(*E,E)*-2,6-nonadienal	Vegetal, green cucumber, fatty[j]	
	(*E,E)*-2,4-decadienal	Cooked fat, deep-fried fat[a,j]	0.03-0.11[a]
Strecker aldehydes	2-methylbutanal	Almond, apples, malty[a]	35-1250[a], 500[b]
	3-methylbutanal (isovaleraldehyde)	Malty, chocolate, sour cherry[h]	46-600[a], 1250[b]
	2-phenylacetaldehyde	Sweet, nutty, floral[h]	<1-1600[a], 1600[b]
	Benzaldehyde	Almonds, marzipan[h]	515-2000[a], 1925[c] (air)
	3-(methylthio)propionaldehyde (Methional)	Cooked potatoes, cooked vegetables [a,b,j]	4.2-250[a, j]
	5-hydroxymethylfurfural		1000000[b]
Ketones	*(E)*-β-damascenone	Red fruits, blackcurrant, Tom cat urine[a,b]	2.5-203[a]
	3-methyl-2-butanone	Camphor-like odour/cognac	400[b]
	4-methyl-2-butanone		-
	4-methyl-2-pentanone		-
	2,3-butanedione	Butter, butterscotch[a,b,d,j]	17-150[a]
	2,3-pentanedione	Cream, butter, toffee	-
Cyclic acetals	2,4,5-trimethyl-1,3-dioxolane		900[d]
	2-isopropyl-4,5-dimethyl-1,3-dioxolane		-
	2-isobutyl-4,5-dimethyl-1,3-dioxolane		-
	2-*sec*-butyl-4,5-dimethyl-1,3-dioxolane	Fruity, ethereal[b]	-
Heterocyclic compounds	Furfural	Caramel, bread-like, cooked meat[h]	15000- 150000[a], 150000[b]
	5-hydroxymethyl-furfural	Caramel, bread-like[h]	35784- 1000000[a]
	5-methyl-furfural	Almond, marzipan[a,b]	1174-20,000[a] 90000-97000 in ales, 4000-12000 lagers[b]
	2-acetyl-furan	Nutty, almond, burnt[a]	513-80000[a]
	2,5-Dimethyl-4-hydroxy-3(2H)-furanone	Sweet, caramel[a,b]	160[a], 190-2730[b]
	2-propionylfuran		-
	Furan		-
	Furfuryl alcohol (furanmethanol?)		3,000,000[d]

	Furfuryl ethyl ether		6[d]
	2-ethoxymethyl-5-furfural		-
	2-ethoxy-2,5-dihydrofuran		-
	Maltol	Cotton candy, caramel, fresh-baked bread	35000[b]
	Dihydro-5,5-dimethyl-2(3H)-furanon		-
	2-acetylpyrazine	Popcorn, corn chip, nutty, breadcrust[b]	-
	2-methoxypyrazine		-
	2,6-dimethylpyrazine		-
	Trimethylpyrazine		-
	Tetramethylpyrazine		-
Ethyl esters	Ethyl 3-methylbutyrate		1300[b]
	Ethyl- 2-methylbutyrate (ethyl isovalerate)	Apples, apricot, orange, fruity[a]	-
	Ethyl 2-methylpropionate		5000[b]
	Ethyl nicotinate	Medicinal, solvent, anise[a]	4555-6000[a]
	Ethyl acetate	Nail varnish remover, solvent[a,k]	21000[e], 3800[k]
	Diethyl succinate		1200[d]
	Ethyl lactate	Fruity, buttery[a]	Aprox. 250000-353553[a]
	Ethyl phenylacetate	Roses, honey[k]	3800[b,k]
	Ethyl hexanoate	Red apple, solvent[b], fruity[k]	230[b,k]
	Ethyl octanoate	Apple, aniseed[k]	900[k]
	Ethyl formate		150000[b]
	Ethyl cinnamate		2000[e]
	Isoamyl acetate	Banana, pear-drops[b,k]	
Lactones	γ-nonalactone	Peach, apple-like[i]	11.2-607[a]
	γ-hexalactone	Sweet, creamy, coconut, coumarin, tobacco[j]	1600[c]
	4,5-Dimethyl-3-hydroxy-2(5H)-furanone (Can also be classed as cyclic esters of hydroxyacids[b])	(*R)*-form= rancid, walnut, Madeira (S)-form= curry, walnut[f]	5-42ug/L, (>600ug/L) rancid odour in French wine[f]
	Dihydro-2(3H)-Furanone (Can also be classed as cyclic esters of hydroxyacids[b])		-
S-compounds	Dimethyl trisulfide	Onion, rotting fruit, cabbage, sulphurous	0.012-0.15[a]
	3-methyl-3-mercaptobutylformate		-
	2-sulfanethyl acetate	Gas grill[g]	-
	3-sulfanpropyl acetate	Charcoal grill[g]	-

[a]Saison *et al*., (2009), [b]Briggs *et al*., (2004), [c]Bononi *et al*., (2012), [d]Vanderhaegen *et al*., (2003), [e]Grosch, (2001), [f]Scholtes *et al.*, (2015), [g]Thu Hang Tran *et al.*, (2015), [h]Baert *et al*., (2012), [i]Bravo *et al*., (2008), [j]Moreira *et al*., (2013), [k]Saerens *et al.*, (2010).
*Table adapted from (Vanderhaegen *et al.*, 2005)

2.4.5 Raw Material Impact to Beer Quality

2.4.5.1 Barley and Malt Protein Effect on Colloidal Stability

Barley and malt proteins extracted in the brewing process cause increases in turbidity (Asano *et al.*, 1982; Bamforth, 1999; Iimure *et al.*, 2009; Steiner *et al.*, 2011; Jin *et al.*, 2012). Barley proteins are divided into four subcategories- albumin, globulin, hordein and glutelin (Ye *et al.*, 2016). Hordeins are the most common contributors to beer haze. In barley, hordeins are storage proteins, in the highest abundance (approximately 40-50% of the total protein composition) (Osman *et al.*, 2003) and contain proline and glutamine (Iimure *et al.*, 2009). Hordeins are classified into four different groups- B, C, D, and γ hordeins (Jin *et al.*, 2012). When examining groups of hordeins, sulphur-rich hordein-B and a sulphur poor hordein-C are the two largest fractions, accounting for approximately 70-80% of the total hordein fraction. Previously, hordeins (barley prolamines) have been proposed to be a culprit of non-biological haze formation due to their high proline content (Steiner *et al.*, 2010). Protein research has observed large amounts of proline in precipitated beer haze (Siebert, Carrasaco, *et al.*, 1996; Bamforth, 1999, 2011; Steiner *et al.*, 2010; Kotlikova *et al.*, 2013; Cejnar *et al.*, 2016). In addition, recent research suggests that haze formation is a result of trypsin inhibitor CMe precursor (BTI-CMe) acting as a haze-active protein (Iimure *et al.*, 2009; Schulte *et al.*, 2016; Ye *et al.*, 2016). Additionally, the haze positive activity of the CMe precursor contains haze-active haplotypes. Haze-active haplotypes included alpha-amylase/trypsin inhibitor CMa, CMb, and CMd in addition to BDAI-1 (Jin *et al.*, 2012; Schulte *et al.*, 2016; Ye *et al.*, 2016).

Hydrophobic low molecular weight polypeptides also encourage beer haze formation (Jin *et al.*, 2012). These polypeptides originate from protein breakdown within barley, forming low-molecular weight polypeptides and amino acids during malting and brewing (Ye *et al.*, 2016). Although minor contributors, low molecular weight proteins, horedein B and gamma 3 hordein have the ability to form small haze networks, providing structure for the creation of large networks of colloidal haze. Finally, it is also important to note that hexoses derived from Maillard reactions as well as other sugars are potentially haze-positive and relevant in regard to beer haze formation (Jin *et al.*, 2012; Kotlikova *et al.*, 2013; Schulte *et al.*, 2016).

However, both Iimure *et al.,* (2009) and Schulte *et al.,* (2016) suggest that prolamines or amino-acid derived hazes, are not the main culprits of beer turbidity. For a prolamine haze to form, the sample must contain high hordein levels (ca. 20 mol%) with other proteins in trace amounts. This is highly unlikely because proline found in permanent hazes of several barley varieties have only been observed in small proportions (6 mol %). It is suggested that proteomic-focused beer haze research should be broadened due to these findings (Schulte *et al.,* 2016).

2.4.5.2 Hops

Hop-derived components are sensitive to oxidation, are not in a state of chemical equilibria upon extraction and contribute to the chemical instability of beer (De Almeida *et al.,* 2015). Therefore, it is recommended that hops are stored cold to prevent the oxidation of fatty acids which are detrimental to late-addition or dry-hop flavour (Almaguer *et al.,* 2014). If stored improperly, components such as alpha-acids, beta acids, and essential oil compounds (aldehydes, acids, ketones, epoxides, esters, etc) may oxidise (Ashurst *et al.*, 1966; Mikyška *et al.,* 2012). Best practices recommend storing hops at 5°C or lower, in a sealed non-air-permeable pack, packaged with an inert gas, and stored away from light (Biendl *et al.*, 2014).

Dry-hopping, originally developed in the production of British ales, is a practice now commonly used around the world (Stevens, 1967; Vollmer *et al.,* 2016). Dry-hopping is usually defined as the addition of hops on the 'cold side' of production, typically at the end of fermentation, conditioning, or maturation to extract aromatic terpenes, esters, sulphur-containing compounds and aldehydes within hop oil to enhance hop flavour. Dry-hopping increases the risk of oxygen influx leading to oxidation and extracts more polyphenols from vegetative material. However, the presence of some oxidised constituents before the dry-hop addition, such as free carboxylic acids, may negatively affect the organoleptic perception of the beverage (Wang *et al.,* 2008; Xu *et al.,* 2013; Biendl *et al.,* 2014; Ting *et al.,* 2017; Rettberg *et al.,* 2018). Turbidity increases with more dimeric-polyphenol extraction from vegetative hop material (Aron *et al.,* 2010).

2.5 Understanding Beer Quality- Analytical Chemistry

Modern brewing research requires the use of analytical instrumental assessment to measure concentrations of various analytes. Common techniques are- Gas Chromatography Mass/Mass Spectroscopy (GC/MS) to measure volatile aroma-active

components, liquid chromatography to measure non-volatile beer components, and electron paramagnetic resonance (EPR) to assess reducing power in beer.

2.5.1 Mass Spectrometry

Mass spectrometry is used in the identification and quantification of inorganic and organic compounds. A mass spectrometer is beneficial in analysing samples with very low concentrations of the analyte of interest as a mass spectrometer has a high mass accuracy. Mass spectrometers are required in modern 'omics' applications (i.e.- proteomics, metabolomics, transcriptomics, etc.) and are used more frequently in industrial brewing research, regardless of scale (Hughey *et al.*, 2016).

Mass spectrometers consist of three main components- an ionisation source, mass analyser, and an ion detector. In mass spectrometry analytes pass through an ion source through which they are ionised. The mass analyser will separate the ionised analytes based upon their mass-to-charge ratio (*m/Z*) values and are passed onto the detector. Detectors are dependent upon the particular type of analysis (proteins, carbohydrates, terpenes, etc.) and the instrument used (GC, HPLC, etc). Similar to the detector, different mass analysers will generate different types of signals.

2.5.2 Liquid Chromatography

Liquid chromatography is a form of separation chemistry designed to separate non-volatile components from complex mixtures (Niessen *et al.*, 1995; Harris, 1999). In beer, liquid chromatography is used to separate components such as acids, proteins, carbohydrate residues and polyphenolic material (Hughey *et al.*, 2016). In simple terms, separation in liquid chromatography is achieved by ionic interactions between sample and column.

In High Performance Liquid Chromatography (HPLC), the principles of separation are based upon the binding interaction of a targeted analyte within a sample to a column (solid-phase) and is selectively washed off with a solvent (mobile phase) (Niessen *et al.*, 1995).

During analysis, the targeted analyte suspended in its liquid medium binds to the column. The column is washed to remove any component of the liquid medium that is not critical to analysis. Following a washing step, the targeted analyte, which has bound to the column, is washed off with solvent or elution buffer (mobile phase). As the target analyte washes/elutes off of the column, the sample passes through a detector and produces a signal in linked software. In high performance liquid chromatography

(HPLC) refractive index (RI) and ultraviolet detectors (UV) are the two most popular methods of detection (Niessen *et al.*, 1995; Harris, 1999).

Several types of chromatographic techniques exist. Normal-phase (or adsorption) chromatography utilises a polar stationary phase and a less polar solvent while reversed-phase chromatography utilises a non or weakly polar stationary phase and a more polar mobile phase (**Figure 1.8**). Size-exclusion chromatography acts as a molecular sieve, excluding particles by size. Separation in ion exchange chromatography functions through the attraction of solute to charged stationary phase material. Analytes are selectively washed out of the column by acid-base chemistry. Elution is performed by either isocratic elution or gradient elution. Isocratic elution utilises one solvent at a constant strength to elute analytes bound to the stationary phase. Gradient elution utilises a mixture of solvents increasing in strength to create a gradient for better separation (Harris, 1999).

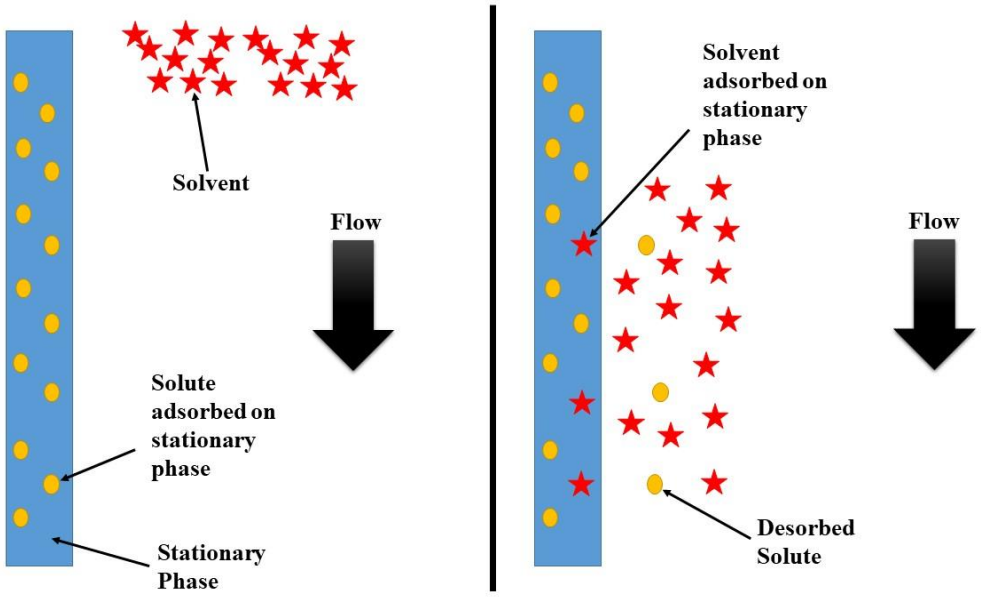


Figure 1.8- Normal phase chromatography. Solvent competes for binding sites on stationary phase. Adapted from (Harris, 1999).

2.5.3 Liquid Chromatography/Mass Spectrometry (LC/MS)

Liquid Chromatography/Mass Spectrometry (LC/MS) utilises HPLC as a separation technique but is paired to a mass spectrometer to identify various compounds based with spectral libraries.

The most popular ionisation technique in LC/MS is electrospray ionisation. Electrospray ionisation is a technique that is commonly used to ionise large biomolecules without fragmentation (Fenn, 2002). Electrospray ionisation is used to

ionise and simultaneously convert samples from a liquid phase to gaseous phase without fragmenting the targeted analytes. A positive charge is applied to a capillary needle and the sample is forced through the capillary, through a Taylor cone by an increasing potential gradient (Harris, 1999). The aerosol sample is ionised, dried of solvent, and passed through a sampling capillary. The sample then passes through a skimmer cone to preferentially sample gas phase ions and reduce the overall load on the mass analyser (**Figure 1.9**). The gas-phase sample is then passed through an ion guide, focusing lenses, and mass analyser (Quadrupoles). Gas phase ions are sorted according to either a particular mass to charge ratio (m/Z) or to 'scan' all mass to charge ratios (m/Z) in the sample. One to three quadrupoles may be used, depending on the level of precision required. The ionised samples then pass to a detector where the ions are detected, resulting in a signal of reported abundance in a chromatograph (**Figure 1.10**) (Harris, 1999; Griffiths *et al.*, 2001; Fenn, 2002; Li *et al.*, 2013).

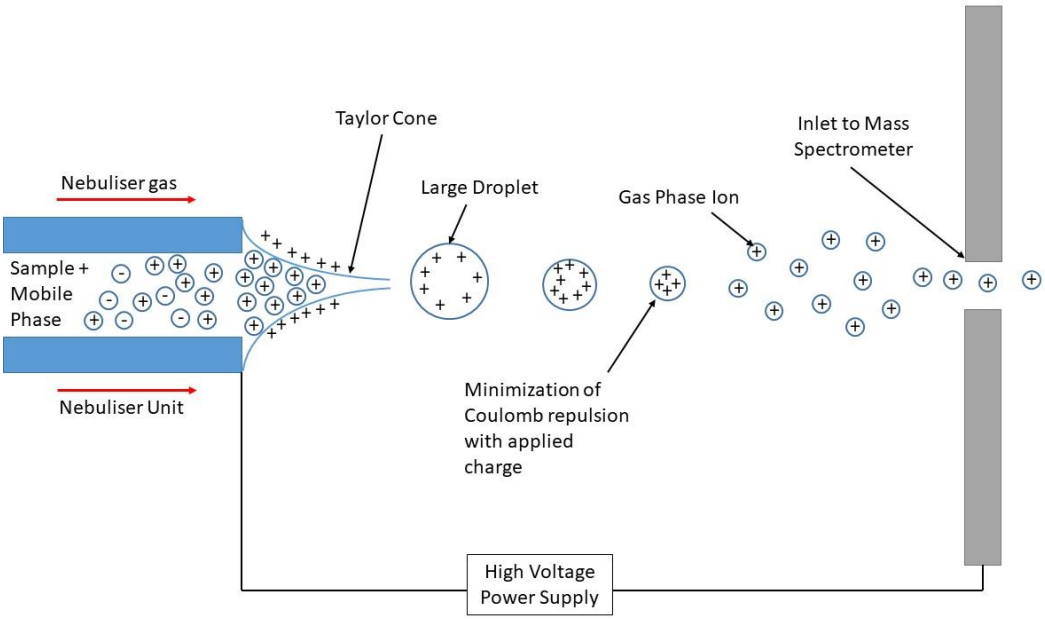


Figure 1.9- Process of ion evaporation in electrospray ionisation. Adapted from (Harris, 1999).

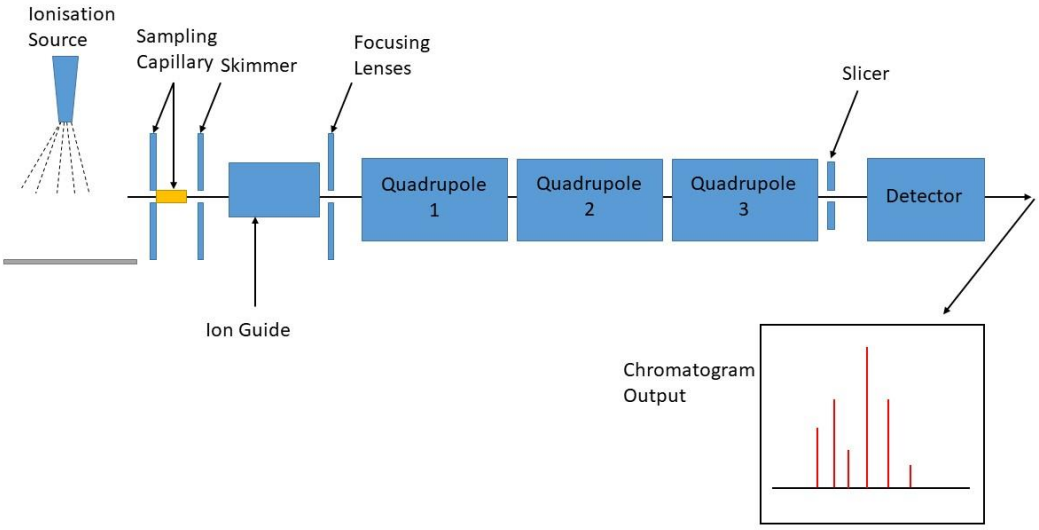


Figure 1.10- Schematic of LC/MS functions utilising a triple quadrupole. Adapted from (Harris, 1999).

2.5.4 The Use of Gas Chromatography in Assessing Beer Stability

Gas chromatography utilises similar principles to liquid chromatography in that a mobile phase and a stationary phase are utilised. The mobile phase in gas chromatography is a carrier gas, commonly helium, nitrogen or hydrogen (Harris, 1999). The material that lines the inside of the chromatography column is known as the stationary phase. Gas chromatography columns are typically made out of borosilicate glass but differ stationary phase material. Stationary phase materials isolate different

analytes, depending on the chemistry of the targeted compound(s). In gas chromatography, analytes in a gaseous phase (mobile phase) are injected into a column (stationary phase) and separated by the interaction of the analyte(s) with the stationary phase with the aid of a carrier gas. In simple terms, gas chromatography separates compounds based upon the volatility of the target analyte(s). Highly volatile compounds pass through the column rapidly and low volatility compounds stick to the stationary phase, requiring higher elution temperatures. Throughout the sample run, the temperature of the gas chromatography oven raises to increase the volatility of the analytes within the sample. The analytes pass through the column, reaching a detector at higher temperature than the peak oven temperature to ensure that all analytes are in a gaseous phase. The detector produces a signal as the analytes elute from the column, respective to their m/Z value. Similar to liquid chromatography, multiple modes of detection are utilised, depending on the target analyte measured (Harris, 1999).

In GC/MS, analytes are ionised as they pass through an ionisation source, prior to reaching the detector. The ionised analytes hit the detector and generate a mass spectrum. The primary horizontal axis of the mass spectra represents mass fragments and provides the molar mass of the ions, the primary vertical axis shows the relative intensity of signal given the ionisation conditions. The relative intensity is generally represented as a percentage relative to the base peak (100% intensity). The intensity scale shows the frequency of occurrence under the ionisation conditions (Hübschmann, 2015).

A plethora of gas chromatography techniques are employed in the brewing industry (Eri *et al.*, 2000; Ochiai *et al.*, 2003; Santos *et al.*, 2003; Oritz *et al.*, 2010; Rodrigues *et al.*, 2011; Aberl *et al.*, 2012; Praet *et al.*, 2014; Riu-Aumatell *et al.*, 2014; da Silva *et al.*, 2015). Gas chromatography is commonly utilised to assess the evolution of beer flavour and aroma during the ageing process (Rodrigues *et al.*, 2011). Table 1.4 displays various chromatographic techniques and their applications in beer analytics. The targeted analyte is the determining factor in the selection of a chromatographic technique. Factors such as polarity, chemical species, boiling point and molecular weight must be considered when selecting an analytical technique (**Table 1.4 and**

Table 1.5) (Andres-Iglesias *et al.*, 2014).

Table 1.4- Common extraction techniques utilised in beer aroma/flavour analysis*

Extraction Technique	Compounds	Advantages	Disadvantages
Headspace (HS)	Volatiles, thermolabile compounds	Good repeatability, fast, sensitive, small sample volume, minimal sample prep, used in combination with other extraction techniques	Headspace (HS) trap system reduces detection limit/sensitivity
Solid Phase Extraction (SPE)	Semi-volatile to non-volatile, polar to non-polar, and ionizable analytes	Broad use, fast, minimal use of organic solvents, good reproducibility and recovery	Use of solvents, difficult to automate
Solid Phase Micro-Extraction (SPME)	Volatiles and semi-volatiles (alcohols, esters, vicinal diketones, carbonyl compounds, fatty acids, sulfur compounds, monophenols)	Simple, reproducible, solvent-free, fast analysis, low cost, highly sensitive, easy automation	Fragile fibre, expensive to maintain, careful manipulation of instrument, poor recovery of long-chain fatty acids, limited extraction efficiency with charged analytes
Stir-Bar Sorptive Extraction (SBSE)	Volatiles and semi-volatiles (sulfur compounds, esters, carbonyl compounds, medium-to long-chain fatty acids, terpenoids)	Robust, solvent free (thermal desorption) or small volume of organic solvents, low cost, no trace volatiles, good sensitivity	Poor recovery for long-chain alcohols, time consuming

*Adapted from Andrés-Iglesias *et al.*, (2014)

Table 1.5- Common analytical techniques used in beer flavour/aroma analytics*

Analytical Technique	Compounds	Advantages	Disadvantages
Gas Chromatography- Flame Ion Detection (GC-FID)	Flavour Compounds	Robust, reproducible, low cost	Sample preparation, fresh standards required for identification, cannot measure highly volatile analytes
Gas Chromatography- Mass Spectrometry (GC-MS)	Flavour Compounds	Robust, reproducible, compound library allows identification without standards	Sample preparation, fresh standards required for calibration curves, cannot measure highly volatile analytes
Liquid Chromatography-Mass Spectrometry (LC-MS)	Hop acids, aflatoxins, amines, oligosaccharides, semi-volatile compounds	Linearity, good reproducibility	Solvents required and volatile compounds must be derivatised
Nuclear Magnetic Resonance (NMR)	Hop acids, carbohydrates, oligosaccharides, aromatic profiling	Limited sample preparation, non-destructive sampling, rapid analysis	Expensive, extensive training required for operation and data analysis
Extractive Electrospray Ionization- Mass Spectrometry (EESI-MS)	Volatile and semi/non-volatile compounds	No sample pre-treatment, reduced time, possible automation	Avoid foaming, extraction efficiency is dependent upon flowrate of desorption gas, gradual signal loss of volatile compounds
Electronic Nose (EN)/GC-Olfactometry	Aroma profiling for simple or complex mixtures	Highly sensitive, small sample volume required, rapid analysis	Aroma response depends upon sensor used and not selective to all compounds

*Adapted from Andrés-Iglesias *et al.*, (2014)

2.6 Aims and Objectives

The purpose of this research was to further understand the aromatic stability of dry-hopped beer and to identify possible sources of unfilterable beer haze for the improvement of beer quality. Consistency, stability and quality of products are the greatest challenges faced in breweries. If batch to batch variation in aroma profiles and turbidity levels are large, consumer product perception is skewed and may result in product rejection.

Maintaining consistent turbidity levels is essential to customer brand loyalty. If a beer known to be brilliantly bright is poured with high levels of turbidity, a consumer would typically reject the product and question the quality (**Figure 1.11**). Efforts are made to reduce turbidity by efficient brewery and packaging processes. However, further issues arise when turbidity is unable to be removed by clarification methods of centrifugation or filtration.

The first objective of this research was to understand the cause of random, unfilterable beer haze, specific to the sponsoring brewery. Each step in the brewing process may detrimentally impact the final product and variations in brewery processes often contribute to subtle batch to batch variation. It was hypothesised that brewery processes could be attributed to occurrences of increased turbidity during packaging. This was examined by statistical analysis calculating Spearman's Rank- Order correlation and step-wise regression (Chapter 3). As these statistical results were ambiguous (Chapter 3), extensive diagnostic studies were conducted utilising samples collected in real time. 'Normal'/'low' haze in addition to 'high' haze samples were collected with the hypothesis that certain macromolecules within beer (proteins, polyphenols, or β-glucans) were the culprit of the unfilterable haze (Chapter 4). Previous literature has briefly discussed unfilterable turbidity derived from yeast but further research was required to confirm this phenomenon (Chapter 4) (Chlup, Conery and Stewart, 2007; Omura and Nakao, 2009; Kupetz *et al.*, 2015).

Figure 1.11- Demonstration of sporadically turbid samples (right) compared to samples within specifications (left), source-industry partner.

Aromatic stability is also important when considering consistency, quality and the brand loyalty of consumers. Flavour and aroma detection are dependent on the concentration of aroma active compounds in solution and if they are above or below sensory thresholds. In some cases, compounds may be above sensory threshold concentrations but are not perceived due to flavour masking by other compounds (Diaz, 2004; Mac Namara *et al.*, 2007; Guido, 2016). Heavily dry-hopped products will realise changes in the concentration of aroma active compounds by various chemical reactions (Vanderhaegen *et al.*, 2006).

Due to these reactions, it is essential for brewers and brewing scientists to understand dry-hop aroma and how compounds, such as terpenes, change over time. As the experimental samples in this thesis were centrifuged, filtered, and packaged on a professional packaging line, the influence of yeast activity was not considered. It was hypothesised that in packaged products, terpenes would decline over time or be adsorbed into the foam liner of the bottle cap (Chapter 5).

When considering the stability of dry-hop aroma, it is crucial to understand how terpenes/terpenoids are primarily extracted in the beer. Chapter 6 details methods used to understand which factors affected dry-hopping (temperature, ethanol content, exposure time, dose) by using a model solution to measure what remained in the spent

hop material, the efficiency of terpene extraction in dry-hop conditions (GC/MS-SPME), and cross-varietal differences in terpene extraction.

Methods utilised to quantify hop aroma are expensive. To date, there are no methods available to quantify hop aroma without the utilisation of instrumental analysis. Developing an alternative to instrumental analysis to quantify hop aroma would be cost-effective and increase accessibility to brewers, large and small. The Vanillin assay was a technique utilised by Cacho and Ferreira, (1990) to assess monoterpenols at low levels (<12mg/L) in 'low aromatic' or 'non-aromatic' muscat grapes. As terpene/terpenoid concentrations range anywhere from ng/L- mg/L, it was hypothesised that the vanillin assay could be optimised and updated to measure monoterpenes and terpene alcohols in dry-hopped beer (Chapter 7).

Chapter 2- Methodology

2.1 Statistical Software

SYSTAT version 13.1 (Systat Software Inc., Chicago, US), Design-Expert® DOE Software version 11 (Stat-Ease, Minneapolis, US), Origin 2018b (OriginLab, Northampton, US) and RStudio version 1.1.463 (R Core Team, Vienna, Austria) were used for all statistical examination. The details of the statistical tests performed are described in detail in Chapters 3 to 7, as appropriate.

2.2 Haze Experiments

2.2.1 Samples

The beer samples used in haze experiments were provided by the industrial partner. Samples were collected during regular packaging runs at the beginning, middle, and end of the run. At each sample point, three cases were collected from the packaging line and a total packaged oxygen (TPO) reading was taken with a Haffmans Automatic InPack TPO/CO_2 Meter (Pentair, Enschede, Netherlands).

The control sample collected was a lager that had not been dry-hopped. The experimental beer was a dry-hopped India Pale Ale (IPA) observed to throw exceedingly high haze values (>5.0 EBC) at random. Due to the sporadic occurrence of the haze, parameters for high and low haze samples were selected. Any beer ≥5.0 EBC haze units were considered to be 'high' haze sample. Any beer ≤5.0 EBC were part of the 'low/normal' sample group. Low/normal haze samples were collected once per month, control samples were collected for two months, and high haze samples were collected as observed. If turbidity did not decrease during maturation, the batch was 'flagged' by the brewery laboratory and collected. Control, high haze, and low haze samples were collected over a total of nine months.

2.2.2 Haze Determination

An Anton Paar DMA 4500M density meter with attached HazeQC ME turbidity module, (Anton Parr, St. Albans, UK) was used to measure EBC haze, light scatter at 25°, and at 90°. The instrument was used for all haze analysis as it is approved by Mitteleuropäische Brautechnische Analysenkommission (MEBAK) and EBC guidelines with measurement standard deviation of ± 0.02 EBC (0.08 NTU) (Anton Paar, 2015). Turbidity in samples was measured by selecting the 'Beer Turbidity- 20°C' method. The method measured the light scatter of the sample at 20°C at 25° and 90° angle of incidences to the light source. The reported values were 25° nephelometric turbidity

units (NTU) and 90° (NTU) from which European Brewing Convention (EBC) turbidity units were calculated.

The instrument's measuring cells were washed with distilled water before and after use. The measuring cells of the instrument were flushed and stored in 80% ethanol, following use. A 50/50 (v/v) solution of bottle Milton Sterilising Fluid (Procter & Gamble, Newcastle, UK) and distilled water were used for cleaning. The solution was flushed through the instrument, left for five minutes, and rinsed with distilled water.

Instrument checks were run once per month according to manufacturer guidelines. If the instrument failed a check after cleaning, adjustments/recalibrations were completed with deionised water.

2.2.3 Enzymatic Digestion of Beer Haze

High, low/normal, and control triplicate 330 mL samples (Section 2.2.1) were each decanted into a 500 mL beaker with a magnetic stir bar and spun for at least one hour at 500 RPM. The pH was measured and 25 mL samples of each beer were drawn into a syringe and injected into the Anton Paar QCMe and HazeQCMe (Section 2.2.2).

Following this procedure, three 100 mL aliquots were decanted into three separate 250 mL Duran bottles (Sigma Aldrich, Poole, UK). One enzyme treatment was dosed per bottle as followed: pepsin (≥97%, Porcine Gastric Mucosa, EC 3.4.23.1) (Merck, Darmstadt, DE), amyloglucosidase (from *Aspergillus niger*, >260 U/mL) (AldrichChemio, Steinheim, DE), and UltraFlo® Max (Novozymes, Bagvaerd, DK) in volumes of 0.1g, 30µL and30 µL were added, respectively. Each bottle was swirled to mix and the amyloglucosidase and UltraFlo® Max samples were placed into a 20°C incubator while pepsin samples were placed into a 40°C waterbath. All samples were incubated for 18 hours.

Samples were removed from incubation and left to settle for one hour. All samples were injected into the Anton Paar QCMe and HazeQCMe units using the established method for 'Beer Turbidity at 20°C' (Section 2.2.2). The values for EBC Haze, 25° NTU, and 90° NTU were recorded for each enzyme treatment.

2.2.4 Microscopy

2.2.4.1 Preparing Beer Samples

Beer particles were concentrated by decanting a room-temperature beer samples (see Section 2.2.1) into clean 500 mL centrifuge bottles and samples were centrifuged

with an Avanti® J-26 XP centrifuge (Beckman Coulter®, Brea, US) for 15 min at 14,000 RCF. All but 10 mL of the supernatant was discarded and the pellet was resuspended in the remaining supernatant and decanted into a clean, 50 mL centrifuge tube (Corning, Deeside, UK).

2.2.4.2 Preparing Dyes

Staining methods as described by Glenister, (1970, 1977, and 1978) and Steiner, Becker and Gastl, (2010) were used to prepare dyes for microscopy analysis. Dyes were prepared before each microscopy session according to **Table 2.1**. A Zeiss Axio Scope.Al microscope and an AxioCam ERc 5s camera was utilised for recording and processing still images (Zeiss International, Oberkochen, DE).

2.2.4.3 Preparing microscope slides

Concentrated beer particles were vortexed to resuspend any settled particulate materials. Approximately 15 µl of concentrated beer sample and 15 µl of dye was placed onto a clean glass microscope slide and mixed with a pipette tip. A glass cover slip was placed on top of the mixture and the sample was immediately analysed under the 10x and 40x objective of the Zeiss Axio Scope.A1 microscope.

After the image was focused and centred, the software was used to capture an image for subsequent processing (2.3 Lite software- Zeiss, Oberkochen, DE).

Table 2.1- Dyes, concentrations and descriptors of each dye used in microscopy tests.

Dye	Concentration (mg/mL)	Targeted Particles Stained	Background Information	Microscopy
Congo Red	12.5[a]	β-glucan	Stains oxalate crystals	Brightfield
Eosin Yellow	0.2[b]	Protein- skins and flakes Joined hemispheres- dextrins	Negative charge (pink) bonds to protein groups with positive charge (orange) by electrostatic adsorption[c]	Brightfield or phase
Methylene Blue	0.1[b]	Polyphenols, oily droplets will stain green	Stains fibres, tannins and polyphenols a very intense blue colour[c]	Phase, brightfield
Thionine	2.0[b]	Presence of dextrins/starch particles	Neutral polysaccharides- violet, Acidic polysaccharides- pink. Dark circular particles- carbohydrate material[c]	Brightfield
Iodine	0.1 (0.1 K: 0.05 I) in Sat. NaCl[c]	Starchy particles	Starch particles- Blue/purple colour	Brightfield
No dye	N/A	Oil droplets	Easy to visualise hop oil or tannins	Brightfield or fluorescent microscope using FITCI filter

[a]Skinner, Hardwick and Saha, (1993), [b]Glenister, (1975), [c]Glenister, (1977)

2.2.5 Gallery™ Plus Beermaster Analysis

2.2.5.1 Start-up Procedures

A Gallery™ Plus Beermaster Automated Photometric Analyser (ThermoFisher Scientific, Perth, UK) measured protein, polyphenol and β-glucan content in samples. The Beermaster contained pre-programmed wet chemical analytical tests for beer samples. Start-up procedures and operating procedures were followed according to manufacturer instructions (ThermoScientific, 2016). After the start-up procedures were completed, water blank and temperature settings were manually checked. This concluded all start up proceedures and calibrations for individual tests were ready to be run.

2.2.5.2 Calibrations

Each calibration standard was prepared at least 12 hours before analysis. To set-up a calibration, a volume of 500 µl of each standard was pipetted into a 1 mL sample cup and placed into a sample rack. A 3 mL sample cup was inserted into the rack with a 2.5 mL volume of water for water checks and the sample rack was placed into the Beermaster.

Reagent kits for each test were purchased from ThermoFisher Scientific (Perth, UK). Each reagent kit was stored at the required conditions and immediately inserted into the instrument. After all standards and reagents were inserted into the instrument, the calibrations were ready to begin.

The appropriate calibration methods,'Protein' 'Pphenol', and 'Bgluc' were selected and run in the software. The instrument software allowed review of the calibrations. If the coefficient of determination was ≥0.99, the calibration was accepted. If the calibration was outside of the range, the calibration was rejected and rerun. Upon acceptable calibrations for each test, sample analysis could proceed. All calibration curves can be found in **Appendix A**.

2.2.5.3 Preparing Standards for Calibrations- Protein

Total Protein standards were made by making a stock solution of Bovine Serum Albumin (BSA) (Sigma-Aldrich, >99%) at a concentration of 10 g/L. The solution was prepared by adding 100 mg of BSA in 10 mL of distilled water in a volumeteric flask. Five milliliters of distilled water were used to rinse the BSA into the volumetric flask and topped up with an additonal 2 mL of water. The solution was mixed and placed in the refrigerator to allow all bubbles to settle. On the morning of testing, the solution was taken out of the fridge and made up to 100 mL with distilled water and mixed. The remaining standard was divided into 700 µl aliquots and frozen at -20°C. When calibrations were required, an aliquot was removed from the freezer and thawed at room temperature before use.

2.2.5.4 Preparing Standard for Calibrations- Polyphenol

A water based gallic acid standard of 500 mg/L was prepared by weighing out 0.051g of pure anhydrous Gallic acid standard (purity 98%, Thermo Fisher Diagnostics, Perth, UK) into a 100 mL volumetric flask and reconstituted with 10 mL of analytical grade ethanol (99.8%, Fisher Scientific, Loughborough, UK) and up to volume with distilled water. The standard was divided into 700 µl aliquots and frozen at -20°C.

2.2.5.5 Preparing Standards for Calibrations- β-glucan (High MW)

Beta-Glucan Standard at 500 mg/L (Thermo Fisher Diagnostics, Perth, UK) was prepared by reconstituting the lyophilizate in a 120 mL glass beaker with 5mL of absolute ethanol (Thermo Fisher Diagnostics, Perth, UK). The tube and cap was lightly rinsed with distilled water to remove any excess lyophilizate. A stir-bar was placed in the beaker and approximately 80 mL of distilled water was added. The beaker was stirred and heated to 120°C for one hour. A visual inspection was performed to ensure all beta-glucan had dissolved into solution and the mixture was allowed to cool to room temperature. The solution was decanted into a 100 mL volumetric flask and lightly rinsed with distilled water to ensure all standards were transferred. The volumetric flask was made up to 100 mL with distilled water and was mixed. The standard was divided into 700 µl aliquots and frozen at -20°C for up to a maximum of six months.

2.2.5.6 Sample Analysis

Samples were prepared by degassing 330ml of beer from the beginning, middle, and end of packaging (Section 2.2.1) by stirring with a magnetic stir bar at 450 RPM for a minimum of 30 minutes. Aliquots of 30 mL were collected from each sample point, labelled and frozen at -20°C for analysis. Samples were frozen in triplicate.

Beer samples were slowly thawed at ambient room temperatures (16°C lab temperature). Up to four racks at a time were used for running samples. Three millilitre sample cups were placed into the racks and 2 mL of sample was pipetted into each sample cup. Samples were analysed in duplicates.

2.2.6 Protein Precipitation

To prepare beer samples for mass spectrometry, a high haze sample (12.57 EBC average) and low haze sample (0.51 EBC average) (Section 2.2.1) were degassed and prepared in triplicate using methods described by Schulz *et al.*, (2018) and Pink *et al.*, (2010). The following methods were tested to select a method with the greatest protein precipitation.

Using the method described by Schulz *et al.* (2018), a 10 mL volume of beer was pipetted into a 50 mL centrifuge tube and proteins were precipitated by the addition of 1 mL of sodium deoxycholate (Sigma Aldrich, Poole, UK) in 100% (w/v) trichloroacetic acid (TCA) (Sigma Aldrich, Poole, UK). The mixture was incubated for 30 minutes at 0°C in an ice bath. The tube was then centrifuged at 14,000 RCF for 10 minutes. The supernatant was discarded, pellet resuspended in 10 mL of ice-cold

acetone, and incubated at 0°C for 15 minutes. The tube was centrifuged for 10 minutes at 14000 RCF. The supernatant was discarded and the pellet was air-dried (Schulz *et al.*, 2018).

In the second protein precipitation method this time described by Pink *et al.* (2010), a 6 mL volume of sample was combined in a 50 mL centrifuge tube with 0.4 mL of ice-cold 50% (w/v) TCA. Samples were vortexed and incubated on ice for 10 minutes. The tubes were centrifuged at 14,000 RCF for 5 minutes. The supernatant was carefully discarded and the pellet was washed with ice-cold acetone. The tube was vortexed to mix, centrifuged at 14,000 RCF for five minutes, and the acetone-wash was repeated. After centrifugation and removal of acetone, the pellets were dried at 95°C on a heat block for approximately five minutes until the sample was dry (Pink *et al.*, 2010).

2.2.7 SDS-PAGE Analysis

To assess the concentration and success of protein precipitation in Section 2.2.6, the air-dried pellet was resuspended in 50 µl of 0.5 M Tris-HCl, pH 6.8 buffer (Sigma Aldrich, Poole, UK). To ensure the buffer covered the pellets in the bottom of the tubes, the tubes were placed in a microcentrifuge and spun for ten seconds. A 50 µl volume of Laemmli sample buffer (Sigma Aldrich, Poole, UK) was added to each tube and spun again for ten seconds to mix and cover the pellets. Samples were heated at 90°C for five minutes in a heating block, cooled to room temperature in an ice bath, and centrifuged for ten seconds.

To separate proteins based on molecular weight, a precast 4-20% Bio-Rad Mini-PROTEAN Tris-Glycine (TGX) polyacrylamide gel was used with a Bio-Rad Mini-Protean Tetra Cell System for precast mini gels (Bio-Rad Laboratories, Herts, UK). A 10x concentrated Tris-Glycine running buffer (Bio-Rad Laboratories, Herts, UK) was diluted 1/10 with ultrapure water (Merck-Millipore, Livingston, UK) before use. Cassettes were rinsed with distilled water, placed in the buffer tank, and filled with Tris-Glycine running buffer. Each well of the cassette was washed by gently pipetting 20 µl of running buffer into each well three times. The outer wells (1 and 10) were loaded with 5 µl of 2-250 kD Precision Plus Protein Dual Extra Standard protein ladder (Bio-Rad Laboratories, Herts, UK) to estimate sample protein molecular weights and the remaining wells were loaded with 20 µl of sample. The tank lid and appropriate electrodes was attached and the sample was run at 120V for one hour and fifteen minutes.

Following electrophoresis, the gel was removed from the cassette frame and rinsed three times with distilled water. The gel was placed into a weigh boat and covered with enough Colloidal Coomassie Blue stain (5% (w/v) aluminium sulphate hydrate (14-18 degree of hydration), 10% (v/v) ethanol, 0.02% (w/v) Coomassie Brilliant blue G-250 and 8% (v/v) orthophosphoric acid) (Thermo Fisher Scientific, Perth, UK) to cover the gel and was incubated overnight at room temperature with gentle agitation.

After staining, the gel was removed from the incubator and rinsed four times with distilled water to remove any stain residue. The gel was placed back into the weigh boat and enough destaining solution (10% ethanol and 2% phosphoric acid) (Thermo Fisher Scientific, Perth, UK) was added to cover the gel. The gel was destained with gentle agitation for two hours. After destaining, the gel was rinsed with distilled water until all background stain was removed. Finally, the gel was placed onto the white-backed gel reading tablet and visually analysed with a Bio-Rad GelDoc EZ imaging system (Bio-Rad Laboratories, Herts, UK).

2.2.8 Analysis of Protein Digests Utilising Liquid Chromatography-Quadrupole Time of Flight-Mass Spectroscopy (LC-QTOF-MS)

In order to resolubilise and denature proteins, the air-dried pellets utilising the precipitation method as described by Pink *et al.* (2010) were resuspended in 100 µl of Urea (8 M) (Sigma Aldrich, Poole, UK) and incubated at room temperature for two hours. The samples were then reduced by adding 5 µl of 1 M dithiothreitol (DTT) (Sigma Aldrich, Poole, UK) and incubated at room temperature for 30 minutes. A 500 µl volume of ammonium bicarbonate (50 mM) was added to each sample. Samples were alkylated by adding 30 µl of 0.5 M iodoacetamide (Sigma Aldrich, Poole, UK) and incubated in the dark for 60 minutes. The alkylation reaction was then quenched by adding 15 µl of 1 M DTT. To each sample, 2 µl of trypsin (1 µg/µL) (Sigma Aldrich, Poole, UK) was added and the samples were vortexed for 30 seconds before being transferred to a 37°C heating block and incubated overnight.

The resulting peptide mixtures were purified using 100 µl C18 solid-phase tips (OMIX) (Agilent Technologies, Edinburgh, UK) and desalted by washing with 0.1% formic acid (Sigma Aldrich, Poole, UK). Peptide mixtures were eluted in 100 µl of a 60:40 (v/v) acetonitrile: water solution containing 0.1 M formic acid (Sigma Aldrich, Poole, UK). The acetonitrile was then removed from the sample using a speed vacuum

centrifuge. The sample was transferred to an amber glass vial (Agilent Technologies, Edinburgh, UK) for analysis by LC-QTOF-MS.

Samples were analysed using an Agilent Technologies 1260 HPLC coupled to a 6530 qTOF mass spectrometer vial (Agilent Technologies, Edinburgh, UK). Samples were analysed in positive ion mode, with mobile phase A: water (0.1% formic acid) and B: acetonitrile (0.1% formic acid) (**Table 2.2**). The samples were separated using a Waters column (XSelect Peptide 100 Å, 2.5 µm, 4.6 x 100 mm) (Waters Corporation, Milford, US) on a 45 minute gradient (**Table 2.3**), at 0.5 mL/minute flow rate.

Table 2.2- LC-QTOF-MS instrument parameters.

HPLC Conditions	
Column	Waters Xselect peptide 100Å, 2.5 µm, 4.6 x 100 mm
Mobile Phase A	LC-MS grade water (0.1% formic acid)
Mobile Phase B	LC-MS grade ACN (0.1% formic acid)
Flow Rate	0.5
Column Temp	35°C
UV Scan	214 nm
Injection Volume	10 µL
Total Run Time	40 minutes
Mass Spec Conditions	
Ionisation Mode	POS
Gas Temp	300°C
Gas Flow	4 L/minute
Nebuliser	35 psig
Sheath Gas Temp	350°C
Sheath Gas Flow	10 L/minute
Capillary Voltage	4000 V
Nozzle Voltage	500 V
Wash	First two minutes to waste as wash
Reference Mass	922.0481
Fragmentor	150 V
Skimmer	65 V

Table 2.3- Gradient composition throughout LC-QTOF-MS run.

Time (minutes)	%A (H_2O: 0.1% formic acid)	%B (Acetonitrile: 0.1% formic acid)
2.000	97	3.0
7.960	97	3.0
27.00	85	15
27.15	64	36
29.71	40	60
32.00	5.0	95
34.00	5.0	95
35.00	97	3.0
30.00	97	3.0

2.2.9 Protein Fractionation ÄKTA Avant Liquid-Chromatography System

To concentrate and quantitatively determine differences between protein fractions, an ÄKTA Avant Liquid Chromatography system was used (GE-Healthcare, Chicago, US). Fractions were collected in 5 mL aliquots during the elution step. Each

fraction was collected in a 10 mL tube (BD Biosciences, Franklin Lakes, US) for further analysis.

A 1 mL HiTrap SP Sepharose FF (GE-Healthcare, Chicago, US) cation exchange column was used to purify the proteins in high and low haze beer samples. Specifications for the Hi-Trap SP FF column are listed in (**Table 2.4**).

Table 2.4- Properties of the Hi-Trap SP FF chromatography column used to purify and isolate beer proteins.

Parameter	HiTrap SP FF
Matrix	6% highly cross-linked beaded agarose
Chromatography	Cation exchange
Loading capacity	High
Column Volume	1 mL

2.2.10 ÄKTA Avant- Liquid Chromatography- Method Development

To determine if better separation could be obtained from a gradient or step elution, two methods were tested. The parameters of both experiments are found in table (**Table 2.5**). Solutions of 1 M citrate 1 M citric acid, 1 M sodium hydroxide, and 1.5 M sodium chloride buffers (Sigma Aldrich, Poole, UK) at pH 4 were made. Experiment 1 tested if a competitive salt elution could be utilised to separate beer proteins based upon their isoelectric point (**Figure 2.1**). Experiment 2 was a further developed version of experiment 1 utilising a high pH (4.0-5.8) gradient and a 0-0.45 M NaCl step increase to fractionate beer proteins (**Table 2.5 and Figure 2.2**).

Table 2.5 - Method development Parameters of ÄKTA Avant- Liquid Chromatography experiments

Step	Experiment 1	Experiment 2
Equilibration	0.1 M Citrate (50 mL)	0.1 M Citrate (50 mL)
Loading	50 mL (50 CV)	50 mL (50 CV)
Washing	0.1 Citrate/0.1 Citric Acid buffer at pH 4 (2 CV)	0.1 Citrate/0.1 Citric Acid buffer at pH 4 (2 CV)
Elution	3.6- 5.2 pH gradient over 12 CV 0-1.5 M NaCl/0.1 Citric Acid gradient every 3 CV	4.0- 5.8 pH gradient over 12 CV 0- 0.45 M NaCl/0.1 Citric Acid step elution after 6 CV followed by a step to 1.5 M NaCl/Citric acid for 6 CV
Cleaning	6 CV of 0.3 M NaOH	6 CV of 0.3 M NaOH

*CV=Column volumes

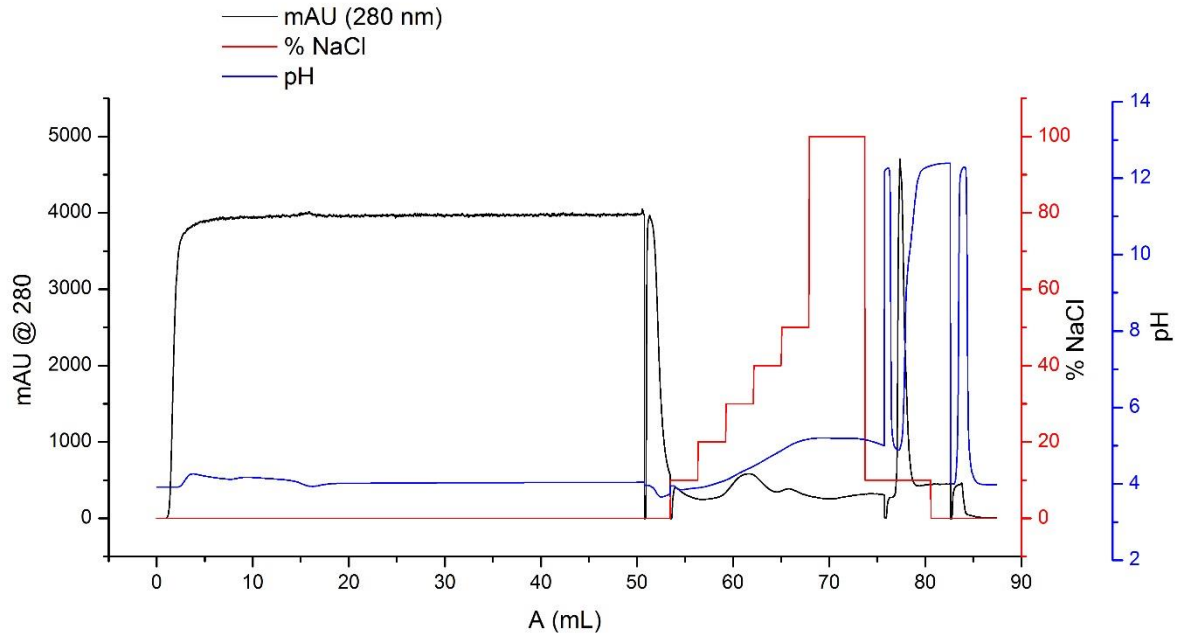


Figure 2.1 - Experiment 1 chromatogram utilising a HiTrap SP Sepharose FF column, a stepwise NaCl gradient (red) and a 3.6- 5.2 pH gradient. Protein fractionations were monitored by measuring light absorbance at 280 nm.

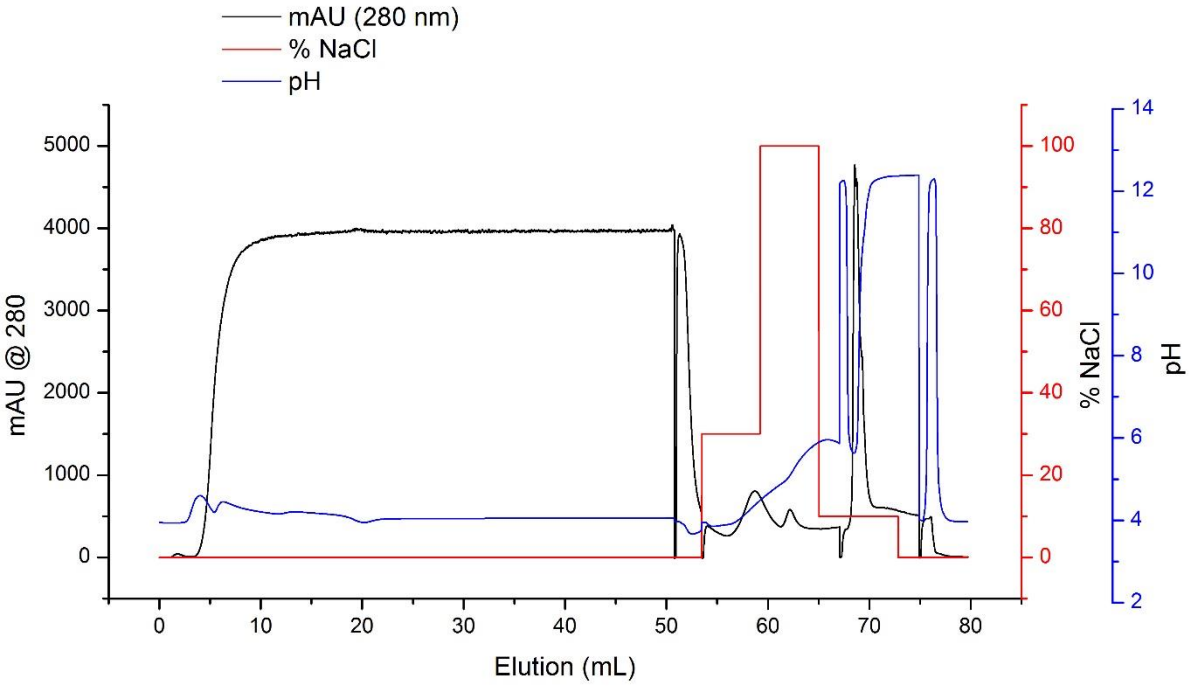


Figure 2.2 - Experiment 2 chromatogram utilising a HiTrap SP Sepharose FF column, a 0.45 M NaCl and a 1.5 M NaCl step increase (red) and a 3.6- 5.2 pH gradient. Protein fractionations were monitored by measuring light absorbance at 280 nm.

2.2.11 Removal of Salt with Dialysis

Dialysis was used to remove salt from protein fractions collected from the reverse-ion phase chromatography (ÄKTA Avant Liquid Chromatography system, GE-Healthcare, Chicago, US). A dialysis buffer was made by preparing a 10mM solution of NaOH (Sigma Aldrich, Poole, UK) with 50 mM Tris (Sigma Aldrich, Poole, UK) and fixed to a pH of 4.5 with a 1 M solution of citric acid (Sigma Aldrich, Poole, UK) with constant stirring in distilled water. Dialysis tubing (Sigma Aldrich, Poole, UK) were cut to 6 cm lengths and rehydrated for two hours in the dialysis buffer before sample was applied. Two-litre graduated cylinders, for each sample, were filled with one litre of dialysis buffer each. The tubing was folded twice, clipped at one end, and filled with sample. All bubbles were removed from the tube by application of a sweeping motion and the open end of the dialysis tube. Following this, the tubes were folded twice and clipped. Each dialysis tube was placed into a graduated cylinder and dialysed for 24 hours at 4°C with constant stirring.

After incubation, each dialysis tube were rinsed with distilled water to remove buffer from the outside of the tube. Following this, the contents of each tube was decanted into a clean microcentrifuge tube (ThermoFisher Scientific, Perth, UK) and snap-frozen with liquid nitrogen.

2.2.12 Determination of D-Mannose, D-Fructose, and D-Glucose

A Megazyme© assay kit for D-mannose, D-fructose, and D-glucose (Megazyme Ltd, Bray, IE) was used to determine D-mannose, D-fructose, and D-glucose in high and low haze beer samples. Suspensions supplied in the kit and used in the assay are listed in (**Table 2.6**).

Table 2.6- List of reagents used in D-mannose, D-fructose, and D-glucose assay kit.

Suspension	Content
1	Buffer (pH 7.6) plus sodium azide (0.02% w/v) as a preservative
2	$NADP^+$ plus ATP
3	Hexokinase plus glucose-6-phosphate dehydrogenase suspension
4	Phosphoglucose isomerase suspension
5	Phosphomannose isomerase suspension

A high haze sample (12.57 EBC average) and low haze sample (0.51 EBC average) were degassed for two hours by stirring in a beaker at 320 RPM. A sample blank was prepared utilising the low haze sample and omitting the addition of suspension 3.

Cuvettes with a 1 cm light path (Fisher Scientific, Loughborough, UK) were used for the assay. A 2.0 mL volume of distilled water, 0.10 mL of degassed beer sample, 0.2 mL of suspension 1, and 0.2 mL of suspension 2 were added into the cuvette, covered with parafilm and inverted to mix. After a three minute incubation, the cuvette(s) were blanked against air and absorbance (A_1) was measured with a Genysis 6 Spectrophotometer (Thermofisher Scientific, Perth, U.K) at 340 nm. A 0.02 mL volume of suspension 3 was added into the cuvettes containing sample and none into the blank. The cuvettes were covered in parafilm, inverted to mix, incubated for approximately five minutes, and absorbance (A_2) was measured. Following this, 0.02 mL of suspension 4 was added to each cuvette, mixed, and incubated for ten minutes. Absorbance (A_3) was measured and a 0.02 mL volume of suspension 5 was added to each cuvette, inverted to mix, incubated for 20 minutes, and measured at 340 nm (A_4).

The absorbance difference of D-glucose ($\Delta A_{D\text{-glucose}}$) was calculated by determining the difference for both blank and sample (A_2-A_1). The total absorbance difference of the blank was subtracted from the absorbance difference of the sample. The absorbance difference of D-fructose ($\Delta A_{D\text{-fructose}}$) was determined by subtracting the absorbance difference of the blank from the absorbance difference of the sample (A_3-A_2). The absorbance difference of D-mannose ($\Delta A_{D\text{-mannose}}$) was determined by subtracting the absorbance difference of the blank from the absorbance difference of the sample (A_4-A_3). To calculate the concentration of D-glucose, D-fructose, and D-mannose in grams per litre the following **Equation 2.1** was used:

$$c = \frac{V_f \text{ x } MW}{\varepsilon \text{ x } d \text{ x } v} \text{x } \Delta A$$

(2.1)

Where V_f represents final volume in mL, MW represents molecular weight D-glucose, D-fructose, and D-mannose (g/mol), ε is the extinction coefficient of NADPH

at 340 nm (6300 ((l x mol^{-1} x cm^{-1})), d is light path (cm), and v is the sample volume (0.10 mL).

2.3 GC/MS-SPME Method Development

2.3.1 Chemicals

All chemicals were purchased from Sigma-Aldrich (Sigma Aldrich, Poole, UK) unless stated otherwise.

2.3.2 Standard Solutions

A master stock was prepared at concentrations appropriate to each terpene/terpenoid's concentration in beer based on the literature (**Table 2.7**). A mixed master stock of 5mg/mL β-myrcene, α-humulene, (-)-caryophyllene oxide; 15 mg/mL (R)-linalool, trans-geraniol, β-caryophyllene, and 25 mg/mL (R)-linalool and trans-geraniol were prepared. The mixed-master stock was diluted to reach the concentration ranges listed in (**Table 2.7**). All stock solutions were stored at -20°C

2.3.3 Internal Standard

Beta-damascone was used as an internal standard (ISTD) for quantitation. A master stock of 35 mg/L β-damascone stock was prepared in absolute ethanol and stored at -20°C. The master stock was diluted to 1000 mg/L in an 8% (v/v) ethanol solution and stored at 4°C. The diluted internal standard was added to each sample to obtain a final concentration of 10 µg/L for analysis.

2.3.4 Calibration and Validation

Each data point in calibration standard curves were averages of three replicates. Standard curves were constructed in Shimadzu Corp. GCMSsolution Post-Run Analysis Software, Version 2.61 (Shimadzu Corp., Milton Keynes, UK) in the assessment of linearity. Curves with an $r^2 \geq 0.998$ were accepted.

After standard curves were accepted, a commercially produced 5.6% alcohol by volume (ABV) dry-hopped ale was adjusted to 8% (v/v) with absolute ethanol (Fisher Scientific, Loughborough, UK). Beer samples were run in SCAN mode to determine relevant hop volatiles, specific to hops in beer. Samples were run in triplicate to determine retention times and optimize sampling parameters. Results were determined to have an error of less than 10%.

2.3.5 Instrumentation

Samples were analysed with a Shimadzu Corp. GCMS-QP2010 Ultra Gas Chromatograph/Mass Spectrometer (GC/MS) (Shimadzu Corp., Milton Keynes, UK) with a PAL-AOC 5000 autosampler (CTC Analytics AG, Zwingen, Switzerland).

Table 2.7- Targeted terpenes and relative concentrations in beer.

Terpene/Terpenoid	Concentration in Beer (µg/L)	Concentration in commercial lager (µg/L)*	Concentration in commercial IPA (µg/L)*	Concentration in commercial stout (µg/L)*	Standard curve range (µg/L)	Recovery test spike concentrations (µg/L)
β-myrcene	[a]30-100, [b]3-1000	0	150	0	1-100	17
Linalool oxide	[c]20, [b]0-500	0	193	1.5	10-500	82
(*R*)-linalool	[d]45 (pilsner), [c]470,[b]1-50, [e]25-44	0	1386	12	1- 500	46
β-citronellol	[c]10, [b]1-90	21	384.9	23	10- 500	84
trans-geraniol	[c,e]5, [b]1-200, [e]69	27	1617	33	1- 500	46
β-caryophyllene	[b]0.2-30, [e]75	2.3	7.8	2.3	10- 150	52
α-humulene	[b]32, [e]27	1.14	16.2	3.6	1- 50	17
(-)-caryophyllene oxide	[b]14	7.13	12.2	7.0	1-50	17

[a]Tressl, R. *et al.,* (1978), [b]Fritsch, H. T. *et al.,* (2005), [c]Rettberg, N. *et al*., (2018), [d]Zunkel, M. and Schoenberger, C. (2012), [e]Peacock, V. E. and Deinzer, M. L. (1981)

*Values are means of triplicate mesurements

2.3.6 Instrument Conditions

Samples were pre-incubated at 50°C and agitated at 500 RPM in five-second bursts for five minutes. Volatiles in the vial headspace were extracted by adsorption onto a polydimethylsiloxane/divinylbenzene (PDMS-DVB) solid-phase microextraction (SPME) (Supelco, Poole, U.K) fibre for 30 minutes. The fibre desorbed in the injection-port for one minute.

Samples were separated utilising an HP-5MS column (30m x 0.25mm x 0.25µm film thickness) (Agilent Technologies, Edinburgh, UK) with a helium carrier gas in splitless mode. The GC oven programme held temp at 50°C for two minutes, ramped to 160°C at a rate of 4°C per minute, followed by a ramp to 320°C of 70°C per minute and held at 320°C for 3.22 minutes for a total oven programme of 35 minutes.

2.3.7 Mass Spectrophotometric Conditions

In selective ion mode (SIM), mass-charge ratios (mu) of 177 (β-damascone-ISTD), 69 (β-myrcene, β-citronellol, and *trans*-geraniol) 59 (linalool oxide), 71 ((*R*)-linalool), and 93 (β-caryophyllene, α-humulene, (-)-caryophyllene oxide) were monitored to identify targeted terpenes (**Table 2.7**). The solvent cut time was two minutes.

2.3.8 Sample Preparation

Beer was fixed to 8% ABV with absolute ethanol (Fisher Scientific, Loughborough, UK). and a 5 mL aliquot was pipetted into a 20 mL glass chromatography vial (Agilent Technologies, Edinburgh, UK), The diluted internal standard (Section 2.3.3) was added to each sample to obtain a final concentration of 10 µg/L for analysis. Chromatography vials were immediately crimp-sealed and placed into the sample queue for analysis.

2.4 Dry-Hop Conditions Effect on Hop Oil and Terpene Extraction

2.4.1 Fractional Four-Factorial Experiment- Sample Preparation

A volume of 4.5 L of water were degassed by sonicating water for 45 minutes in a sonicating water bath. An acidified model beer solution was made by fixing distilled water to a pH of approximately 4.20-4.15 with sodium citrate/citric acid (Sigma Aldrich, Poole, UK) with constant stirring. The simulated beer matrix was split and analytical reagent grade ethanol (99.8%) (Fisher Scientific, Loughborough, UK) was added to create the appropriate % ABV from the output table (**Table 2.8**). Four, one-

litre amber bottles were flushed with nitrogen and 500 mL of the degassed acidified simulated beer matrix was added to the purged bottles. The required hop dose was added to each bottle, and incubated for the dictated time at either 4°C or 20°C depending on the parameters of the run number (**Table 2.8**).

Table 2.8 - Output table from design expert describing total number of tests (Run), hop exposure time (hours), hop dosage (g/L), ethanol concentration (%ABV), and temperature (°C).

Run	Ethanol Concentration (% ABV)	Temperature (°C)	Exposure Time (Hours)	Hop Dosage (g/L)
1	3	20	12	6
2	3	4	12	1
3	10	4	12	1
4	10	4	48	6
5	10	20	12	1
6	3	20	48	6
7	10	4	12	6
8	10	20	12	6
9	3	20	12	1
10	10	20	48	1
11	3	4	48	1
12	10	20	48	6
13	3	4	12	6
14	10	4	48	1
15	3	4	48	6
16	3	20	48	1
17	10	20	12	6
18	3	20	12	1
19	3	20	12	6
20	10	20	12	1
21	10	4	48	1
22	10	4	48	6
23	3	4	48	1
24	10	4	12	1
25	3	4	12	1
26	10	20	48	1
27	3	20	48	6
28	3	20	48	1
29	3	4	12	6
30	10	4	12	6
31	10	20	48	6
32	3	4	48	6
33	6.5	12	24	3.5
34	6.5	12	24	3.5

Following the incubation, the hopped simulated beer matrix was filtered to separate spent hop material from the simulated beer matrix with Whatman Grade 1 filter paper (GE Healthcare Inc., Chicago, US). Prior to filtration, each filter paper was weighed and labelled appropriately. The filter papers with the hop material were air dried for 48 hours. To complete the drying process, the filter papers were placed into an 80°C oven for three minutes. The filter papers were stored in a desiccator for Soxhlet extraction (Section 2.4.2) and the simulated beer matrix was saved for analysis via liquid-liquid extraction (Section 2.4.3).

2.4.2 Soxhlet Extraction

Soxhlet crucibles (Fisher Scientific, Loughborough, UK) were defatted for extraction by soaking in hexane for at least 12 hours (Sigma Aldrich, Poole, UK) and were dried for one hour in a 105°C oven. Dried filter papers (Section 2.4.1) were inserted into a prepared soxhlet crucible. The crucibles were covered with a piece of cotton wool to prevent any hop debris from escaping during the extraction. The filled crucible and 500 mL round bottom flask with boiling chips were weighed prior to extraction. The flask was filled with 200 ml of hexane and the soxhlet crucible was placed in the extraction chamber of the soxhlet extraction apparatus. The extraction was run for a minimum of six hours.

After extraction, the crucible was removed from the extraction chamber and dried in a 105°C oven for a minimum of one hour. The crucible was cooled in a dessicator before weighing. The pre-extraction weight was subtracted from the post-extraction weight to determine the weight of lipids lost from the Soxhlet crucible.

The hexane remaining in the round-bottom flask was removed by rotary evaporation following Buchi's 20/40/60 rule (Hoegge, 1998) left to cool and dry in a laminar flow hood, and was weighed. Pre and post extraction weights of the round-bottom flask were subtracted to quantify total hop oil present in the spent hop material.

2.4.3 Liquid-Liquid Extraction

In order to separate hop oil from the simulated beer matrix collected in Section 2.4.1., liquid-liquid extraction techniques were utilised. The objective was to extract organic hop oil (lipids). Dichloromethane (DCM) (Fisher Scientific International Inc., Loughborough, UK) was utilised as the solvent as oils (lipids) were miscible in the

organic layer of the phase separation. A 300 mL volume of the 500 mL fraction was added to a 1 L glass separating funnel. An equal volume of DCM was added to a separating funnel. The funnel was shaken and allowed to settle to separate organic and aqueous phases. The organic phase was collected, washed once with DCM, and dried with sodium sulphate crystals (Sigma Aldrich, Poole, UK). The solvent was removed by rotary evaporation in a weighed round-bottom flask following Buchi's 20/40/60 rule. The difference between pre and post-extraction weight was calculated to determine the total amount of hop oil extracted in the simulated beer matrix.

2.4.4 Targeted Study- the Effect of Ethanol and Hop Dose on Terpene/Terpenoid Extraction

An acidified simulated beer matrix was prepared by fixing distilled water to a pH of 4.18-4.20 with a 1 M solution of citric acid. Fractions of the acidified simulated beer matrix was then fixed to 3% ABV, 6.5% ABV and 10% ABV, with analytical reagent grade ethanol (99.8%) (Fisher Scientific, Loughborough, UK) and stir bars were added into each bottle. The simulated beer matrix was placed in a glove box, purged with nitrogen three times, and left stirring overnight to remove oxygen from the matrix.

Twelve samples were prepared at a time- six bottles contained stir bars to assess terpenic extraction with agitation and six bottles did not contain stir bars to assess terpenic extraction without agitation. A 200 ml volume of deaerated simulated beer matrix was added to each bottle with the corresponding ethanol content required for each hop dose (**Figure 2.3**). Six of the samples were placed on a multi-position stir plate and stirred at 130 RPM. The bottles were placed into the glove box and the chamber was purged with nitrogen three times to remove oxygen. After the chamber had been filled with nitrogen, the chamber was covered with light barriers and incubated for 48 hours. The experiments were run with Simcoe (John I. Haas, Washington, US) in triplicate and repeated with Chinook (John I. Haas, Washington, US) in triplicate.

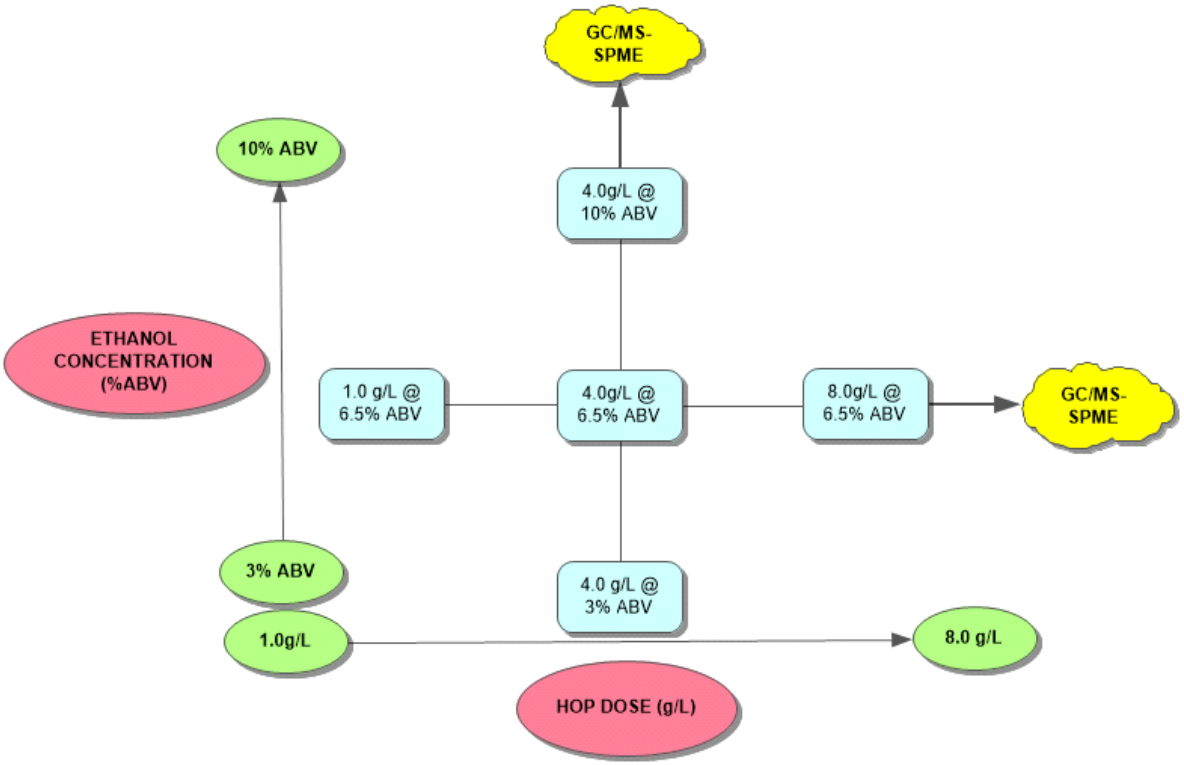


Figure 2.3 - Schematic of targeted study examining the effect of increasing hop dose and increasing ethanol content. Increasing ethanol content is denoted on the y-axis and increasing hop dose is denoted on the x-axis. Each blue square denotes a sample point tested. Each sample point was tested in triplicate in addition to being tested in stirred and unstirred reactions.

Following the incubation, stirring was stopped and the hop debris was allowed to settle to the bottom of the bottle. Samples were prepared for GC/MS- SPME analysis according to Section 2.3.8.

2.5 Terpene Studies in Packaged Beer

2.5.1 Sample Collection

To model and assess terpenes/terpenoids contents in packaged beer, sixteen cases of a dry-hopped ale with less than 0.5 EBC haze value, packaged in 330 mL amber glass bottles (Owens-Illinois, Alloa, UK), were collected during a standard packaging run by the industrial partner. The collected samples were split into two incubation temperatures- 4°C and 20°C for a total of 16 weeks. Sample points for the ageing experiment were collected at day zero (control), 2, 4, and 7 in addition to 2, 4, 6, 8, 10, 12, 14, and 16-week time points.

2.5.2 GC/MS-SPME Analysis

At the time of sampling, samples were brought to 4°C and two, 40 mL aliquots of the 4°C and 20°C incubations were collected in 50 mL centrifuge tubes and frozen at -

20°C for GC/MS-SPME analysis (Section 2.3.8). The remaining samples collected were prepared for sensory panels (Section 2.5.3).

2.5.3 Sensory Panels

The sensory panel developed by the industrial partner was utilised for sensory analysis in this study. Panel design and setup is described in detail in Chapter 5.

2.5.3.1 Panel Preparation

A total of eight beers were collected from each sample point and were chilled to a target temperature of 4°C. Taste panel samples were required to be less than or equal to 10°C. An approximate volume of 100 mL of beer was poured into clear, plastic 200 mL cups for panellist assessment. Beer was prepared and immediately served to panellists to ensure each panellist received identical samples. Panellist assessment took place in individual tasting booths illuminated by red light to remove visual bias.

Panellists required an invitation to be included in the descriptive profiling panel for the experiment and the minimum number of attendees per panel was six people. The panels were held from 10:15- 10:45 in the morning to prevent any food, drink or toothpaste bias. Four samples- two 20°C storage and two 4°C storage, were mixed into a routine daily taste panel to ensure panellists were blindly assessing the samples.

The panel was a descriptive profiling panel based upon hop-attributed sensory descriptors (**Figure 2.4**) to monitor changes in hop flavour and aroma. Panellists were provided with a laptop and a personal login for the Sensecheck™ software (Cara Technology, Leatherhead, UK) to record responses. The software listed each sensory descriptor with a slide-bar for panellists to rate approximate intensities of each flavour. Following the panel, the results from each panellist were collected in a folder specific for the project.

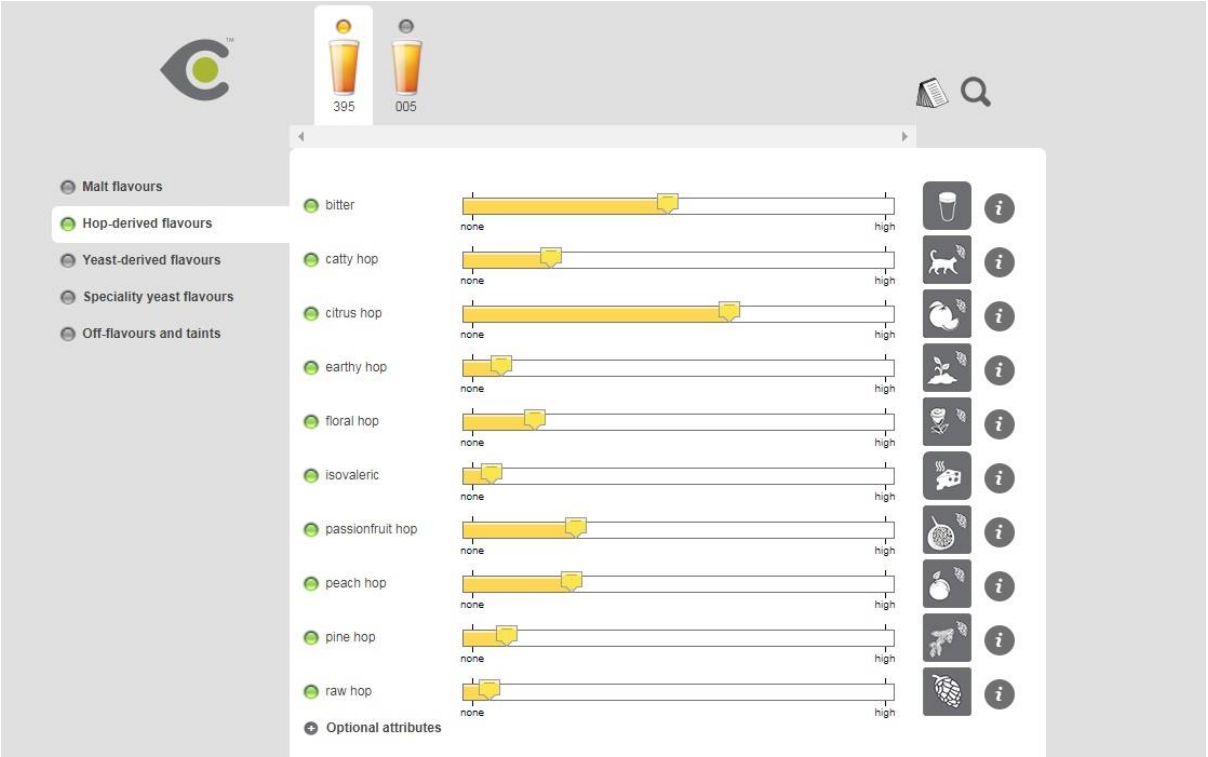


Figure 2.4- SenseCheck™ descriptive profiling form for panellists participating in assessing aged dry-hopped ale (Section 2.5) (Cara Technology, Leatherhead, UK).

2.6 The Vanillin Assay

2.6.1 Vanillin Assay Protocol

Fresh beer from the sponsoring brewery was used as the experimental beer used in Vanillin Assay tests. Terpene and/or beer sample, ice-cold distilled water, 1.6% vanillin (≥97%, Sigma Aldrich, Poole, UK)/sulphuric acid (≥95%, Fisher Scientific, Loughborough, UK) and clean 10 mL glass screw cap culture tubes (Fisher Scientific, Loughborough, UK) were placed into an ice bucket to chill. In a clean test tube, 1.4 mL of terpene isolate and 0.6 mL of ice-cold distilled water were combined. A one-millilitre volume of the 1.6% (v/v) vanillin/sulphuric acid solution was added dropwise while continuously spinning the tube in the ice bucket to prevent localised overheating. Following this, tubes were capped and heated for 20 minutes in a 60°C circulating water bath. After incubation, the tubes were cooled to room temperature and scanned from wavelengths of 300-700 nm on a GENESYS 6 UV-Vis spectrophotometer (Thermo Fisher Scientific, Perth, UK). An ethanol blank corresponding with the ethanol concentration tested in the assay, was prepared simultaneously and used to record a baseline measurement for the spectrophotometer scans.

2.6.2 Solid-Phase Extraction (SPE)

A Solid-Phase Extraction method utilised by (Praet *et al.*, 2014) was optimised for the experiment. Bond Elut C18 cartridges (500 mg, 6 mL, Agilent Technologies Technologies, Lake Forest, US) were placed into a vacuum manifold, per isolate, and conditioned with three column volumes of absolute ethanol, Millipore MQ water (Merck-Millipore, Livingston, UK), and a 70% (v/v) ethanol solution. The columns were loaded with 5 mL of terpenoid isolate and isolates were drawn through the column by a vacuum. Columns were washed with three volumes of Millipore MQ water (Merck-Millipore, Livingston, UK) and eluted with three volumes of analytical reagent grade ethanol (99.8%) (Fisher Scientific, Loughborough, UK). The eluate was collected in a 15 mL chromatography vial and sealed with a crimp-silicone cap (VWR International, Leicestershire, UK). The terpene isolate eluate was tested in the vanillin assay as described in Section 2.6.1.

2.6.3 Beer Test Solid-Phase Extraction (SPE)

Room temperature beer was degassed by stirring with a stir bar in a 500 mL beaker for one hour at 200 RPM on a stir plate and covered with a watch glass. Bont Elut C18 cartridges (Mega Bond Elut Flash, 1g, 60 mL, 40µm, Agilent Technologies Technologies, Lake Forest, US) were conditioned with three volumes of analytical grade ethanol (99.8%, Fisher Scientific, Loughborough, UK), three volumes of Millipore MQ- water (Merck-Millipore, Livingston, UK), and three volumes of 70% (v/v) ethanol/Millipore MQ-water solution (Merck-Millipore, Livingston, UK). A 50 mL beer sample volume was pipetted onto each column and drawn through the column by a vacuum pump. The eluate was discarded and three volumes of Millipore MQ-water (Merck-Millipore, Livingston, UK) were added to the column to remove any non-terpene/terpenoid substances. The compounds were eluted with 20 mL of analytical grade ethanol (99.8%, Fisher Scientific, Loughborough, UK) and collected in glass vials. The eluates were diluted by 50% with Millipore MQ-water (Merck-Millipore, Livingston, UK). The diluted eluates underwent a second SPE using Bond Elut C18 Cartridges (500mg, 6 mL, Agilent Technologies Technologies, Lake Forest, US). The columns were conditioned with three volumes of analytical grade ethanol (99.8%, Fisher Scientific, Loughborough, UK), Millipore MQ-water (Merck-Millipore, Livingston, UK), and 50 % ABV ethanol/Millipore MQ water (Merck-Millipore, Livingston, UK). A 5 mL volume of diluted eluate was pipetted onto and drawn through

the column with a vacuum pump. The columns were washed with three volumes of Millipore MQ water (Merck-Millipore, Livingston, UK). Finally, two 5 mL volumes of analytical grade ethanol (99.8%, Fisher Scientific, Loughborough, UK) were drawn through the column to elute the compounds that had adsorbed to the column material. The eluates were tested in the vanillin assay as described in Section 2.6.1.

Chapter 3- Historical Process Data as a Diagnostic Tool

3.1 Introduction

The use of statistical methodology to study brewing process variables is not commonly reported in the literature (Speers and Stokes, 2009). However, there are a limited number of reports utilising statistics in the optimisation of the brew house, cellaring, and packaging processes (Mayer, Morton and Laufer, 1953; Tighe *et al.*, 2003; Speers *et al.*, 2003; Hughey, McMinn and Phung, 2016). At its core, statistical methods allow one to clearly view a *signal* or trend through a *cloud* of variability (Speers, personal communication, 2015). Presumably, in-depth statistical process control is undertaken by breweries as it is often required in process operations (statistical process control). These statistical results can be used to inform decisions to cut losses in processing/transferring, increase efficiencies, and reduce waste (Mayer *et al.*, 1953).

As discussed in Chapter 1 (Section 1.4.2), there are various reasons for increased beer turbidity to occur. In some seemingly sporadic cases, breweries have observed turbidity to be persistent immediately after filtration or centrifugation (F. Gormley, personal communication, 2015). To understand the occurrence of random unfilterable beer haze, attention was turned to procedures and processes within the brewery. It was found that increased turbidity was observed to sporadically occur, with no discernible variation in raw materials, brewing procedures, conditioning or packaging. As part of the industrial partner brewery's quality programme, EBC haze levels were monitored throughout fermentation, conditioning, and packaging processes.

Brewing procedures were tailored to ensure that clear wort was produced as clear wort is critical for later beer clarification (Jin *et al.*, 2004). Kettle finings were added during the boil to aid in the polymerization and precipitation of proteins and polyphenols in addition to hot-break formation (Bamforth, 1999). Yeast pitching rates and fermentation conditions were regulated as additional measures to reduce beer turbidity. Finally, a minimum of three days of cold-conditioning at -1°C were carried out to aid in beer clarification. Beer that did not reach ≤5.0 EBC haze units after three days of conditioning was left at -1°C for further maturation until turbidity requirements were met or the beer met flavour/aroma standards.

Despite these measures, the variability in the appearance of haze was high. One batch produced EBC haze values at approximately 13 EBC with subsequent batches reporting values as low as 0.13 EBC (**Figure 3.1**). In this case, inferential statistics can be a powerful diagnostic tool to investigate various factors that cause haze to occur.

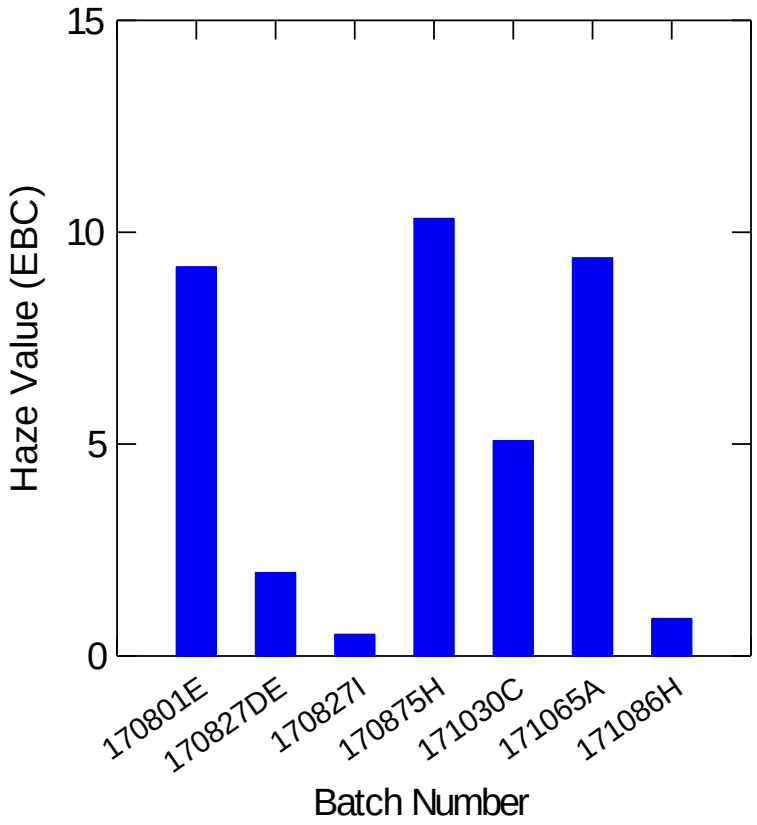


Figure 3.1 EBC Haze values between brew numbers of an identical brand.

3.1.1 Pearson Correlation Coefficient

The Pearson Correlation Coefficient is used to determine if a linear relationship exists between two variables. The Pearson Correlation Coefficient was developed by Karl Pearson in the 1920's using information and ideas introduced by Francis Galton in the 1800's (Pearson, 1896, 1920). The test measures the linear relationship between two variables in a normally distributed data set. Pearson correlation values range between -1 to +1. Any value above zero indicates a positive correlation. Any value below zero indicates a negative correlation. The closer the Pearson correlation coefficient, *r*, is to +1 or -1, the better the data fits the line of best fit (Miller, 2012). The closer the Pearson correlation coefficient is to zero the more the variation around the line of best fit exists and the weaker the relationship is between the two variables tested. The test is based upon the assumptions that variables are interval or ratio measurements, the data is

normally distributed, that the data contains few outliers and finally that the data is homoscedastic (Miller, 2012). The Pearson correlation is a suitable test in determining if one variable in upstream processes has an effect or relationship with a different variable in downstream processes.

3.1.2 Spearman's Rank-Order Correlation Coefficient

For data that is not-normally distributed and does not fit the assumptions of the Pearson correlation statistic, the Spearman's rank-order correlation coefficient is commonly used in its place (Dalgard, 2002; Miller, 2012). The Spearman rank-order correlation coefficient is used to assess the strength of linear relationship (positive or negative) between two variables with data that is not normally distributed. Similar to the Pearson Correlation Coefficient, values closer to 1 indicate a strong positive correlation, values closer to -1 indicate a strong negative correlation, and values closer to zero indicate that there is no relationship between the two variables tested (Dytham, 2015). The test is based on two essential assumptions. First, the data must be on an ordinal, interval, or ratio scale and second, that the data has a monotonic relationship between the two variables tested (Stevens, 2015).

It was hypothesised that incidences of increased beer turbidity were due to variances in brewery processes. The objective of this work was to utilise statistical tests as a tool to ascertain the cause of sporadic spikes in beer turbidity. Historical brewery data was analysed in search of significant correlations between increased turbidity and brewery data. Therefore, Pearson correlations and Spearman's Rank-Order Correlation Coefficients were utilised as correlations in this experiment.

3.1.3 Stepwise Regression

A different way of assessing the relationship between cause-and-effect variables in a large dataset is to utilise stepwise regression. Stepwise regression builds the best-fit model regarding multiple correlations. The model is built in stages, assessing the fit of the model as variables are added in or taken out. It is beneficial when attempting to highlight specific variables that could build a more robust model (Dytham, 2015). In the case of this experiment, it is useful to identify variables that build a strong model when related to haze. The variables that build the most robust model are useful to inform and monitor in future analysis.

To test the fit of the model, the Akaike Information Criterion (AIC) is used to determine the 'goodness of fit' as variables are added or deleted from the model. The lower the AIC value, the better the model fits the data (Dytham, 2015). The model can also be checked solely utilising 'forward selection' or 'backward elimination'. These methods only utilise a forward progression or backward elimination of selecting variables to build the 'best fit' model. Stepwise regression combines these methods and, in building the model, assesses the fit of the model as variables are added or eliminated. It is important to note that in forward selection, backward elimination, and stepwise regression, only one variable may added or deleted at a time (Dalgard, 2002; Dytham, 2015).

3.2 Experimental Design

3.2.1 Collection of Data

Brewery data collected over two years, as part of routine quality assurance procedures, were obtained from the sponsoring brewery. The brew kit produced 50 hL of wort per brew and the fermentation vessels (FV) were of 100 hL capacity. Therefore, two batches of beer were required to fill a fermentation vessel (FV). Due to this, each beer was grouped by FV number.

3.2.2 Parameters for Statistical Analysis

The parameters in each batch of beer were assessed using the Pearson correlation statistic. The parameters included: mashing time (minutes), mash pH, strike water volume, first running gravity, last running gravity, total lauter time (minutes), pre-boil volume (hL), pre-boil gravity (SG), post-boil gravity (SG), dilution water, cold wort total, total minutes casting, oxygen volume added, original gravity (SG), final wort volume (hL), density, alcohol by volume (%ABV), pH value (bottle), International Bitterness Units (IBU), and total brewing time (minutes).

3.2.3 Statistical Analysis

Upon analysis, the data was checked for normality using a quantile-quantile (Q-Q) plot obtained using the linear model syntax and plotted in RStudio (R Core Team, Vienna, Austria). If the data was normally distributed, the *p*-value was calculated from the summary of the linear model. If the data was not normally distributed, the Spearman's rank-order correlation coefficient was used to calculate the *p*-value. The *p*-value was found to be significant at $p<0.05$.

To measure the degree of linear relationship between variables, Pearson Correlations were calculated utilising SYSTAT 13.1 statistical software (Systat Software Inc., Chicago, US). The probability of the correlation coefficient being significant (*p*-value) was calculated utilising the necessary syntax for linear regressions on RStudio (R Core Team, Vienna, Austria). Backward elimination, forward selection, and stepwise linear regressions were also calculated utilising RStudio (R Core Team, Vienna, Austria).

3.3 Results

Each parameter was checked for normal distribution. If the parametric data was normally distributed, a simple linear regression could have been used to calculate the *p*-value. However, the Q-Q plots proved that none of the parameters were normally distributed (**Appendix B**). Therefore, it was not appropriate to use Pearson's product-moment correlation and the Spearman's rank-order correlation were used to calculate the correlation coefficient, ρ. The correlation coefficient, ρ, was used to calculate the *p*-value (Dalgard, 2002) (**Table 3.1**).

Results close to 1 or -1 indicate a perfect positive or negative correlation, respectively. The significant results in the study all pointed to the early steps of the process- mashing, lautering, and boiling. The parameters of mash pH, strike water, mash in volume, and total lauter time reported negative correlations and were significant ($p<0.05$) (**Table 3.1**). Strike water and total lauter time were the closest reported values to -1 at $\rho= -0.278$ and $\rho= -0.288$, respectively. These values may indicate that small mash volumes are related to high haze values. However, the large sample number, $n= 322$, may have caused the low correlation coefficients to be artificially significant.

Parameters of first runnings gravity, pre-boil volume, pre-boil gravity, post-boil gravity oxygen volume added to wort, original gravity, final wort volume, density, alcohol by volume, and international bitterness units reported positive correlation coefficient values and were significant ($p<0.05$) (**Table 3.1**). The correlation coefficient, ρ, for pre-boil gravity showed the closest reported value to 1 at $\rho= 0.325$. This indicated that pre-boil gravity values might have increased with EBC haze in beer. First-runnings gravity appeared to closely follow a similar relationship with $\rho= 0.294$.

Again, the high probability of the correlation coefficients significance may have been artificially induced by the large sample number (n= 322).

The results were based upon non-parametric correlations and were not based upon the same assumptions as a simple linear regression. Results from Spearman's rank-order correlation are not as conservative as Pearson's product-moment correlation and the interpretation of the results from this test may not always be clear (Dalgard, 2002; Dytham, 2015).

The stepwise regression proved that the strongest model was constructed by backward elimination. However, the AIC value was high at 253.87 and the difference between backward elimination, forward selection, and stepwise regression was 4.61 as forward selection and stepwise regression reported the same values (**Appendix C.16 and C.17**). In backward selection, the variables that constructed the most robust model were mash temperature, mash pH, total lautering time, pre-boil volume, dilution water added, total wort volume, total minutes casting, density, present gravity (PG), and EBC colour. In forward selection, the variables that constructed the most robust model were mash temperature, cold wort total, total casting time, total lauter time, dilution water, and mash pH. Finally, in stepwise regression, the variables that constructed the most robust model were mash temperature, cold wort total, total casting time, total lauter time, dilution water added, and mash pH (**Appendix C.15- C.17)**.

Table 3.1 Spearman correlation values of various brewhouse parameters compared to EBC haze values[a].

Parameter	Spearman Coefficient (ρ)	*p*-value
Mash pH	**-0.129[b]**	**0.023[b]**
Mash Temp (°C)	0.036	0.525
Strike Water Volume (hL)	**-0.278[b]**	**<0.001[b]**
Mash in Volume (hL)	**-0.198[b]**	**<0.001[b]**
First Runnings Gravity (SG)	**0.294[b]**	**<0.001[b]**
Last Runnings Gravity (SG)	0.005	0.930
Total Lauter Time (minutes)	**-0.288[b]**	**<0.001[b]**
Pre-Boil Volume (hL)	**0.121[b]**	**0.033[b]**
Pre-Boil Gravity	**0.325[b]**	**<0.001[b]**
Post-Boil Gravity	**0.277[b]**	**<0.001[b]**
Dilution Water (hL)	-0.100	0.080
Casting Time (minutes)	0.019	0.741
Oxygen Volume Added	**0.120[b]**	**0.034[b]**
Original Gravity (SG)	**0.203[b]**	**<0.001[b]**
Final Wort Volume (hL)	**0.136[b]**	**0.016[b]**
Density (SG)	**0.196[b]**	**<0.001[b]**
Alcohol by Volume (ABV)	**0.121[b]**	**0.033[b]**
pH Value (bottle)	0.042	0.05
International Biterness Units (IBU)	**0.129[b]**	**0.023[b]**
Total Brewing Time (Minutes)	-0.094	0.098

[a]n=322
[b]Bolded values indicate statistical significance ($p<0.05$)

3.4 Discussion

The data suggests that no relationship exists between brewery processes and EBC haze as correlation coefficients were not close to 1 (**Table 3.1**). Several of the correlation coefficients reported probability values with high significance (**Table 3.1**). However, after assessing the raw data in scatter plots, it was concluded that the reported correlations were not significant and do not have a strong relationship (**Appendix C.1-C.14).** The significance could be induced by the large sample size (n= 322).

The previous statement was further confirmed by the stepwise linear regression. Backward elimination, forward selection, and stepwise regression reported high AIC values despite selecting variables to build the best model (**Appendix C.15, C.16 and C.17**). The forward selection and stepwise regression reported identical optimum AIC values of 258.48 (**Appendix C.16 and C.17**). Stepwise regression is often used with caution as some statisticians regard stepwise regression as form of 'data mining' by hand selecting variables to build the best model. However, it was utilised in this experiment to identify what brewery parameters might affect each other, inducing sporadic turbidity. The stepwise regression found the combined variables of mash temperature, cold wort total, total casting time, total lauter time, dilution water added and mash pH to build the most robust model. From this, speculations can be made with correlation coefficients regarding the cause of random, unfilterable beer haze.

In this study, two 50 hL brews were required to fill one fermenter and ultimately, introduced two possible points of variation in the fermentation process. The Spearman rank-order correlation coefficient value suggested that a positive linear relationship existed between EBC haze and first runnings gravity, oxygen volume added to wort, original gravity, final wort volume, density, alcohol by volume, and international bitterness units (p<0.05). Significant negative linear relationships (p<0.05) were observed between EBC haze and mash pH, strike water, mash in volume, total lauter time, pre-boil volume, pre-boil gravity, and post-boil gravity (**Table 3.1**).

Despite their low values, the strongest correlation coefficients observed point to issues in mashing/lautering. The highest correlation coefficients were reported in pre-boil gravity (ρ= 0.325) and first runnings gravity (ρ= 0.294). The values tie in well with the high negative correlation coefficients reported in total lautering time (ρ= -0.288) and

strike water volume (ρ= -0.278). Too little strike water would alter the mash in volume and ultimately, impact the scheduled liquor to grist ratio. Lower strike volumes would affect the mashing process and result in incomplete conversion of starches into fermentable sugars (Muller *et al.*, 1994). Incomplete conversion would result in a weaker wort, leading to a shorter scheduled lautering time. With an altered brewing schedule, short lautering time may have resulted in the collection of a slightly higher gravity wort. The data suggests that slight variations in mashing regimes detrimentally impact downstream processes.

In incidences of high turbidity, it is possible that lower volumes of strike water affected the liquor to grist ratio. Low liquor to grist ratios (less than 2:1) or concentrated mashes have an impact on starch conversion causing the concentrated dextrins and other carbohydrates to inhibit amylase activity (Home *et al.*, 1993; Muller *et al.*, 1994; Briggs *et al.*, 2004). Limited solubilisation of β-glucans, α-glucans and starch hydrolysis occurs in thicker mashes as a portion of starch molecules are bound by water. This binding process thickens the mash, lowering the liquor to grist ratio (Home *et al.*, 1993; Cooper *et al.*, 1998; Stoupis *et al.*, 2002). Amylolytic enzymes are inhibited with the reduction of free water as sugars function as competitive inhibitors (Muller *et al.*, 1994; Briggs *et al.*, 2004). Jin *et al.*, (2004) also noted that increased incidences of high molecular weight β-glucans increase turbidity in high gravity wort and beer. This may explain the significant relationship between increased turbidity when compared to pre-boil gravity, first runnings gravity, and total lauter time. The wort collected from the mash may have been more concentrated with starches and carbohydrates surviving the brewing process, which ultimately, might have had an effect on beer turbidity.

The time spent in the lautering process greatly depends upon the brew kit, raw material and the style of milling employed (Briggs *et al.*, 2004; Priest *et al.*, 2006). In the theory of wort separation, a modified application of Darcy's Equation **(Equation 3.1)** is used to explain the flow of wort through a bed of grist (Briggs *et al.*, 2004; Priest *et al.*, 2006). The equation is:

$$V = \frac{K A \Delta P}{L \eta}$$

(3.1)

Flow rate through a bed of particles is referred to as V, K represents bed permeability, A represents bed area, ΔP is the pressure difference through the bed, L is bed thickness or the path through the bed, and Ƞ is the rheological term for the apparent viscosity of the wort (Briggs *et al.*, 2004; Priest *et al.*, 2006). This equation indicates the theoretical flow of wort through the bed and can be used to understand the factors involved in mash filtration. However, a number of these variables change during lautering making the exact computation of flow difficult. The act of sparging displaces wort from the bed of grist and higher flow is observed with lower viscosity (Ƞ) causing faster runoff. The diffusion coefficient of particles in the grist bed is dependent upon particle size (or diameter). As particle sizes will naturally differ in the grist bed, a short sparging time could affect extract recovery ultimately, as the extraction of desirable and undesirable nutrients could be influenced, affecting fermentation. Wort clarity may also be influenced by reduced sparging time as undesirable compounds (ungelatinised starches and carbohydrates) may be extracted and transferred into the boiling stage. This would affect the nutrient content of the wort, ultimately causing high turbidity in downstream processes (Briggs *et al.*, 2004). Higher mash gravities have also been reported to yield higher values in turbidity, viscosity and β-glucan concentrations (Budde *et al.*, 2005).

As two brews were required to fill the fermentation vessel, the higher volume of wort, containing slight inconsistencies across batches of beer may ferment differently than a smaller tank volume containing only one batch of beer (Boulton and Quain, 2006; Speers and Stokes, 2009; Bamforth, 2017). Fermenter volume may have an effect on apparent degree of fermentation (ADF) which may impact the health of the yeast cells in the fermenter (Speers and Stokes, 2009). Poor yeast cell heath may lead to cell wall disruption and may ultimately have an impact on beer turbidity (Stoupis *et al.*, 2002).

As the fermenter vessels in this study contained roughly 105 hL of wort for each fermentation, hydrostatic pressure may have had some impact fermentation performance and ultimately, the resulting beer (Shimada *et al.*, 1993; Boulton *et al.*, 2006). In fermentation vessels, yeast excrete amino acids, peptides, and phosphates in differing concentrations at the bottom of the vessel as opposed to the top due to sedimentation over the course of fermentation (Masschelein and Van Der Meersche 1976). As yeast cells circulate throughout the tank during fermentation and flocculation, the pressure on

the yeast cells changes depending on their tank position. Stratified layers within the fermenter may cause osmotic shock that yeast cell walls encounter in the cells to excise mannan complexes (Williams *et al.*, 1973; Shimada *et al.*, 1993; Chlup, Bernard, *et al.*, 2007). Due to the size of peptides and mannan complexes, the complexes may be difficult to remove by common centrifugation and filtration techniques (Chlup, Bernard, *et al.*, 2007; Stewart, 2018).

It is also possible that thermometers were not properly calibrated and that fermentation temperatures were higher than programmed. With increased temperature and ethanol content, flocculation and yeast health may be negatively impacted (Claro *et al.*, 2007; Soares, 2011). As pH measurements are normally corrected for temperature, the actual pH values in tank may have also affected yeast health and ultimately, adversely impacted the quality of the final beer (Jin *et al.*, 2000; Briggs *et al.*, 2004; Priest *et al.*, 2006).

Undermodified malt may have also been a cause for increased turbidity. Small granules of starch easily form complexes with protein that may oxidise. The oxidation reaction firmly binds the starch particles to protein forming a network for other particles, such as polyphenols to complex with and form a haze network (Briggs *et al.*, 2004).

It is important to note that as the *p*-value for final wort volume was obtained utilising Spearman's rank correlation coefficient, the significance of the results may not be reliable as the value only assesses the rank of independently ranked variables (Kallner, 2014). The high probability of the correlation coefficients significance may have been artificially induced by the large sample number, $n = 322$.

No robust conclusions can be drawn from the Spearman correlation coefficient analysis, therefore these results warrant further investigation. It is possible that slight alterations in mashing/lautering processes, fermentation performance, yeast health and modification of malt may induce the formation of sporadic hazes. It is hypothesised that excess starch/β-glucan, polyphenol or protein from the mashing process may be a culprit of sporadic haze formation. It is also speculated that fermentation procedures might also have an impact on haze formation. These hypotheses were tested in later experiments, which are presented and discussed in Chapter 4.

Chapter 4- Identification and Elucidation of Unfilterable Sporadic Beer Haze

4.1 Introduction

Most beer in the world is sold as 'bright' meaning it is clear, with no apparent colloid formation, or visual turbidity (Stewart, 2004). Physical stability is the term used to describe the maintenance of this desirable state. The occurrence of turbidity is undesirable, and, is more commonly referred to as 'haze'. Increased beer turbidity is the result of various 'culprits' such as microbial growth, poor extraction during mashing, protein-polyphenol complexes, β-glucans, inorganic materials from packaging, the formation of calcium oxalate, haze active proteins, and yeast cell-wall material (Chapter 1, Steiner *et al.*, 2010; Neugrodda *et al.*, 2014).

As previously reported, (primarily craft) breweries in Scotland and around the globe have observed persistent turbidity immediately following clarification procedures (F. Gormley, personal communication, 2015).

When historical and statistical data provide little insight into diagnosing a problem, investigative diagnostic studies are methods used to identify the source of the issue. This monitors specific macromolecules to find the root cause of the issue investigated. Research has linked proteins, polyphenols, protein-polyphenol complexes and β-glucans to be involved in the formation of colloids that increase turbidity levels (McMurrough *et al.*, 1996; Siebert, Carrasaco, *et al.*, 1996; Bamforth, 1999, 2011; Jin *et al.*, 2004; Iimure *et al.*, 2009; Steiner *et al.*, 2010).

Polyphenols in brewing are derived from plant material, and therefore in the case of beer from malt and hops. Polyphenols provide antioxidative stability but encourage the formation of colloids when, at minimum, dimeric polyphenols cross link with protein in beer (Qureshi *et al.*, 1979; Siebert, Carrasaco, *et al.*, 1996; Aron *et al.*, 2010). Catechins cross-link with proteins rich in proline residues and form small flocs that grow with further polymerisation (Siebert, Carrasaco, *et al.*, 1996). Although polyphenols are typically removed by filtration techniques, the compounds are important to measure in the case of any turbidity concerns.

Protein in beer is derived from cereals (Fasoli *et al.*, 2010; Steiner *et al.*, 2011; Jin *et al.*, 2012; Colgrave *et al.*, 2013), yeast cells (Fasoli *et al.*, 2010; Berner *et al.*, 2013; Colgrave *et al.*, 2013), or in smaller quantities, hops (Neugrodda *et al.*, 2014).

Protein levels as little as 2mg/L have been reported to cause haze in beer (Kaersoaard *et al.*, 1979; Ye *et al.*, 2016). While the impact of haze- active proteins derived from barley has been extensively studied (Iimure *et al.*, 2009; Jin *et al.*, 2012; Schulte *et al.*, 2016; Ye *et al.*, 2016) few definitive links between yeast protein influence on increased turbidity have been made (Lewis *et al.*, 1991; Chlup, Bernard, *et al.*, 2007; Chlup, Conery, *et al.*, 2007; Omura *et al.*, 2009).

Yeast cell walls are made up of a branched β-1,3-glucan and β-1,6-glucan network held together by hydrogen bonds. Glycosylated mannoproteins are linked to the cell wall β-1,3-glucan network. Two classes of glycosylated mannoproteins form the outer layer of the cell wall (Klis *et al.*, 2002). Glycosyl-phopatidylinositol (GPI)-dependent mannoproteins are linked to β-1,3-glucan within the cell wall via β-1,6-glucan and Pir proteins (proteins with internal repeats) are directly linked to β-1,3-glucan by an alkali-sensitive linkage (Chapter 1). Expression of one GPI-mannoprotein in regard to haze stabilisation in beer has been studied and links between production of GPI-CWP, Cwp1, to cell wall stress have been found (Ram *et al.*, 1998; Jung *et al.*, 1999; Terashima *et al.*, 2000; Klis *et al.*, 2002; Omura *et al.*, 2009). A link between yeast cell wall proteins and increased beer turbidity has been found (Chlup, Bernard, *et al.*, 2007; Chlup, Conery, *et al.*, 2007). However, a total fingerprint of excised cell-wall mannoproteins present in high haze and low haze beer is yet to be elucidated.

Historical data was collected and statistically analysed utilising Spearman's Rank-Order correlation coefficient and stepwise regression with a hypothesis that specific brewery processes could be correlated with haze values to identify trends and relationships (Chapter 3). As the results of Chapter 3 were inconclusive, the purpose of this study was to use diagnostic techniques to selectively eliminate factors contributing to sporadic haze formation. As high turbidity levels occurred seemingly at random and could not be removed with conventional clarification methods, 'high' haze and 'normal/low' haze samples were collected from the sponsoring brewery for diagnostic studies. An initial hypothesis was proposed that increased turbidity was a result of protein, polyphenol, or β-glucan content. This informed later targeted studies.

4.2 Experimental Design

4.2.1 Sample Collection

Samples were collected from the industry sponsor of a brand exhibiting sporadic haze formation. An industrially produced ale dry hopped between 0.2-0.8 kg/hL with sporadically high turbidity levels were used as the experimental samples for the study. A non dry-hopped lager was used for a control, as the product had consistently low turbidity. As elevated turbidity levels occurred at random, samples were collected for nine months to ensure suitable sample set was created. Samples were collected as described in Section 2.2.1.

At each sample collection, three cases were collected at the beginning, middle, and end of a packaging run and total packaged oxygen values were recorded simultaneously. After three months, only one case from the beginning, middle, and end of the packaging run were collected to reduce beer waste and to reduce sample-processing time.

4.2.2 Enzymatic Digestion of Beer Haze

Techniques for enzymatic degradation were adapted for purpose in the study (Steiner *et al.*, 2010). A full schematic of the experiment can be found in **Figure 4.1**. Enzymes were added according to the method described in Section 2.2.3 and incubated at the appropriate temperature for 18 hours (**Figure 4.1**).

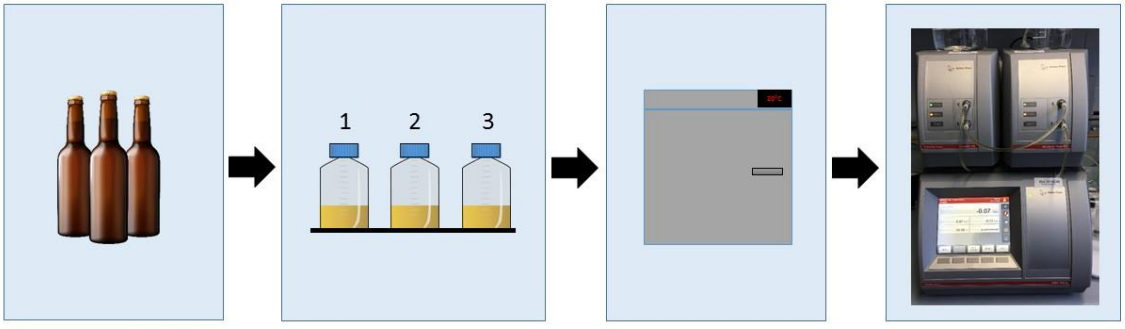


Figure 4.1. Schematic of steps required for haze degradation experiments.

Samples were removed from incubation and left to settle for one hour prior to analysis. A sample was carefully collected with a syringe, taking care not to draw up

any precipitate, from each digestion and a post digestion turbidity reading was recorded (Section 2.2.3).

4.2.3 Haze Characterisation- Microscopy

Haze microscopy techniques were adapted from (Steiner *et al.*, 2010). High haze and low haze beer samples were prepared according to the method described in Section 2.2.4 prior to microscopic analysis. Each dye was prepared according to **Table 2.1** with distilled water. Each slide was prepared immediately prior to analysis to ensure particles were properly stained and that non-selective staining did not occur.

4.2.4 Wet Chemical Analysis-Gallery™ Plus Beermaster Automated Photometric Analyser

Two 50 mL aliquots of beer were collected from each batch collected (Section 2.2.1) and stored at -20°C until wet-chemical analysis. A Gallery™ Plus Beermaster Automated Photometric Analyser (ThermoFisher Scientific, Perth, UK) was used for all protein, polyphenol and β-glucan measurements. All operating and calibration procedures are described in Section 2.2.5 and calibration curves are found in **Appendix A**.

Beer samples were thawed and pipetted into 1 mL sample cups and placed into a sample tray for analysis. The sample numbers and tests were logged in the paired Beermaster software and the concentrations of protein, polyphenol and high molecular weight (MW) polyphenols were measured (Section 2.2.5). Results were collected, collated, and analysed using SYSTAT statistical software (Systat Software Inc., Chicago, US) to create summary bar charts. RStudio (RCore Group, Vienna, Austria) was used to calculate probability values ($p< 0.5$) using Tukey's Post-Hoc Test (Section 2.1). The analysis of the protein, polyphenol, and β-glucan data was used to inform later analytical experiments.

4.2.5 Molecular Determination of the Origin of Sporadic Beer Haze

Upon initial analysis of wet-chemical and haze degradation data, proteins and β-glucan residues were suspected sources of sporadic beer haze. As cross-linked, polymerised barley proteins and β-glucans are easily removed with filtration techniques, it was hypothesised that yeast glucans and/or yeast cell wall proteins were a culprit of increased turbidity. Further investigation was conducted utilising LC/ESI-QTOF-MS analysis and a Megazyme© assay kit for D-mannose, D-fructose, and D-glucose

(Megazyme Ltd, Bray, IE) to determine if yeast cell wall components were contributing to the increased turbidity values that were observed (Section 2.2.8 and Section 2.2.12).

4.2.5.1 Intact Protein Precipitation

Proteins were precipitated from the samples reporting the highest haze values (12.57 EBC average) and the sample reporting the lowest haze values (0.51 EBC average) that were collected during the experiment. As target proteins were expected to be in low abundance, two precipitation methods were tested- according to Pink *et al.*, (2010) and Schulz *et al.*, (2018) were tested (Section 2.2.6). To assess if the protein precipitations were successful, a sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) was run and analysed in a light box (Section 2.2.7).

4.2.5.2 Protein Fractionation ÄKTA Avant Liquid-Chromatography System

An ÄKTA Avant Liquid Chromatography system (GE-Healthcare, Chicago, US) method was developed to fractionate beer proteins in high (12.57 EBC average) and low haze (0.51 EBC average) samples (Section 2.2.10).

Methods previously described in Chapter 2 (Section 2.2.10- **Experiment 1 and 2**) were assessed as to their suitability to fractionate proteins in beer samples. First, a test was carried out to determine if proteins could be separated by isoelectric point with a competitive salt elution (**Experiment 1- Table 2.4**). As the goal was to isolate proteins unique to high haze samples, high haze beer was utilised in method development for **Experiment 1 (Table 2.4).** A second optimised method used a NaCl step-elution in addition to a higher pH gradient to alter protein-binding affinity to the column to selectively wash out proteins (**Experiment 2- Table 2.4)**. Chromatographs were collected and assessed in real- time during method development. The optimised method (**Experiment 2- Table 2.4**) was used to fractionate proteins in high and low haze samples for LC/ESI-QTOF-MS (Section 2.2.9 and 2.2.10).

Following extraction via cation-exchange chromatography using the ÄKTA, a high concentration of salt was present in the protein fractions. Salt required removal as sodium corrodes the electrospray ionisation interface in LC/MS analysis. Dialysis, as described in Section 2.2.11, utilised the principles of osmosis to remove salt from the fractions collected.

4.2.5.3 LC/ESI-QTOF-MS

High and low haze protein fractions collected from the ÄKTA Avant Liquid Chromatography system (GE Healthcare, Chicago, US) (Section 2.2.10) in addition to high and low haze intact protein precipitations (Section 2.2.6) were analysed using LC/ESI-QTOF-MS (Section 2.2.8).

As mannoproteins were speculated to be unfilterable due to their small size, several structural mannoproteins and flocculation proteins were selected for targeted analysis based on the literature (**Table 4.1**) (Chlup, Conery and Stewart, 2007). The FASTA formatted sequences for the proteins, specific to *Saccharomyces cerevisiae* were loaded into the coupled LC/ESI-QTOF-MS software. By loading FASTA formatted sequences, the peptide fragments in samples could be matched to partial sequences of proteins suspected to be present in the samples (**Table 4.1**). Agilent Technologies Bioconform (Agilent Technologies, Edinburgh, UK) software was used for post-run analysis to compare percent sequence coverage of proteins obtained from the tryptic digests in high and low haze samples, respectively.

Table 4.1 - Selected mannoproteins and flocculation proteins assessed in LC-QTOF-MS.

Protein	Cellular Function
Cell Wall Mannoproteins	
Uth1	Involved in aging, oxidative stress response, and regulation of mitochondrial biogenesis[a] Anchored to the cell by disulphide bridge[a] Involved in remodelling of cell wall during culture development and stress/degradation responses[a]
Sim1	Cell wall remodelling during culture development[b,c]
Hpf1p	Haze protective mannoprotein[d]
Ecm33	Required for cell wall integrity and assembly of the mannoprotein outer layer of the cell wall[e]
Cwp1	Linked to β-1,3 and β-1,6-glucan through a phosphodiester bond[f] Does not require GPI anchor[f]
Cis3	Component of outer cell wall layer for cell wall stability and optimal growth[c]
Flocculation Proteins	
Flo1	Flocculation inhibited by mannose residues[g]
Flo5	Loss of gene FLO5, requires propagation culture to be replaced[g]
Flo9	Small flocculation protein[g]
Flo10	Small flocculation protein[g]
Flo11	Involved in filamentous, chain formation growth and flor forming than flocculation[g]

[a]Berner, Jacobsen and Arneborg, (2013), [b]Fasoli *et al.*, (2010), [c]Colgrave *et al.*, (2013), [d]Lewis and Poerwantaro, (1991), [e]Jung and Levin, (1999), [f]Omura and Nakao, (2009), [g]Stewart, (2018)

4.2.5.4 Determination of D-Mannose, D-Fructose, and D-Glucose

A Megazyme© assay kit for D-mannose, D-fructose, and D-glucose was used to measure the respective carbohydrates in high haze (12.57 EBC average) and low haze (0.51 EBC average) samples. The assays were run according to the manufacturer's guidelines (Section 2.2.12) and the values for D-mannose, D-fructose, and D-glucose

were calculated with the equation found in Section 2.2.12. All samples were run in triplicate.

4.3 Results

4.3.1 Enzymatic Digestion of Beer Haze

The purpose of the enzymatic digestion of beer haze was to assess if enzymes were able to degrade specific macromolecules in beer and to assess the change in turbidity pre and post-enzymatic digestion. Pepsin degrades proteins in beer, amyloglucosidase degrades dextrins and starches and Ultraflo® Max degrades β-glucans. Tables of pre and post-digestion averages and the probability that the difference of the pre and post-digestion values were significant (p<0.05) are found in **Table 4.2**, **Table 4.3** and **Table 4.4**. When analysing the data, the difference between pre and post-digestion and the analysis of variance (ANOVA) were calculated in RStudio (R Core Group, Vienna, Austria). In addition, summary bar charts were constructed using SYSTAT (Systat Software Inc., Chicago, US) to visually display differences in digestions (**Figure 4.4, Figure 4.5, Figure 4.6**). In all cases, the ANOVA p-values comparing normal to control samples were not significant (p>0.05) (**Table 4.5** and **Appendix D.1**). All other cases were significant (p<0.05) (**Table 4.5**).

A paired t-test was used to assess pre and post digestion values in the high, normal, and control samples (R Core Group, Vienna, Austria). All pre and post digestion differences were significant (p<0.05), with the exception of Ultraflo® Max at the 25° angle of incidence in the control samples (**Table 4.2**, **Table 4.3**, **Table 4.4**, and **Appendix E**). Ultraflo® Max digestion at the 25° light angle in the control samples was the only case where pre and post digestion values were not significantly different from one another reporting a p-value of 0.0924 (**Table 4.4**).

Table 4.2- Averages, standard deviation (S.D.), and *p*-values obtained from a paired t-test of normal/low haze pre and post digestion values (n=7 brews). The *p*-values determine if significant differences exist between pre and post digestion values of amyloglucosidase, pepsin, and Ultraflo®Max at 25° and 90° light angles from which EBC units were calculated from.

Haze Measurement	Pre-Digestion[a]	S.D.[b]	Post-Digestion[a]	S.D.[b]	P-value
EBC					
Amyloglucosidase	1.38	±0.70	0.92	±0.59	<0.0001
Pepsin	1.38	±0.70	0.67	±0.34	<0.0001
Ultraflo® Max	1.38	±0.70	1.09	±0.77	<0.0001
25°					
Amyloglucosidase	6.79	±3.18	4.55	±2.44	<0.0001
Pepsin	6.79	±3.18	3.23	±2.52	<0.0001
Ultraflo® Max	6.79	±3.18	4.56	±2.45	<0.0001
90°					
Amyloglucosidase	5.37	±2.74	3.59	±2.24	<0.0001
Pepsin	5.37	±2.74	2.69	±1.56	<0.0001
Ultraflo® Max	5.37	±2.74	4.03	±2.68	<0.0001

[a]Pre and post digestion values are average values for the data collected.
[b]Standard deviation

Table 4.3 - Averages, standard deviation (S.D.), and *p*-values obtained from a paired t-test of high haze pre and post digestion values (n=7 brews). The *p*-values determine if significant differences exist between pre and post digestion values of amyloglucosidase, pepsin, and Ultraflo®Max at 25° and 90° light angles from which EBC units were calculated from.

Haze Measurement	Pre-Digestion[a]	S.D.[b]	Post-Digestion[a]	S.D.[b]	*P*-value
EBC					
Amyloglucosidase	8.69	±2.39	5.59	±2.47	<0.0001
Pepsin	8.69	±2.39	5.00	±1.24	<0.0001
Ultraflo® Max	8.69	±2.39	5.38	±2.41	<0.0001
25°					
Amyloglucosidase	35.61	±10.13	2.70	±8.41	<0.0001
Pepsin	35.61	±10.13	8.24	±4.12	<0.0001
Ultraflo® Max	35.61	±10.13	10.39	±8.67	<0.0001
90°					
Amyloglucosidase	33.86	±9.21	5.61	±9.51	<0.0001
Pepsin	33.86	±9.21	18.79	±4.76	<0.0001
Ultraflo® Max	33.86	±9.21	21.53	±9.20	<0.0001

[a]Pre and post digestion values are average values for the data collected.
[b]Standard deviation

Table 4.4- Averages, standard deviation (S.D.), and *p*-values obtained from a paired t-test of control haze pre and post digestion values (n=7 brews). The *p*-values determine if significant differences exist between pre and post digestion values of amyloglucosidase, pepsin, and Ultraflo®Max at 25° and 90° light angles from which EBC units were calculated from.

Haze Measurement	Pre-Digestion[a]	S.D.[b]	Post-Digestion[a]	S.D.[b]	*P*-value
EBC					
Amyloglucosidase	0.21	±0.07	0.12	±0.07	<0.0001
Pepsin	0.21	±0.07	0.10	±0.03	<0.0001
Ultraflo® Max	0.21	±0.07	0.13	±0.07	<0.0001
25°					
Amyloglucosidase	0.95	±0.91	0.69	±0.62	0.0015
Pepsin	0.95	±0.91	0.61	±0.52	0.0043
Ultraflo® Max	0.95	±0.91	0.76	±0.72	0.0924
90°					
Amyloglucosidase	0.81	±0.26	0.42	±0.24	<0.0001
Pepsin	0.81	±0.26	0.39	±0.12	<0.0001
Ultraflo® Max	0.81	±0.26	0.52	±0.27	<0.0001

[a]Pre and post digestion values are average values for the data collected.
[b]Standard deviation

Table 4.5 - ANOVA of the differences in turbidity values, post-digestion, of high, normal, and control beer samples after each enzyme treatment at each angle of incidence measured.

Amyloglucosidase	*p*-value	Pepsin	*p*-value	Ultraflo® Max	*p*-value
EBC		**EBC**		**EBC**	
High-Control	<0.0001*	High-Control	<0.0001*	High-Control	<0.0001*
Normal-Control	0.464	Normal-Control	0.733	Normal-Control	0.858
Normal-High	<0.0001*	Normal-High	<0.0001*	Normal-High	<0.0001*
25°		**25°**		**25°**	
High-Control	<0.0001*	High-Control	<0.0001*	High-Control	<0.0001*
Normal-Control	0.464	Normal-Control	0.125	Normal-Control	0.570
Normal-High	<0.0001*	Normal-High	<0.0001*	Normal-High	<0.0001*
90°		**90°**		**90°**	
High-Control	<0.0001*	High-Control	<0.0001*	High-Control	<0.0001*
Normal-Control	0.734	Normal-Control	0.263	Normal-Control	0.875
Normal-High	<0.0001*	Normal-High	<0.0001*	Normal-High	<0.0001*

*Indicates a significant value

Overall, pepsin was the only enzyme to digest colloid particles to a value below 5.0 EBC (**Figure 4.2, Figure 4.3, Figure 4.4**). When assessing pre and post digestion values, all cases (EBC, 25° angle (NTU), and 90° angle (NTU) utilising the enzyme pepsin were significant *and* showed the greatest difference, pre-post digestion.

Ultraflo® Max did not digest β-glucans below 5.0 EBC/20 NTU qualifier to classify these samples as low haze (**Figure 4.2-Figure 4.4**). Finally, digestion with amylglucosidase did not have a significant impact on the digestion of dextrins or starch residues and did not digest particulates in the beer to levels below 5.0 EBC/20 NTU (**Figure 4.2, Figure 4.3, and Figure 4.4**).

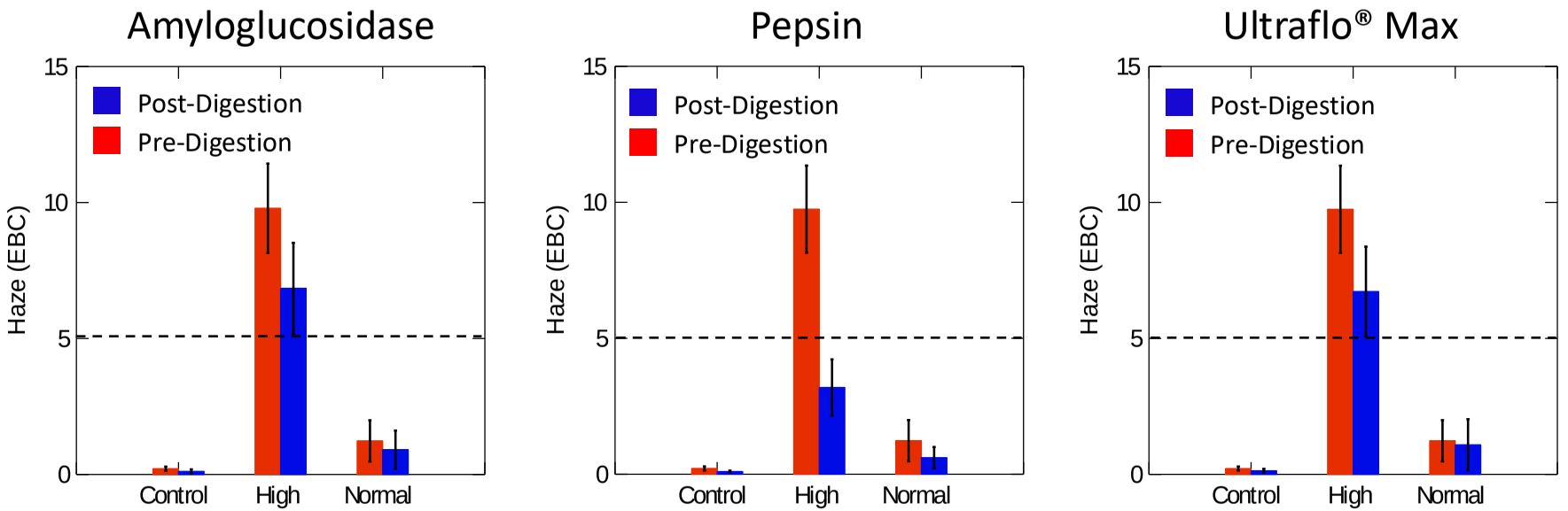


Figure 4.2 - Comparison of pre & post digestion values of amyloglucosidase, pepsin, and Ultraflo® Max in control, normal, and high haze beer utilising EBC units. The dotted line represents the value at which samples were classified as high haze. Normal haze samples contained n=7 brews, high haze samples contained n=5 brews, and control samples contained n=2 brews. Error bars indicate standard deviation.

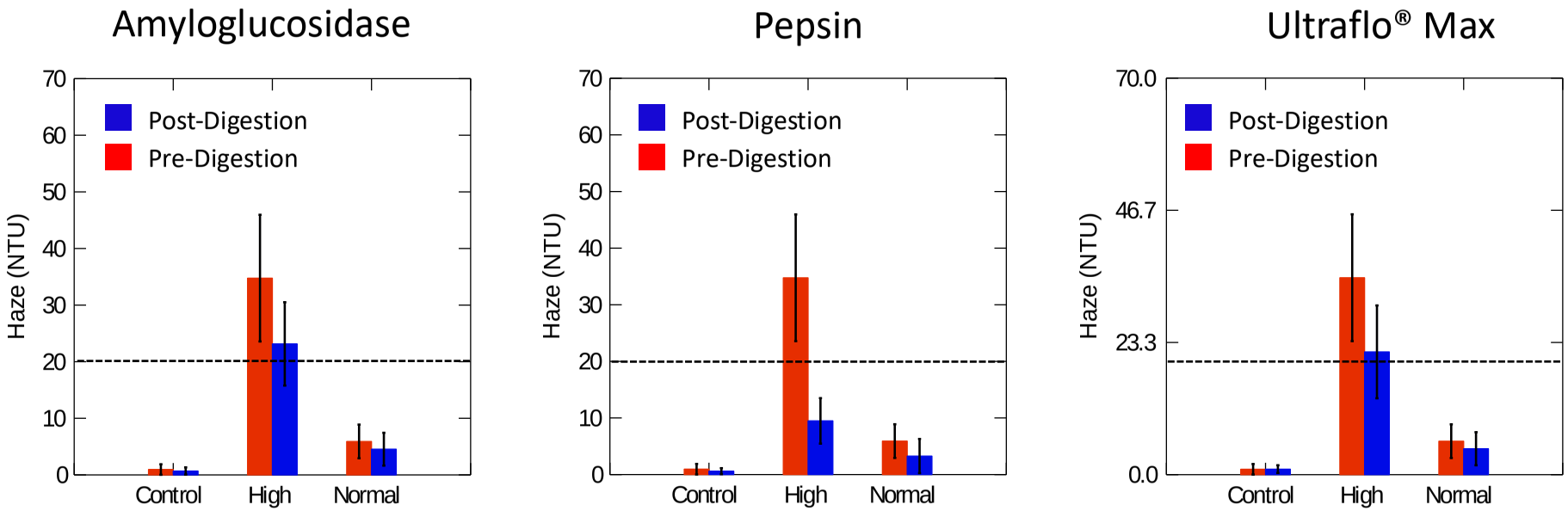


Figure 4.3 - Comparison of pre & post digestion values of amyloglucosidase, pepsin, and Ultraflo® Max in control, normal and high haze beer measured at a 25° angle compared to the light axis. The dotted line represents the value at which samples were classified as high haze. Normal haze samples contained n=7 brews, high haze samples contained n=5 brews, and control samples contained n=2 brews. Error bars indicate standard deviation.

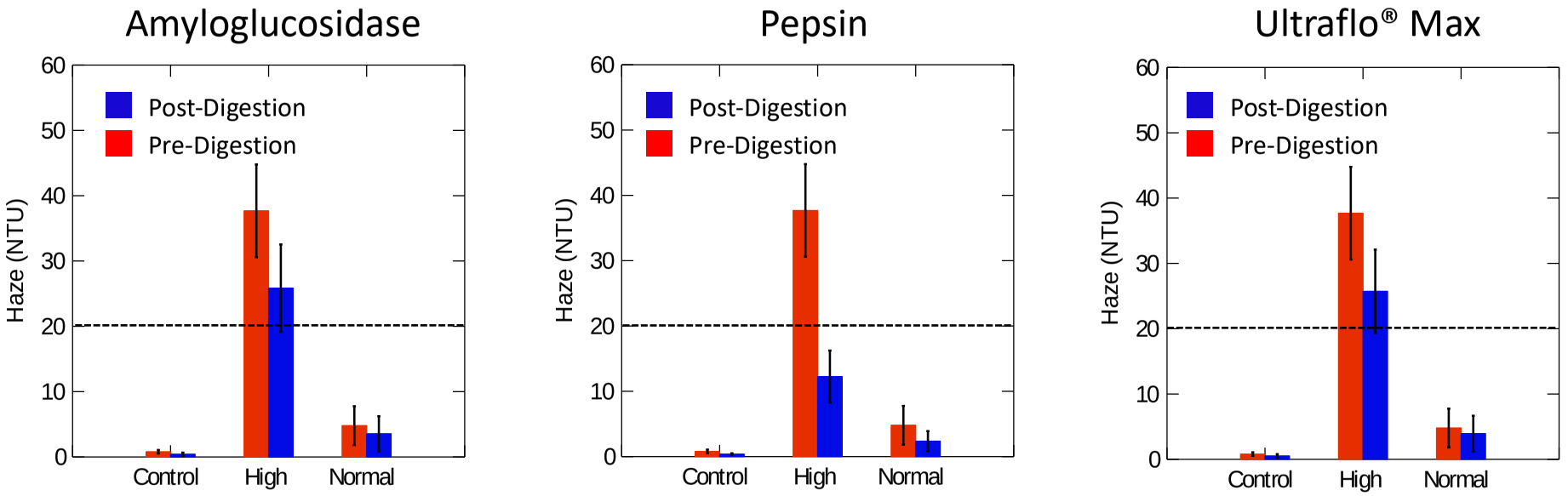


Figure 4.4- Comparison of pre & post digestion values of amyloglucosidase, pepsin, and Ultraflo® Max in control, normal, and high haze beer measured at a 90° angle compared to the light axis. The dotted line represents the value at which samples were classified as high haze. Normal haze samples contained n=7 brews, high haze samples contained n=5 brews, and control samples contained n=2 brews. Error bars indicate standard deviation.

4.3.2 Haze Characterisation- Microscopy

Observational differences were recorded when microscopically assessing high and low haze samples. The particles were successfully stained with the methods described (Section 2.1.4). In all cases, high haze samples showed large flocs of particulate matter while low/normal haze samples showed smaller, but more particulate matter.

In **Figure 4.5(A)**, the high haze beer sample contained larger flocs of proteinaceous haze material. Dark spots within the samples show negatively charged particles adsorbing to positively charged protein groups by electrostatic adsorption- accounting for the differences in colour in the sample (Glenister, 1975). The lightly stained particles indicate a low level of protein present in the sample (Skinner *et al.*, 1993). The low haze sample contained some of these flocs, but fewer than high haze samples **(Figure 4.5 (B)).** Large aggregates were observed in the high haze sample (**Figure 4.5 (A))** and small aggregates were observed in the low haze sample, but an increased amount of fine particulate matter was observed **(Figure 4.5 (B)).**

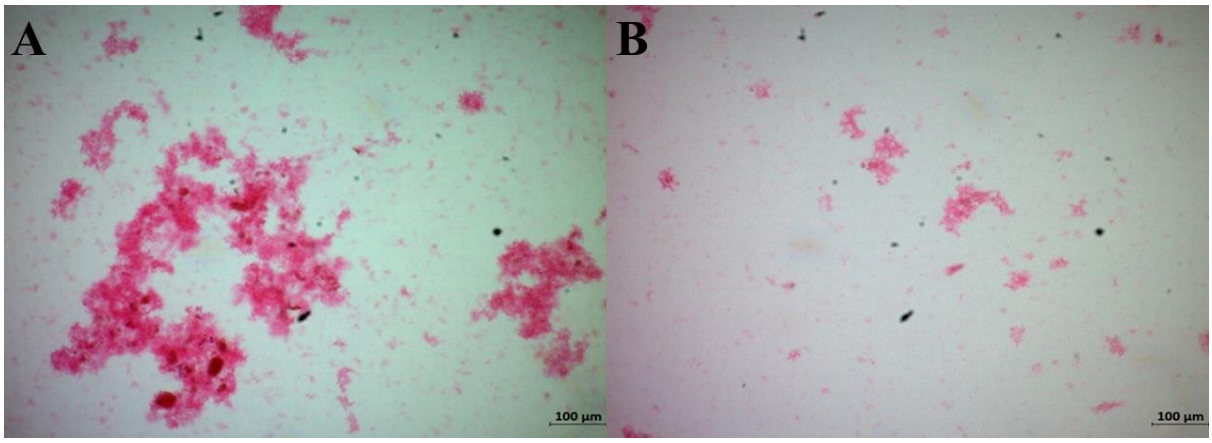


Figure 4.5 (A) Eosin Yellow, high haze, brightfield, 100x magnification and (B) Eosin Yellow, normal haze, brightfield, 100x magnification.

Polyphenols and tannins were selectively stained with methylene blue are observed in **Figure 4.6**. The high haze sample saw large stained polyphenol particles but less small polyphenolic and tannic material (**Figure 4.6(A)** and **Figure 4.6(B)).** As previously observed, fine particulates were observed in low haze samples, but not observed in high haze samples (**Figure 4.6**).

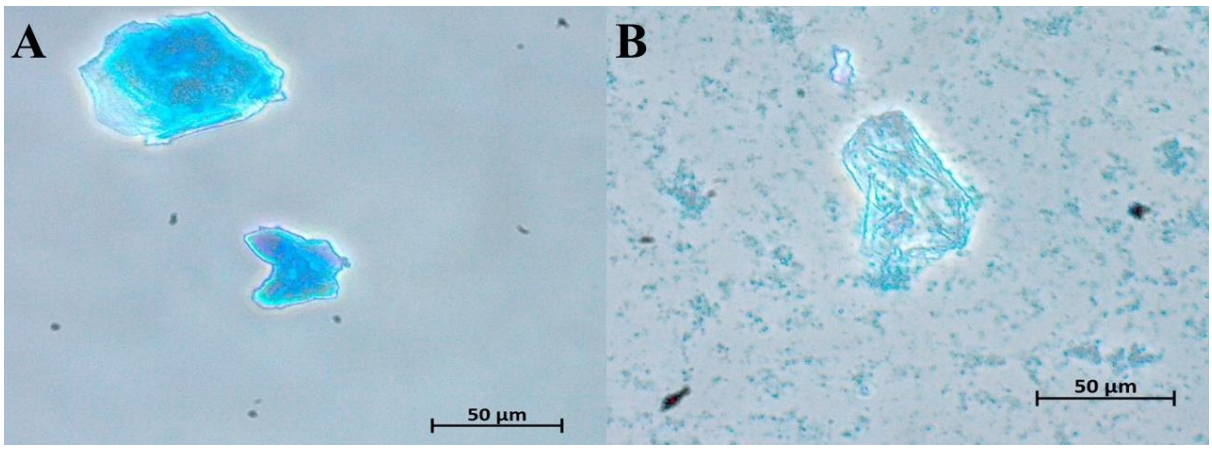


Figure 4.6 (A) Methylene Blue, high haze, phase contrast, 400x magnification and (B) Methylene Blue, low haze, phase contrast, 400x magnification.

Thionin stain targeted the presence of dextrins and starch particles in the high and low haze samples. The black staining in **Figure 4.7(A)** was speculated to be carbohydrate material (**Table 2.1**). The purple stained particles in both samples was hypothesised to be neutral polysaccharides as these particles typically stain a violet colour (Glenister, 1975) (Section 2.1.4). When comparing samples, the low haze sample contained smaller, but more dextrin/starch material than the high haze sample (**Figure 4.7(A) and Figure 4.7(B)).** Unsurprisingly, as previously determined, fine particulates were observed in low haze samples that were not present in high haze samples (**Figure 4.7).**

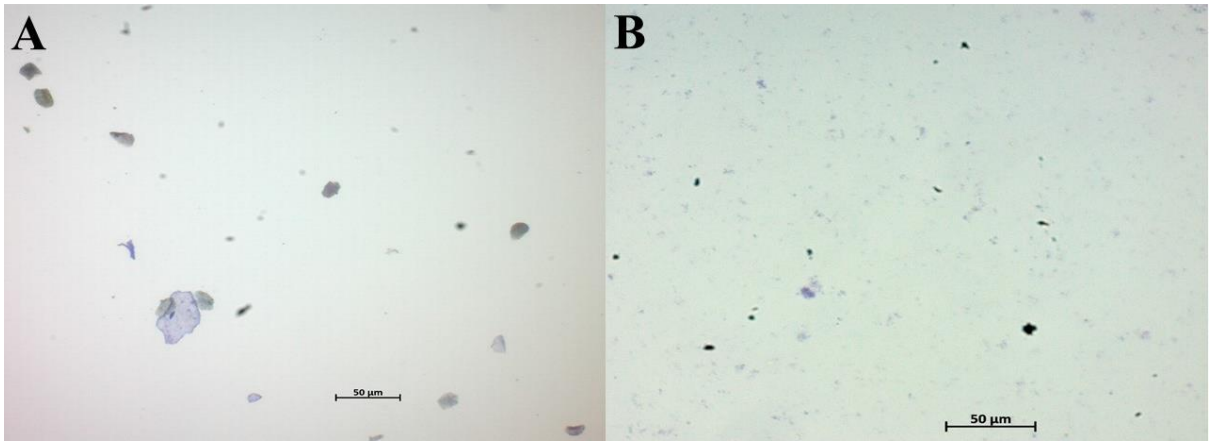


Figure 4.7. (A) Thionin, high haze, brightfield, 400x magnification and (B) normal haze, brightfield, 400x magnification.

The Congo Red dye did not stain calcium oxalate crystals but did stain some particulate matter, potentially β-glucan. The aggregates stained in the high haze samples were darker and more condensed than the low haze samples (**Figure 4.8**). The darker stained material in sample A indicated a higher β-glucan concentration than the lightly

stained particulate matter in sample B (**Figure 4.8**). High levels of small particulate matter were observed in low haze samples but not observed in high haze samples.

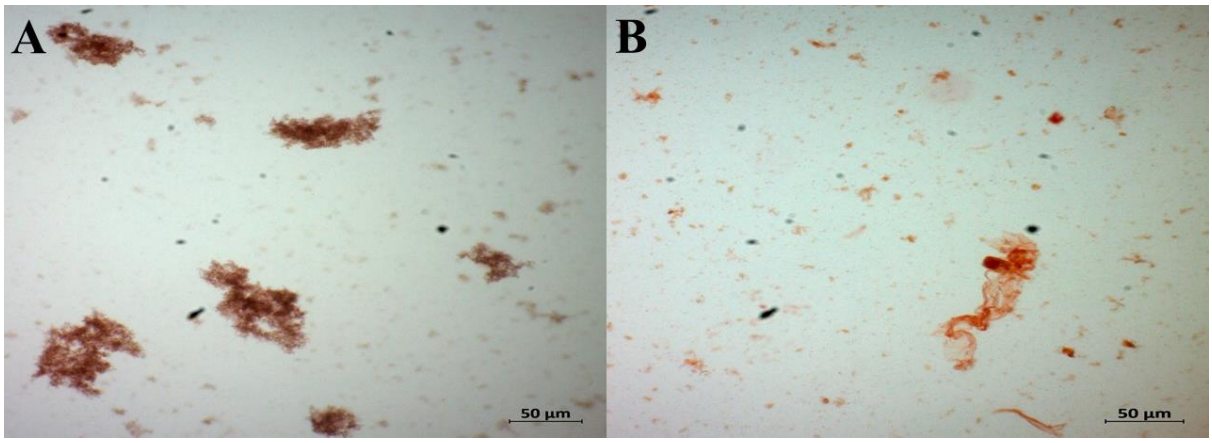


Figure 4.8 – (A) Congo Red, high haze, brightfield, 400x magnification and (B) normal haze, brightfield, 400x magnification.

Finally, the samples stained with iodine and the unstained beer particles did not show any stained starchy particles or hop oil, respectively and are not presented, as there were no stained particles of interest (**Table 2.1)**.

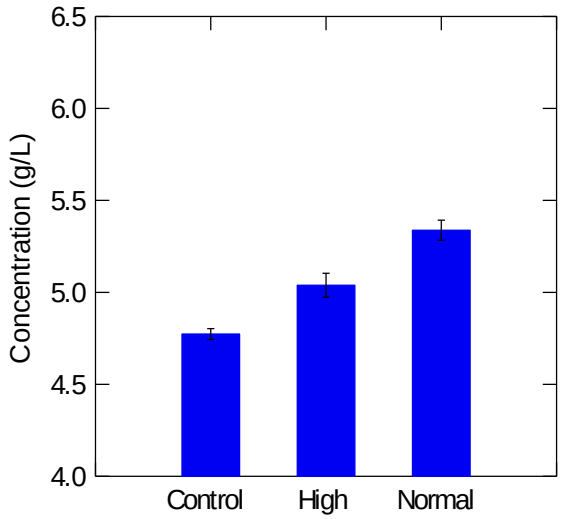
4.3.3 Wet-Chemical Analysis

Summary bar charts were created from replicate values of the Beermaster data utilising SYSTAT (Systat software Inc., Chicago, US) and P-values were calculated in RStudio to assess significant differences between samples (R Core Group, Vienna, Austria). The polyphenol and protein concentrations were not significantly different in control and high haze samples with control values at 476.87 mg/L and 4.77 mg/L and high haze values at 500.23 mg/L and 5.04 mg/L, respectively (**Table 4.6**, **Figure 4.9** and **Figure 4.10**). Interestingly, all normal and control sample values, were significantly different (**Figure 4.9**, **Figure 4.10, and Figure 4.11**). The β-glucan, polyphenol, and protein concentrations for normal haze samples were 28.18 mg/L, 524.40 mg/L, and 5.34 g/L, respectively while the control values were 81.97 mg/L, 476.87 mg/L, and 4.77 g/L, respectively (**Table 4.6**). Excluding the control samples, high molecular weight β-glucan was the only macromolecule recorded at higher concentrations in high haze beer (34.73 mg/L) than in low haze beer (28.18 mg/L) (**Table 4.6** and **Figure 4.11**). The high haze samples reported lower values of polyphenol and protein at 500.23 mg/L and 5.04 g/L, than the low haze samples at 524.40 mg/L and 5.34 g/L, respectively (**Table 4.6**). It was suspected that results of the wet chemical tests may not have been representative of the actual concentrations of proteins and polyphenols as the materials

may have formed complexes. Therefore, the compounds were not detectable at their true concentration in the assays.

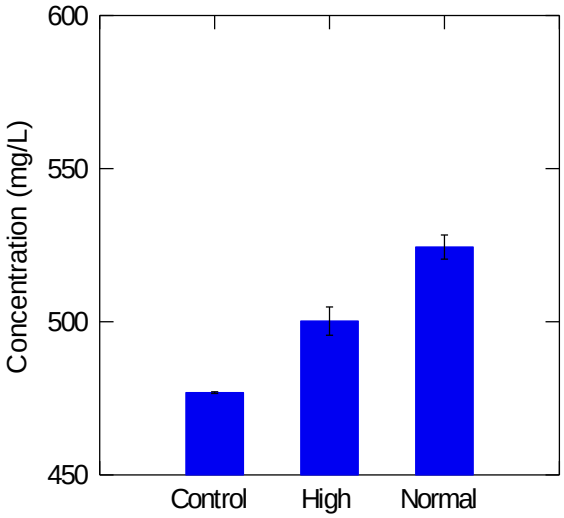
Table 4.6- Average values of high molecular weight β-glucan, total polyphenol, and total protein in normal, high, and control samples with corresponding standard deviations (S.D.).

Macromolecule	Normal	S.D.	High	S.D.	Control	S.D.
High Molecular Weight β-glucan (mg/L)	28.18	±14.19	34.73	±3.151	81.97	±7.773
Total Polyphenol (mg/L)	524.40	±35.98	500.23	±41.44	476.87	±1.345
Total Protein (g/L)	5.34	±0.498	5.04	±0.530	4.77	±0.144



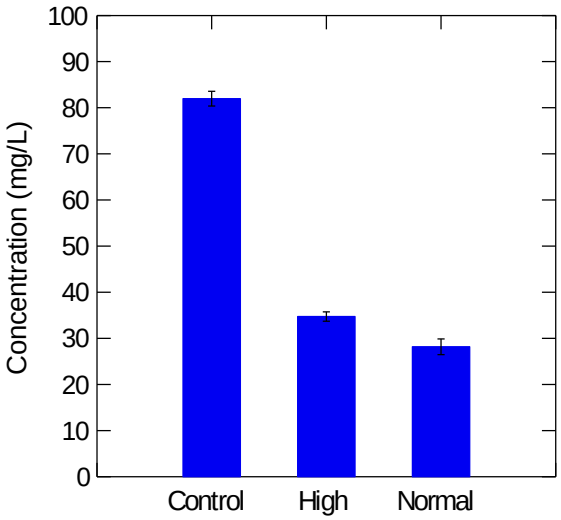
Sample	p-value
High/Control	0.054
Normal/Control	0.000
Normal/High	0.000

Figure 4.9. Concentration of total proteins in control, high, and normal haze samples with corresponding p-values calculated by Tukey's Significant Different Test. Normal haze samples contained n=7 brews, high haze samples contained n=5 brews, and control samples contained n=2 brews. Error bars indicate standard deviation.



Sample	p-value
High/Control	0.012
Normal/Control	0.000
Normal/High	0.000

Figure 4.10. Concentration of total polyphenols in control, high, and normal haze samples with corresponding p-values Tukey's Significant Different Test. Normal haze samples contained n=7 brews, high haze samples contained n=5 brews, and control samples contained n=2 brews. Error bars indicate standard deviation.



Sample	p-value
High/Control	0.000
Normal/Control	0.000
Normal/High	0.002

Figure 4.11. Concentration of high molecular weight β-glucans in control, high, and normal haze samples with corresponding p-values Tukey's Significant Different Test. Normal haze samples contained n=7 brews, high haze samples contained n=5 brews, and control samples contained n=2 brews. Error bars indicate standard deviation.

4.3.4 Molecular Determination of the Origin of Sporadic Beer Haze

4.3.4.1 Protein Purification and Fractionation with ÄKTA Avant Liquid Chromatography

Elution methods were optimized to elute beer proteins on the ÄKTA Avant Liquid Chromatography system. Gradient and step-elution methods were tested and the step elution method was selected to purify, concentrate and isolate proteins in high and low haze samples (**Figure 4.12**, **Figure 4.13** and **Figure 4.14**) (Section 2.2.9 and Section 2.2.10).

The gradient method was successful in determining the optimum point at which the proteins of interest eluted and if differences in protein content between high and low haze samples existed (**Figure 4.12**). Two distinct peaks were observed in the high haze samples utilising the gradient method. This the method was used to fractionate proteins in high and low haze samples (**Figure 4.13**). To increase peak signal, a step elution from 30% NaCl to 100% NaCl with a higher pH range (4.0-5.8) were used in high and low haze samples. The liquid chromatography techniques showed that different proteins existed in high and low haze beers, proving the hypothesis to be partially correct (**Figure 4.13** and **Figure 4.14**). The protein fractions were collected, desalted with dialysis, and utilised in LC-QTOF-MS to specifically identify the proteins present (or absent) in each sample.

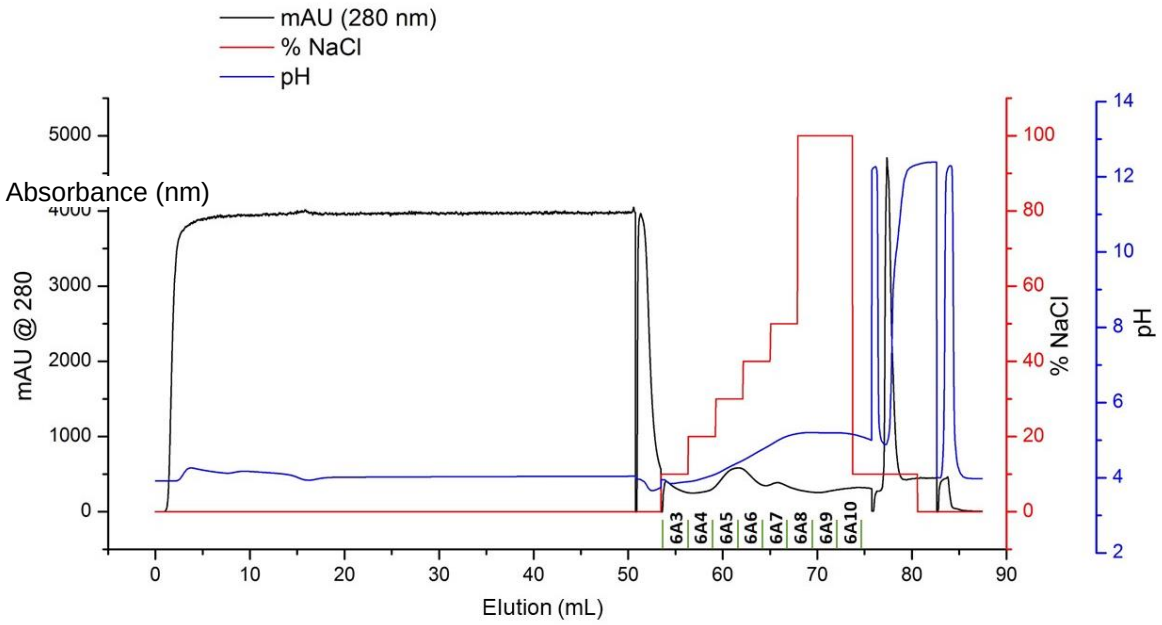


Figure 4.12- Chromatogram of high haze beer protein fractionation by gradient utilising an ÄKTA Avant Liquid Chromatography System (GE Healthcare, Chicago, US). The absorbance of proteins was measured at 280 nm (black line) and is reported on the primary vertical axis. Sodium chloride concentration (red line) and pH (blue line) were measured and are reported on the secondary axes. Fractions were labelled (6A3-6A10) as the fractions eluted.

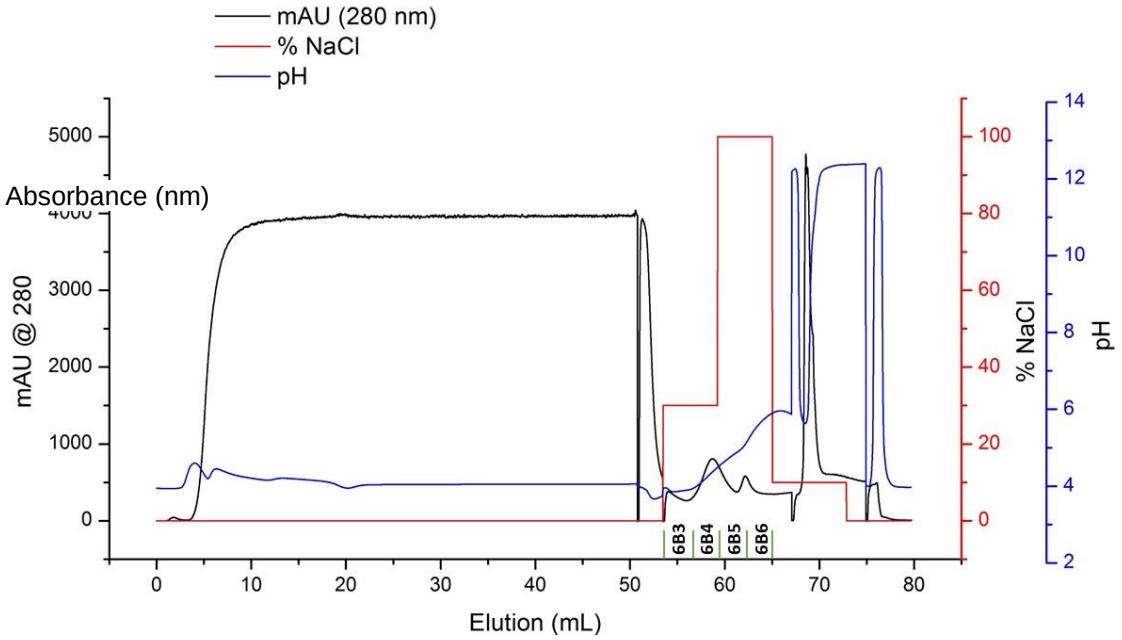


Figure 4.13- Chromatogram of high haze beer protein fractionation by step elution utilising an ÄKTA Avant Liquid Chromatography System (GE Healthcare, Chicago, US). The absorbance of proteins was measured at 280 nm (black line) and is reported on the primary vertical axis. Sodium chloride concentration (red line) and pH (blue line) were measured and are reported on the secondary axes. Fractions were labelled (6B3-6B6) as the fractions eluted.

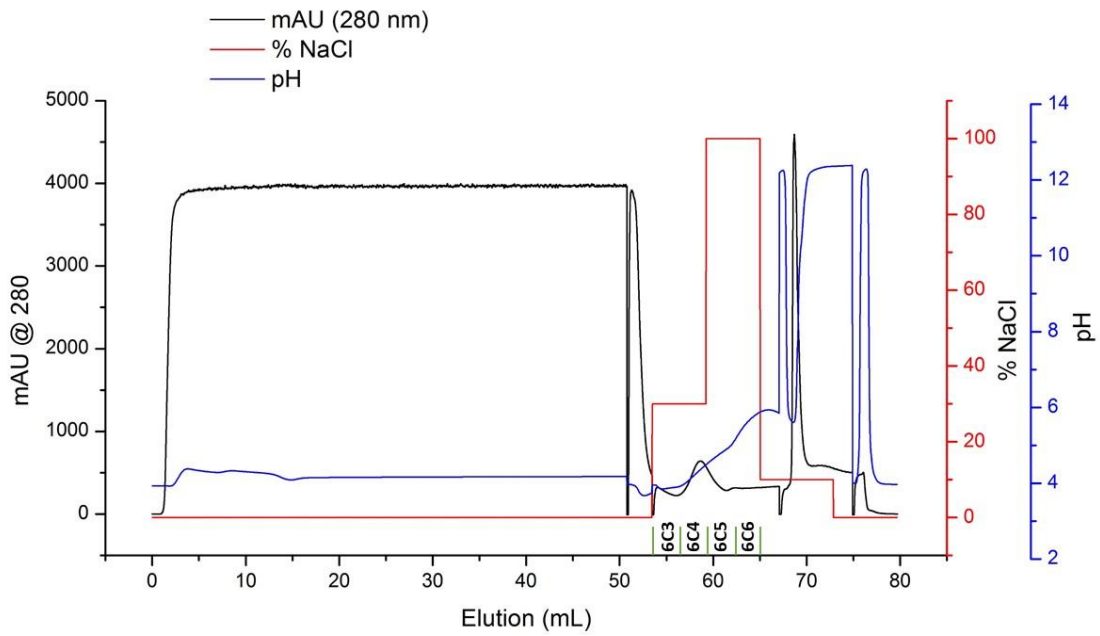


Figure 4.14- Chromatogram of low haze beer protein fractionation by step elution utilising an ÄKTA Avant Liquid Chromatography System (GE Healthcare, Chicago, US). The absorbance was measured at 280 nm (black line) and is reported on the primary vertical axis. Sodium chloride concentration (red line) and pH (blue line) were measured and are reported on the secondary axes. Fractions were labelled (6C3-6C6) as the fractions eluted.

4.3.4.2 Intact Protein Precipitation

In testing the two protein precipitation methods, the method described by Schulz *et al.*, (2018) (Section 2.2.6) was selected for precipitation as more protein bands were observed on the polyacrylamide gel (**Figure 4.15**).

The smearing of the bands in **Figure 4.15** was likely due to polyphenolic/tannic material present in the beer as polyphenol levels in all samples were high (**Figure 4.10**). To aid in the removal of polyphenols, further purify, isolate and concentrate the proteins, an ÄKTA Avant Liquid Chromatography system was used (GE-Healthcare, Chicago, US) as previously described in Section 4.3.1. Dialysis was used to remove salt from the protein fractions prior to SDS-PAGE analysis (Section 2.2.11).

The SDS-PAGE gel showed proteins in low abundance with faint bands. The normal/low haze fractions (wells 1 and 2) did not show a great difference from high haze samples (**Figure 4.16**). If the gel was over-exposed, very faint bands were observed in the high haze sample at approximately 25 kDa (**Figure 4.17**). The fraction 6B6 (wells 7 and 8) only showed a faint band, observed at approximately 37 kDa. However, these bands were observed in all of the samples (**Figure 4.16**). The faint

bands observed indicated that protein concentration in the samples was low. Due to the faint bands in the high haze samples and the low concentration of proteins in the samples, LC/ESI-QTOF-MS was required to identify proteins and protein differences in high and low/normal haze samples.

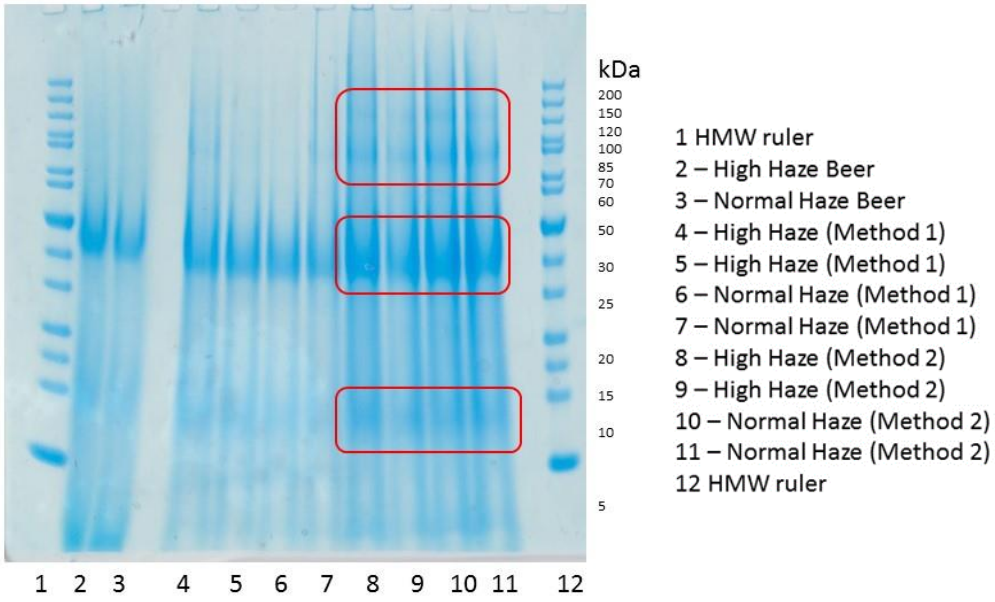


Figure 4.15- SDS-PAGE gel of proteins in high and low haze beer precipitated by two methods. HMW= high molecular weight, *Method 1= Pink et al., (2010), and Method 2= Schulz et al., (2018).

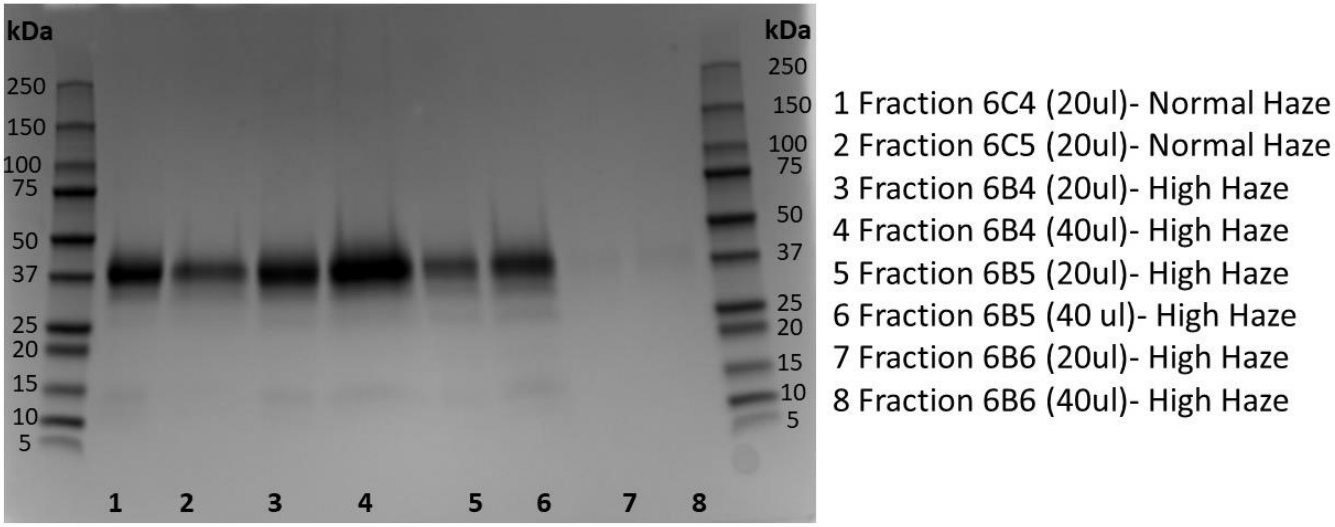


Figure 4.16- SDS-PAGE gel comparing high and low haze beer samples post purification, fractionation, and dialysis. To see specific fractions, see **Figure 4.13** and **Figure 4.14**.

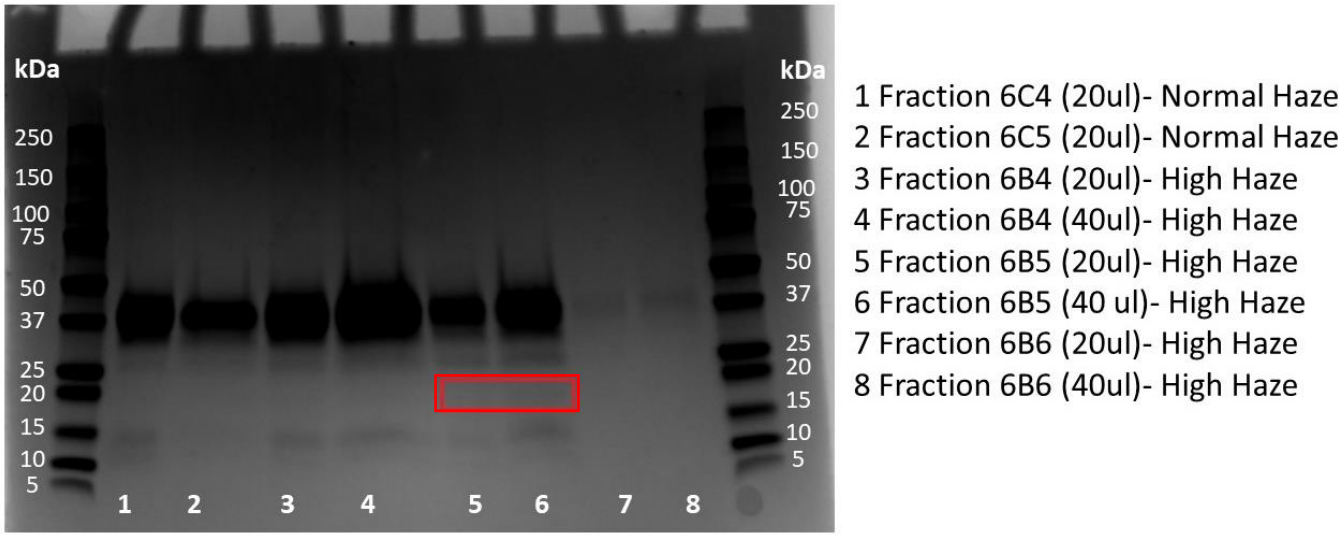


Figure 4.17- Over-exposure of SDS-PAGE gel comparing high and low haze beer samples post purification, fractionation, and dialysis. Faint bands observed in the high haze fractions are highlighted with a red focus box.

4.3.4.3 Liquid Chromatography-Quadrupole Time of Flight- Mass Spectroscopy (LC-QTOF-MS)

Intact protein digests and fractionated protein samples were analysed by liquid chromatography/electrospray ionisation-quadrupole time of flight-mass spectrometer (LC/ESI-QTOF-MS). The peptide hits and percent sequence coverage was collected and assessed in post-run analysis. The data was presented as percent sequence coverage by the software. Percent sequence coverage refers to the number of amino acid residues in all identified peptides divided by the number of amino acid residues in the corresponding FASTA sequence. The greater the percent sequence coverage, the greater chance of the presence of the protein in the sample. An example of a high haze ion chromatogram and a low haze ion chromatogram can be found in **Appendix F.**

As **Table 4.7** and **Table 4.8** indicate, there was greater percent sequence coverage of mannoprotiens but larger differences in flocculation proteins. Protein Ecm33 and Uth1 contain relatively high sequence coverage for spectral data at 76.92% and 38.02% sequence coverage, respectively (**Table 4.7**). Despite the low sequence coverage, protein Flo9 was observed in all high haze samples with an average sequence coverage of 5.3% and was absent in the low haze samples analysed (**Table 4.8**). The difference of protein Flo1 (>1000%) may have been an indication of the release of flocculation proteins related to a cell wall stress response (**Table 4.8**). In general, the high haze samples contained greater protein concentrations than low haze samples (**Table 4.7 and Table 4.8**). The evidence suggests that cell wall proteins may have impacted turbidity levels in high haze beer.

Table 4.7- Percent sequence coverage of cell wall mannoproteins in high and low haze samples obtained from LC-QTOF-MS analysis.

Protein	Accession Number (UniProt)	High Haze (% coverage)	Low Haze (% coverage)	% Difference
Uth1	B3LRC	38.02	26.52	43.4
Sim1	P40472	18.07	6.93	160.8
Hpf1p	A0A0L8VIV9	10.29	8.48	21.3
Ecm33	P38248	76.92	35.9	114.3
Cwp1	YKL09	13.81	18.41	25.0
Cis3	B5VL27	21.59	14.54	48.5

Table 4.8- Percent sequence coverage of targeted flocculation proteins in high and low haze samples obtained from LC-QTOF-MS analysis.

Protein	Accession Number (UniProt)	High Haze (% coverage)	Low Haze (% coverage)	% Difference
Flo1	P32768	13.6	0.91	1394.5
Flo5	P38894	9.21	2.6	254.2
Flo9	P39712	5.3	absent	100
Flo10	P36170	9.5	5.05	88.1
Flo11	P08640	4.97	4.39	13.2

4.3.4.4 Determination of D-Mannose, D-Fructose, and D-Glucose

The D-glucose concentration of the high haze and low haze samples were comparably close to each other in values with an average difference of 0.02 g/L (**Table 4.9**). However, D-fructose and D-mannose concentrations in high haze beer samples were 1.22 g/L, nearly three times the concentration of 0.54 g/L noted in the low haze samples (**Table 4.9**). As the samples were the same colour, colour interference did not have an effect on the assay. The average concentration of D-mannose in the high haze sample (1.22 g/L) was significantly (p<0.05) different from the concentration of D-mannose in the low haze samples at 0.54 g/L (**Table 4.9**).

Table 4.9- Calculated concentrations of D-glucose, fructose and mannose concentration in low and high haze beer samples. Samples were run in triplicate and the average value and standard deviation are reported.

Carbohydrate	Low Haze (g/L)	S.D.	High Haze (g/L)	S.D.
D-Glucose	0.43	±0.00	0.45	±0.00
D-Fructose	0.54	±0.00	1.22	±0.00
D-Mannose	0.54	±0.00	1.22	±0.01

4.4 Discussion

4.4.1 Enzymatic Digestion of Beer Haze

Diagnostic studies were useful in the determination of sporadic beer haze. Differing angles of incidence (NTU), as well as EBC values, were considered when analysing the pre and post-digestion results. Assessing these values provided information on colloid particle size, which was useful in speculating what was present in the samples. A guide on the cross comparison of EBC haze units and nephelometric turbidity units (NTU) or formazin turbidity units (FTU) as it was formerly called, is discussed in Briggs *et al.* (2004). Measuring particle sizes at a 90° angle to the light axis measures small particles such as proteins and carbohydrates. Measuring particle sizes at a 25° angle to the light axis measures larger particles in beer such as yeast cells and inorganic material (Gales, 2000). Protein particles in beer tend to be quite small as complexes of protein and polyphenol tend to range from 0.1-1.0 µm in diameter (Bamforth, 1999). However carbohydrate residues, such as high molecular weight β-glucan (31-433 kDa) are reported to be even smaller as sizes range from 0.01-0.1 µm in diameter (Jin, 2002).

Overall, pepsin was the only enzyme to digest the colloid particles to a value below the 5.0 EBC/20 NTU accepted turbidity values (**Figure 4.4**). When assessing pre and post digestion values, all cases (EBC, 25° angle (NTU), and 90° angle (NTU)) utilising the enzyme pepsin were significant ($p<0.05$) and showed the greatest difference, pre-post digestion.

Only one-third of proteins survive the brewing process into final product beer as most proteins are lost in boiling, fermentation, and filtration processes (Steiner *et al.*, 2011). Beer contains approximately 500 mg/L of total protein and only 2 mg/L is required to form hazes in beer (Steiner *et al.*, 2010). The significant ($p< 0.0001$) drop in turbidity with the addition of pepsin indicates that proteins in the high haze samples

were at a higher concentration than beer proteins present in low haze samples. The drop also indicates that proteins were, most likely, causing the increased turbidity. It is possible that some proteinaceous particles were far less than 1.0 µm in diameter. If this were the case, these particles could have easily passed through a 0.45 µm filter sheet. The partner brewery utilised 0.45 µm pore size filter sheets as part of normal beer packaging processes. Therefore, it was proposed that β-glucans could have also contributed to the increased turbidity values.

Beta-glucan turbidity values, post-digestion via Ultraflo® Max at the 25° light angle were close to the 20 NTU 'low/normal haze' acceptance limit. Additionally, while the difference of pre and post digestion values via amyloglucosidase were significant (p<0.05), the difference in overall values pre and post digestion, were not as great as pepsin. Despite residual starches and β-glucan's decreased likelihood of contributing to sporadic increased turbidity, these components may have contributed slightly the turbidity issue. The contributions could be malt-derived as undermodified malts increase β-glucan levels or it is possible that malt α-amylase activity was insufficient at degrading fine starch particles (Steiner *et al.*, 2010; Ye *et al.*, 2016). However, if these speculations are correct, the variations would be so minor that they would, most likely, large deviations in friability or iodine tests would not be observed.

After reviewing all of the enzymatic digestion results, it was hypothesised that proteins contributed to sporadic beer turbidity.

4.4.2 Haze Characterisation- Microscopy

Haze microscopy was used to visually inspect and identify discernible differences between high and low haze samples. In all samples, a greater amount of particulate matter was observed in low haze samples but all particle sizes were very small (<50 µm). The high haze samples contained larger, darker stained particles, but did not show a great quantity of small particulate matter (**Figure 4.5 to Figure 4.8**).

In **Figure 4.5 (A)**, Eosin Yellow was used to stain proteinaceous material. The high haze sample stained a brighter and darker pink, indicating that a dense concentration of proteinaceous material was present. The high haze sample also contained material that stained a dark orange colour. Particles stained in the low haze sample were a very light pink colour and did not contain the thick, dark orange-stained spots that the high haze samples contained (**Figure 4.5 (B))**. The difference in colour

indicates that additional proteinaceous material was present in high haze samples. Proteolysis during the wort-boiling steps could have been deficient in high haze samples, contributing to the dark protein flocs observed in the samples. Additionally, the flocs observed in the samples could have cross-linked with polyphenols forming large, dark agglomerations of complexed proteins and polyphenols in the high haze samples.

Methylene blue stained large tannic particles a dark blue colour in high haze samples. In low haze samples, the tannic material was light blue and an abundance of small particles were stained (<50 μm). It is possible that, in the high haze samples, protein and phenolic material had complexed, reducing small particles and increasing large particle formation that contained a high concentration of tannic material (**Figure 4.6**) (Steiner *et al.*, 2010). These visual observations coincide with the wet-chemical results discussed in Section 4.3.3.

Thionin was used to stain carbohydrate material- staining neutral polysaccharides a purple colour, acidic polysaccharides pink, and carbohydrate material a black colour (**Table 2.1**). In the samples, some particles stained a dark-ashy colour. It is possible that the dark-stained particles may have been stained slightly pink coloured (an acidic polysaccharide) but the staining reaction was quenched before the samples were assessed (**Figure 4.7**). The purple stained particles showed the presence of dextrins in the samples (Skinner *et al.*, 1993). These microscopy results do not provide strong evidence linking polysaccharide content to haze.

Congo red has traditionally been used to study the presence of β-glucans in beer deposits and to detect undermodified endosperm in barley (Glenister, 1975; Skinner *et al.*, 1993; Briggs, 2002). The dye is now frequently used in assays to detect weakening of yeast cell walls as it is a destabilising agent (Omura *et al.*, 2009). In **Figure 4.8**, darker stained particles indicated that higher concentrations of β-glucans were present in the high haze samples as opposed to the low haze samples (Skinner *et al.*, 1993). Again, particles in high haze samples stained much darker than particles in low haze samples. The particles stained in high haze samples were a dark brown colour while particles in the low haze sample were a much lighter and brighter red colour. The darker staining indicated that the particle concentration was more dense in the high haze samples.

Selective-staining microscopy is a quick, low-cost method to perform a qualitative turbidity identification without the use of wet chemical tests. If used in conjunction with diagnostic enzyme studies, the culprit of most filterable beer hazes can be identified. However, the enzymatic digestion and microscopy results in this study still indicated that protein and β-glucan could have been a culprit of turbidity, raising the question of whether one or both of these macromolecules were responsible for sporadic haze formation. To continue to narrow the focus of the study, wet-chemical experimental testing was required to determine the concentration of the macromolecules in solution.

4.4.3 Wet Chemical Analysis

The samples were selected for the study to assess the difference in macromolecule content between high and low haze samples (Section 2.2.1) and the difference between samples consistently low in turbidity (control). The control and test samples were two different brands containing different base malts but it was desired to determine if the macromolecule content of the control samples greatly differed from the test samples as this may provide an explanation as to why turbidity spikes were observed in 'test' beers. From the samples, there were no distinct differences between control and test samples that were related to haze formation (**Figure 4.9**-**Figure 4.11**). The elevated protein and polyphenol content in the 'normal' haze beer, when compared to the 'control' could explain a potential reason as to why elevated turbidity levels were observed. This may also explain the lower levels of protein and polyphenol in high haze beer, as these macromolecules were likely complexed, increasing turbidity. However, during separation procedures (i.e.- centrifugation and filtration), the complexes should have easily been removed. Some small complexes <0.1 μm may have still been able to pass through the filter sheets.

The high standard deviation for polyphenol measurements, across all sample groups, was expected as polyphenol concentrations will vary per brew and dry-hop. Hop-derived and malt-derived polyphenols vary in slightly with each brew due to process parameters and variations in raw materials. As the largest uncertainty in the study were the samples, the standard deviations of the natural-products were less of a concern (**Table 4.6**).

Interestingly, β-glucan was the only macromolecule present in elevated concentrations in high haze samples compared to low haze samples. The large

degradation of protein in the enzymatic digestion results and the wet chemical analysis results appeared to contradict each other. However, the results are not contradictory when the structure of a yeast cell wall is taken into consideration (**Figure 4.18**).

It is hypothesised that stressed storage conditions, propagation, or fermentation conditions may affect the configuration and structure of yeast cell walls. The yeast cell wall composition has been found to be dependent upon the growth conditions of the media the cells are suspended in (Aguilar-Uscanga *et al.*, 2003; Kwiatkowski *et al.*, 2009).

Yeast cell walls consist of three groups of polysaccharides: mannose polymers (mannoproteins, approx. 40% dry cell mass), glucose polymers (β-glucan, 60% of dry cell mass), and *N*-acetylglucosamine (chitin, 2% of dry cell mass) (Aguilar-Uscanga *et al.*, 2003). Cell wall β-glucan is split into two categories based upon the degree of polymerisation. Long-chain β-glucans consist of approximately 1500 β-1,3-glucose monomers (85% of β-glucan units) while short chain β-glucans consist of approximately 150 β-1,6-glucose monomers (15% of β-glucan content) (Klis *et al.*, 2002). Short-chain β-1,3-glucans exist within the cell wall and form a network by hydrogen bonding between molecules. Long-chain β-1,6-glucan molecules are highly branched, extracellular to the cell wall, anchored by bonds to β-1,3-glucans and are water soluble (Lipke *et al.*, 1998; Klis *et al.*, 2002). The water solubility of β-1,6-glucans allow glycosylphosphatidylinositol (GPI)-cell wall proteins (CWP) to be tethered to β-1,3-glucans within the cell wall. Cell-wall proteins (CWP) or mannoproteins are glycosidically linked to these polysaccharides, forming the outer cell wall layer (**Figure 4.18)** (Klis *et al.*, 2002).

Cell wall proteins attached to cell wall β-glucans are divided into two categories based upon covalent bonding properties. The first group of covalently linked cell wall proteins observed in *Saccharomyces cerevisiae* are the GPI modified intermediate proteins. Cell wall proteins can attach to a GPI anchor by hydrolysation of an oligomannosyl group of the GPI lipid anchor that results in a trans-mannosylation. Interestingly, the GPI-CWP can be excised from the cell wall by β-1,6 and β-1,3 glucanases as the GPI-CWP are linked to β-1,6-glucan tethered to the β-1,3-glucan network in the cellular membrane (Klis *et al.*, 2002; Ecker *et al.*, 2006).

Second, putative proteins with internal repeats, or Pir proteins, are conserved and consist of at most, ten repeating units (Ecker *et al.*, 2006). Pir proteins are directly linked to β-1,3-glucan and do not contain a GPI-addition signal at the C-terminus end. As the Pir cell wall proteins are immediately linked to β-1,3-glucan and these cell wall proteins can be excised from the cell wall by β-1,3-glucanase. Multiple classes of Pir proteins exist. However, some phenotypes of Pir proteins are expressed as a result of nitrogen starvation and heat stress potentially aiding in the formation of sporadic hazes in beer (Ecker *et al.*, 2006).

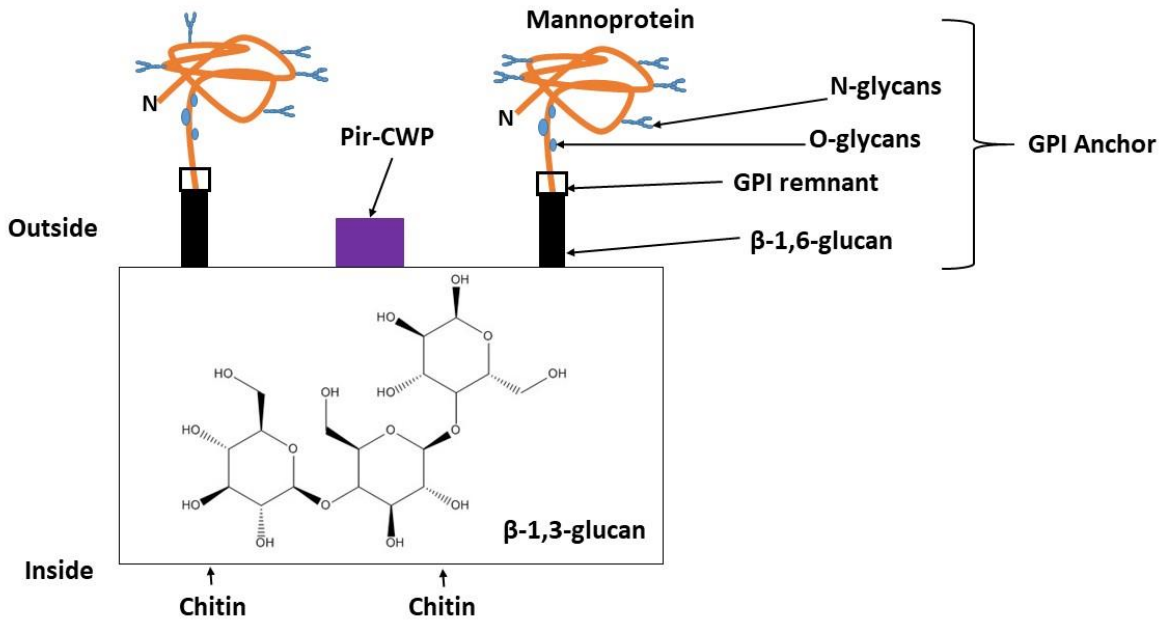


Figure 4.18- Molecular organisation of *Saccharomyces cerevisiae* cell wall adapted from Klis *et al.*, (2002) and Lipke and Ovalle, (1998). The mannoprotein is orange and N-linked or O-linked oligosaccharides are blue. The intercellular β-glucan is in the box and chitin is inside the cell.

Attached to the GPI or Pir protein anchors, cellular mannoproteins have a variety of different functions. Cell wall mannoprotein, Cwp1, is observed on yeast cells in rich medium with a GPI-link (Dean, 1999; Vladimir Mrsa *et al.*, 1999; Klis *et al.*, 2002). When considering beer haze, the absence of mannoprotein Cwp1 and gene CWP1 may have detrimental effects to beer quality as the pair strengthen yeast cell walls. Cwp1 can also bind directly to β-1,3-glucan by an alkali-sensitive bond via Pir-CWP binding mechanism (Toh-E *et al.*, 1993; Kapteyn *et al.*, 1999; V Mrsa *et al.*, 1999; Klis *et al.*, 2002). Double-linked GPI-CWP's such as Cwp1 are reported to be relevant

to the cellular-response to cell wall stress while Pir encoding genes are upregulated in the instance of cell wall stress (Ram *et al.*, 1998; Jung *et al.*, 1999; Terashima *et al.*, 2000; Klis *et al.*, 2002).

The links of β-1,6-glucan, β-1,3-glucan, chitin, and mannan stabilise the cell wall, causing the wall to be insoluble (Kwiatkowski *et al.*, 2012). However, it is hypothesised that under situations of induced cell stress (low nutrient and low oxygen environments), the glucan network/bonds will lose their structure causing the cell walls to become more soluble, releasing intracellular components. The cell wall glucans released from mannoproteins remain as an insoluble fraction which is hypothesised to also cause insoluble, unfilterable hazes (Kwiatkowski *et al.*, 2012).

Finally, mechanical damage from pumps in pitching steps and tank transfers may have induced shear damage to yeast cells contributing to increased turbidity. Lab scale and pilot scale studies confirmed the release of mannan from lager and ale strains by means of mechanical agitation (Chlup, Bernard, *et al.*, 2007). In lab scale studies, the release of mannan increased the turbidity of the supernatant the yeast was suspended in (Chlup, Conery, *et al.*, 2007). In pilot scale studies, after beer was subjected to centrifugation, mannan was observed in the haze material originating from the exterior of yeast cells and was reported to be unfilterable (Chlup, Bernard, *et al.*, 2007).

The evidence from the literature supports the hypothesis that cell wall mannoproteins were related to unfilterable turbidity (Siebert *et al.*, 1987; Van Der Vaart *et al.*, 1995; V Mrsa *et al.*, 1999; Chlup, Bernard, *et al.*, 2007; Chlup, Conery, *et al.*, 2007). Considering the enzymatic digestion, haze microscopy and wet-chemical analysis results, it was hypothesised that yeast cells in propagation, storage, transfers, and acid washing were occasionally exposed to stress inducing environments, causing sporadic increases in turbidity. This hypothesis informed the final study of this chapter.

4.4.4 Molecular Determination of Beer Haze

Intact protein precipitation techniques were required to isolate and extract beer proteins for LC-QTOF-MS analysis. Two methods were tested (Section 2.2.6) in order to select a method sensitive enough to extract differing yeast proteins in each sample SDS-PAGE gels (**Figure 4.15**) (Section 4.3.4.2). However, the methods of precipitation left very streaked bands on the gel, hindering clear band separation **Figure 4.15**. The heavy streaks were most likely due to the polyphenol content of the beer as the addition

of phenolic material induces streaking (Figure 1-Maria *et al.*, 2013). Due to this, protein purification and fractionation was used to purify, concentrate, and isolate beer proteins.

An ÄKTA Avant liquid chromatography system was used to purify, concentrate, and isolate proteins in high and low haze samples (Section 2.2.10). The first experiment utilised an NaCl gradient to determine at which concentration proteins with differing isoelectric points would elute (**Figure 4.12**). Following the first experiment, a step elution with a higher pH gradient was utilised to properly isolate and fractionate the proteins of interest in low and high haze samples (**Figure 4.13** and **Figure 4.14**).

The protein fractionation was successful at isolating beer proteins as differences were observed between high and low haze samples. Two distinct peaks were observed in the fractionation of the high haze beer sample (**Figure 4.14**) and only one peak was observed in the low haze sample (**Figure 4.13**). Based on the ÄKTA results, it can be confidently stated that the protein content between high and low haze samples was different. However, LC/ESI-QTOF-MS analysis was required to confirm and measure how the protein content differed.

The LC/ESI-QTOF-MS results were able to identify differences in protein content in high and low haze samples. The total ion chromatograms were visually different and the percent sequence coverage was also substantially different (**Appendix F, Table 4.7, and Table 4.8**).

Osmotic shock and shearing cause the excision of yeast cell wall fimbrae (Klis *et al.*, 2002; Chlup, Conery and Stewart, 2007). Because of this, it was hypothesised that stressed storage, propagation, or fermentation conditions caused *Saccharomyces cerevisiae* strains to excise structural mannoproteins. Depletions of these proteins show increased sensitivity to Congo red and a much less electron-dense outer cell wall (Van Der Vaart *et al.*, 1995). Additionally, mannan does not impair filterability and as the hazes observed were unfilterable, the case for the excision of mannan from cell walls was supported (Kupetz *et al.*, 2015).

Following guidelines for proteomic data interpretation, the reported proteins were observed with 95% confidence. Meaning that, there was a 95% chance that the protein was present in the sample with the given percent sequence coverage. Protein Sim1, which is required for remodelling the cell wall during culture development, was detected in high abundance with nearly 90% sequence coverage (**Table 4.7**). The yeast-

derived protein has been recovered in other proteomics studies (Colgrave *et al.*, 2013) and observed at a much lower low percent coverage in lager beers (9.1%) (Fasoli *et al.*, 2010). Although this study cannot quantify the protein, it can be speculated that the protein is present in relatively high abundance in the sample with a robust sequence coverage and 160% increase in coverage compared to a low haze sample.

Protein Ecm33 was another structural cell wall protein with high sequence coverage. The mannoprotein is required for cell wall integrity and for the assembly of the outer mannoprotein layer of the cell wall (**Table 4.7**). In lager beers, a percent sequence coverage of 5.1% has been reported (Fasoli *et al.*, 2010). In this study, high haze realised a 76.92% sequence coverage and a low haze percent sequence coverage of 35.9% leading to 114% overall percent difference between the two (**Table 4.7**). Again, absolute concentrations cannot be determined but it can be speculated that mannoproteins in high haze samples were present in greater abundance than low haze samples.

The flocculation proteins reported large percent differences between high and low haze samples. Flocculation protein 9 (Flo9) was of interest as the protein was absent in low haze samples but present in high haze samples. It is possible that the GPI-anchored protein is cleaved off by β-1,6-glucanase but further research is required to prove this hypothesis. According to the lectin-like flocculation theory, the flocculation proteins extend as fimbrae from the cell wall, waiting for calcium ions to activate their conformation and interact with mannose residues on neighbouring cells (Soares, 2011). As flocculation proteins/mannoproteins are located at the yeast cell surface, it is hypothesised that flocculation proteins are excised from the cell wall under stressed storage conditions, shearing by mechanical agitation, or by stressed propagation/storage conditions (Cunningham *et al.*, 1998; Van Bergen *et al.*, 2004). This would increase turbidity in the propagation medium and in the beer produced (Stoupis *et al.*, 2002). The data and literature support this current theory, however, confirmatory studies are required (Lewis and Poerwantaro, 1991; Chlup, Conery and Stewart, 2007; Siebert, 2009).

Finally, the results of the D-mannose, D-glucose, and D-fructose assay further justified the proposed theories that cell wall mannoproteins contributed to increased turbidity. The mannose concentrations in high haze samples were significantly different at nearly three times the concentration when compared to low haze samples. Overall, it

can be speculated that yeast cell walls were altered due to stress or damage during the brewing process. The alteration of the yeast cell wall caused an increase in turbidity that was unfilterable as other factors of turbidity could be easily removed by separation aids.

Chapter 5- Terpene Studies in Packaged Beer with Paired Sensory Analytics

5.1 Introduction

Beer stability post-packaging remains a difficult and complex topic for brewers and brewing scientists to understand. During storage, major changes in beer flavour occur as the result of oxidation. There are several, well studied oxidative mechanisms that lead to a major alteration of beer flavour. The first being the formation of carbonyl compounds. An example of this is the oxidation of unsaturated fatty acids, yielding *(E)*-2-nonenal, experiencing a cardboard or papery flavour (Drost *et al.*, 1990; Uchida *et al.*, 1996). Another flavour-altering reaction is degradation of amino acids via reactive oxygen species to yield Strecker aldehydes (Saison *et al.*, 2009; Wietstock *et al.*, 2016). Bitter acids also oxidatively degrade during raw hop (pelleted, flowers, etc.) storage or in beer yielding various carboxylic acids that impart cheesy, sour, and rancid aromas (Williams *et al.*, 1979; Rakete *et al.*, 2014). Esterification of carboxylic acids may contribute to increases in sherry-like attributes during beer ageing. Finally, the oxidation of higher alcohols in aged beer by melanoidins also alter beer flavour by enhancing aldehydes that contribute caramel and stale flavours (Hashimoto *et al.*, 1977). Although, the formation of aldehydes by oxidation of iso-acids is now reported to minimally contribute to aged beer flavours (De Clippeleer *et al.*, 2010).

Dalgliesh, (1977) was a pioneer in the discussion of flavour stability in beer. Dalgliesh suggested that understanding flavour consists of three main concepts. First, that physical chemistry of the compounds and the reactions that alter them must be understood. Next, sensory response/flavour thresholds of the compounds should be established and finally, that compound-specific anosmia's and/or aversions be taken into consideration (Dalgliesh, 1977). The same author also suggested that flavour active compounds in beer range in concentration and that their contribution to flavour is dynamic, proposing a figure to explain the general change in aroma as beer ages (**Figure 5.1**).

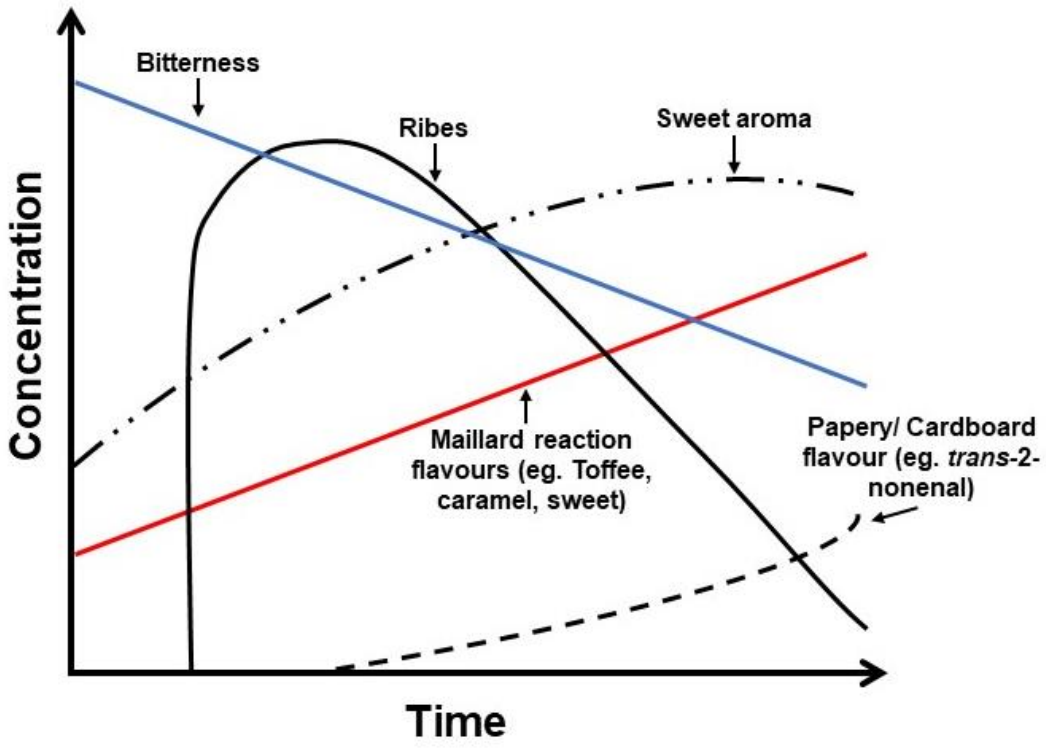


Figure 5.1- The general evolution of beer flavour over time. Adapted from Dalgliesh, (1977).

It is clear that an extensive amount of research has been dedicated to understanding the stability, or instability, of beer aroma. However, little information has been reported regarding the stability of hop-derived compounds in heavily dry-hopped beers and their sensory impact.

Hops provide bitterness and an array of different aromas, depending on the variety used. One of the key components of hop aroma are terpenes/terpenoids, contained within the essential oil of hops (Section 1.3.4). Hop terpenes and terpenoids contribute a variety of aromas in beer ranging from cooked vegetables to citrus aromas (Guillaume Lermusieau *et al.*, 2001).

Terpenes/terpenoids are volatile and able to react with other molecules in beer (King *et al.*, 2003; Karabin *et al.*, 2015). Due to this, hop-derived terpene concentrations change in beer over time in fermentation, conditioning and storage conditions (Takoi *et al.*, 2012; Biendl *et al.*, 2014). However, limited research has been dedicated to understanding the link between the change of these compounds and the potential sensory impact.

Sensory analysis is an essential component of food production, ensuring that a food and beverage products meet set standards of colour, texture, flavour and aroma for the producer and the consumer (O'Sullivan, 2017). In the brewing industry, sensory analysis is a crucial component of product quality assessment, consistency, and market acceptance. Sensory assessment evaluates palate, aroma, texture, mouthfeel, the impact of various ingredients added to beer and process change (Drost *et al.*, 1990; Vanderhaegen *et al.*, 2003; Van Opstaele *et al.*, 2010; Mizuno, 2013; De Almeida *et al.*, 2015; Praet *et al.*, 2016a; Schnaitter *et al.*, 2016; Vollmer *et al.*, 2016, 2017). The orthonasal and retronasal senses in the human nose are a powerful tool in sensory analysis as the human senses detect compounds in exceptionally low concentrations (ng/L) that are difficult to detect in chemical analysis (Diaz, 2004; De Schutter *et al.*, 2009).

When evaluating beer quality, it is beneficial to compare and correlate sensory data with analytical data. This immediately allows breweries to monitor product quality and the likelihood of consumer acceptance. Correlating sensory and analytical data may provide further insight into the evolution of beer flavour to enhance understanding of beer aroma, post-packaging.

The purpose of this study was to pair analytical data with sensory data to begin to understand the link between changes of terpene/terpenoid concentration in beer and the sensory impact that compound shifts may impose. The study also assessed the change in terpenes/terpenoids over time in packaged beer. Finally, the study was conducted at two different storage temperatures to assess the impact temperature may have on sensory and analytical profiles.

5.2 Experimental Design

5.2.1 Samples

The goal of the experiment was to measure the changes in terpenes/terpenoid concentrations in a dry-hopped ale, in addition to correlating sensory and analytical data. Sample bottles of an ale, that was dry-hopped between 0.2 and 0.8 kg/hL, packaged in 330 mL amber glass bottles (Owens-Illinois Inc., Alloa, UK) were collected from the packaging line of the collaborating brewery. The beers were split and incubated at two different temperatures, 4°C and 20°C for a total of 16 weeks. Samples

were collected at day zero, two, four, and seven in addition to two, four six, eight, 10, 12, 14, and 16 week time points with four repetitions of the study over two years.

5.2.2 Sample Collection

At the time of sampling, the temperature of the samples were brought to 4°C and two, 40 mL aliquots of each storage temperature were collected in 50 mL centrifuge tubes (Corning, Deeside, UK) and frozen at -20°C for later GC/MS-SPME analysis. The remaining samples collected were prepared for assessment by sensory panels.

5.2.3 Sensory Training

The sensory training programme for the project was adapted from the Aroxa™ Beer Sensory Programme (Cara Technology, Leatherhead, UK). The training programme is described in Chapter 2, Section 2.5.3.

5.2.3.1 Preparation for Sensory Training

A dry-hopped ale (dry-hopped between 0.2 and 0.8 kg/hL) was selected as the base beer for the study. The base beer was spiked with flavour standards for beer sensory training purposes. A 500 mL volume of room-temperature beer was decanted into a 1.5 litre jug. Flavour standard capsules were added to each jug, per manufacturer guidelines (Cara Technology, Leatherhead, UK) and an additional 500 mL of beer was added to each jug to dissolve and mix the flavour standard. A table listing all flavours used in basic sensory training are found in **Table 5.1**. Descriptive profiling flavours are not listed due to confidentiality. The beer was poured into another jug once to completely mix and dissolve the standard. Samples were prepared immediately prior to panellist training to ensure freshness.

Table 5.1- Flavours used for basic sensory training paired with respective chemical names. The 'Flavour' column presents all of the flavour names utilised by Aroxa™.

Flavour	Chemical name	CAS registry number
Acetaldehyde	Acetaldehyde	75-07-0
Acetic	Acetic acid	64-19-7
Bitter	Iso-alpha acids	25522-96-7
Burnt sugar	Furaneol	3658-77-3
Butyric	Butyric acid	107-92-6
Caprylic	Octanoic acid	124-07-2
Catty hop	4-mercapto-4-methyl-penatan-2-one	38462-22-5
Chlorophenol	2,6-dichlorophenol	87-65-0
Citrus Hop	β-linalool	n/a
Damascenone	β-damascenone	23696-85-7
Diacetyl	2,3-butanedione	431-03-8
DMS	Dimethyl sulphide	75-18-3
Ethyl acetate	Ethyl acetate	141-78-6
Ethyl butyrate	Ethyl butyrate	105-54-4
Ethyl hexanoate	Ethyl hexanoate	123-66-0
Freshly cut grass	Cis-3-hexanol	928-96-1
Geraniol	Geraniol	106-24-1
Grainy	Isobutyraldehyde	78-84-2
H_2S	Hydrogen sulphide	7783-06-4
Isoamyl acetate	Isoamyl acetate	123-92-2
Isovaleric	Isovaleric acid	503-74-2
Leathery	Isobutylquinoline	1333-58-0
Lightstruck	3-methyl-2-butene-1-thiol	5287-45-6
Limonene	(S)-(-)-limonene	1195-92-2
Malty biscuity	2-acetylpyridine	1122-62-9
Mercaptan	Methanethiol	74-93-1
Metallic	Ferrous sulphate	7782-63-0
Musty	2,4,6-trichloroanisole	87-40-1
Onion	Dimethyl trishulphide	3658-80-8
Papery	*trans*-2-nonenal	18829-56-6
Phenolic 4EP	4-ethyl phenol	123-07-9
Phenolic 4VG	4-vinyl guaiacol	7786-61-0
Raw hop	B-myrcene	n/a
Smoky	Guaiacol	90-05-1
Sour	Citric acid	77-92-9
Sulphitic	Sulphur dioxide	7757-83-7
Sweet	Sulcralose	56038-13-2
Vanilla	Vanillin	121-33-5

Each panellist was provided with the correct number of cups for the session and each panellist was encouraged to arrange sample cups in a sequential preferred fashion.

Beer was decanted into clear 200 mL plastic cups for each panellist as the training session progressed. A ‘base beer’ reference beer sample was provided at each training session for reference.

5.2.3.2 Training Panellists

Basic beer sensory training was required for sensory panellist participation. The training consisted of 15 sessions over three days, each lasting approximately one hour. A 15 minute break was provided at the end of each session for panellists to rest. The first three sessions were tutored training sessions with 10 spiked samples, spiked at three times the respective sensory threshold. Each sample was poured individually and described in regard to common sensory descriptors, the origin of the flavour/aroma, sensory threshold, and impact to beer quality. This was followed by prompting panellists to describe what aromas were perceived. Panellists were encouraged to speak to each other to develop a common sensory lexicon. After the tutored sessions, the following twelve sessions contained a mix of recognition tests and ranking tests.

In recognition tests, panellists were required to leave the sensory suite, allowing samples to be placed out of order at their station. Panellists were instructed to return and identify what compound each beer had been spiked with, using an unspiked sample as a reference. In ranking tests, panellists were provided with 10 different samples containing various spike levels of the same flavour standard. Panellists were required to rank the intensity of each characteristic for each beer from 0-10.

At the end of each training day, panellists were required to sit a recognition test that could utilise any flavour spike taught during the training day. At the conclusion of the three days of training, panellists were required to sit a further recognition test that could contain any of the flavour standards taught over the course. To pass basic training and to be included in regular taste panels, panellists were required to pass with a minimum score of 75%.

5.2.4 Descriptive Profiling Training

If a panellist passed the basic sensory training, descriptive profiling training was provided to assess the quality of tasters within the panel. In descriptive profiling, panellists were required to describe a random beer sample by utilising descriptions from a provided list of attributes, assigning an intensity score to each attribute.

A total of 17 sessions were provided over the course of three days. Descriptive profiling training sessions comprised two tutored tastings, six recognition tests, five rank tests, two descriptive profiles, and one product recognition test. Data from these sessions were collected to determine how accurately panellists could identify aged flavours, the overall cohesiveness of descriptive profiling, and panellist sensitivity to various beer flavours (**Figure 5.2, Figure 5.3 and Table 5.2**). To take part in the experimental panel and pass descriptive profiling, panellists were required to pass with a minimum score of 80%.

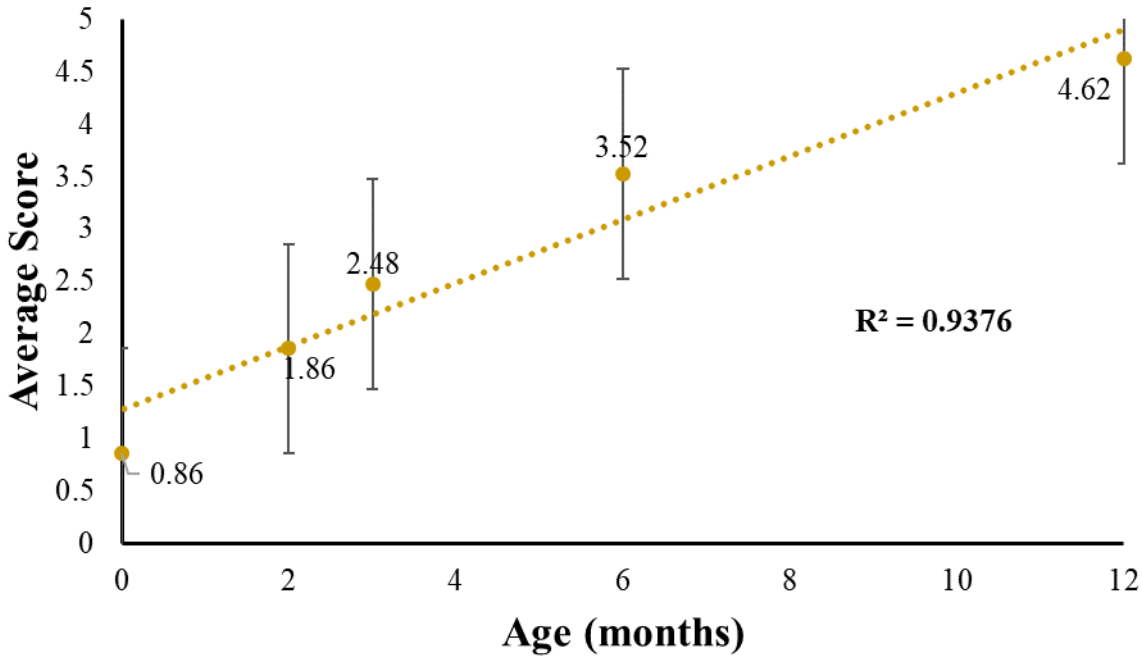


Figure 5.2- Aged beer rank-rating sensory panel results. Panellists (n=21) were instructed to score beers and place them in order of 'fresh= 0' to 'aged=5'. Error bars indicate standard deviation of panellists.

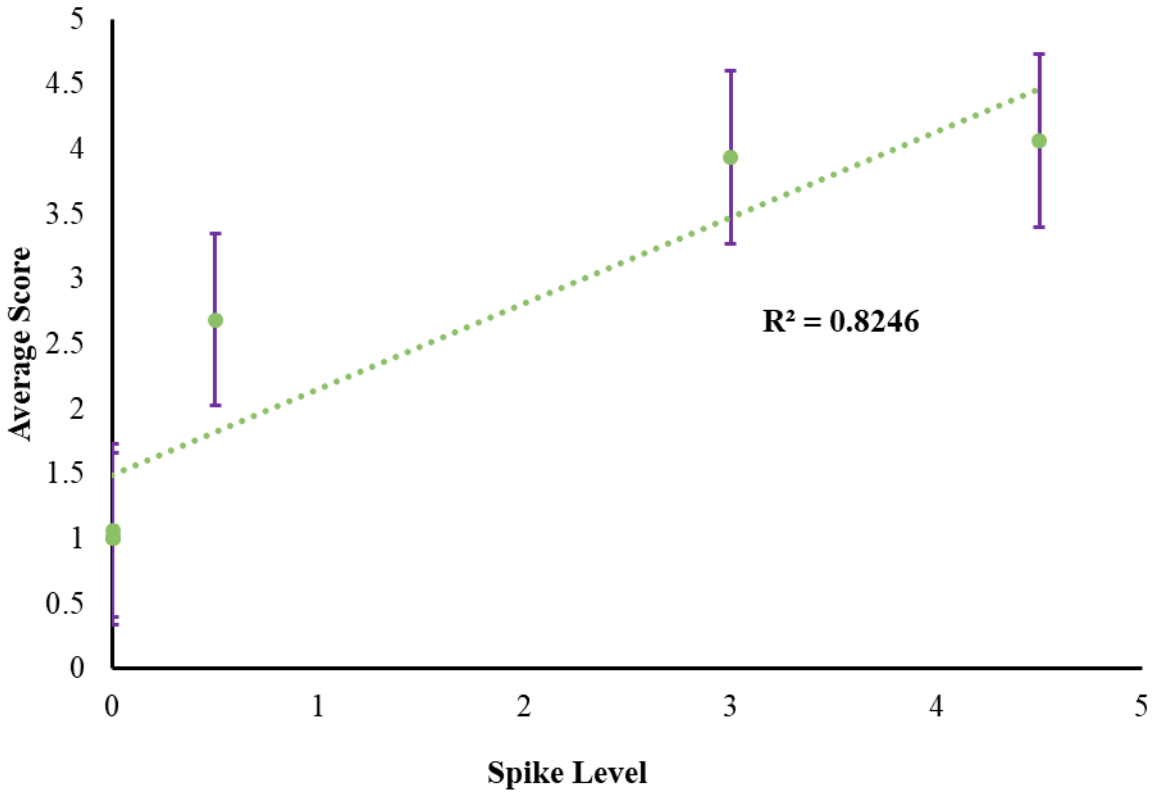


Figure 5.3- Beta-damascenone rank-rating sensory panel results. Panellists (n=21) were instructed to score beers and place them in order of intensity (0= Lowest, 5= highest). Spike levels correlated to sensory threshold of each analyte tested. Error bars indicate standard deviation of panellists.

5.2.4.1 Refresher Courses

To ensure panellists continued to provide accurate results, several annual training sessions were held. Conformance scoring, brand recognition, and rank rating tests were completed by panellists and followed by a group discussion as to why each panellist chose their score for conformance, true-to-type for brand, and rankings. This provided a recalibration of the panellists and enforced panellist accountability.

Panellists were also required to participate in a monthly taster validation test as a final check. For validation, panellists were provided with two samples of an 'unknown' flavour standard spike at an unknown level. Descriptive profile panellists were required to identify what each beer was spiked with and to estimate the approximate spike concentration. Spike tests were double-blind, and results checked using the Aroxa™ website. If scores were out of range and the panel did not pass, panellists were retrained on the particular compound that failed.

5.2.5 Selection of Panellists

Panellists were selected for the descriptive profiling panel for the experiment based upon their job role, training level, weekly attendance, weekly training averages, and average training score. Based upon these matrices, each panellist was scored using a ten point system. Points were awarded based upon taste panel attendance, regular training attendance, and an annual training scores.

5.2.5.1 Panel

Panellists required an invitation to be included in the descriptive profiling panel. Panellists were advised not to consume any food or drink prior to the panel.The panels were held from 10:15- 10:45 to prevent any bias from food or drink that panellists had consumed in the morning. A total of four samples, two 20°C storage and two 4°C storage, were mixed into routine daily taste panel samples to ensure the panel was blind and to prevent bias. The minimum number of attendees for a panel to be of an appropriate size for statistical validation was six people.

The panel was a descriptive profiling panel based upon sensory descriptors found in **Table 5.2**. The purpose of the panel was to monitor changes in hop flavour and aroma. Panellists were provided with a laptop and a personal login for the Sensecheck™ software (Cara Technology, Leatherhead, UK) to record and collate sensory data. The software listed each sensory descriptor with a slide-bar for panellists to rate approximate intensities of each flavour. Following the panel, the results from each panellist were collected and assessed for accuracy. Panellists scored sensory descriptors on a scale of 0-10, based on intensity.

Table 5.2- Sensory descriptors with commonly associated chemical compound(s) found in literature.

Sensory descriptor	Common compound association
Astringent	Polyphenols[a], nonanal[b]
Bitter	β-myrcene[c], citral, nonanal, 2-undecanone[d]
Catty Hop	4-mercapto-4-methyl-penatane-2-one[e]
Citrus Hop	β-linalool[c], limonene, β-citronellol, linalool oxide[b]
Damascenone	*p*-menthane-8-thiol-3-one [f]
Earthy Hop	β-myrcene[c], α-humulene, β-caryophyllene[b]
Floral Hop	β-linalool[c], *trans*-geraniol, β-citronellol, caryophyllene oxide, α-humulene, β-caryophyllene[b]
Freshly Cut Grass	β-myrcene[c], nerol, α-humulene[b]
Isovaleric	isovaleric acid[b]
Passionfruit Hop	β-citronellol[b]
Peach Hop	3-mercapto-octanol, β-damascenone[b]
Pine Hop	α-pinene, α-terpinene, β-myrcene[b]
Raw Hop	β-myrcene, caryophyllene oxide, linalool oxide[b]

[a]Aron and Shellhammer, (2010), [b]Zunkel, (2015), [c]Peltz and Shellhammer, (2017), [d]Aberl and Coelhan, (2012), [e]Vanderhaegen *et al.*, (2006) [f]Clapperton, Dalgliesh and Meilgaard, (1976)

5.2.6 GC/MS-SPME

All GC/MS-SPME method development and sample preparation is described in Section 2.4. The compounds analysed are found in **Table 5.3**.

Table 5.3- Chemical compounds measured paired with their commonly associated sensory descriptor

Compound	Sensory descriptor
β-myrcene	Woody, earthy, vegetal, raw hop, peppery, resinous[b]
Linalool oxide	Raw hop, green, citrus[a,b]
β-linalool	Floral, fruity, citrus, coriander[a,b]
β-citronellol	Floral, citrus, rose, lime, waxy[b]
trans-geraniol	Floral, rose, geraniums, perfume[a]
β-caryophyllene	woody, floral, spicy, cloves, sweet[a,b]
α-humulene	Woody, herbal spicy, grassy[a,b]
caryophyllene oxide	Musty, Spicy, floral[b]

[a]Peltz and Shellhammer, (2017), [b]Zunkel, (2015)

5.2.7 Constructing Principal Component Analysis Plots (PCA)

Principal Component Analysis (PCA) plots were created to examine the relationship between sensory and analytical data. Each replicate, at each incubation

temperature, was assessed in Origin 2018b (Origin Lab, Northampton, US). Plots were made of paired sensory and analytical data, solely sensory data, and solely analytical data as 'days' were plotted as observational variables.

All PCA plots were constructed utilising RStudio (R Core Group, Vienna, Austria). To aid visualisation of the PCA plots, codes were assigned to each sensory and analytical descriptor assessed. Each sensory and analytical descriptor were assigned a code to construct clear PCA plots (**Table 5.4**). The values obtained from the analytical data for both storage temperatures were collated (**Table 5.5** and **Table 5.6**). The mean scores of sensory analysis data were calculated for each sample point at both temperatures. **Table 5.7** and **Table 5.8** display the collated sensory results from the 4°C and the 20°C storage temperature. The values in **Table 5.5**, **Table 5.6**, **Table 5.7**, and **Table 5.8** were normalised and used in the creation of PCA plots.

Table 5.4- Descriptors for factors assisted in PCA plots. Non-bolded compounds and codes are compounds measured by GC/MS-SPME and bolded compounds are sensory descriptors assessed and scored by sensory panellists.

Compound	Code
β-myrcene	M
Linalool oxide	LO
β-linalool	L
β-citronellol	CIT
Trans-geraniol	TG
β-caryophyllene	BC
α-humulene	AH
Caryophyllene oxide	CO
Astringent	**A**
Bitter	**B**
Catty Hop	**CAT**
Citrus Hop	**CIT.1**
Damascenone	**D**
Earthy Hop	**EH**
Floral Hop	**FH**
Freshly Cut Grass	**FCG**
Isovaleric	**I**
Passionfruit Hop	**PH**
Peach Hop	**PEH**
Pine Hop	**PIH**
Raw Hop	**RH**

5.2.8 Measuring Terpene/Terpenoid Concentration in Aged Beer

The GC/MS-SPME data described in Section 5.2.6, was collated and plots comparing the 4°C and 20°C results of each compound were created with Origin 2018b (OriginLab, Northampton, US).

5.3 Results

5.3.1 Correlating Sensory and Analytical Data

Discrepancies were observed between each of the four repeats of the experiment performed over the two year time period. One replicate was omitted due to the incomplete set of analytical samples collected. The discrepancies are explained by slight alterations to the recipe and brewing procedures by the collaborating brewery across the trial period. In the analytical data, all peaks were manually checked and re-integrated, if necessary, for accuracy. The compounds α-humulene and β-caryophyllene, at both storage temperatures, were omitted from the PCA plots either as the compounds were not detected and/or due to analytical errors (**Table 5.5** and **Table 5.6**).

In the first sample set, citrus hop, linalool oxide, β-citronellol, β-linalool and damascenone had the greatest impact on the model (**Figure 5.4**). A relationship between β-myrcene and bitterness and β-myrcene and citrus hop was found, these compounds clustered with 'fresh' beer samples of both ageing temperatures. For the purposes of the experiment, 'fresh' beer was considered to be 14 days or less. At 28 days of ageing in the 4°C group, β-citronellol was found to be prominent, possibly due to storage temperature. At 14 days within the 20°C group, linalool oxide was prominent- possibly due to the level of packaged oxygen and warmer storage temperatures encouraging oxidation. The damascenone sensory descriptor, in both ageing temperatures, was grouped with aged samples. The damascenone character was stronger in the 20°C sample set at 84 and 112 days but was still apparent at 112 days at 4°C. Other than these observations, the sensory and analytical data did not demonstrate strong trends in the evolution of beer flavour over time (**Figure 5.4**).

In the first sample set, the most important correlations of analytical and sensory data were β-myrcene and bitterness in addition to, β-myrcene and citrus hop. Earthy hop and β-linalool were also related but shared a weaker relationship than the previous stated as the earthy hop sensory descriptor was purple contributing less weight to the model (**Figure 5.4**).

Table 5.5- Analytical data extracted from GC/MS-SPME analysis assessing sample set one at 4°C. The compounds β-caryophyllene and α-humulene were omitted from all analysis, as the compounds were not detected in GC/MS-SPME. Error is accounted for in GC/MS-SPME calibration and validation steps (Section 2.3.4).

Days	β-myrcene (µg/L)	Linalool oxide (µg/L)	β-linalool (µg/L)	β-citronellol (µg/L)	*trans*-geraniol (µg/L)	β-caryophyllene (µg/L)	α-humulene (µg/L)	Caryophyllene oxide (µg/L)
0	1.19	0.00	28.90	19.80	67.70	n.d.*	n.d.*	1.59
4	1.79	17.80	29.50	24.00	66.70	n.d.*	n.d.*	1.33
7	1.73	15.00	32.50	25.10	81.10	n.d.*	n.d.*	1.77
14	1.56	13.70	27.60	24.10	66.70	n.d.*	n.d.*	1.15
28	1.40	16.60	38.20	27.30	97.80	n.d.*	n.d.*	1.64
42	1.25	6.08	29.40	24.00	66.00	n.d.*	n.d.*	0.85
56	1.22	7.72	34.90	25.20	78.40	n.d.*	n.d.*	1.12
70	1.26	7.06	34.00	24.80	71.50	n.d.*	n.d.*	0.97
84	1.28	7.34	31.80	24.10	63.80	n.d.*	n.d.*	0.87
98	1.61	11.60	30.40	26.10	87.20	n.d.*	n.d.*	1.59
112	1.28	7.17	34.60	24.90	72.30	n.d.*	n.d.*	1.00

Table 5.6- Analytical data extracted from GC/MS-SPME analysis assessing sample set one at 20°C. The compounds β-caryophyllene and α-humulene were omitted from all analysis, as the compounds were not detected in GC/MS-SPME. Error is accounted for in GC/MS-SPME calibration and validation steps (Section 2.3.4).

Days	β-myrcene (µg/L)	Linalool oxide (µg/L)	β-linalool (µg/L)	β-citronellol (µg/L)	*trans*-geraniol (µg/L)	β-caryophyllene (µg/L)	α-humulene (µg/L)	Caryophyllene oxide (µg/L)
0	1.18	2.60	27.40	19.70	60.00	n.d.*	n.d.*	1.35
4	1.68	10.48	25.35	22.1	63.95	n.d.*	n.d.*	1.175
7	1.53	10.80	25.60	23.30	52.90	n.d.*	n.d.*	0.83
42	1.37	16.20	39.80	27.90	103.00	n.d.*	n.d.*	1.82
56	1.34	11.80	33.20	27.00	93.70	n.d.*	n.d.*	3.70
70	1.27	11.40	31.90	24.90	63.10	n.d.*	n.d.*	n.d.*
84	1.20	11.10	36.10	24.60	72.50	n.d.*	n.d.*	0.77
98	1.21	5.99	28.90	24.10	67.70	n.d.*	n.d.*	0.81
112	1.19	7.59	35.60	25.40	78.70	n.d.*	n.d.*	0.95

*n.d. indicates that the GC/MS-SPME did not detect any signal at these sample instances due to analytical errors.

Table 5.7- Collated sensory data from all assessment points from sample set one at 4°C. All values are ranked out of 10. The values represent the means of each sample point. Panellist attendance ranged from n=6 to n=15. Standard deviations are found in **Appendix G.**

Days	Astringent	Bitter	Catty Hop	Citrus Hop	Damascenone	Earthy Hop	Floral Hop	Freshly Cut Grass	Isovaleric	Passionfruit Hop	Peach Hop	Pine Hop	Raw Hop
0	0.75	3.10	0.85	2.90	0.45	0.05	1.15	0.80	0.25	0.45	0.40	0.35	0.35
4	0.55	3.35	1.25	3.60	0.70	0.40	1.55	1.15	0.35	1.15	1.20	0.50	0.70
7	0.80	3.20	1.35	3.10	0.55	0.25	1.85	1.00	0.10	0.65	0.35	0.55	0.15
14	1.20	3.70	1.40	3.50	0.50	0.20	1.70	0.60	0.30	1.00	0.70	0.70	0.50
28	1.60	3.00	1.80	3.00	1.80	0.80	1.10	0.80	0.90	0.90	0.70	0.90	0.50
42	1.30	1.95	1.25	2.00	1.15	0.25	0.85	0.85	0.25	0.55	0.45	0.35	0.30
56	0.75	2.25	0.80	1.60	1.95	0.20	1.20	0.50	0.70	0.35	0.35	0.45	0.20
70	1.05	2.35	1.50	1.70	2.10	0.45	1.30	0.60	1.15	0.15	0.20	0.00	0.20
84	1.60	3.15	1.45	2.45	1.20	0.45	1.00	0.65	0.60	0.70	0.35	0.20	0.05
98	0.10	2.90	1.35	3.15	0.00	0.30	1.15	0.55	0.55	0.80	1.25	0.45	0.60
112	1.75	2.65	1.45	2.40	1.35	0.20	0.85	0.70	0.85	0.70	0.80	0.35	0.40

Table 5.8- Collated sensory data from all assessment points from sample set one at 20°C. All values are ranked out of 10. The values represent the means of each sample point. Standard deviations are found in **Appendix G.**

Days	Astringent	Bitter	Catty Hop	Citrus Hop	Damascenone	Earthy Hop	Floral Hop	Freshly Cut Grass	Isovaleric	Passionfruit Hop	Peach Hop	Pine Hop	Raw Hop
0	0.55	3.4	0.7	2.9	0.25	0.25	0.75	1	0.45	0.4	0.25	0.2	0.3
4	0.45	2.9	1	2.95	0.9	0.5	1.4	0.75	0.35	0.65	0.65	0.45	0.45
7	1.15	2.55	1.4	2.25	1.05	0.3	1.35	0.55	0.1	0.7	0.45	0.25	0.05
42	1.45	3.7	1.8	3.05	0.8	0.5	1.25	1.05	0.95	0.95	0.5	0.75	0.5
56	1.25	3.45	1.35	2.6	0.75	0.3	1.4	0.95	0.75	0.45	0.4	0.35	0.45
70	0.9	2.8	2	1.9	1.15	0.45	1.05	0.85	0.95	0.3	0.15	0	0.25
84	1.4	2.15	1.1	1.4	2.95	0.75	0.9	0.15	0.6	0.55	0.65	0.15	0
98	0.5	1.9	1.15	1.4	1.15	0.8	0.6	0.45	1	0.25	0.3	0.05	0.05
112	1.75	1.95	1.2	1.5	2.6	0.55	0.75	0.5	1	0.4	0.55	0.4	0.5

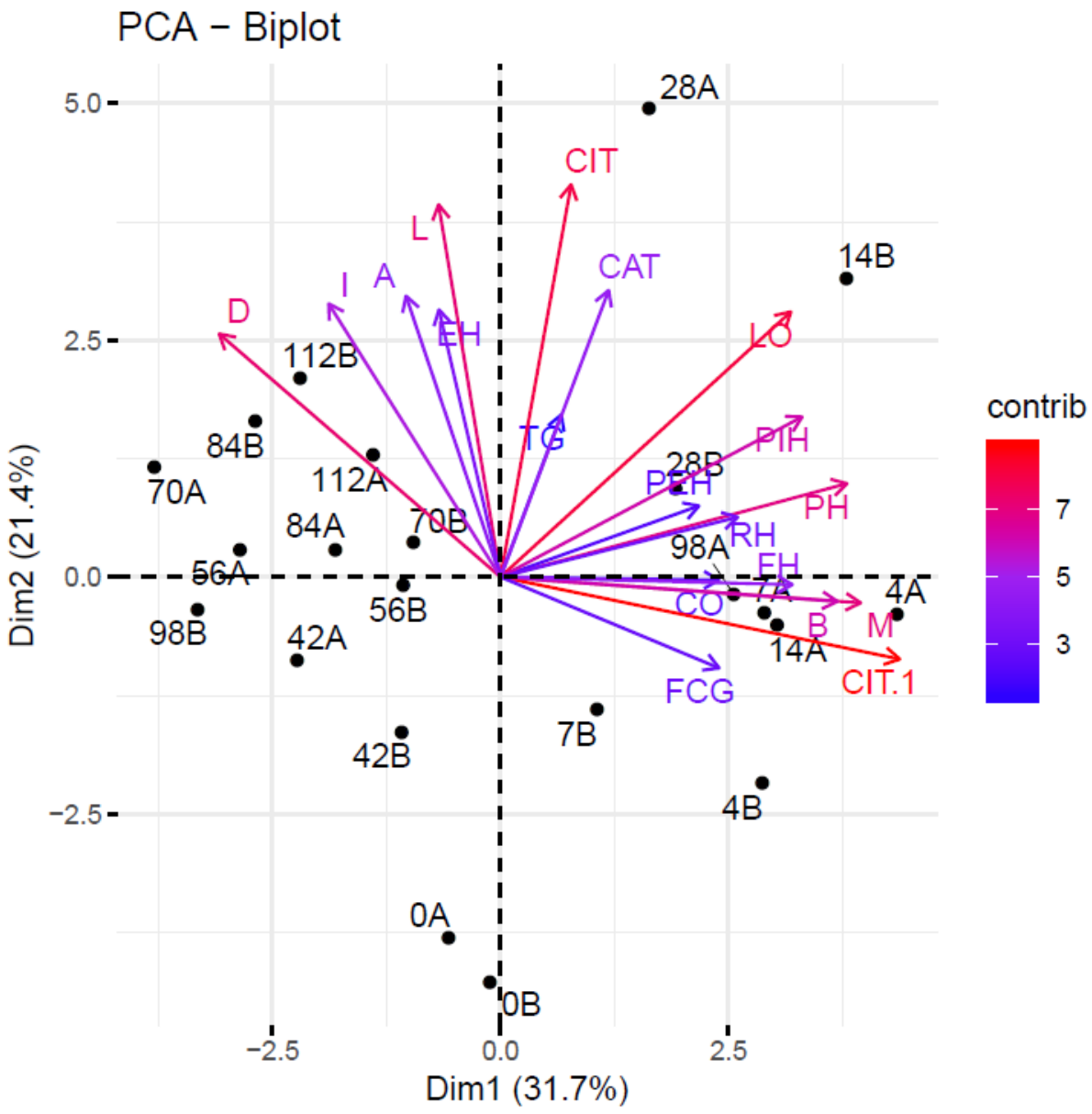


Figure 5.4- Sample set 1, a PCA plot assessing the relationship of sensory and analytical data over time. Days are labelled with data black data points. The label 'A' following a number indicates a 4°C storage temperature and 'B' denotes a 20°C storage temperature during the experiment. The weighted colour scale (right), indicates the contribution of the factor to the model. Red indicates the strongest impact, while blue indicates the weakest impact on the model.

The results of the second sample set were different to the first sample set. The model did not contain as many 'days' data points due to unforeseen circumstances in the sample collection of GC/MS-SPME samples. The peach hop, citrus hop, and bitterness sensory descriptors had the greatest impact on the model as their labels were

coloured red. It was apparent that, despite the weak influence on the model, β-myrcene, coloured in blue, was observed in fresh beer samples of the 4°C and 20°C ageing group. Interestingly, the compound was clustered with 28 days of ageing in both sample groups and could be grouped with 42 days in the 4°C group (42A data point) and 56 days in the 20°C group (56B data point). It is possible that with changes to the recipe and brewing procedures, the samples may have contained a greater concentration of β-myrcene. Variation between hop harvests may have also contributed to the differences in terpene concentration (**Figure 5.5**).

Pine hop and caryophyllene oxide shared a strong relationship as they were found on the same vector. The relationship was also clustered with fresh beer samples of the 4°C sample set as the vector was close to the 7A and 4A data points. Passionfruit hop was observed at 7 days of ageing in the 4°C sample set as the vector was close to the 7A data point. The sensory panel detected floral hop characteristics at 70 days of ageing in both groups as the vector was close to the 70B and the 70A data points. However, floral hop shared a stronger relationship in the 20°C group as the vector was close to the 70A data point (**Figure 5.5**). Bitterness and citrus hop sensory descriptors were apparent in fresh beers in the 20°C age group as the respective vectors passed through the 4B data point. Overall, floral hop, freshly cut grass, astringent, catty hop, and earthy hop characteristics were associated with aged beer at both temperatures (70A and 70B). Pine hop, passionfruit hop, caryophyllene oxide, raw hop, and peach hop characteristics are associated with fresh beer stored cold (7A and 4A) (**Figure 5.5**).

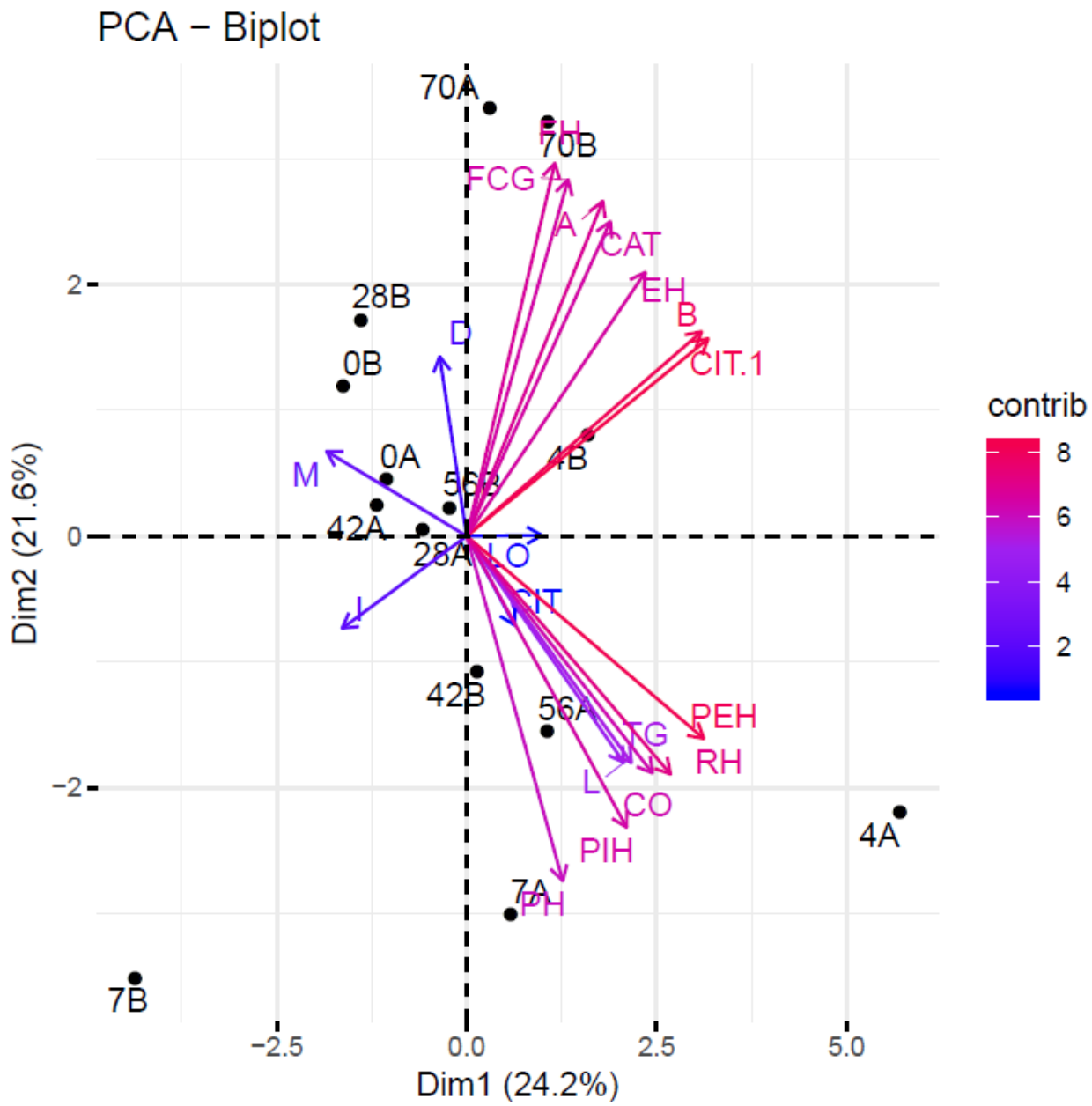


Figure 5.5- Sample set 2, a PCA plot assessing the relationship of sensory and analytical data over time. Days are labelled with data black data points. The label 'A' following a number indicates a 4°C storage temperature and 'B' denotes a 20°C storage temperature during the experiment. The weighted colour scale (right), indicates the contribution of the factor to the model. Red indicates the strongest impact, while blue indicates the weakest impact on the model.

Finally, the third sample set showed a small number of similar trends compared to the previous sample sets, however, a majority of the trends were different. Citrus hop, floral hop, bitterness, raw hop, β-linalool, and β-citronellol all had the greatest weight on the model. Beta-myrcene appeared to be present in fresh beer but was present in higher concentrations in the 20°C group (2B and 7B) than the 4°C group (7A and 14A). The most dramatic difference as a result of ageing was observed at 42 days. In the 20°C

group, linalool oxide was present. In the 4°C group, earthy hop aromas were present. The figure suggests that in the 20°C stored samples, linalool oxide begins to form as the beer hits a 'midpoint' of age as the vector passes through part of the 28B datapoint and ends at the 42B datapoint (**Figure 5.6**).

There was no strong correlation of sensory and analytical data in the third sample set. The correlation observed was β-myrcene and astringent in the right-hand side of principal component 1. Sensory and analytical descriptors though, could easily be clustered together. Beta-linalool, β-citronellol, and *trans*-geraniol shared the same vector line and were strongly related to each other. The previous compounds were also present in samples aged for 84 days in both temperature groups (**Figure 5.6**).

Overall, the earthy hop sensory descriptor was present in all of the samples aged above 42 days. Additionally, β-myrcene was also observed in all of the 'fresh beer' samples, regardless of ageing temperature. In each of the PCA plots constructed, the citrus hop descriptor had the greatest impact on the models (**Figure 5.4**-**Figure 5.6**). In the second and third sample set, raw hop was found to be a prominent sensory characteristic in fresh beers less than 14 days old (**Figure 5.5 and Figure 5.6**).

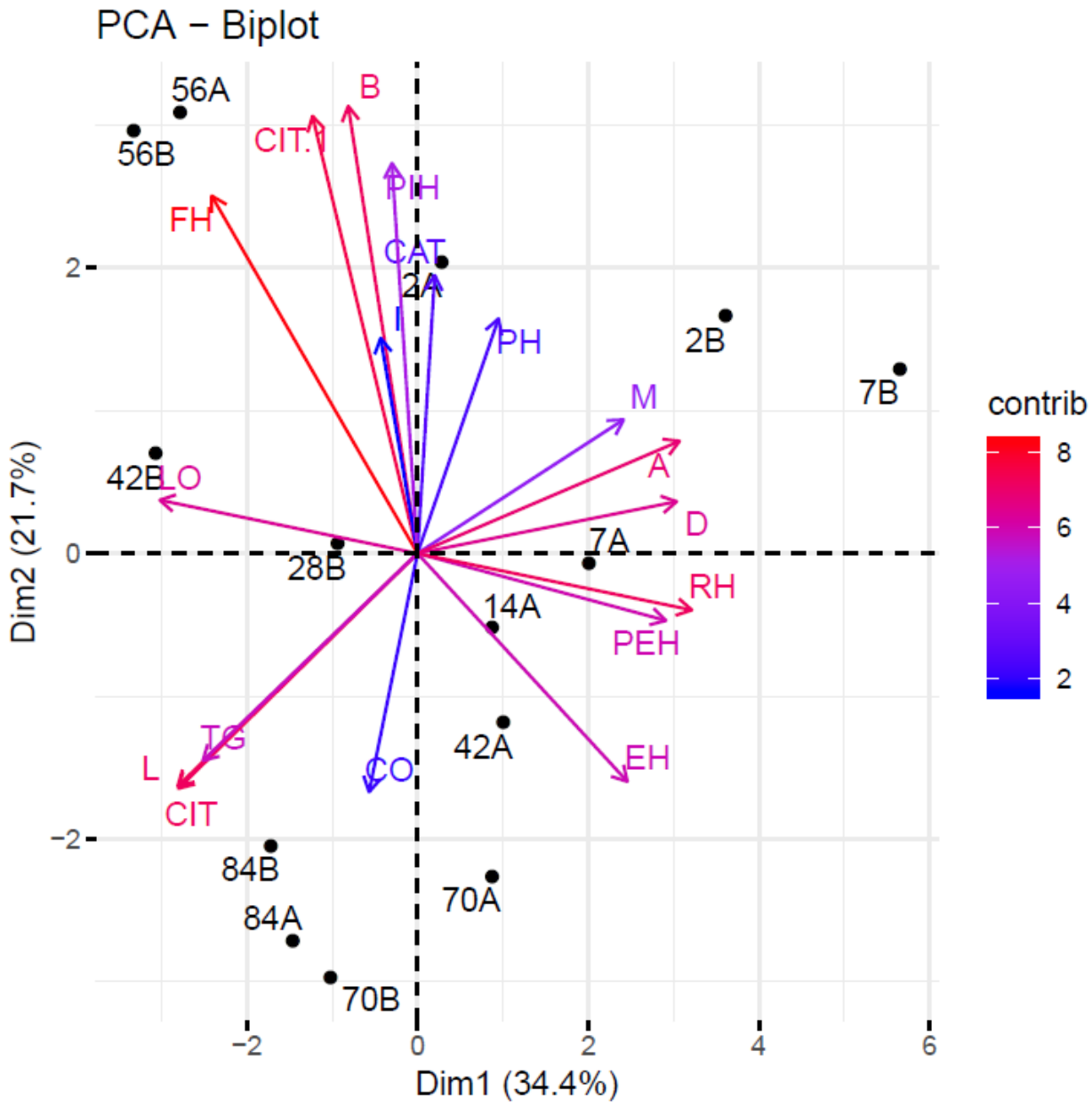


Figure 5.6- Sample set 3, a PCA plot assessing the relationship of sensory and analytical data over time. Days are labelled with data black data points. The label 'A' following a number indicates a 4°C storage temperature and 'B' denotes a 20°C storage temperature during the experiment. The weighted colour scale (right), indicates the contribution of the factor to the model. Red indicates the strongest impact, while blue indicates the weakest impact on the model.

5.3.2 Changes in Terpene/Terpenoid Concentration in Aged Beer

Overall, the compounds measured followed similar trends. Scatterplots were created for only sample set one as this sample set as sample set contained the most comprehensive data set collected. It is important to note that days 14 and 28 are absent from the 20°C data set due to a GC/MS-SPME analytical error. Despite observing small changes overall, interestingly, β-myrcene concentrations reached the highest overall

concentration of 1.79 µg/L after four days of ageing at 4°C while 20°C was at 1.68 µg/L after four days. The β-myrcene concentration declined at a slower rate at 20°C than 4°C as the concentrations were at 1.37 µg/L and 1.25 µg/L, respectively (**Table 5.5** and **Table 5.6**). A spike in β-myrcene at 4°C was observed at 98 days as values rose from 1.28 µg/L to 1.61 µg/L. This spike was not observed in the 20°C sample set (**Figure 5.7** and **Table 5.6**). The rapid increase and decrease could be due to analytical error as β-myrcene concentrations generally declined and the subsequent data point returned to 1.28 µg/L (**Table 5.5**).

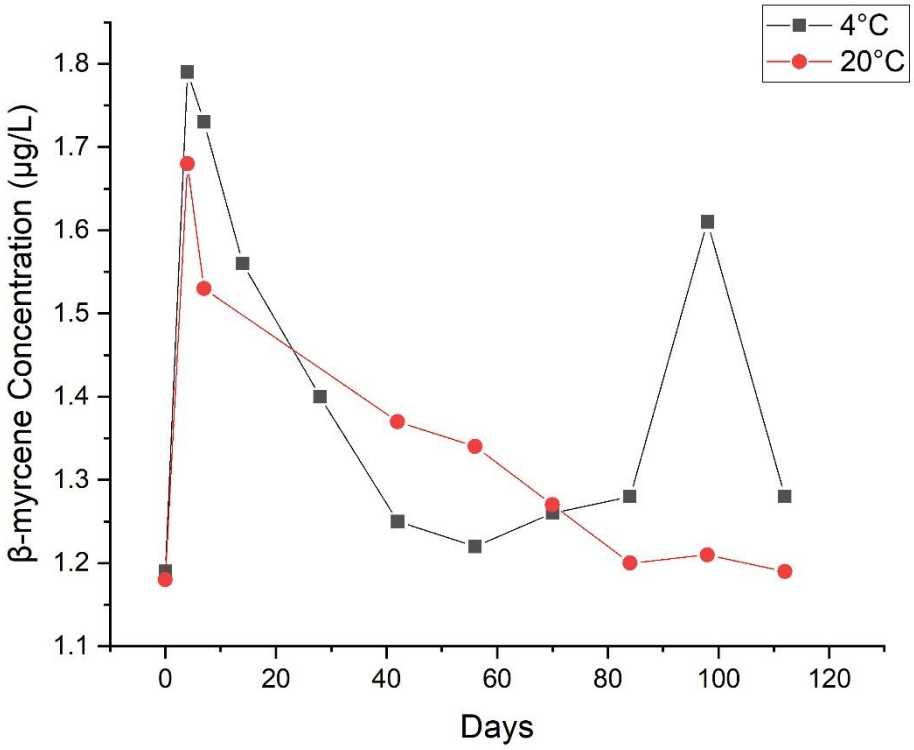


Figure 5.7- A comparison of β-myrcene values in an aged India pale ale over a 112-day time course aged at 4°C and 20°C. Error is accounted for in GC/MS-SPME calibration and validation steps (Section 2.3.4).

The concentrations of linalool oxide, described by raw hop, green, and citrus flavours, increased at 4°C rapidly after two days reaching 17.80 µg/L (**Table 5.3, Table 5.5** and **Figure 5.8**), while 20°C samples were lower at 10.48 µg/L. Both storage temperatures spiked in concentration of linalool oxide however, the concentrations in the 20°C sample declined at a slower rate than the 4°C sample (**Figure 5.8**). Between 56 and 84 days the concentration of linalool oxide in the 20°C samples declined from 11.80 to 11.10 µg/L while the 4°C samples declined from 7.73 to 7.34 µg/L (**Table 5.5 and**

Table 5.6). The 4°C samples increased in concentration from 7.34 to 11.60 µg/L at 98 days but at 112 days, the concentrations levelled off at similar concentrations of 7.17 µg/L (4°C) and 7.59 µg/L (20°C) at 112 days (**Table 5.5, Table 5.6** and **Figure 5.8**). These trends were similar to trends observed in **Figure 5.7**.

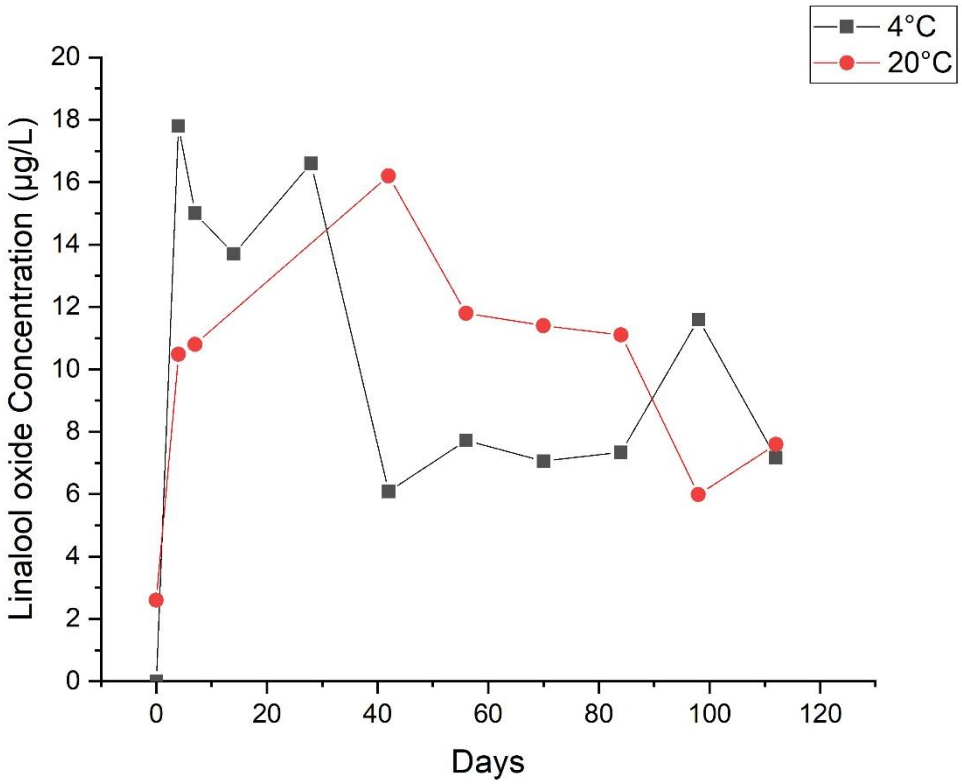


Figure 5.8- A comparison of linalool oxide values in an aged India pale ale over a 112-day time course aged at 4°C and 20°C. Error is accounted for in GC/MS-SPME calibration and validation steps (Section 2.3.4).

Beta-linalool, similar to linalool oxide, is described by floral, fruity, citrus, and coriander-like aromas (**Table 5.3**) When examining both temperatures, the trends of β-linalool initially demonstrated opposite patterns as the 20°C samples declined in concentration while 4°C samples increased in concentration from days zero to seven (**Figure 5.9**). The concentration of β-linalool at 20°C after 42 days of ageing were approximately 10 µg/L higher in concentration than the 4°C samples at 39.80 µg/L (**Table 5.5, Table 5.6**, and **Figure 5.9**). The concentration of β-linalool at 20°C consistently varied in concentration throughout the ageing trial as three spikes in concentration were observed at 42 days, 84 days, and 112 days (**Figure 5.9**). Interestingly, the values of β-linalool after 112 days of ageing at both temperatures were

similar in concentration at 34.60 µg/L (4°C) and 35.60 µg/L (20°C) (**Table 5.5, Table 5.6**, and **Figure 5.9**).

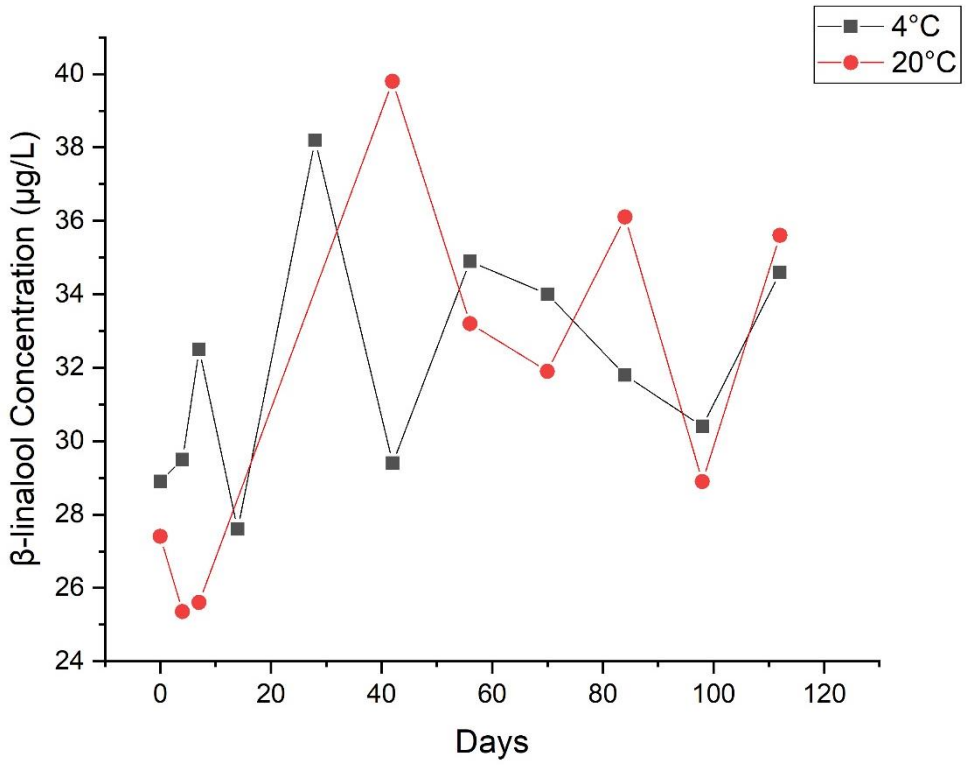


Figure 5.9- A comparison of β-linalool values in an aged India pale ale over a 112-day time course aged at 4°C and 20°C. Error is accounted for in GC/MS-SPME calibration and validation steps (Section 2.3.4).

Beta-citronellol is a compound that is described as containing floral, citrus, rose, lime, and waxy-characters (**Table 5.3**). Beta-citronellol concentrations saw greater increases in 20°C samples than 4°C samples reaching peak concentration of 27.90 µg/L after 42 days of ageing (**Table 5.6** and **Figure 5.10**). Following this, the concentration of β-citronellol slowly decreased until the 98 day timepoint. At this point, the concentration rose from 24.10 µg/L to 25.40 µg/L at 112 days (**Table 5.6** and **Figure 5.10**). The concentration of β-citronellol declined to 24.00 µg/L after a peak of 27.30 µg/L at 28 days in the 4°C samples (**Table 5.5** and **Figure 5.10**). Following this, the 4°C samples saw slight decreases in concentration reporting values at 24.90 µg/L after 112 days of ageing (**Table 5.5** and **Figure 5.10**).

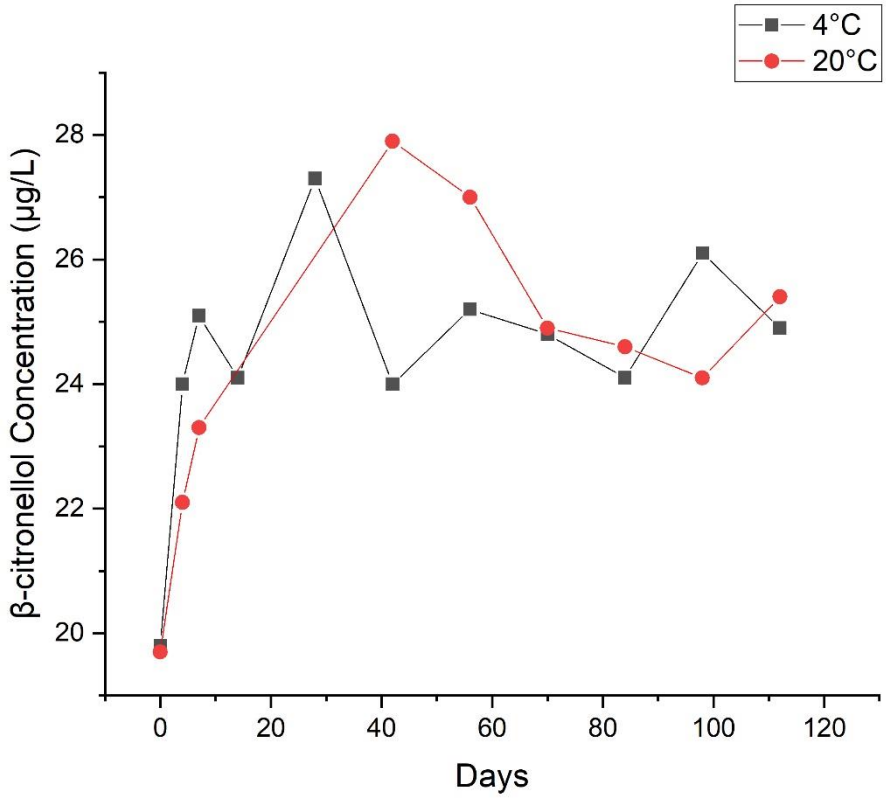


Figure 5.10- A comparison of β-citronellol values in an aged India pale ale over a 112-day time course aged at 4°C and 20°C. Error is accounted for in GC/MS-SPME calibration and validation steps (Section 2.3.4).

Described by floral, rose, geranium, and perfume-like attributes, *trans*-geraniol reported erratic changes in concentration (**Table 5.3, Table 5.5** and **Table 5.6**). After seven days of ageing, *trans*-geraniol concentrations in 4°C and 20°C samples reported contrasting trends as the 4°C samples increased from 66.70 µg/L to 81.10 µg/L (**Table 5.5** and **Figure 5.11)**. While the 20°C samples declined from 63.95 µg/L to 52.90 µg/L (**Table 5.6** and **Figure 5.11**). A large spike was noted in the 20°C samples from seven to 42 days as the concentrations increased from 52.90 µg/L to 103 µg/L (**Table 5.6** and **Figure 5.11**). The 4°C samples followed similar trends but a peak in concentration at 28 days reported values of 97.80 µg/L. Following this, the concentration of *trans*-geraniol substantially decreased to 66.00 µg/L (**Table 5.5** and **Figure 5.11**). The gradual decrease in concentration in the 20°C samples first dropped to 93.70 µg/L at 56 days followed by a drop to 63.10 µg/L at 70 days. Following the 70 days, the 20°C samples overall, increased in concentration at 112 days reporting values at 78.70 µg/L (**Table 5.6** and **Figure 5.11**). Following the decline from day 56, erratic changes in the 4°C

samples were observed as samples repeatedly increased and subsequently decreased (**Figure 5.11**).

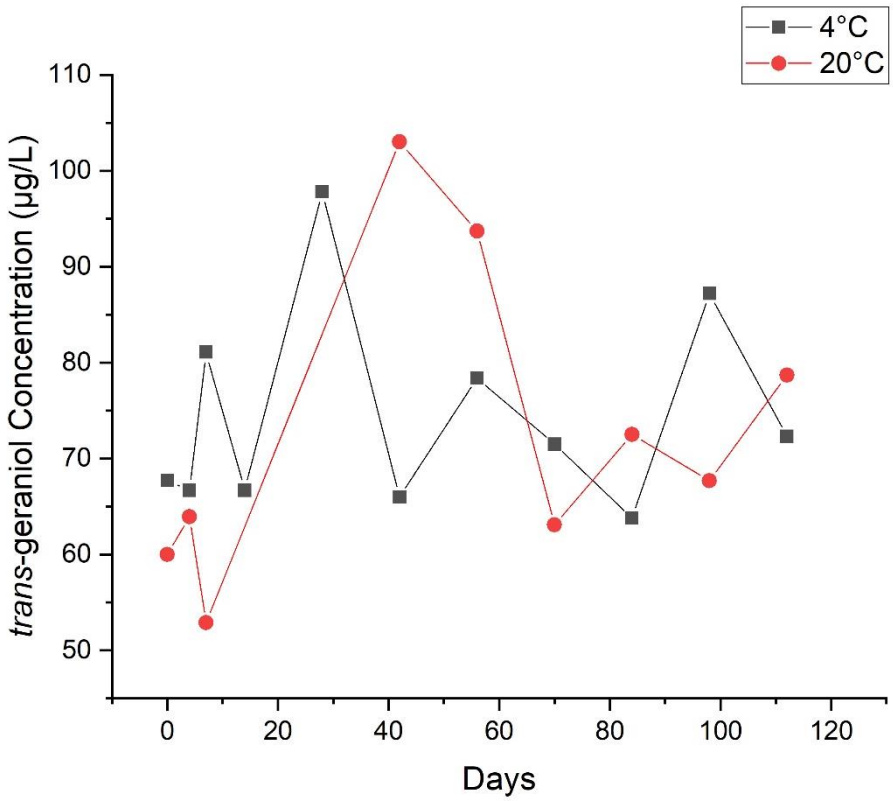


Figure 5.11- A comparison of *trans*-geraniol values in an aged India pale ale over a 112-day time course aged at 4°C and 20°C. Error is accounted for in GC/MS-SPME calibration and validation steps (Section 2.3.4).

Oxygenated sesquiterpenoid, caryophyllene oxide, is a common 'spicy hop' descriptor with descriptors of musty, spicy, and floral (**Table 5.3**) (Praet *et al.*, 2016b). Caryophyllene oxide concentrations aged at 4°C, did not show any robust trends or changes during the ageing trial (**Figure 5.12**). Overall, the caryophyllene oxide concentration decreased from 1.59 µg/L to 1.00 µg/L in the 4°C samples (**Table 5.5**). However, the 20°C samples reported a substantial increase in concentration from day seven to 56 with values increasing from 0.83 µg/L to 3.70 µg/L. Following the considerable increase in concentration, the concentration dropped to zero at day 70- though this was likely due to analytical error. Following this error, values rose from day 70 to 0.95 µg/L at 112 days (**Figure 5.12**).

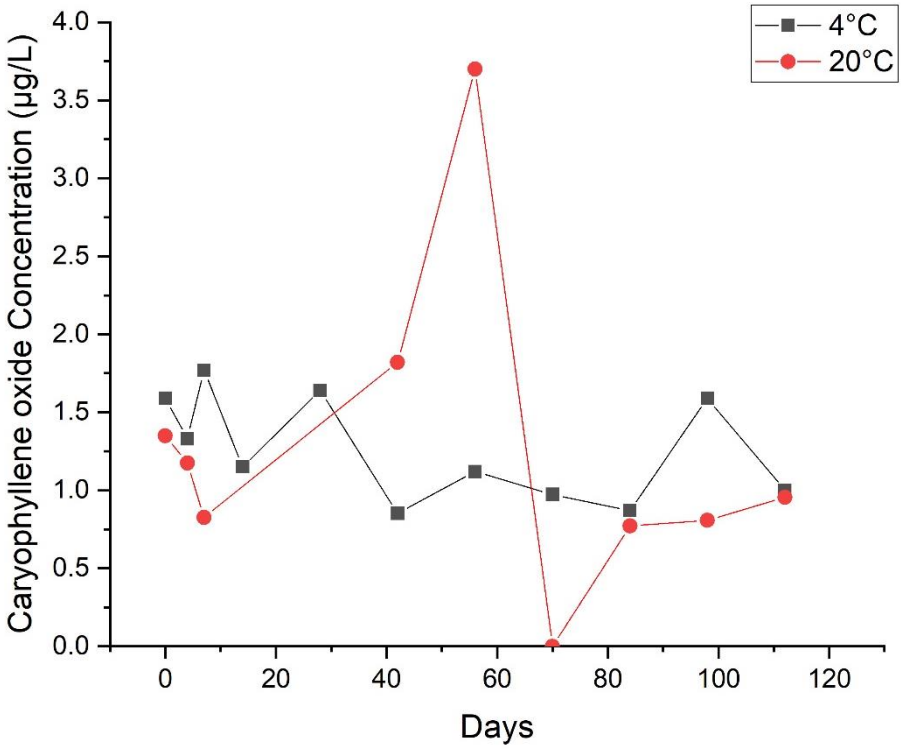


Figure 5.12- A comparison of caryophyllene oxide values in an aged India pale ale over a 112-day time course aged at 4°C and 20°C. Error is accounted for in GC/MS-SPME calibration and validation steps (Section 2.3.4).

5.4 Discussion

5.4.1 Correlating Sensory and Analytical Data

The results show that it is not always possible to pair sensory data with analytical data as typical sensory descriptors do not always pair with their described/respective chemical compound, particularly in a mix of compounds. Beta-caryophyllene and α-humulene were required to be removed from all of the analyses as the compounds were not detected in GC/MS-SPME. This could be due to the low concentrations of the compounds in the varieties used for dry-hop additions and poor solubility of the compounds (**Table 5.5**).

Despite robust sensory training, the erratic results show that some sensory data may not always correlate to analytical data. If sensory and analytical data correlations are used as a quality check for overall beer aroma, both scores must be consistently checked and measured upon analysis.

Sensory and analytical data are difficult to pair for several reasons. First, the human senses are extremely subjective and are influenced by factors such as diet,

hormones, stress, and health status (Goldstein *et al.,* 2014). Second, a consequence of the high sensitivity of instrumental analysis is the variability of results obtained from replicate samples. Another difficulty of correlating sensory and analytical data in this particular experiment was the concentration of the volatiles. Hop volatiles in finished beer exist at low levels, causing difficulties in quantification (Peppard *et al.,* 1989). Another limitation of the study was the alteration of recipes and brewing regimes throughout the study. Because of this, cross-comparison of the results was not entirely representative.

Hop-derived compounds are present in beer at a range of concentrations from ng/L to mg/L (Rettberg *et al.,* 2018). However, hop compounds are present in beer in concentrations that are difficult for sensory panellists to detect due to factors such as compound-specific anosmia, high sensory threshold values, or environmental desensitisation (Meilgaard, 1993). Compounds such as linalool have been reported to only provide minor impacts to dry-hopped beer aroma (Peacock *et al.,* 1981) but to exist above sensory threshold values with kettle-hopping and dry-hopping techniques (Biendl *et al.*, 2014).

It well is established that each hop variety will consist of a different composition of essential oils and bitter acids (Sharpe *et al.*, 1981; Biendl *et al.*, 2014; Almaguer *et al.,* 2015). As the raw materials were derived from various agricultural origins, each harvest year is compositionally different which, most likely, affected the results (Likens *et al.*, 1967). In addition, hops are grown in various countries and climates causing each hop variety to be compositionally different in essential oil content, even when growing the same varieties. Studies have confirmed existing differences in essential oil content in European and non-European varieties (Kenny, 1987; Perpète *et al.*, 1998). Finally, when hops are aged, terpene content slowly declines over time (Lam *et al.*, 1986).

Only sample set one exhibited an increase in the damascenone sensory characteristic. Typically, β-damascenone increases as beer ages, it has a distinctive, sweet, berry, honey-like flavour with a low flavour threshold in water (20-90 ng/L) contributing a strong sensory impact (De Schutter *et al.,* 2009; Rettberg *et al.,* 2018). Glycosidically-bound β-damascenone found in malt and hops is released upon ageing, it is present due to biotransformation via β-glucosidase or acid-catalyzed conversions at low pH (Chevance *et al.*, 2002; Vanderhaegen *et al.*, 2006; Biendl *et al.*, 2014). In the first sample set, it was apparent that β- damascenone was closely related to age as the

sensory descriptor was clustered with longer ageing times. It was interesting that sample sets two and three did not show strong relationships between age and damascenone characters. It is possible though, that the change in recipe/brewing parameters had a great enough impact on aroma that as beer aged, other analytes synergistically changed the aroma of damascenone to represent a different beer aroma such as 'floral hop' (**Figure 5.5** and **Figure 5.6**).

A compound that correlated with 'catty hop' in sample set 1 was *trans*-geraniol (**Figure 5.4**). The results were curious as *trans*-geraniol is typically associated with floral, rose-like aromas (Zunkel, 2015). However, it is possible that *trans*-geraniol may have had synergistic or additive effects with another aromatic component in beer to produce a 'catty hop' aroma. This postulation is supported by the fact that the monoterpene oxide has been proven to influence the aroma of other compounds. Studies show that less than 10 µg/L of *trans*-geraniol in addition to β-citronellol influence the aromatic characteristics of β-linalool (Biendl *et al.*, 2014). Additionally, aromatic compounds have also been proven to cover up or 'mask' other flavours/aromas in beer (Kaltner *et al.*, 2013). This phenomenon occurs when a compound containing a very low sensory threshold is present in beer at a high concentration, completely overpowering and masking other aromatic components present. Due to this, sensory panellists may not be able to detect all of the compounds present in the beer due to an abundance of one particular compound.

Despite this, some of the observations from the study were expected. For example, the monoterpene β-myrcene is usually present in very low levels in dry-hopped beer but is a principal component of the essential oil in hop cones (Biendl *et al.*, 2014; Rettberg *et al.*, 2018). In sensory analysis, the compound β-myrcene is commonly described as a vegetable-like, raw hop, resinous, or grassy aroma and has an impactful contribution to 'fresh' hop aromas (**Table 5.3**). The monoterpene is extremely volatile and unstable in packaging undergoing autoxidation, scavenging into crown cap liners, and in some cases, biotransformation (Rettberg *et al.*, 2018). Due to this, the compound is only present in freshly packaged products. Generally, a relationship between β-myrcene and fresh beer (beer less than 14 days aged) was observed in a majority of the samples (**Figure 5.4**- **Figure 5.6**).

The change in β-myrcene's impact across the sample sets could be due to the changes to the recipe and brewing procedures by the collaborating brewery. The

changes may have impacted the concentration of β-myrcene in the final product. It is also possible that the sample collection procedures were fine-tuned, and samples were immediately placed in the freezer following collection. The fast-freezing prevented the loss of detectable, volatile β-myrcene contained within the sample.

The variance of panellist assessment is an important factor to consider when examining the results. The sensory lexicon for each panellist will be unique as smells, tastes, and flavour are perceived in a unique sense by each panellist. Training is put in place in an attempt to remove or mitigate against bias however, a panellist's sensitivity will vary day to day as aromatic compounds are detected differently in various products (Goldstein *et al.*, 2014; Peltz *et al.*, 2017).

Another possible explanation for the discrepancies in the replicates and the lack of cohesive, firm conclusions could be a result of the concentration of the hop compounds in the sample. Sensory threshold values are different for each compound tested. In some cases, threshold values persist at exceptionally low levels in the ng/L range, such as β-damascenone. Other thresholds are quite high, such as β-myrcene, ranging from 30-100 µg/L (Rettberg *et al.*, 2018). As panellists input more efforts to detect aromas at low concentrations, sensory fatigue or selective adaptation, is another factor that should be taken into consideration.

Selective adaptation (sometimes called sensory fatigue) is the psychological observation that as a stimulus is continuously presented, the response to the stimulus is reduced. In this case, the beer sample is presented and neurons in the brain fire in response to the stimulus (beer). As the beer is continually assessed by the panellist, the neuron firing rate of the panellist will decrease or the neurons will fire less when the sample is presented (Goldstein *et al.*, 2014). With this, panellists continuous assessment of the beer may have altered individual sensory performances. This theory paired with high/low sensory threshold values provide further evidence that the compounds present in the beer may have altered panellist performance. Further work is required to properly explain and pair sensory data with analytical data.

Finally, across all of the PCA plots constructed, the citrus hop descriptor had the greatest impact as the descriptor was coloured red in each of the models (**Figure 5.4**-**Figure 5.6**). This may indicate that the citrus hop descriptor provided more information as to how beer ages than previously anticipated. It is postulated that the disappearance

of the citrus hop descriptor could mark the point at which beer no longer contains 'fresh' aromas (**Figure 5.4**-**Figure 5.5**). Alternatively, the appearance in citrus hop characteristics could also be linked to important biochemical reactions in bottle (**Figure 5.6**). It would be advantageous in future analyses to trace the evolution of the citrus hop aroma characteristic to understand its reactions in bottle.

5.4.2 Changes in Terpene/Terpenoid Concentration in Aged Beer

When the concentration of individual terpenes and terpenoids in packaged beers were assessed at 4°C and 20°C over a 112-day time course, various trends were observed. Aside from a spike of 1.28 μg/L to 1.61 μg/L in the 4°C sample at 98 days, β-myrcene slowly declined in concentration after four days ageing at both temperatures (**Figure 5.7**). The drastic increase in *trans*-geraniol and β-linalool at 42 days of ageing at both temperatures was intriguing (**Figure 5.9** and **Figure 5.11**). During kilning and aerobic hop storage β-myrcene autoxidises, forming terpenoids such as *trans*-geraniol and β-linalool (Rettberg *et al.*, 2018). It is possible that β-myrcene reacts with packaged oxygen in bottles to yield an increase in *trans*-geraniol and β-linalool concentrations.

As discussions regarding the diastatic power of hops have recently resurged, it is possible that enzymes contained within the lupulin glands of hops may have an effect on the change in hop aroma over time (Kirkendall *et al.*, 2018; Kirkpatrick *et al.*, 2018). It is hypothesised that residual enzymes derived from hops may be present in the solution at very low concentration levels. While the hypothesised enzymes will not cause a dramatic effect, it is possible that enough activity is initially present to alter terpene/ terpenoid concentrations.

Linalool oxide and β-linalool concentrations both increased after 42 days of ageing in the 20°C samples. The increase of β-linalool could have resulted from the hydration of β-myrcene and linalool oxide from an oxidation reaction (Peacock *et al.*, 1981; Almaguer *et al.*, 2014). The 4°C sample saw a spike of linalool oxide after only four days of ageing while β-linalool spiked after 42 days of ageing. In this case, it is possible that a small portion of the β-linalool oxidised shortly after bottling. The oxidation contributed to the spike in linalool oxide observed (Peacock *et al.*, 1981) (**Figure 5.8** and **Figure 5.9**).

Yeast biotransformation during fermentation has documented changes in terpene/terpenoid concentration in beer (King *et al.*, 2003; Praet *et al.*, 2012). Geraniol

is reduced by yeast to form citronellol and in some cases, linalool (King *et al.*, 2003; Takoi *et al.*, 2010). It is possible that residual yeast-derived enzymes converted *trans*-geraniol to β-citronellol, as the concentration of β-citronellol increased in the first four data points at 20°C and 4°C (**Figure 5.10** and **Figure 5.11**). However, the event is highly unlikely with the filtration and packaging processes at the brewery concerned in this study.

Caryophyllene oxide was suspected to be in dry-hopped beer as the compound is an oxidation product of β-caryophyllene. Additionally, caryophyllene oxide is susceptible to hydrolysis, oxidation or isomerisation during wort boiling, which may yield other chemical compounds (Yang *et al.*, 1993; Praet *et al.*, 2014; Praet *et al.*, 2016a). This could explain the consistent values that were observed in the samples stored at 4°C.

It was interesting that sample set one and sample set two correlated well with the sensory descriptors (pine hop and earthy hop) of caryophyllene oxide precursor compound, β-caryophyllene (**Table 5.3**, **Figure 5.4** and **Figure 5.5**) (Praet *et al.*, 2016b).

However, caryophyllene oxide is reported to form upon boiling of hop-oil in a laboratory scale (Praet *et al.*, 2015). While the storage temperature of samples were far from boiling point and the study was conducted in beer, the extended storage time (56 days) at 20°C may have encouraged the formation of the sesquiterpene oxide, caryophyllene oxide. The storage at cold temperatures (4°C) prevented the formation of sesquiterpene oxides to occur. The reported value of zero at 70 days was due to analytical sampling errors in GC/MS-SPME analysis (**Figure 5.12**).

There were several occasions where a chromatogram appeared blank, indicating that the ion source in the GC/MS-SPME had failed. It is for this reason why some ‘days’ data points are missing in the figures. The issue was assessed post-run to ensure future analyses ran smoothly. Unfortunately, destructive sampling upon GC/MS-SPME analysis resulted in samples that could not be reused.

Finally, while sampling and studying products produced by an industry partner is beneficial for practical application, difficulties exist in collecting complete and cohesive data sets. Changes in products and recipes are expected in the brewing industry as availability and quality of raw materials will vary each year. However, this poses a

challenge in producing true replicates for cross comparison. Also, sample collection/integrity can be compromised with inconsistent sampling and laboratory techniques.

The results demonstrated that temperature influenced the change in terpene/terpenoid concentration but overall, substantial differences did not exist between 4°C and 20°C samples. Total packaged oxygen was also kept to a minimum during the study (TPO values did not exceed 50 ppb) and should have had less of an impact than previous studies with the advancement of brewing technologies (Peacock *et al.*, 1981). It would be beneficial to rerun the study to confirm the trends observed in addition to searching for new trends and correlations between replicates.

Chapter 6- Dry-Hop Conditions Effect on Hop Oil and Terpene/Terpenoid Extraction

6.1 Introduction

Hops add complexity to beer providing foam stability, bitterness, flavour/aroma, and can also contribute to mouthfeel (Section 1.3) (Ting *et al.*, 2017). The main component contributing to flavour/aroma in hops are terpenes and terpenoids, these are crucial components of hop aroma. Without terpenes, beer, particularly dry-hopped beer would not have the characteristic fruity, spicy, or herbal aromas that it has been reported to contain (Inui *et al.*, 2013; Almaguer *et al.*, 2014; Praet *et al.*, 2014; Praet *et al.*, 2016a; Ting *et al.*, 2017; Rettberg *et al.*, 2018).

Terpenes consist of three main classes of compounds- hydrocarbons, oxygenated compounds, and sulphur-containing compounds. All of these are contained in hop oil fraction (Almaguer *et al.*, 2014). Terpenes are defined in Section 1.3.4. While sulphur-containing compounds are highly odour-active hop constituents, they are also very volatile and, therefore, difficult to measure with accuracy (Almaguer *et al.*, 2014; Rettberg *et al.*, 2018). Typically, the oxygenated compounds and hydrocarbon fractions have lower aroma threshold values but can be quantified more accurately utilising analytical chemistry techniques.

In the steam distillation of hops, monoterpenes (i.e. β-myrcene, limonene, etc.) and sesquiterpenes (i.e. β-caryophyllene, α-humulene, etc.) are found in the hydrocarbon fraction of hop essential oil while terpene alcohols are found in the fraction containing oxygenated terpene/terpenoid compounds (Almaguer *et al.*, 2014; Biendl *et al.*, 2014). Due to the lack of hydroxyl functionality, monoterpenes and sesquiterpenes are less polar than terpene alcohols and have been reported to be less soluble in water than terpene alcohols (Haslbeck *et al.*, 2018; Rettberg *et al.*, 2018). Despite this, solubility of hydrocarbons and some oxygenated components may increase in solutions containing ethanol as ethanol can solubilise both polar and non-polar constituents (Section 1.3).

The purpose of dry-hopping (Section 1.2.9) is to extract volatile aromatic components from hops into beer to enhance hoppy flavours/aromas in beer. The volatile aromatic components mainly consist of several classes of terpenes/terpenoids. Hop volatile compounds are reduced during the kettle boil (De Keukeleire, 2000). These

components provide citrusy, spicy or resinous characteristics to beer that otherwise, could not be obtained (Goiris *et al.*, 2002; Wang *et al.*, 2008; Takoi *et al.*, 2012; Almaguer *et al.*, 2014).

Studies have begun to try to explain the rate at which terpene and terpenoid components are extracted into wort and beer, however, many factors influence the transfer of these compounds (Haslbeck *et al.*, 2018). Ethanol concentration, extraction time, extraction temperature, and dose are factors that impact the extraction of terpene/terpenoid compounds in beer (Wolfe *et al.*, 2012).

As a weakly polar compound, ethanol demonstrates both hydrophilic and lipophilic properties which aid in extracting terpenes and terpenoids (Haslbeck, Minkenberg and Coelhan, 2018, Hinz *et al.*, 2016). It is postulated that increasing ethanol concentration increases the solubility of terpene alcohols and decrease the solubility of monoterpene and sesquiterpenes.

The length of dry-hopping is a difficult parameter to set for industry wide 'best practices' as shape, design, and volume of each brewery are different. This directly effects the hop dose rate, temperature of extraction and dry-hopping duration. As previously discussed in Chapter 5, the terpene/terpenoid composition of each hop variety will undoubtedly be different. Due to this, it is crucial that the desired aroma profile and the chemistry of the aroma-active compounds remain at the forefront during dry-hop selection. If brewers schedule dry-hopping procedures with this in mind, there is a greater potential for targeted aroma-profiles to be obtained.

With regard to dry-hop duration, Wolfe (2012) noted that, terpene and terpenoid extraction did not increase with time but rather, remained stable. This indicates that a large portion of the aroma-active terpenes and terpenoids were not extracted but remained in the spent hop material after extraction. However, this could also indicate that the compounds had been fully extracted.

Temperature is important in every function in the brewery, and plays a key role in dry-hopping (Briggs *et al.*, 2004; Heuberger *et al.*, 2012). Dry-hopping at warmer temperatures may encourage yeast-mediated biotransformation from residual yeast in the fermenter. This releases glycosidically bound terpene alcohols, but also encourages the activity of dextrin hydrolysing enzymes within the hops (King *et al.*, 2003; Tamura *et al.*, 2005; Sharp *et al.*, 2017; Haslbeck *et al.*, 2018; Kirkpatrick *et al.*, 2018; Rettberg

et al., 2018). Solubility is also increased with the increase of temperature and could impact terpene/terpenoid extraction.

Finally, dose rates have been long debated in dry-hopping regimes. Recent studies have found that not all terpenes and terpenoids are extracted linearly with respect to increasing hop dose. The same study found that overall hop oil content does not correlate to beer with stronger perceived hoppy aromas (Vollmer *et al.*, 2016). Additionally, the extraction rate of some terpenic compounds have been observed to decrease with increased hopping rates (Wolfe *et al.*, 2012; Haslbeck *et al.*, 2018).

As discussed in Chapter 5, cross-varietal differences of terpene composition and concentration can be determined. This should be considered when selecting dry-hops. Dry-hopped beers depend upon the terpene/terpenoid containing essential oil fraction to boost floral, spicy, and citrus hop aromas in beer (Section 1.3.2). For example, hop varieties such as Chinook contain more than three times the concentration of the cyclic monoterpene, humulene. Humulene may be more soluble in ethanol than the terpene alcohol, linalool, due to differences in polarity (Kenny, 1987). Thus, certain aroma compounds could be selectively extracted by ethanol to influence flavour.

To understand the stability of hop aroma it is important to understand how hops are extracted into beer and how their chemistry might affect this process. However, the inherent question with dry-hopping lies in the efficiency of the process. How much of the terpene-containing hop oil is transferred into beer and how much hop oil remains in the spent hops? It is important to consider these factors when designing an aroma profile. Further studies can assess the aromatic stability of the terpenes/terpenoids extracted from hop oils. Ultimately, factors such as ethanol content, exposure time, temperature and dose rate can influence each other. In this study, a fractional four-factorial experiment was designed using a design of experiments (DoE) software package, Design-Expert® (Stat-Ease Inc., Minneapolis, US). A similar industry-based study has been conducted assessing how dry-hopping is affected by various process parameters in beer (Holbrook, 2015). However, this study did not undertake preliminary studies in beer matrices and did not consider how the solubility of terpenes/terpenoids would be affected by their respective chemical composition. The aim of this chapter was to understand how ethanol content, exposure time, temperature and dose rate affect the dry-hopping system as a whole and to assess how terpene extraction might be affected

by this process. Later studies assessed how hop dose and ethanol might affect terpene/terpenoid solubility.

6.2 Experimental Design

6.2.1 Developing a Fractional Four-Factorial Experiment Using Design of Experiments

The objective of the experiment was to understand how ethanol content, exposure time, temperature and dose influence the amount of hop oil extracted into an acidified beer matrix. A fractional four-factorial experiment was designed utilising Design of Experiments (DoE) software, Design Expert, Version 8 (Stat-Ease Inc., Minneapolis, US). The software calculated a minimum of 15 tests required for the model. Replicates and midpoints were added to bring the total number of tests to 34, making the model more statistically robust (**Figure 6.1** and **Table 2.7**).

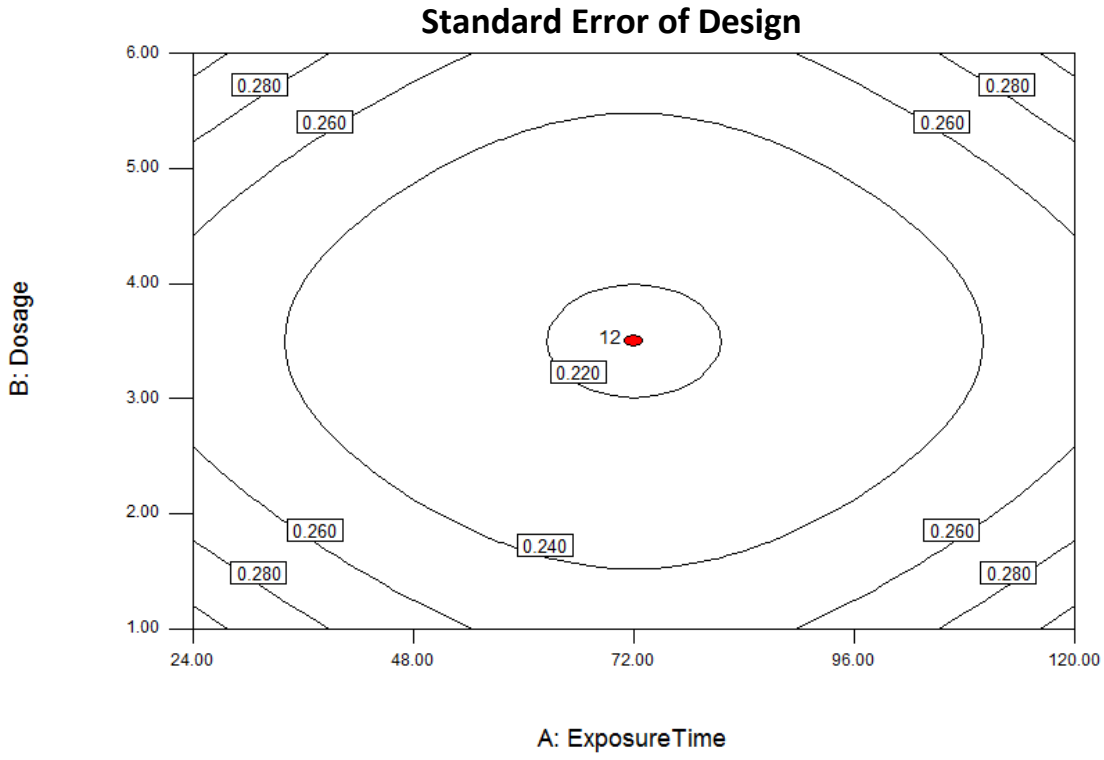


Figure 6.1- Exposure time-dosage response figure demonstrating statistical robustness (Standard Error) of experiments comparing (A) exposure time in minutes and (B) dosage in g/L.

6.2.2 Sample Preparation

Based on the DoE, a workflow for experiments was established (**Figure 6.2**). A simulated beer matrix was made by degassing and acidifying 4.5 L of distilled water with a 1 M solution of citric acid (Sigma Aldrich, Poole, UK). The simulated beer

matrix was fixed to the appropriate ethanol concentration (%ABV) in Duran bottles (Sigma Aldrich, Poole, UK) from which the headspace had been purged with nitrogen to remove oxygen. The appropriate amount of hops, as determined by the DoE, were added to each bottle and incubated (**Figure 6.2**). Following this, each sample was filtered using Whatman Grade 1 filter paper (GE Healthcare Inc., Chicago, US). The filtrate and the filter paper were retained for further analysis (Section 2.2.1).

6.2.3 Measuring Hop Oil in Simulated Beer Matrix

Liquid-liquid extraction was utilised to separate the hop oil from the aqueous fraction collected in Section 6.2.2. Dichloromethane (DCM) (Fisher Scientific International Inc., US) has better extraction efficiency than ionic liquids and was selected as the solvent (Xinmei *et al.*, 2006). As the hop oils under consideration are largely non-polar it was suspected that the compounds were soluble in organic solvents, DCM was considered to be a suitable solvent. As the simulated beer matrix consisted mainly of water and a low percentage of ethanol, DCM was able to remove hop oils from water. Liquid-liquid extraction was used to chemically separate soluble terpenes within the organic (DCM) phase from other, immiscible components found in the simulated beer matrix. The full protocol is described in Section 2.4.3. The difference pre and post-extraction was calculated as an estimation of the total amount of hop oil extracted in the simulated beer matrix.

6.2.4 Measuring Residual Hop Oil in Spent Hops- Soxhlet Extraction

Soxhlet extractions were utilised to assess the lipid content remaining in spent hops (e.g. hop oil). The filter paper containing the spent hop material from Section 6.2.2 was dried, folded and inserted into a Soxhlet crucible that had been defatted in hexane (Sigma Aldrich, Poole, UK). The crucible was weighed and placed into a Soxhlet extraction apparatus. A round-bottom flask was weighed and 200 mL of hexane was decanted into the round-bottom flask for extraction. The Soxhlet apparatus was continuously run for 6 hours to extract the hop oil that remained in the spent hops (Section 2.2.2).

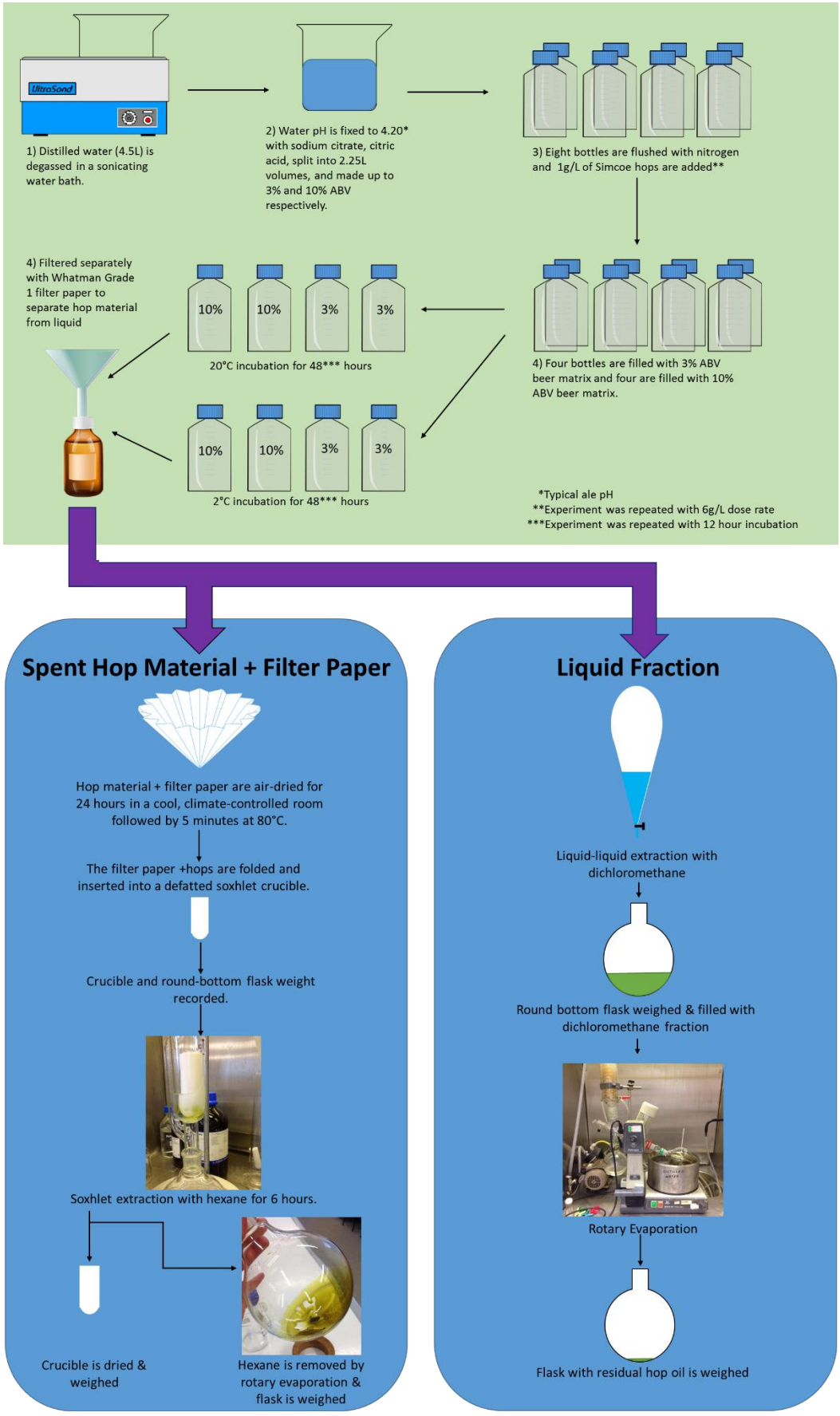


Figure 6.2- Experimental workflow of fractional four-factorial experiments.

Following extraction, the crucible was removed and dried. The crucible was weighed and the difference pre minus post extraction weight was calculated, to assess the lipids lost from the crucible. As a secondary measurement of lipids lost in the crucible, the hexane was removed by rotary evaporation and the round-bottom flask was weighed to assess the hop oil recovered from crucible in hexane (Section 2.4.2).

6.2.5 Targeted Study-Impact of Hop Dose and Ethanol Content on Terpene Extraction

The data gathered from the fractional four-factorial study provided results that contained a high amount of uncertainty (Section 6.3.1). Due to this, a second study was designed. In the targeted study, two of the parameters- ethanol content (%ABV) and hop oil extracted from the crucible- showed a slight positive correlation (**Figure 6.3**), indicating that a relationship may exist between the two variables. Therefore, a targeted study was designed to measure ethanol's effect on terpene extraction in relation to hop dose.

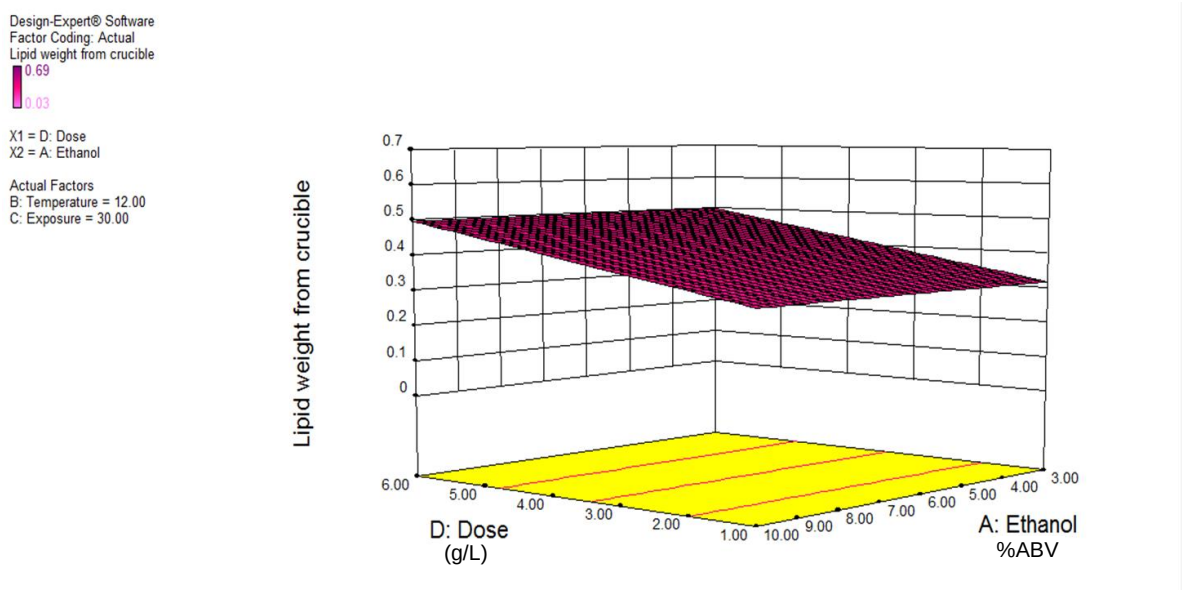


Figure 6.3- Surface response diagram comparing hop dosage (g/L), ethanol content (%ABV), temperature (°C), and exposure time (hours).

The experiment was designed to test the effect of increasing hop dose and ethanol content in both stirred and unstirred settings, across two hop varieties, to assess the effectiveness of terpene and terpenoid extraction in closed systems (**Figure 6.4**).

Samples were prepared according to the method outlined in Section 2.4.4. The experiment was conducted using Simcoe and Chinook hop varieties as their respective terpene composition differ substantially (**Figure 6.5**).

Following incubation, samples were collected and the concentration of hop terpenes/terpenoids were measured by GC/MS-SPME analysis (Section 2.3.6) and assessed by one-phase decay.

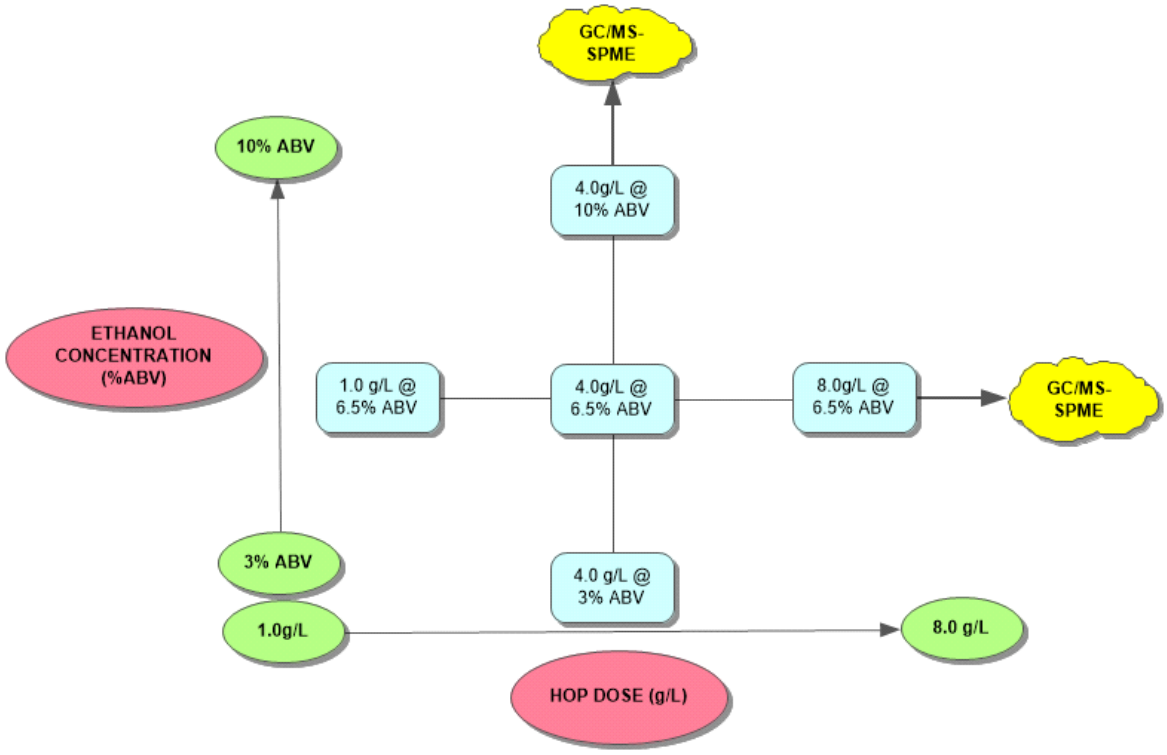


Figure 6.4 Schematic of targeted study examining the effect of increasing hop dose and increasing ethanol content. The y-axis denotes the increase in ethanol concentration (%ABV) and the x-axis denotes the increase in hop dose (g/L). Each blue square denotes a sample point tested. Each sample point was tested in triplicate in addition to being tested in stirred and unstirred reaction

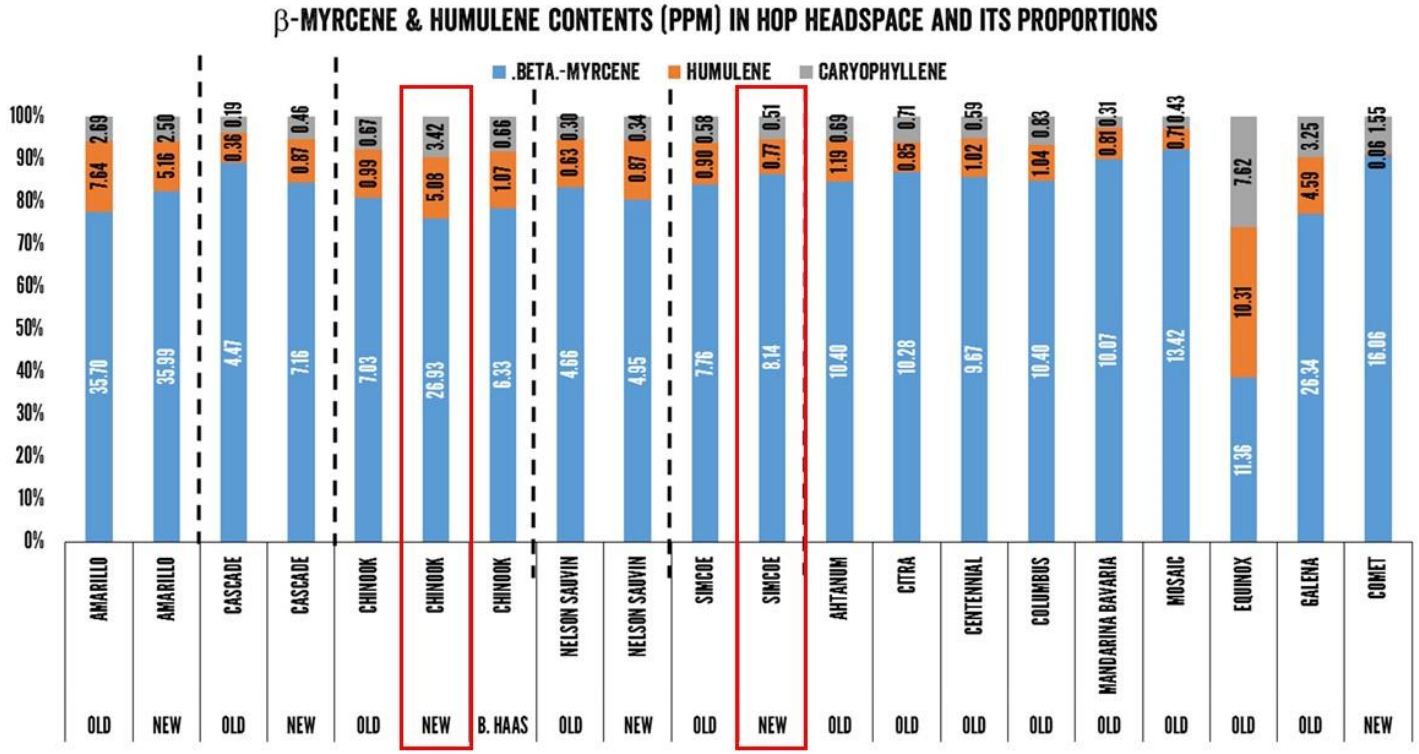


Figure 6.5- GC/MS-SPME Profiling of β-myrcene, α-humulene and β-caryophyllene. The hops that were used in the experiment are indicated with red focus boxes. The term 'new' refers to the hops from the 2017-harvest period. (Unpublished data, Personal Communication, D. Zait, 2017).

6.3 Results

6.3.1 Fractional Four-Factorial Experiment

Data was collected and entered into Design Expert, Version 8 (Stat-Ease Inc., Minneapolis, US) to produce three different models. The three output models were- hop oil extracted into the simulated beer matrix, hop oil recovered from crucible in hexane, and lipid weight extracted from the crucible.

All variables were assessed by checking two-way responses against each variable and assessing the fit of the model. Overall, the data for 'hop oil extracted into the beer matrix' and 'hop oil recovered from the crucible in hexane' did not fit the models as there was an overall low lack of fit in both models (**Figure 6.6**). For example, in **Figure 6.6**, dose was observed to surpass the t-value limit but not the Bonferroni limit. This implies that the model was only weakly significant.

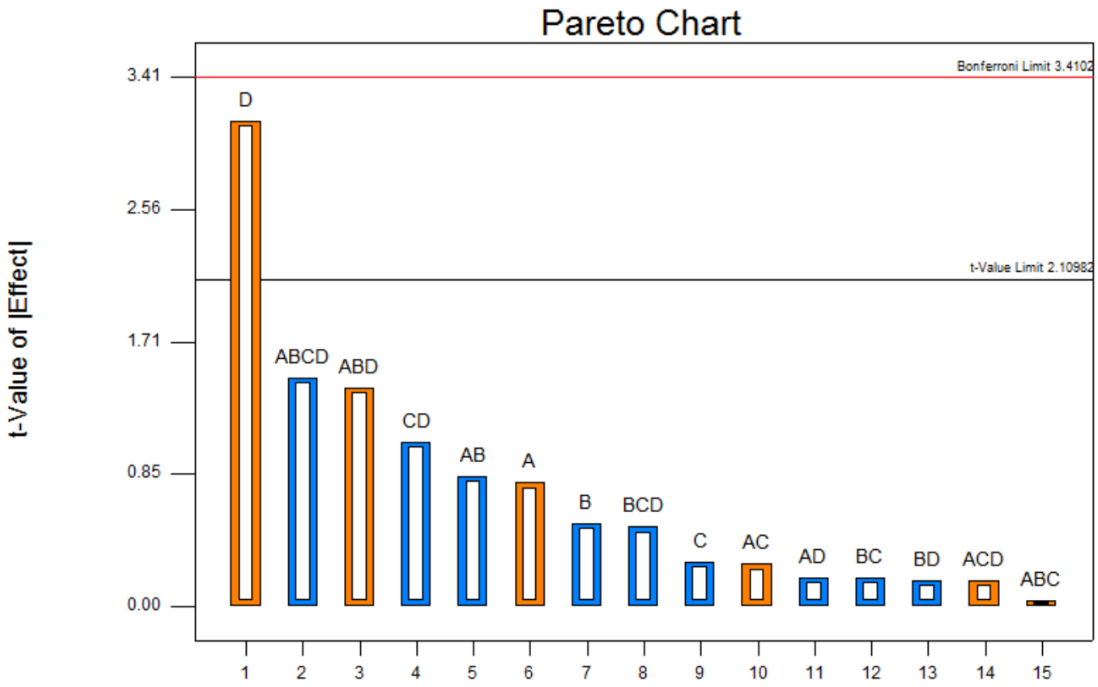


Figure 6.6- Pareto chart comparing hop oil extracted in the simulated beer matrix to all of the variables tested. Factors are labelled as (A) Ethanol content (%*ABV*), (B) Temperature (*°C*), (C) Exposure time (*hours*), and (D) Dose (*g/L).*

A 'lack of fit' F-test determines if the error that exists in the predicted model is significant. If the p-value for the lack of fit test was significant ($p < 0.05$) (i.e.- a low lack of fit), this indicates that the error in the model is significant and the model must be

rejected. The lack of fit p-value, p= 0.87, was observed in the model 'lipid weight extracted from the crucible'. This indicated that the data fits the model. In addition, the p-value for the adjusted model in the model summary was significant (p= 0.0013), further confirming that the 'lipid weight extracted from the crucible' model was significant.

Of all of the models created from the experiment, the model assessing hop dose and lipid extracted from the crucible, was the only significant model in the study. The corresponding Pareto chart for the model showed a response above the Bonferroni acceptance Limit (**Figure 6.7**). As the factor 'dose' was above the Bonferroni Acceptance Limit, it can be assumed that the observations of increasing hop dose is not a random occurrence of an increased extraction of hop oil calculated with 95% confidence. When 'dose' was considered in the 'lipid weight extracted from the crucible' model, the lack of fit for the particular model was not significant (p= 0.87) and the p-value for the adjusted model was significant (p= 0.0016). This confirmed that dose impacted the lipid weight extracted from the crucible.

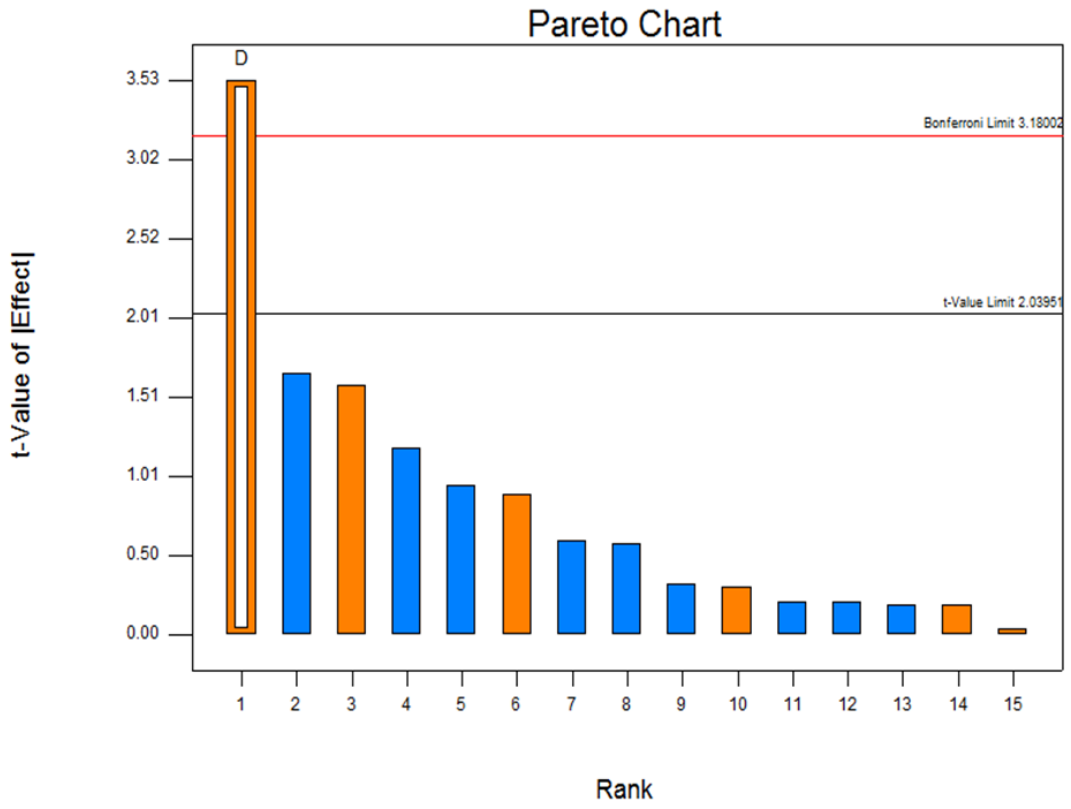


Figure 6.7- Pareto chart examining lipid weight extracted from the Soxhlet crucible against the four variables tested in the experiment. Factors are labelled as

(A) Ethanol content (%*ABV*), (B) Temperature (*°C*), (C) Exposure time (*hours*), and (D) Dose (*g/L).*

The Least Significant Difference (LSD) assesses if the predictions from the model are significantly different in the data. The height of the LSD I-beams are determined by the design of the experiment, model, unexplained variation in the data, and the confidence interval. If the LSD I-beams do not overlap according to the y-axis, the predicted means of the model are significantly different. When assessing a two-way response for the corresponding one-factor plot, hop dose (g/L) was found to significantly affect lipid weight extracted from the crucible (**Figure 6.8**). In the model, the LSD I-beams for hop dose (g/L) and lipid weight extracted from the crucible did not overlap, according to the y-axis (**Figure 6.8)**. Therefore, it can be assumed that the factors of dose and lipid weight from the crucible significantly differ from each other (**Figure 6.8**). The surface response diagram displays the impact of dose on lipid weight extracted (**Figure 6.3**).

In the same model, assessment of the impact of ethanol concentration on hop oil extraction, yielded inconclusive results. In analysing the results, it was challenging to determine whether ethanol had a measurable effect on the model. The factor with the highest contribution to the model was ‘dose’ with an overall 28.63% contribution to the model. Ethanol content only provided a 1.85% contribution to the model. Visual assessment of the surface response model demonstrated that, as ethanol content increased, the concentration lipids extracted from the crucible increased (**Figure 6.9**). As hop oil extraction was influenced by hop dose and loosely by ethanol content, it was desired to explore how the parameters could affect terpene extraction.

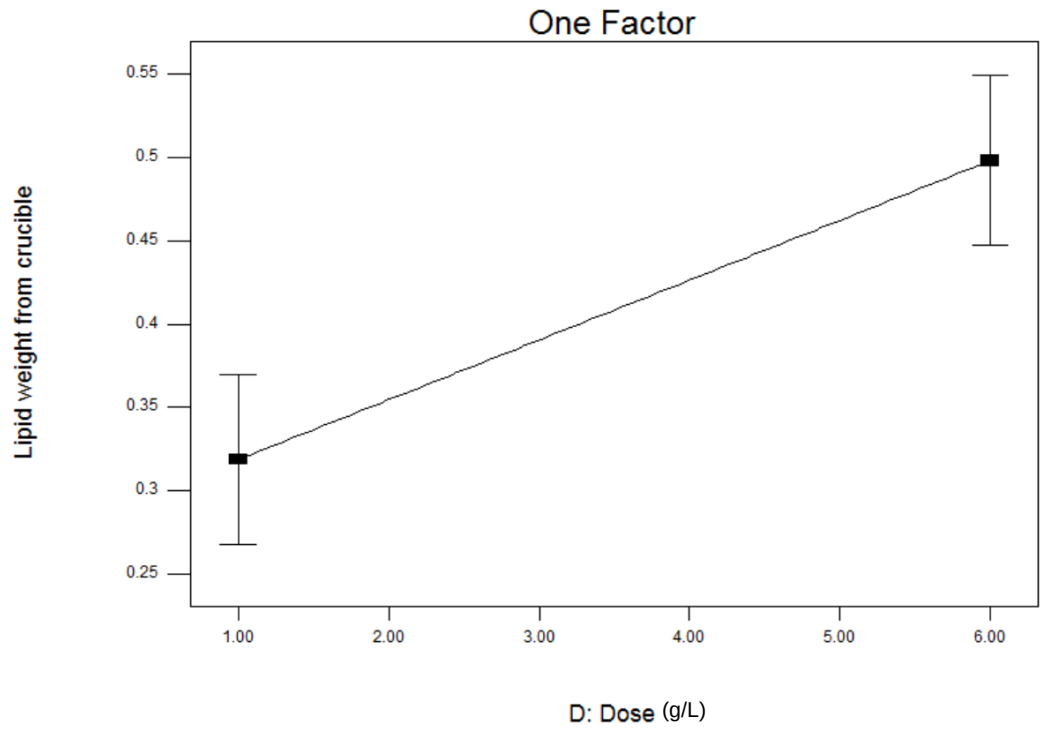


Figure 6.8- One factor plot comparing response of lipid weight extracted from crucible to (D) hop dose (g/L). The least significant difference (LSD) I- beams are found on the far ends of the x-axis.

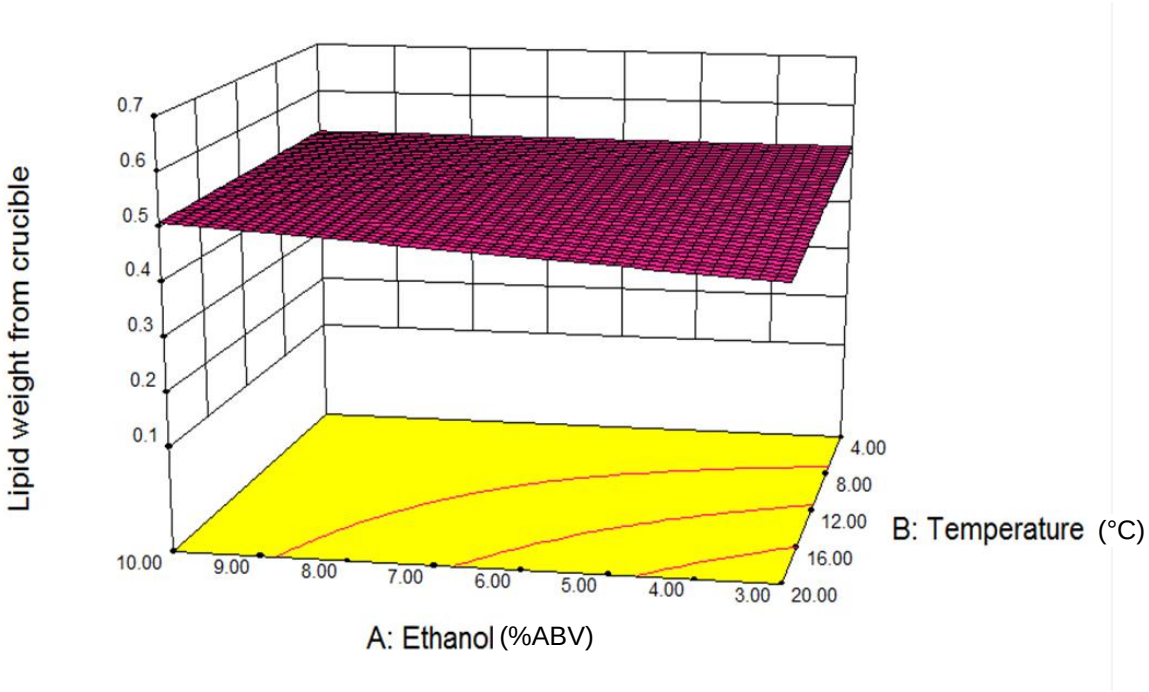


Figure 6.9- Surface response examining lipid weight extracted from the Soxhlet crucible against the four variables tested in the experiment. Factors are labelled as (A) Ethanol content (*ABV*) and (B) Temperature (*°C*).

6.3.2 Targeted Study-the Effect of Ethanol and Hop Dose on Terpene/Terpenoid Extraction

6.3.2.1 Ethanol's Effect on Terpene/Terpenoid Extraction- Chinook

When assessing the stirred samples utilising Chinook hops, an increase in ethanol content showed a first-order kinetic decline in terpene hydrocarbons, β-myrcene (r^2= 0.618) (A), β-caryophyllene (r^2= 0.915) (B), and α-humulene (r^2= 0.995) (C) (**Table 6.1 and Figure 6.10**). No other statistical trends were observed (**Table 6.1**).

Although no other statistical trends were observed, oxygenated compounds, β-linalool (D), caryophyllene oxide (E), linalool oxide (F), and *trans*-geraniol (G) reached peak extraction rates at 6.5% ABV with values reported at 500 µg/L, 2000 µg/L, 150 µg/L and 1500 µg/L, respectively (**Figure 6.10**). Terpenoid, β-citronellol, was the only terpenoid which was best extracted at 6.5% ABV in the unstirred study but was not observed to contain a discernible trend in the stirred study (**Figure 6.10 (H)** and **Figure 6.11** (**H**)). In unstirred reactions, β-caryophyllene (B) and linalool oxide (F) were most soluble at 10 % ABV with final concentrations of 150 µg/L and 375 µg/L, respectively. Compounds β-linalool (D), *trans*-geraniol (G) and β-citronellol (H) were most soluble at 6.5% ABV reporting values of approximately 800 µg/L, 1500 µg/L and 100 µg/L, respectively in the 6.5% ABV solution (**Figure 6.11**).

Table 6.1- Coefficient of determination of the ethanol dose experiments, stirred and unstirred, utilising Chinook hops. All of the r- values were not determined to be significant ($p < 0.05$).

Hop Compound	Coefficient (Stirred)	Coefficient (Unstirred)
β-myrcene	0.618	nr
Linalool oxide	nr	nr
β-linalool	nr	nr
β-citronellol	nr	nr
trans-geraniol	nr	nr
β-caryophyllene	0.915	nr
α-humulene	0.995	nr
Caryophyllene oxide	nr	nr

nr= not reported in the model

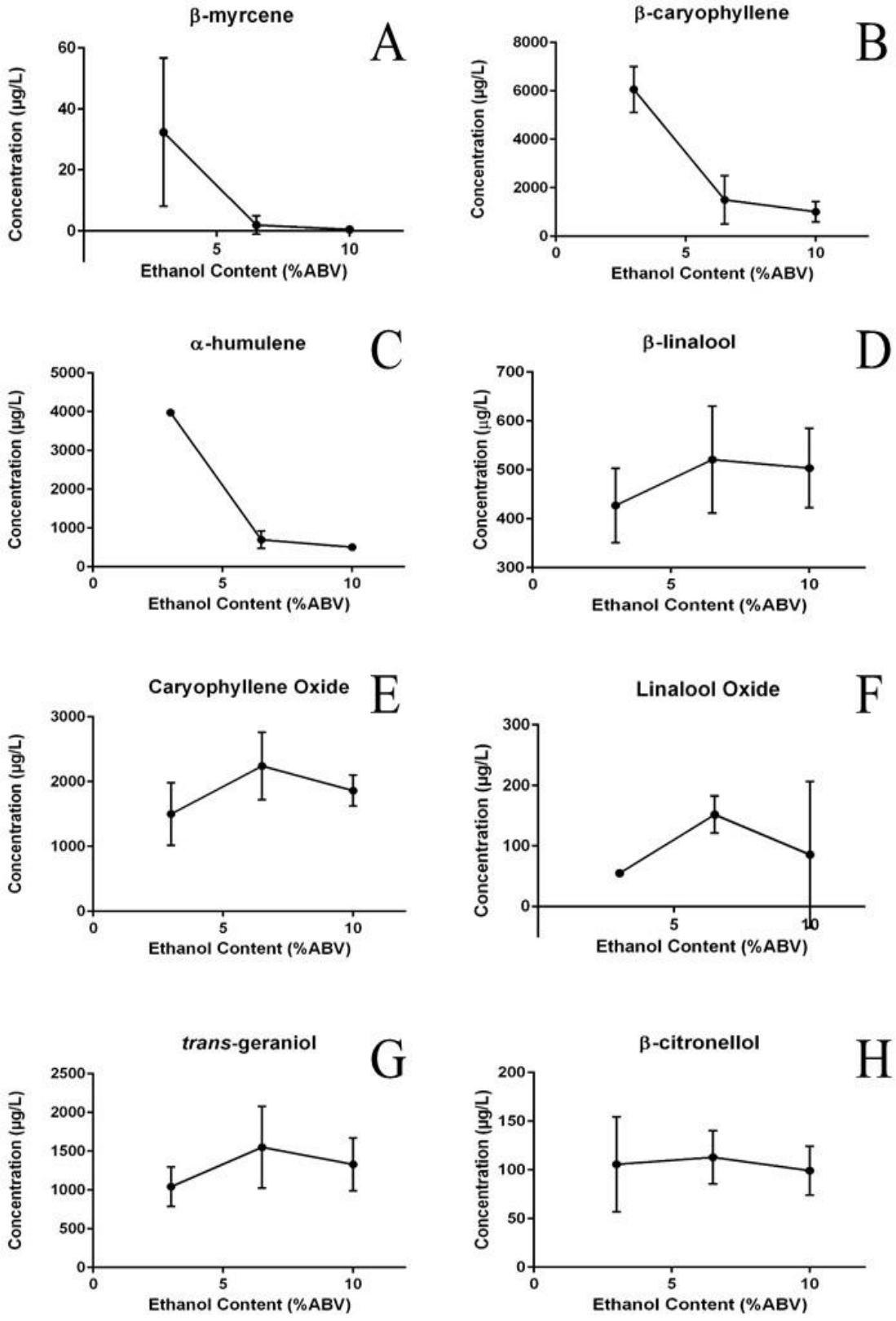


Figure 6.10 - The change in terpene/terpenoid concentration as a function of ethanol content. All samples were stirred and utilised Chinook hops, dosed at a rate of 4.0 g/L.

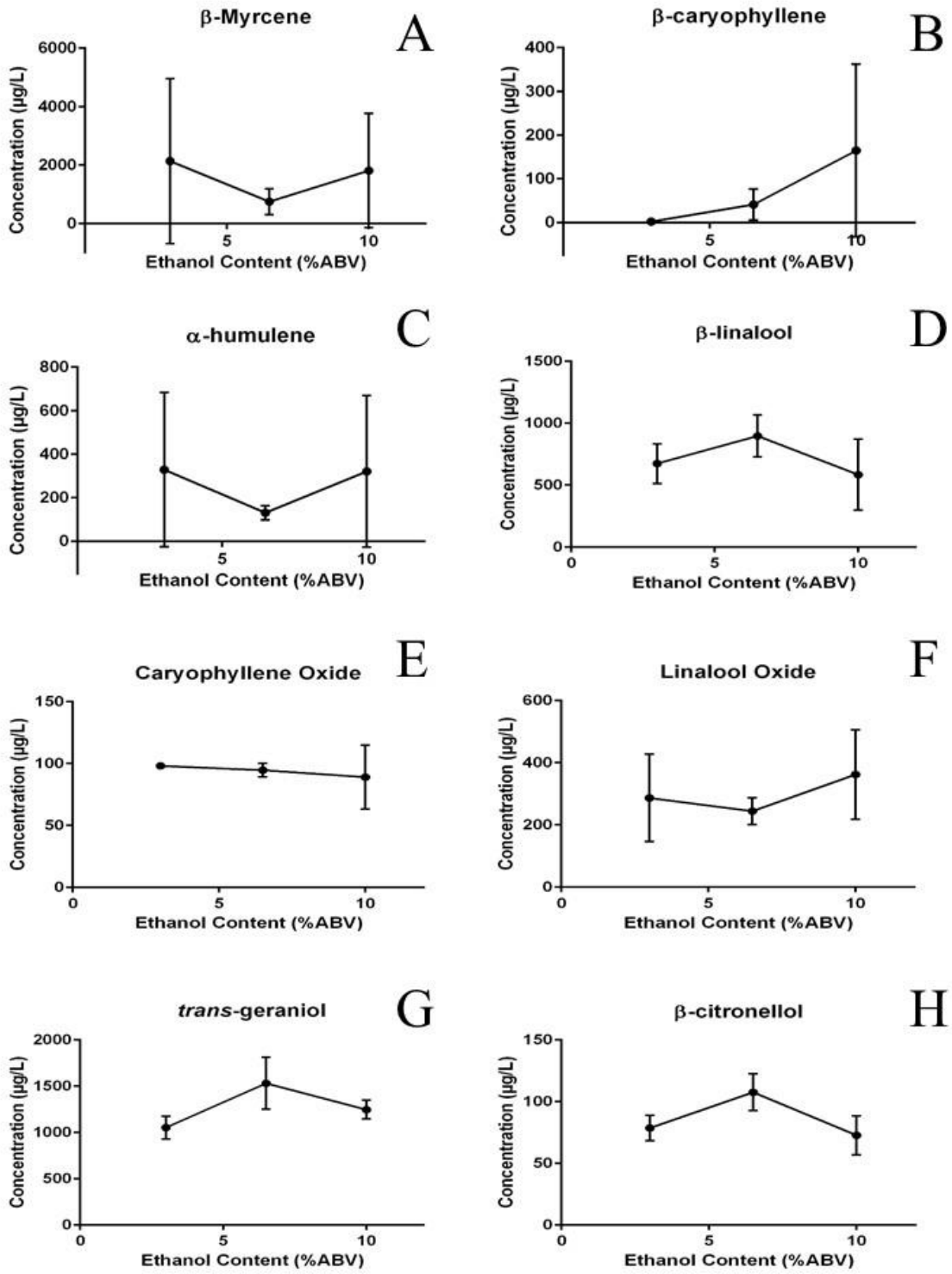


Figure 6.11 - The change in terpene/terpenoid concentration as a function of ethanol content All samples were unstirred and utilised Chinook hops, dosed at a rate of 4.0 g/L.

6.3.2.2 Ethanol's Effect on Terpene/Terpenoid Extraction- Simcoe

The only statistical trends were observed in the stirred study were found when using Simcoe hops (**Table 6.2**). Compounds β-myrcene (r^2= 0.600) (A), caryophyllene oxide (r^2= 0.533) (E), α-humulene (r^2= 0.750) (C), and linalool oxide (r^2= 0.626) (F) declined in a fashion similar to a first-order kinetic decline as ethanol content increased. However, the decline in concentration of the hop compounds were not as robust as Chinook samples (**Figure 6.12**). The compound β-myrcene saw a decline from approximately 30 µg/L to <1 µg/L, caryophyllene oxide from approximately 4500 µg/L to 2500 µg/L, α-humulene from approximately 3500-300 µg/L, and linalool oxide from approximately 500 µg/L to 160 µg/L (**Figure 6.12**).

In unstirred reactions, β-citronellol (H) and β-linalool (D) weakly declined in concentration upon the increase of ethanol concentration from 3-10%, shifting from 150-110 µg/L and 1200-600 µg/L, respectively (**Figure 6.13**).

Interestingly, in unstirred reactions, each of these terpene/terpenoid compounds (in addition to *trans*-geraniol (F)) were least soluble at 6.5% ABV and most soluble 10% ABV (**Figure 6.13**). Terpene hydrocarbons and oxygenated compounds of β-caryophyllene (B), β-citronellol (H), and β-linalool (D) demonstrated a similar trend to the stirred Chinook samples, reaching peak extraction point at 6.5% ABV, reporting values of 32 µg/L, 139 µg/L, and 937 µg/L, respectively (**Figure 6.12**).

Table 6.2- Coefficient of determination on the ethanol dose experiments, stirred and unstirred, utilising Simcoe hops. All of the r- values were not determined to be significant ($p < 0.05$).

Hop Compound	Coefficient (Stirred)	Coefficient (Unstirred)
β-myrcene	0.600	nr
Linalool oxide	0.626	nr
β-linalool	nr	nr
β-citronellol	nr	nr
trans-geraniol	nr	nr
β-caryophyllene	0.550	nr
α-humulene	0.750	nr
Caryophyllene oxide	0.533	nr

nr= not reported in the model

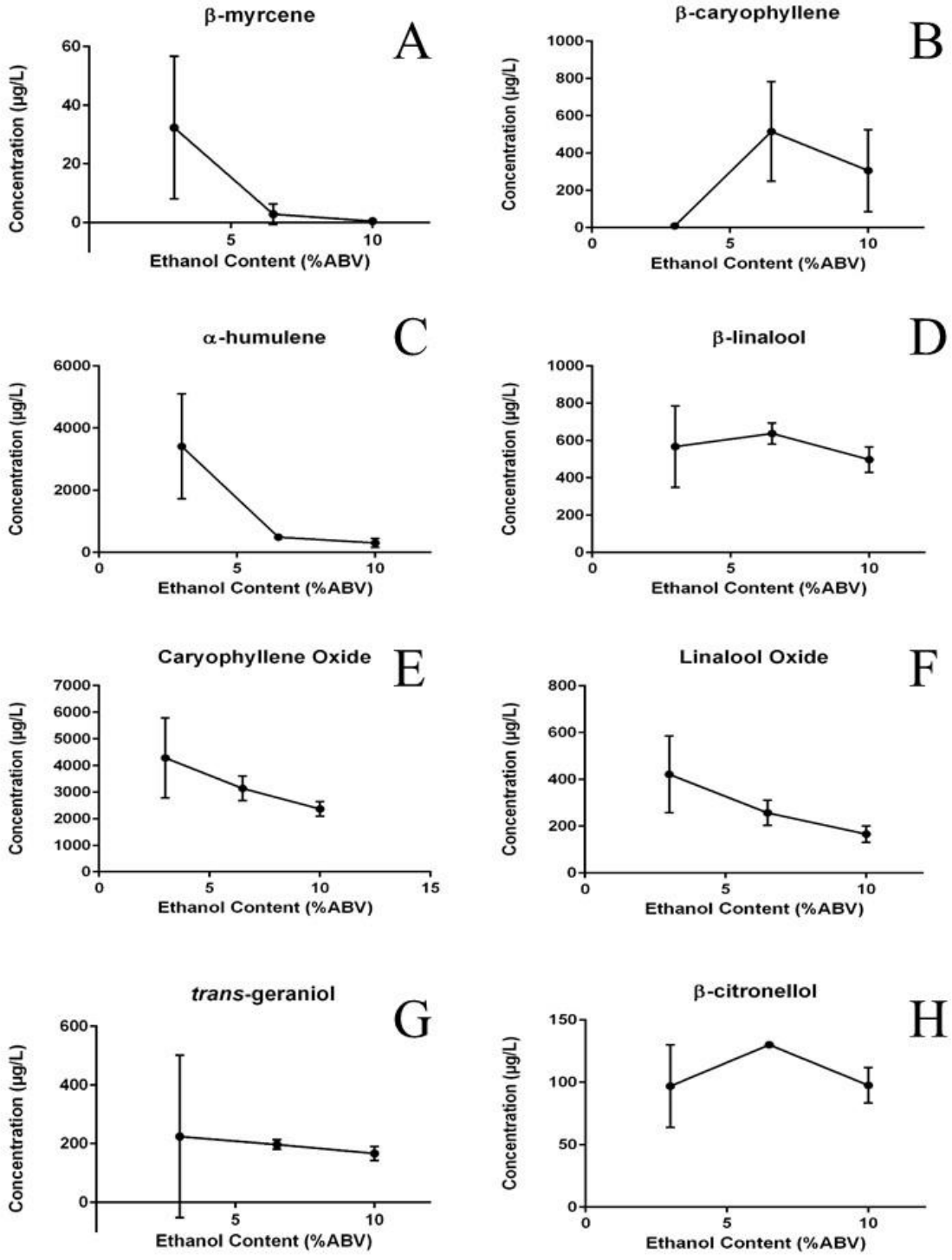


Figure 6.12 - The change in terpene/terpenoid concentration as a function of ethanol content All samples were stirred and utilised Simcoe hops, dosed at a concentration of 4.0 g/L.

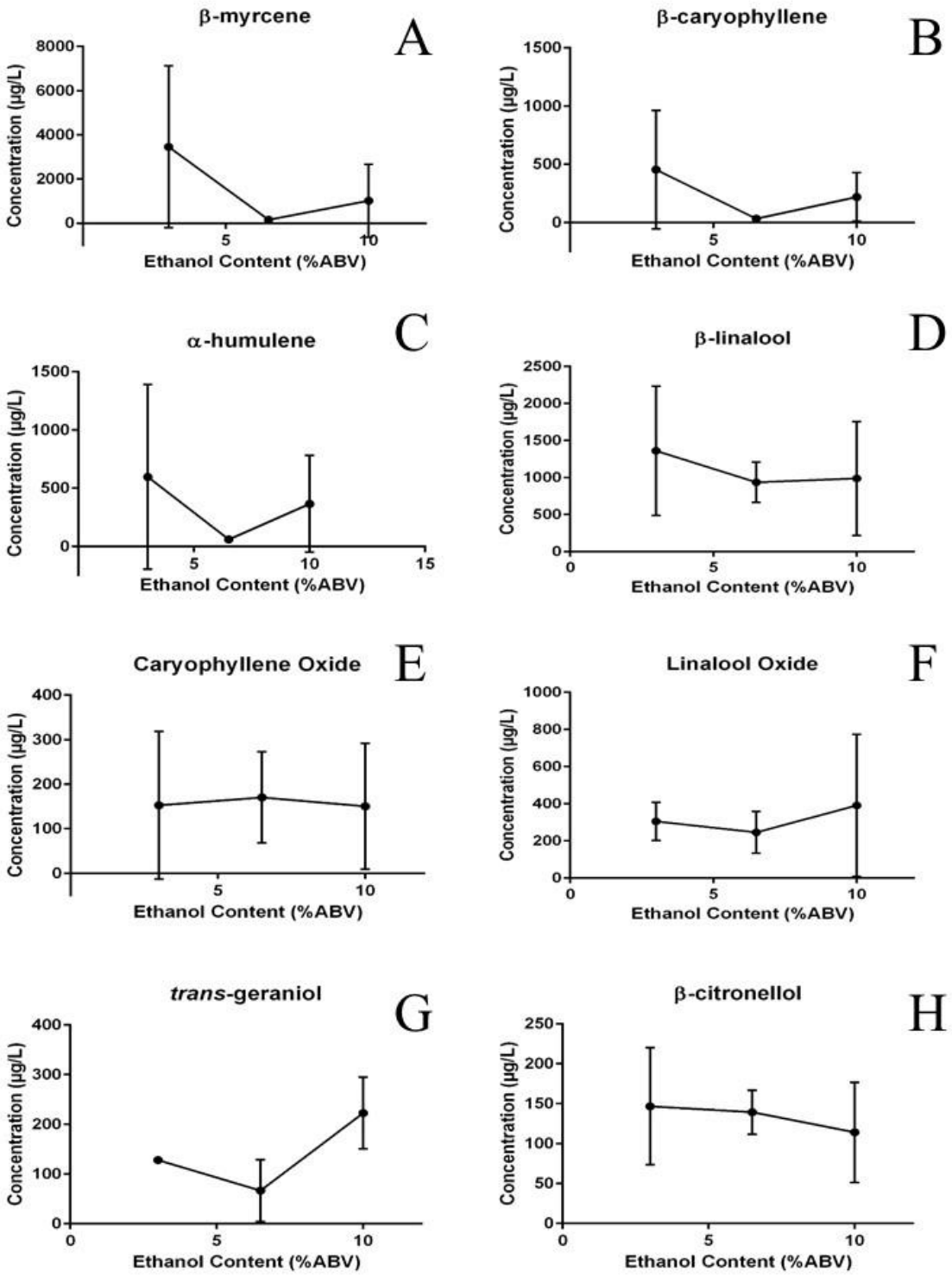


Figure 6.13 - The change in terpene/terpenoid concentration as a function of ethanol content. All samples were unstirred and utilised Simcoe hops, dosed at a concentration of 4.0 g/L.

6.3.2.3 Hop Dose Effect on Terpene/Terpenoid Extraction - Chinook

The stirred study assessing hop dose utilising Chinook hops demonstrated strong linear correlation in all cases (**Figure 6.14**). Terpenes β-linalool (D), β-citronellol (H), and *trans*-geraniol (G) demonstrated linear extraction efficiencies with r-squared values of 0.955, 0.996, and 0.977 and peak extraction rates of approximately 1000, 200, and 2500 µg/L, respectively (**Table 6.3)**.

The unstirred reactions depicted similar trends with terpenes β-linalool (D), β-citronellol (H), and *trans*-geraniol (G) reporting r-squared values of 0.979, 0.757, and 0.928, and peak concentrations of approximately 1000 µg/L, 130 µg/L, and 2000 µg/L, respectively. Caryophyllene oxide was the only exception in stirred and unstirred studies with r-squared values at 0.645 in stirred reactions and 0.777 in unstirred reactions (**Table 6.3**). **Figure 6.15** demonstrates a decline in solubility as hop dose increased as the concentration declined from approximately 240-130 µg/L **(Table 6.3)**.

Table 6.3- Coefficient of determination of the hop dose experiments, stirred and unstirred, utilising Chinook hops. All of the r- values were not determined to be significant ($p < 0.05$).

Hop Compound	Coefficient (Stirred)	Coefficient (Unstirred)
β-myrcene	0.661	0.317
Linalool oxide	0.819	0.181
β-linalool	0.956	0.979
β-citronellol	0.996	0.757
trans-geraniol	0.977	0.928
β-caryophyllene	0.825	0.670
α-humulene	0.042	nr
Caryophyllene oxide	0.646	0.777

nr= not reported in the model

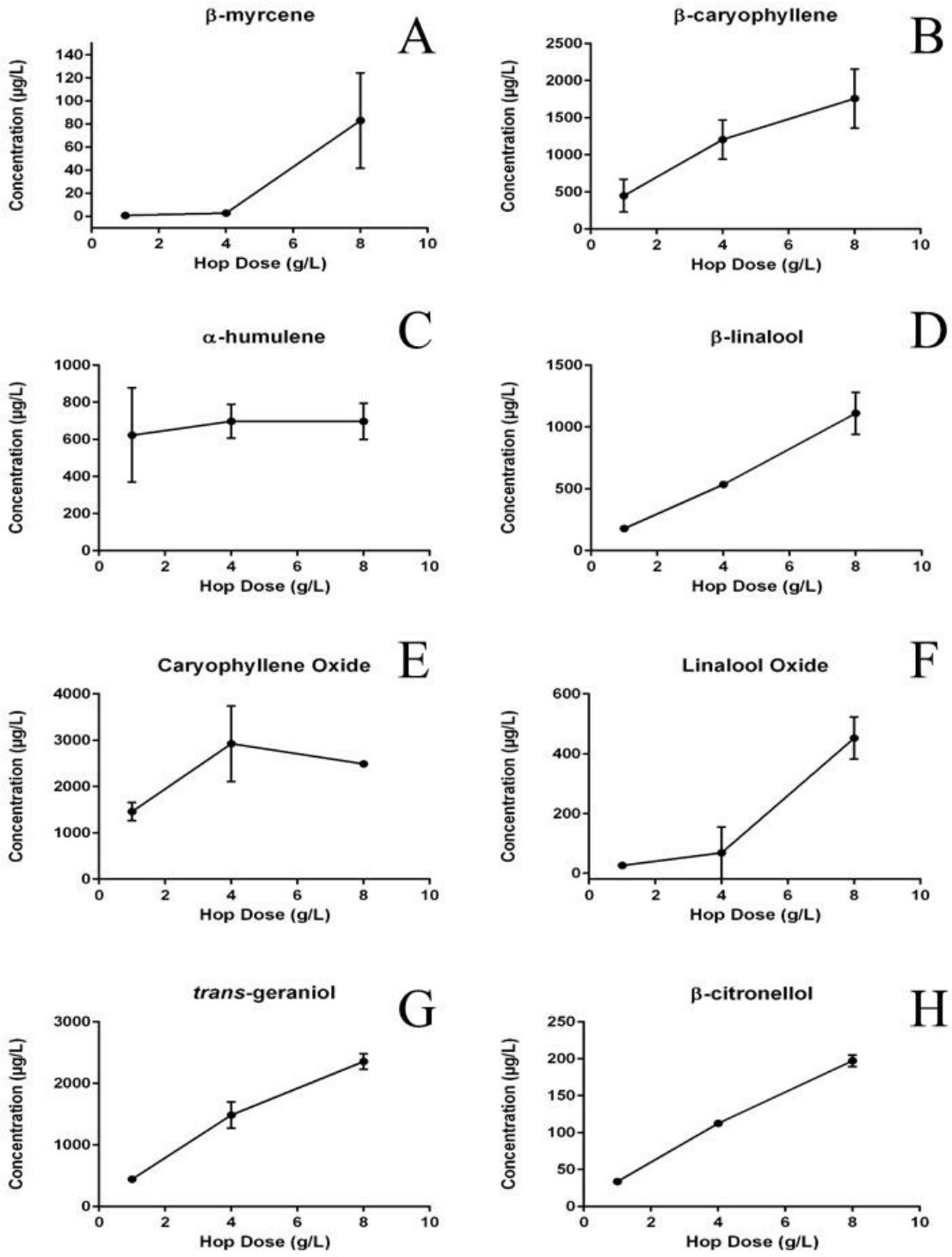


Figure 6.14 - The change in terpene/terpenoid concentration as a function of hop dose. All samples were stirred and utilised Chinook hops, dosed in a 6.5% ABV acidified simulated beer matrix.

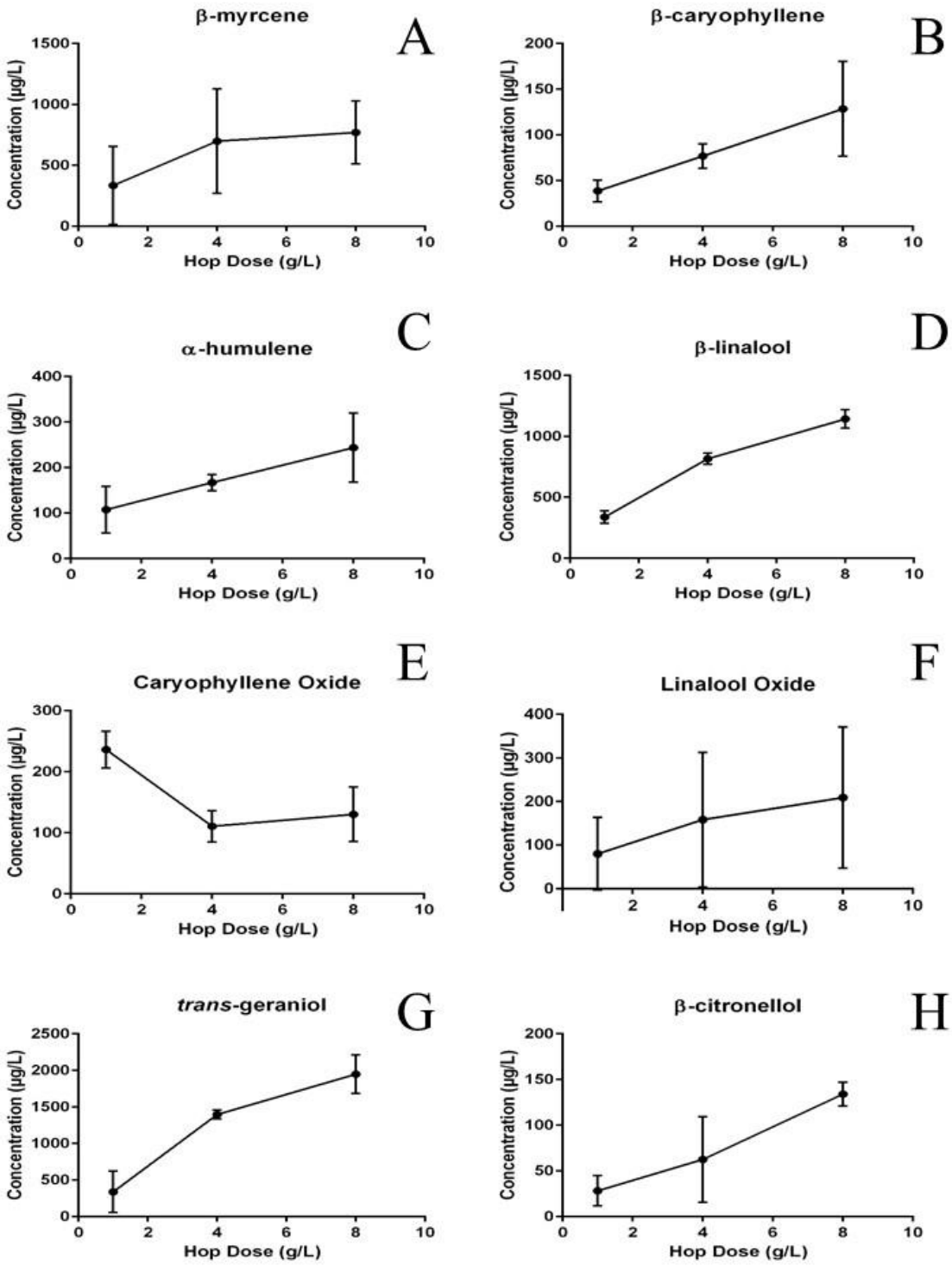


Figure 6.15 - The change in terpene/terpenoid concentration as a function of hop dose. All samples were unstirred and utilised Chinook hops, dosed in a 6.5% ABV simulated beer matrix.

6.3.2.4 Hop Dose Effect on Terpene/Terpenoid Extraction - Simcoe

The study assessing Simcoe hop dosage rates in stirred reactions portrayed strong linear relationships in all cases (**Table 6.4**). The only exceptions being β-myrcene (A) decreasing from approximately 3.0-1.0 µg/L as hop dose increased and caryophyllene oxide (E) spiking at the 4.0 g/L dose with a concentration at approximately 3000 µg/L (**Figure 6.16**). The unstirred reactions utilising Simcoe hops provided nebulous results. Compounds β-myrcene (A) and β-citronellol (H) increased from approximately 20-200 µg/L and 50-200 µg/L, respectively as hop dose increased while caryophyllene oxide (E) decreased from approximately 500-200 µg/L as hop dose increased. Terpene/terpenoids α-humulene (C), β-caryophyllene (B), *trans*-geraniol (G), and linalool oxide (F) were best extracted at a 4.0 g/L dose as the respective concentrations were 168 µg/L, 144 µg/L, 149 µg/L, and 295 µg/L (**Figure 6.17**).

Table 6.4 - Coefficient of determination of the hop dose experiments, stirred and unstirred, utilising Simcoe hops. All of the r- values were not determined to be significant ($p < 0.05$).

Hop Compound	Coefficient (Stirred)	Coefficient (Unstirred)
β-myrcene	0.604	0.567
Linalool oxide	0.898	0.753
β-linalool	0.965	0.887
β-citronellol	0.966	0.809
trans-geraniol	0.973	0.314
β-caryophyllene	0.740	0.434
α-humulene	0.763	0.446
Caryophyllene oxide	0.468	0.279

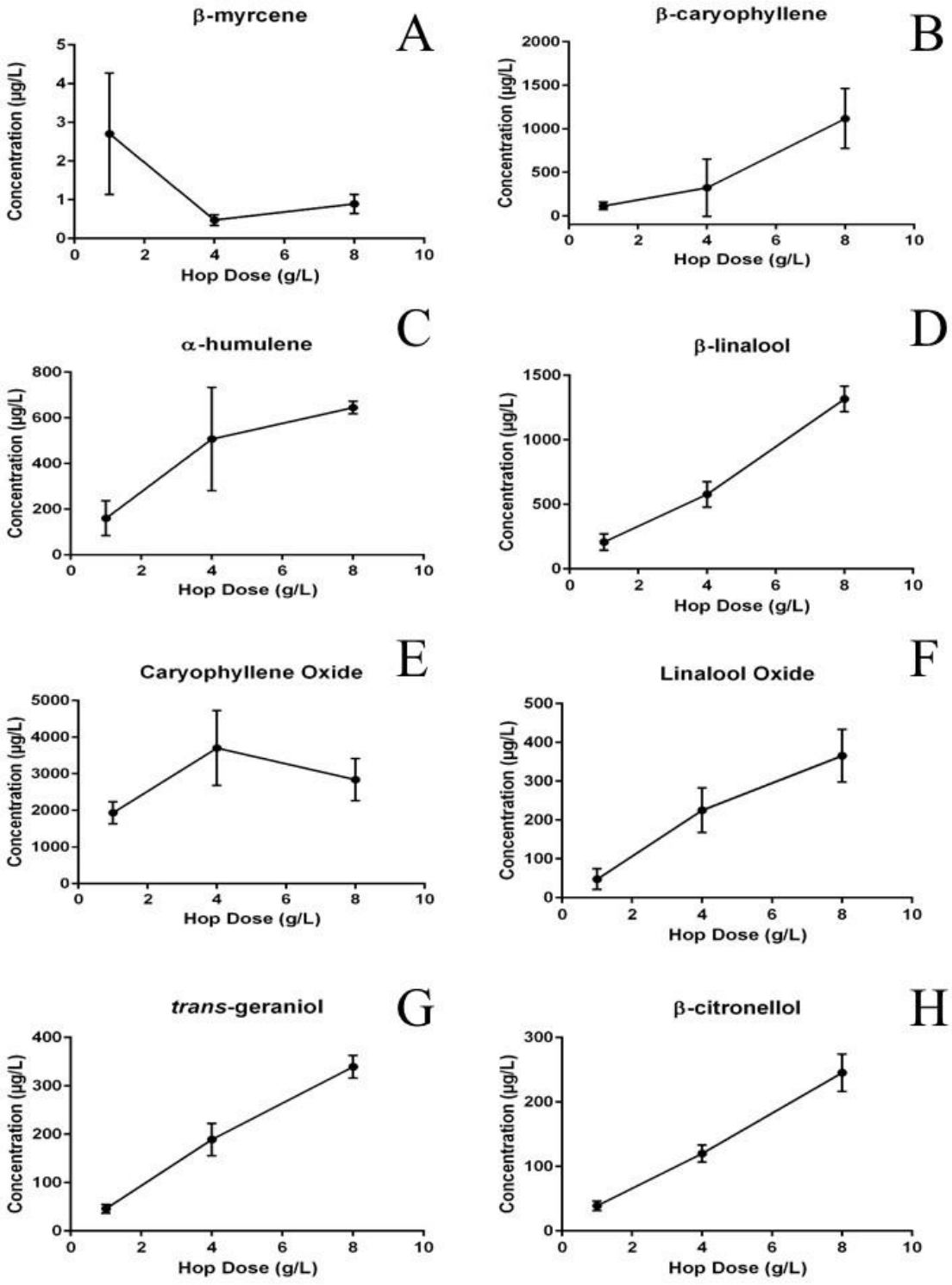


Figure 6.16 - The change in terpene/terpenoid concentration as a function of hop dose. All samples were stirred and utilised Simcoe hops, dosed in a 6.5% ABV simulated beer matrix.

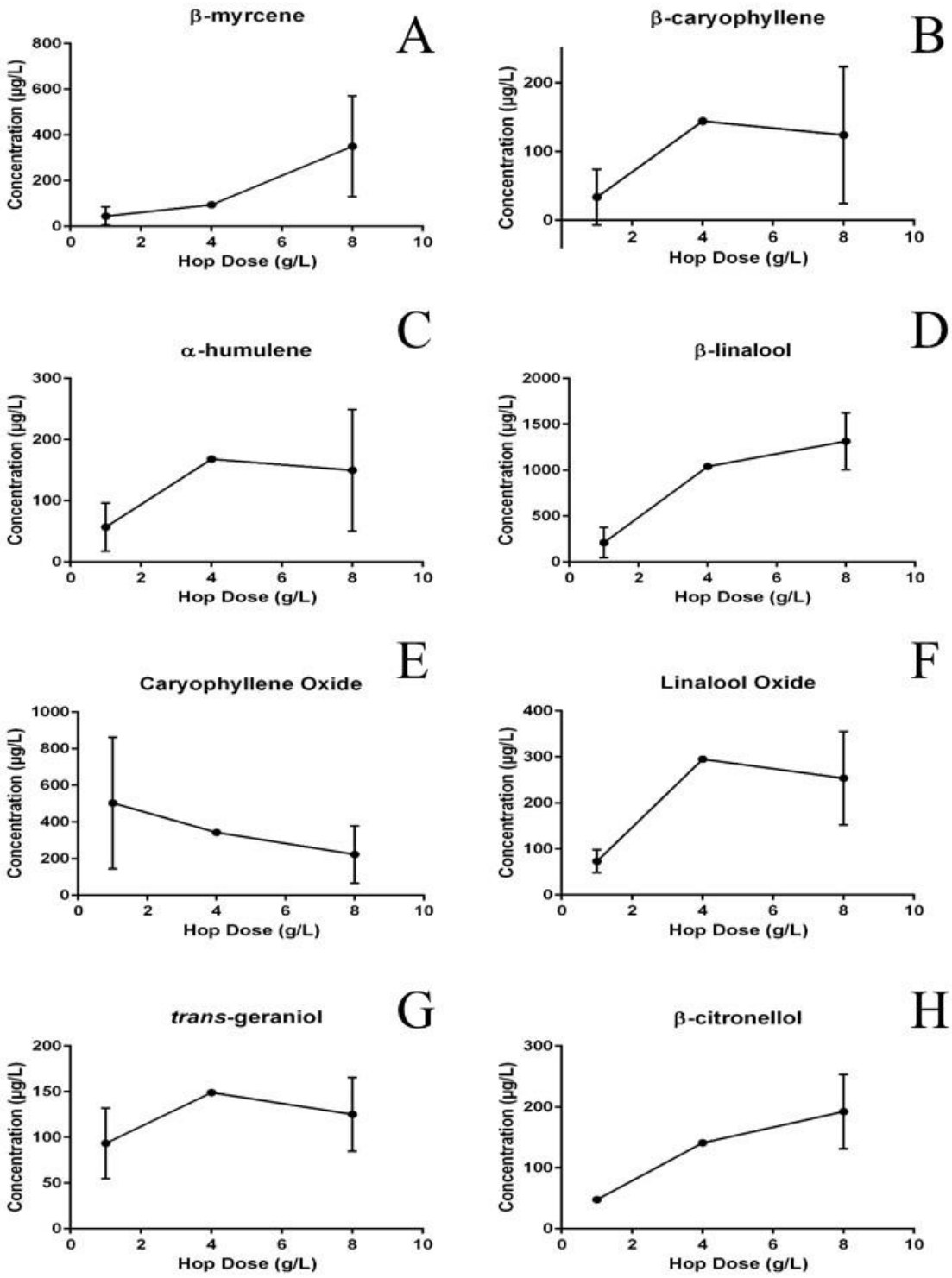


Figure 6.17 - The change in terpene/terpenoid concentration as a function of hop dose. All samples were unstirred and utilised Simcoe hops, dosed in a 6.5% ABV simulated beer matrix.

6.4 Discussion

6.4.1 Fractional-Factorial Experiment

The DoE design assessed dry-hopping as a complete process and the obvious factors that could have an effect on terpene extraction- ethanol content, exposure time, temperature, and dose rate. However, the initial four-factorial experiment did not provide robust conclusions on factors that influence dry-hopping. All models were insignificant except for the model 'lipid weight from crucible' when compared to the factor 'dose'. The data fits the model as there was a high lack of fit for the adjusted model (p= 0.8827). In the surface response models, lipid weight lost from the crucible (pre minus post Soxhlet extraction) was found to be significantly (p> 0.05) dose-dependent (e.g. as more hops are added, more hop oil will be extracted from the spent hop material). This meant that the difference in the weight of the crucible pre minus post extraction as influenced by dose. The greater hop content on the filter paper placed in the crucible, the more hop oil extracted. This is shown by the one factor plot and the surface response diagram found in **Figure 6.8** and **Figure 6.9**.

Logically, these results make sense. With more hop material in the crucible, more (residual) hop oil should be extracted. However, this theory is not supported when assessing hop oil weight in the round-bottom flask following hexane removal as the model was not significant (p>0.05). This could indicate that as hop dose increased, a higher concentration of residual hop oil remained in the spent hops after dry-hopping. This could be due to the limited solubility of non-polar hop oil in in a solution consisting of mainly polar, water.

The insignificance of the model 'hop oil recovered from crucible in hexane' could be explained by several factors. First, the scale that was used for weighing the flask pre-post extraction may not have been sensitive enough to accurately measure the change in weight of the round-bottom flask as the balance only measured to the thousandth of a gram (i.e.- 1.000g). Second, the experimental results for 'hop oil extracted from hexane' may have contained outliers that affected the model (Abraham and Steiner, 2007). Finally, the most reasonable cause for the model's lack of significance was due to an inadequate extraction time. It is possible that the spent hops contained more hop oil than was initially expected causing the extraction time to be insufficient. Additionally, essential oils may take longer to extract as some Soxhlet extractions are continually run for upwards of 12 hours (Ozel *et al.*, 2004).

Ethanol did not impact ‘hop oil extracted from the crucible’, as ethanol’s effect on the model was not significant (p>0.05). However, when visually assessing the surface response diagram, as ethanol content increased, ‘lipid weight from the crucible’ appeared to very slightly increase. The impact of ethanol content on the model proved to be inconclusive as no clear trends/relationships were observed between increasing ethanol content and the increase of hop oil extracted from the crucible. It is likely that, all of the factors studied in the fractional-factorial experiment (dose, ethanol content, temperature, and exposure time) all slightly impacted one another. Due to the lack of a strong relationship directly corresponding to change in variables, this may explain the inconclusive results in the case of ethanol. Overall, the results did not provide enough evidence for any robust conclusion(s) and require further studies.

To further investigate and progress on the topic, the targeted study examining the solubility of terpenes/terpenoids as a function of ethanol content and hop dose was designed.

6.4.2 The Effect of Ethanol on Terpene/Terpenoid Extraction

The two-factor study examining the effect of dry-hop dose and ethanol content was designed to address the inconclusive results from the fractional four-factorial experiment. Additionally, the chemistry of terpenes and terpenoids were considered as respective solubilities may have differed. Simcoe and Chinook hop varieties were assessed in stirred and unstirred reactions as their terpene/terpenoid compositions differed (**Figure 6.5**).

As ethanol is a polar molecule, increasing the polarity of the solution by the addition of ethanol can explain the results in **Figure 6.10 B** and **C**. As the terpene hydrocarbons are non-polar/weakly polar, increasing the ethanol content (polarity) of the solution only limits their solubility. In the stirred samples, the two terpene hydrocarbons β-myrcene and β-caryophyllene decreased as ethanol content was increased. This was not surprising as the compounds are terpene hydrocarbons. Beta-myrcene is a monoterpene hydrocarbon and β-caryophyllene is a cyclic sesquiterpene hydrocarbon compound.

An interesting trend observed were that terpene hydrocarbons and oxygenated terpenoids of β-caryophyllene (B), β-citronellol (H), and β-linalool (D) demonstrated a similar trend to the stirred Chinook samples, reaching peak extraction point at 6.5%

ABV (**Figure 6.12**). The compounds have an optimum solubility point in the beer matrix at 6.5% ABV but further studies are required to confirm if the trend is observed in beer (**Figure 6.12 and Figure 6.13**).

The Simcoe hop samples did not demonstrate the same trend. However, GC/MS-SPME analytical errors were suspected to be the cause as the background signal to noise was large and the peaks from the chromatographs were not well-separated, Gaussian peaks.

Overall, terpene/terpenoid compounds, differ in solubility as compound structure is unique to each compound. Additionally, each variety will contain different compositions of each terpenes and terpenoids. This is important to consider when selecting a hop variety for dry-hop use.

Ethanol may have an effect on the solubility of various terpenes/terpenoids but it may also impact the aroma of the compounds extracted in solution. Recent research has discovered that the presence of ethanol can heighten aroma threshold values for compounds such as *trans*-geraniol and β-linalool (Peltz *et al.*, 2017). The influence of ethanol in dry-hopped beers is complex and may influence dry-hopped beer more than previously anticipated.

6.4.3 The Effect of Hop Dose on Terpene/Terpenoid Extraction

Despite the low r-squared values, the only experiment that contained statistical trends for each terpene/terpenoid was the experiment assessing the effect of hop dose using Simcoe hops. It is speculated that Simcoe hops may have contained a greater amount of essential oil than Chinook. It could also be possible that components exist in Simcoe hops causing the essential oil to be more soluble than other varieties. Future research should study the solubility of essential oil across various hop varieties.

Overall, it appears that hop dose has a greater effect on terpene concentration in beer matrices than ethanol content (**Table 6.1-Table 6.4).** Statistical trends (significant or non-significant) were observed for nearly all groups studying the effect of hop dose (**Table 6.3-Table 6.4**).

The results of this study indicate that increasing the volume of hops should increase the desired terpene/terpenoid compounds. However, further research is still required as studies have found that a higher volume of hop oil does not imply that

greater hoppy aromas will be extracted (Vollmer *et al.*, 2016). Adding more hops with higher essential oil contents may not actually increase the hoppy aromas desired.

6.4.4 The Effect of Stirred and Unstirred Dry-Hop Conditions on Terpene/Terpenoid Extraction

From the results collected, it was concluded that greater extraction efficiencies are obtained by stirring/agitation during dry-hopping. Caryophyllene oxide in stirred and unstirred samples with Chinook hops provided good evidence for this. In stirred samples, the lowest concentration extracted was approximately 1500 ug/L (**Figure 6.14**). Comparatively, in unstirred samples, the lowest concentration was approximately 250 ug/L (**Figure 6.15**).

In other cases, the opposite effect was observed. Additionally, some of the terpene/terpenoids lowest observed concentrations were similar in value regardless if the reaction was stirred or unstirred. For example, the lowest concentration of *trans*-geraniol observed in the matrix was close to 100 µg/L in the unstirred reaction, dosed at 4.0 g/L (**Figure 6.17)**. However, when assessing the stirred reaction, the lowest concentration rested close to 50 µg/L dosed at 4.0 g/L (**Figure 6.16)**.

When assessing models that were dosed in to the 6.5% ABV simulated beer matrix, in the lowest dose rate of 3.0g/L of hops, stirred reactions more efficiently extracted terpenes/terpenoids as observed concentrations were higher in stirred samples (**Figure 6.14** and **Figure 6.16**). However, these results are nebulous as some terpene/terpenoid concentrations at 4.0 g/L were higher in unstirred samples. This is observed in **Figure 6.16** and **Figure 6.17** when comparing β-myrcene concentrations.

The results have raised further questions and future studies are required as large errors were reported in all samples. However, the overall takeaways from the study are that ethanol concentration does not have a great impact on terpene/terpenoid extraction while hop dose does have a notable impact on extraction. Stirring/agitation also enhances the extraction of desired terpene/terpenoid compounds that contribute to beer flavour and aroma and is recommended for dry-hopping procedures. Many dry hopping procedures, as discussed in Chapter 1 (Section 1.2.9), utilise different methods of agitation or passing the beer through hops to extract desired hop flavours and aromas. The results of this study and other studies indicate that dry hopping with agitation increase the extraction of desired hop terpenes/terpenoids.

Chapter 7- The Vanillin Assay: A Potential Method to Determine Total Terpene/Terpenoids in Beer

7.1 Introduction

Hop aroma is exceedingly complex and difficult to analyse due to the multiple chemical constituents that contribute to the overall profile (Almaguer *et al.*, 2014; Ting *et al.*, 2017; Rettberg *et al.*, 2018). Common methods of analysing hop aroma include GC/MS-SPME, GC/MS-HS, GC, or in lesser cases HPLC analysis (Andres-Iglesias *et al.*, 2014; Liu *et al.*, 2017; Ting *et al.*, 2017; Rettberg *et al.*, 2018). Each method of analysis functions by analysing targeted components of hop aroma such as esters, aldehydes, terpenes/terpeniols, thiol compounds or terpenyl glycoside products (Andres-Iglesias *et al.*, 2014; Sharp *et al.*, 2017; Rettberg *et al.*, 2018). Other than these methods, sensory analysis is the only alternative for the quantification of hop aroma.

The vanillin assay was originally designed with the intent of assessing condensed tannins (flavanols) in raw materials in the food industry, including malt and various fruits (Attaway *et al.*, 1967; Broadhurst *et al.*, 1978; Butler *et al.*, 1982; Cacho *et al.*, 1990). Feigl (1947) developed the vanillin assay to quantitatively measure total terpenes in fruit juices or must in the wine industry (Attaway *et al*. 1967; Dimitriadis and Williams 1984; Feigl, 1947). Cacho and Ferreira, (1990) later modified the method to analyse monoterpenols at very low levels (<12mg/L) in low-aromatic or 'non-aromatic' muscat grapes (Cacho *et al.*, 1990).

The assay utilises liquid-liquid extraction to separate monoterpenols from steam distilled homogenized grape juice (Cacho *et al.*, 1990). Liquid-liquid extraction is crude, is time consuming and requires harsh solvents. Solid-phase extraction (SPE) is a fast, clean and efficient, way to purify compounds in complex mixtures with low amounts of solvent and sample preparation (Van Opstaele *et al.*, 2012; Capuzzo *et al.*, 2013; Praet *et al.*, 2014). Solid-phase extraction separates compounds from mixtures based upon physical and chemical properties of the targeted compound(s). SPE separation works by the interaction of a stationary phase with a liquid matrix by polarity, pH, or anion/cation exchange. Liquid is drawn through an SPE cartridge by a vacuum and is collected in a vial (Poole, 2000).

Beer contains terpenes and terpenoids at varying concentrations in the µg/L to mg/L range (De Almeida *et al.*, 2015; Zunkel, 2015). Dry-hopped beer contains more

monoterpenes and monoterpene alcohols than non-dry-hopped beer due to hop additions during the cold-end processing steps (Forster and Gahr, 2013; Rettberg, Biendl and Garbe, 2018). Hop essential oils contain terpene and terpene alcohols, some of which volatilise quickly in hot-end processing due to their low boiling points (Kishimoto *et al.*, 2005). However, these compounds do not volatilise as quickly with conventional dry-hopping methods as the hops are added post-fermentation on the cold-side of processing. As a result, heavily dry-hopped beers should contain low boiling point terpenes and terpenoids (Haley *et al.*, 1983; Fritsch *et al.*, 2005).

A broad range of terpene/terpenoid compounds are present in the essential oil of hops and differ in levels of abundance per hop variety. Each terpene/terpenoid compound will differ in degree of conjugation, cyclization, and hydroxylation (Aberl *et al.*, 2012). Linalool, a monoterpene alcohol, has been proven to contribute to dry-hop aroma in most varieties in beer (Fritsch *et al.*, 2005). It is also one of the very few hop compounds to surpass limits of human aroma detection in non-dry-hopped beer (10 µg/L) (Biendl *et al.*, 2014). Myrcene is a terpene that is present in most hop varieties but is rarely present in beer that is not dry-hopped due to its low boiling point (Kishimoto *et al.*, 2005). Therefore, myrcene is a good target compound to analyse as it will be present in dry-hopped beer but absent in un-dry-hopped beer (Biendl *et al.*, 2014).

Additionally, *trans*-geraniol is also present in raw and processed hops and is important to measure in the assessment of dry-hop aroma (Haslbeck *et al.*, 2018). *Trans*-geraniol is present in raw hops but is also formed by yeast biotransformation or the auto oxidation of myrcene (**Figure 7.1**). Due to this, *trans*-geraniol content may be present in higher quantities in dry-hopped beer than would otherwise be expected (Haslbeck *et al.*, 2018; Rettberg *et al.*, 2018). Biotransformation of terpene/terpenoid compounds are reported to produce chemically similar terpene/terpenoid compounds with different aromatic properties. For example, the biotransformation of *trans*-geraniol has been observed to produce the terpene alcohol, β-citronellol (**Figure 7.1**) (De Almeida *et al.*, 2015). Compounds such as sesquiterpenes, terpene alcohols, and terpene oxides all have the potential to be extracted from essential oil during dry-hopping processes (De Almeida *et al.*, 2015; Haslbeck *et al.*, 2018).

Due to the vast array of terpenes and terpenoids observed after dry-hopping, a broad spectrum of terpene/terpenoid compounds were tested in the vanillin assay. The terpenes/terpenoids selected for the study were β-myrcene, β-linalool, linalool oxide, α-

humulene, β-citronellol, *trans*-geraniol, β-caryophyllene, and caryophyllene oxide. Although the vanillin assay was originally developed to detect monoterpenes and terpene alcohols, the experiment included sesquiterpene hydrocarbons due to its abundance in hop essential oil (15-42%) (Aberl *et al.*, 2012; Biendl *et al.*, 2014).

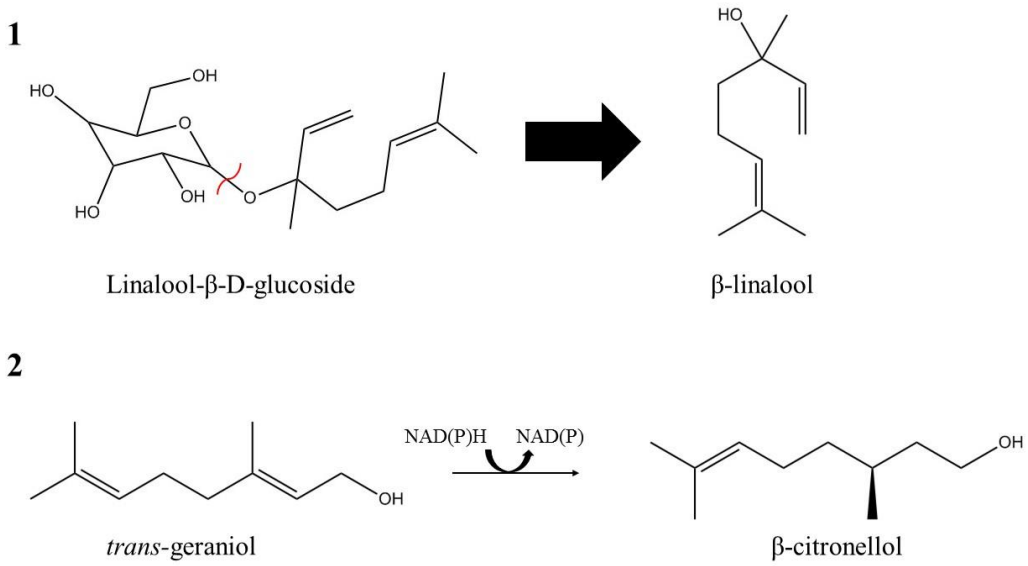


Figure 7.1- Biotransformation of linalool-β-D-glucoside to beta-linalool (1) (adapted from Biendl et al., 2014) and biotransformation (reduction) of trans-geraniol to β-citronellol (2) (King and Dickinson, 2003).

The purpose of this study was to determine the viability of adapting the vanillin assay, as described by Cacho and Ferreira (1990), to assess terpene and terpenoid levels in dry-hopped beer. This objective examined several hypotheses. Firstly, could isolates of terpenes and terpenoids be detected by the vanillin assay? Secondly, can a solid-phase extraction (SPE) method be developed to isolate terpenes from an ethanol solution? Thirdly, with an optimised SPE method, can a serial dilution of a mix of these terpenes be utilised to build a standard curve with the vanillin assay to quantify hop aroma? Finally, can the method be used to detect total terpenoids in beer?

7.2 Experimental Design

7.2.1 Method Development- Terpene/Terpenoid Isolates

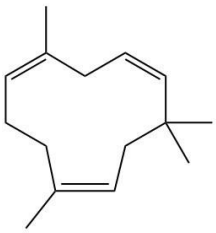
Linalool was utilised as a model compound by Cacho and Ferreira (1990) as its absorbance at 608 nm demonstrated similar results to an analogous molecular mixture of terpenes (Cacho *et al.*, 1990). Due to the differing chemical composition of the targeted terpenes in this study (**Figure 7.2**), terpenoid compounds linalool oxide, β-linalool, α-humulene, and β-caryophyllene (Sigma Aldrich, Poole, UK) were selected as

test compounds with differing chemical compositions to determine an optimum working concentration for the assay. The terpenoids were tested at concentrations of 1 µg/L, 1 mg/L and 100 mg/L in the vanillin assay and scanned on a spectrophotometer to select a wavelength at which most terpenes would produce a signal (peak). A master stock of each terpene isolate was prepared at 100 mg/L in analytical reagent grade ethanol (99.8%) (Fisher Scientific, Loughborough, UK) and in a 70% (v/v) ethanol solution. The master stocks were diluted to concentrations of 1 µg/L and 1 mg/L in a 70% (v/v) ethanol solution and tested in the vanillin assay (Section 2.6.1).

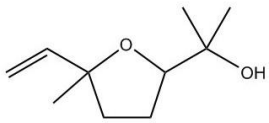
Compounds β-myrcene, β-linalool, linalool oxide, α-humulene, β-citronellol, *trans*-geraniol, β-caryophyllene, and caryophyllene oxide (Sigma Aldrich, Poole, UK) were tested in the vanillin assay at 100 mg/L and scanned in a GENESYS™ 6 UV-Vis spectrophotometer (Fisher Scientific, Loughborough, UK) to assess the reaction of each terpene/terpenoid in the assay.

1

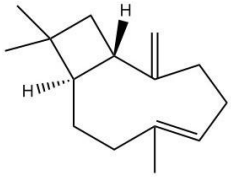
α-humulene



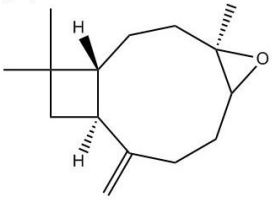
Linalool oxide



β-caryophyllene

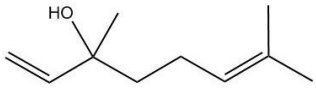


Caryophyllene oxide



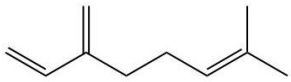
2

β-linalool



β-citronellol

β-myrcene



***Trans*-geraniol**

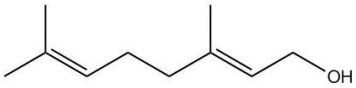


Figure 7.2- Structures of cyclic terpene hydrocarbons (1) and monoterpene hydrocarbons (2) reacted in the vanillin assay.

7.2.2 Solid-Phase Extraction (SPE)

After each terpene isolate was tested in the vanillin assay, each isolate underwent solid-phase extraction (SPE) to determine if SPE was a suitable method to sequester terpene/terpenoid isolates from a liquid matrix and used in the vanillin assay (Section 2.6.2).

7.2.3 Test with Mixed Stocks

Mixed stocks of *trans*-geraniol, β-myrcene, β-caryophyllene, α-humulene, caryophyllene oxide, β-linalool, linalool oxide, and β-citronellol (Sigma Aldrich, Poole, UK) were prepared and serially diluted according to (**Appendix H)**. The level five dilution was chosen according to (**Appendix H)** and the mixed stock was prepared via SPE in (Section 2.6.2) for analysis in the vanillin assay (Section 2.6.1). The effluent from the washing step of SPE was also collected, adjusted to 8% (v/v%) ethanol with analytical reagent grade ethanol (99.8%) (Fisher Scientific, Loughborough, UK) and analysed by GC/MS-SPME (Shimadzu Corp., Milton Keynes, UK.) to examine for terpene/terpenoids washed from the column during the washing steps.

7.2.4 Beer Test

Finally, a commercially produced non-dry-hopped lager and dry-hopped India pale ale were tested utilising SPE and vanillin assay protocols adapted from (Praet *et al.*, 2014) and described in Section 2.6.3 and Section 2.6.1, respectively.

7.3 Results

7.3.1 Terpene/Terpenoid isolates

Preliminary results demonstrated that the vanillin assay could detect terpenes at a concentration of 100 mg/L (**Figure 7.3**). Concentrations below this level showed very low colour formation and were only at or above detection limits on the spectrophotometer (**Figure 7.4).** All experiments following these results were therefore, run at a concentration of 100 mg/L.

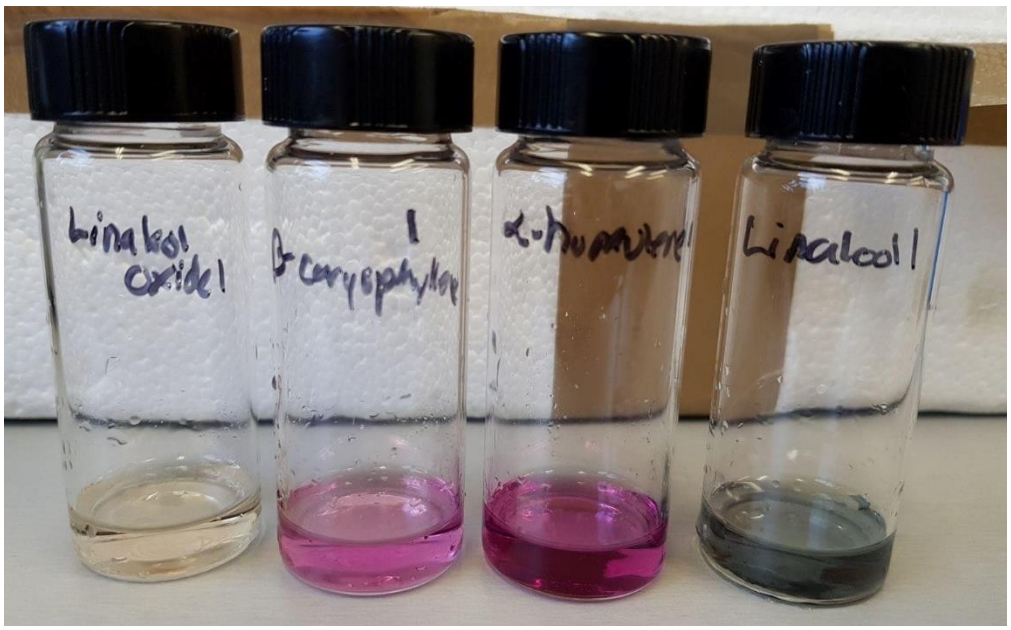


Figure 7.3- Terpene isolates post- vanillin assay, pre-heat treatment at 100 mg/L concentration. Terpene isolate solutions from left to right: linalool oxide, β-caryophyllene, α-humulene, and β-linalool.

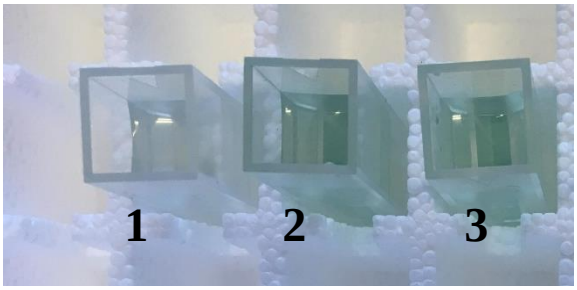
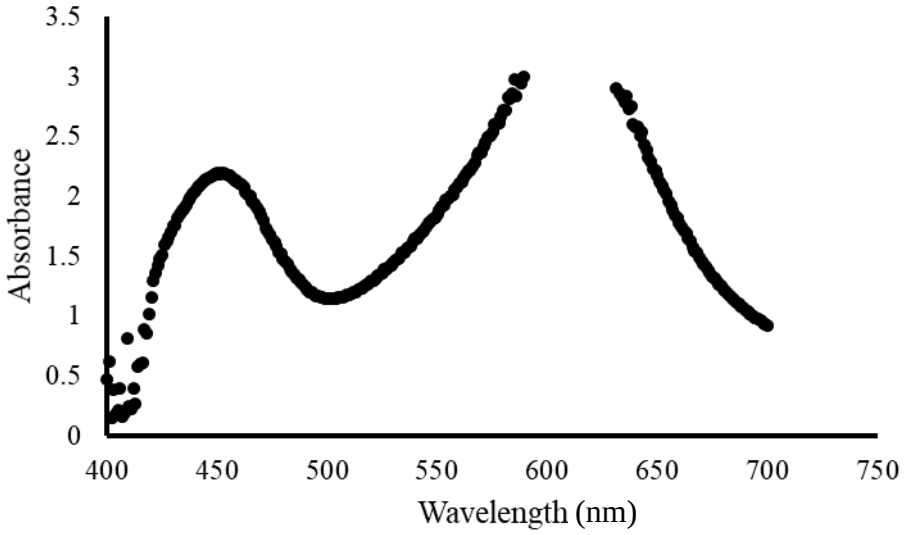


Figure 7.4- Response of β-linalool (2 and 3) compared to an ethanol-blank control (1) from the vanillin assay at 1 mg/L concentration.

Beta-linalool (**Figure 7.5**), linalool oxide (**Figure 7.6**), β-myrcene (**Figure 7.7**), and *trans*-geraniol (**Figure 7.8**) were all observed to produce strong peaks of absorbance at approximately 475 nm. Caryophyllene oxide (**Figure 7.9**) showed a weaker peak of absorbance at approximately 500 nm and β-citronellol (**Figure 7.10**) demonstrated a very weak absorbance around 600 nm. Terpenoids, β-caryophyllene (**Figure 7.11**) and α-humulene (**Figure 7.12**) did not exhibit any trends in absorbance.

A



B

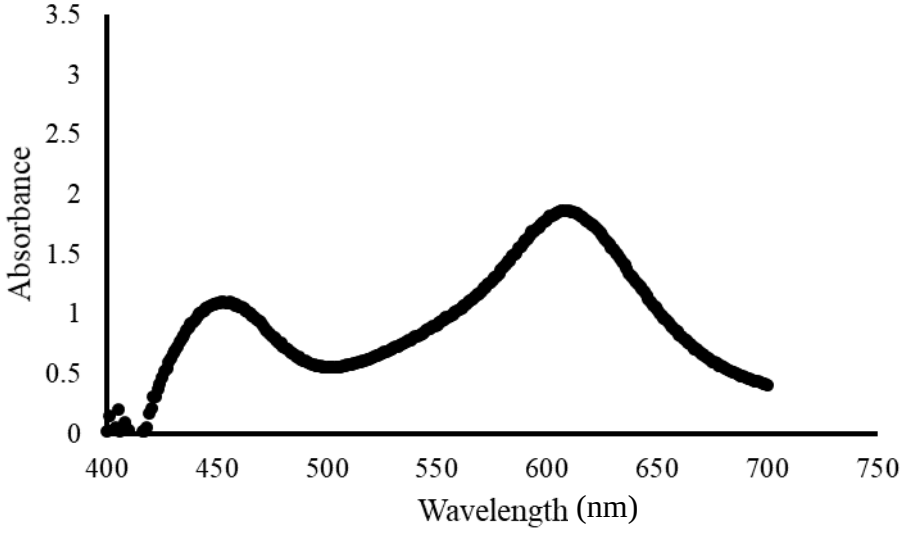
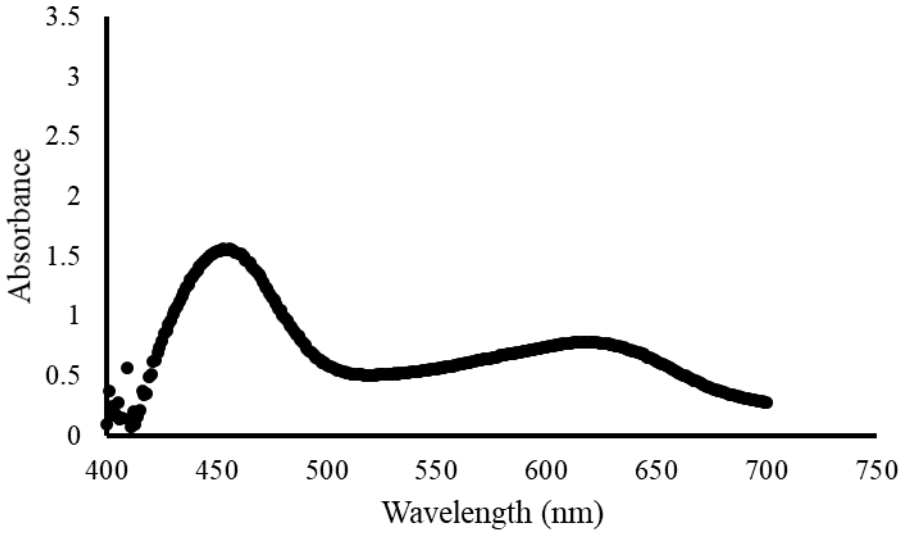


Figure 7.5- Spectrophotometer scans of β-linalool pre-SPE (A) and β-linalool post-SPE (B) at 100 mg/L in 70% ethanol.

A



B

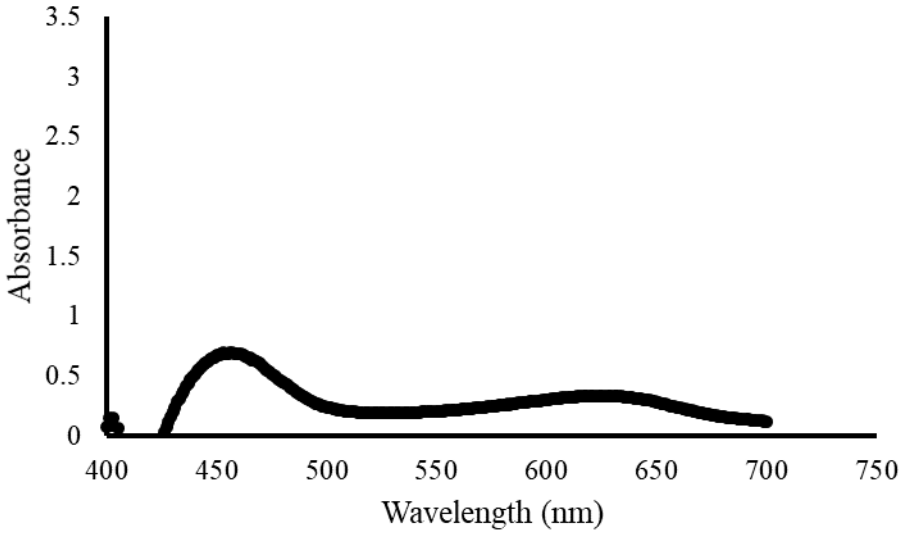
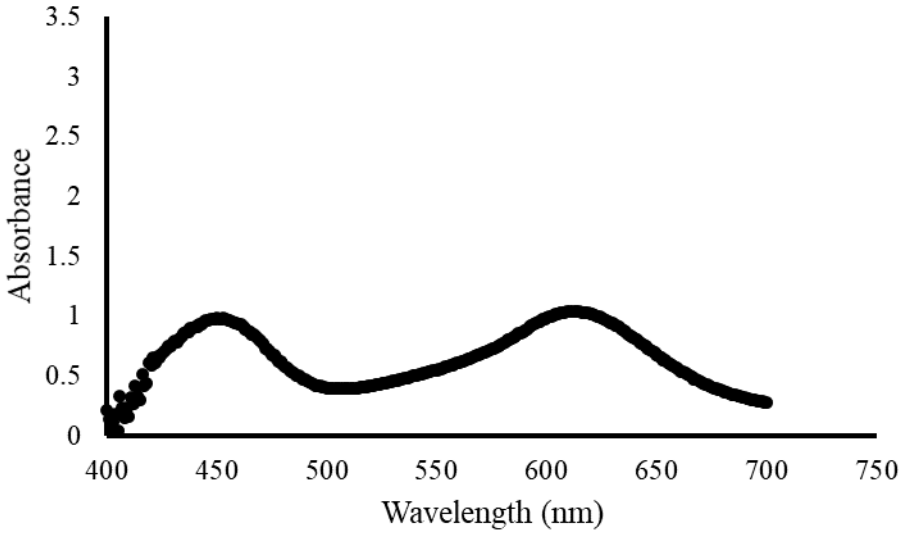


Figure 7.6- Spectrophotometer scans of linalool oxide pre-SPE (A) and linalool oxide post-SPE (B) at 100mg/L in 70% ethanol.

A



B

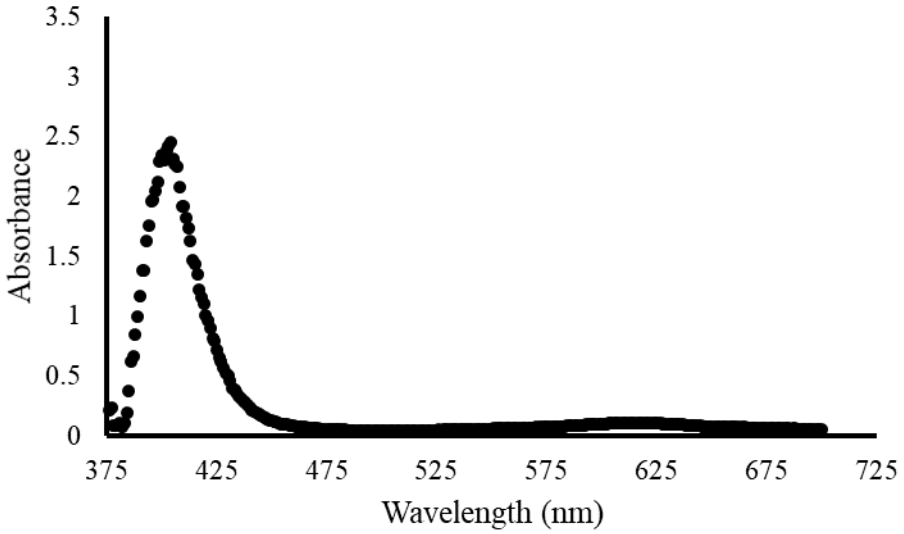
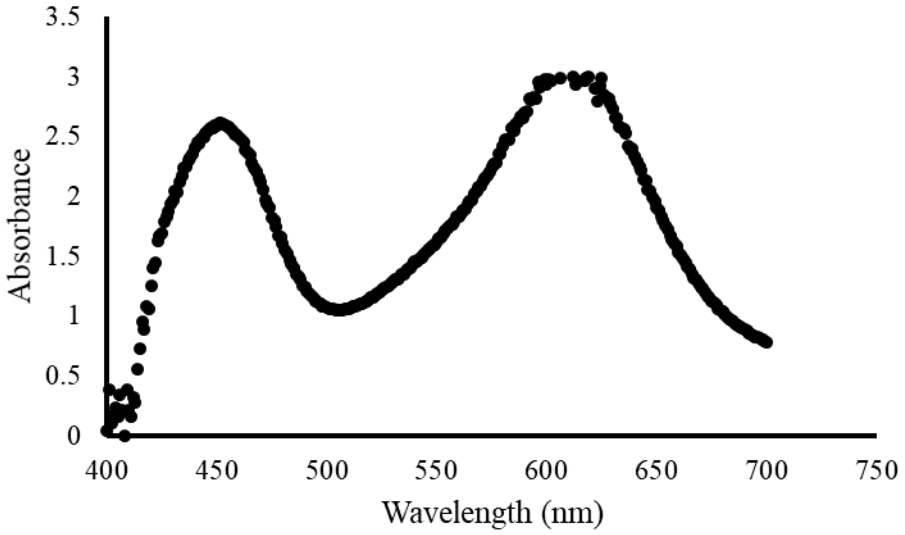


Figure 7.7- Spectrophotometer scans of β-myrcene pre-SPE and β-myrcene post-SPE at 100 mg/L in 70% ethanol.

A



B

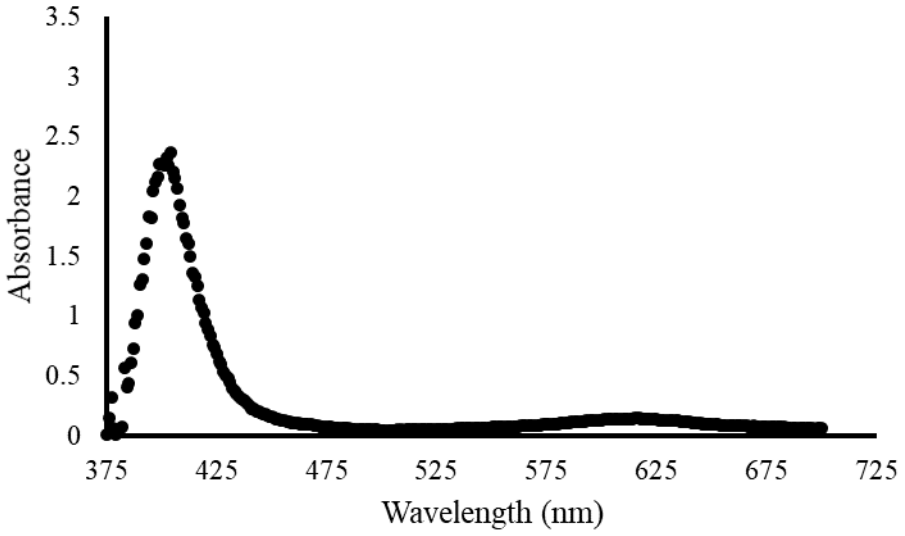
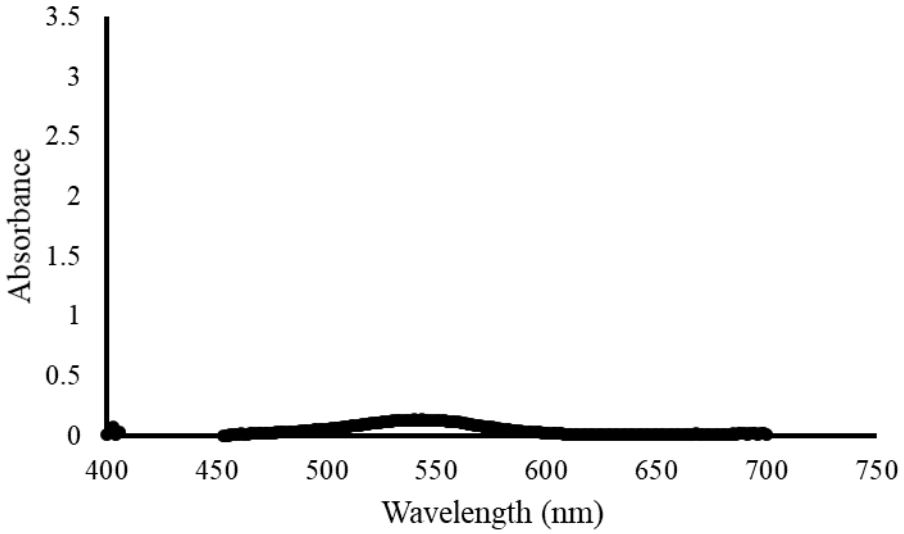


Figure 7.8- Spectrophotometer scans of *trans*-geraniol pre-SPE and *trans*-geraniol post-SPE at 100 mg/L in 70% ethanol.

A



B

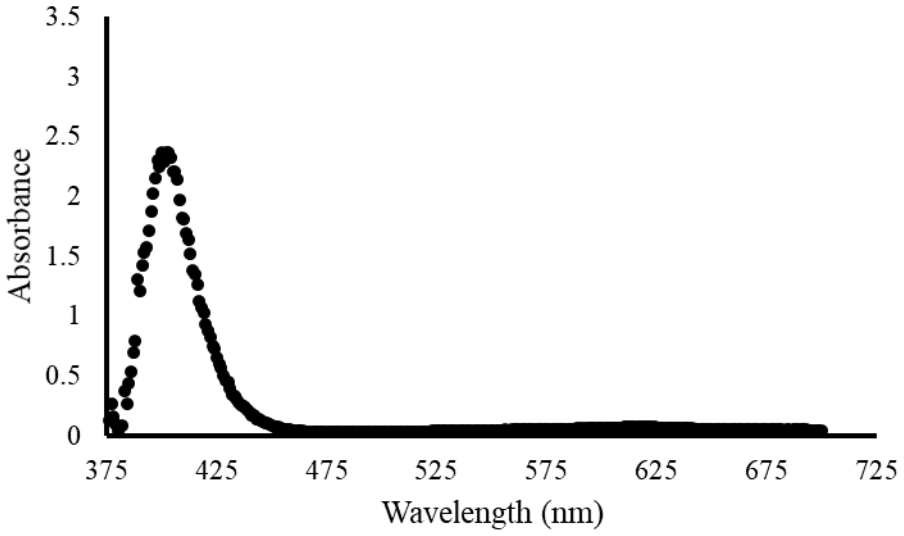
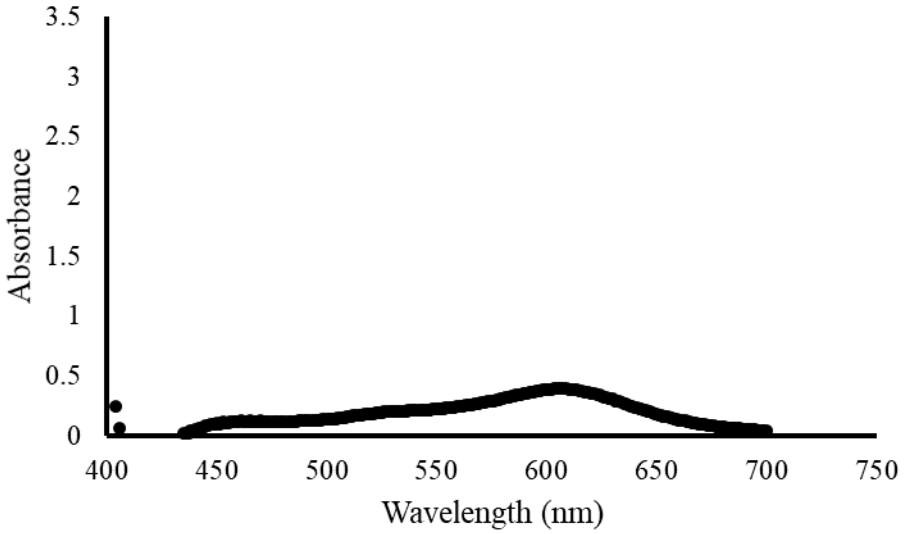


Figure 7.9- Spectrophotometer scans of caryophyllene oxide pre-SPE and caryophyllene oxide post-SPE at 100 mg/L in 70% ethanol.

A



B

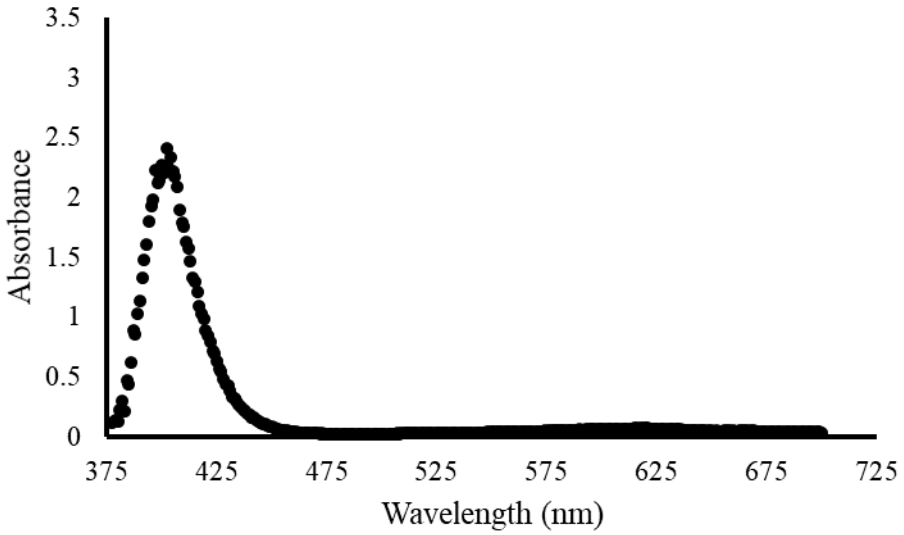
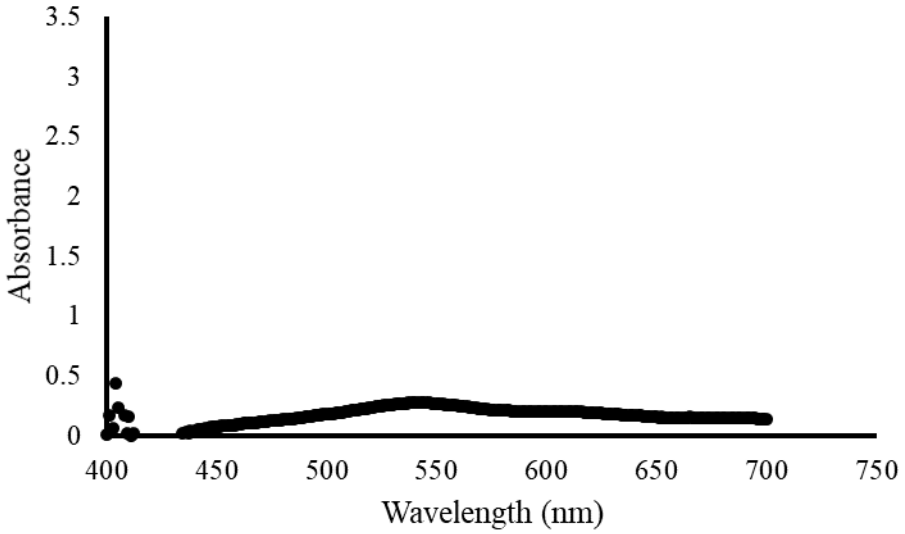


Figure 7.10- Spectrophotometer scans of β-citronellol pre-SPE and β-citronellol post-SPE at 100 mg/L in 70% ethanol.

A



B

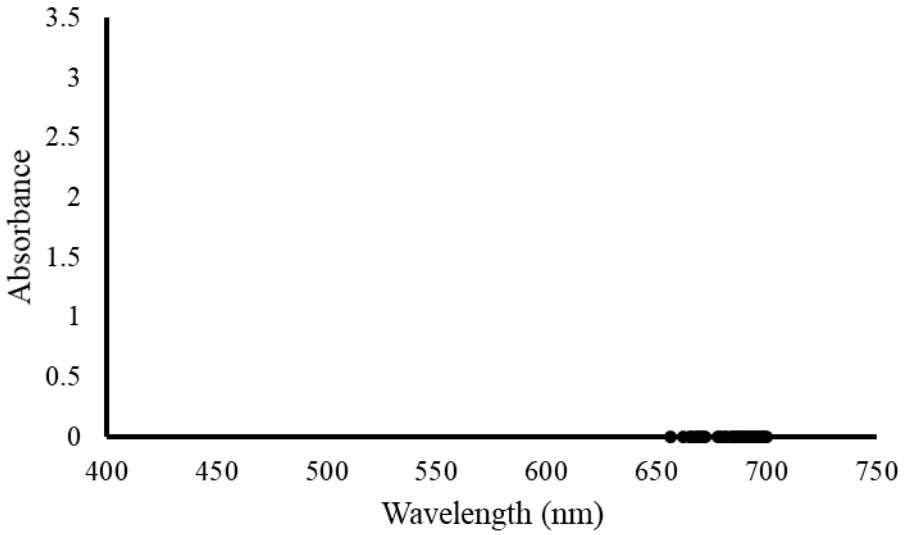
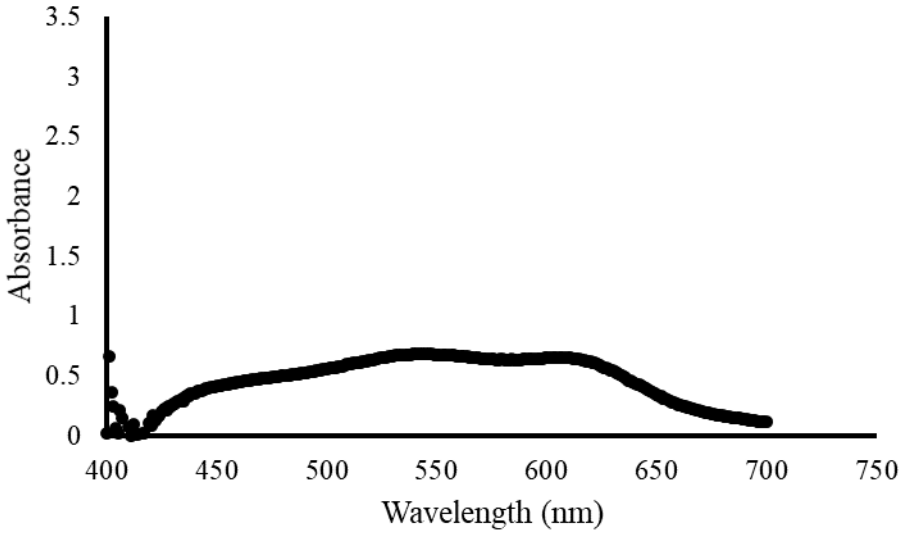


Figure 7.11- Spectrophotometer scans of β-caryophyllene pre-SPE (A) and β-caryophyllene post-SPE (B) at 100mg/L in 70% ethanol.

A



B

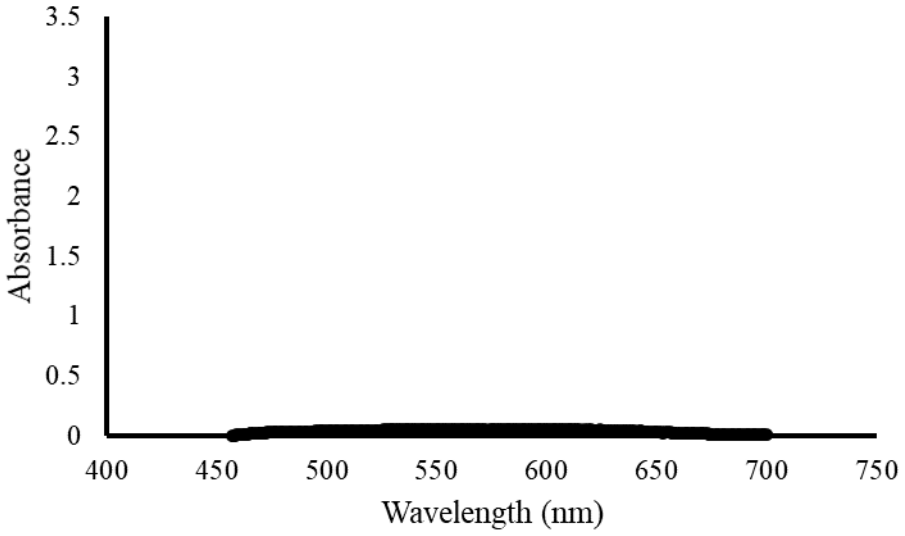


Figure 7.12- Spectrophotometer scans of α-humulene pre-SPE (A) and α-humulene post-SPE (B) at 100 mg/L in 70% ethanol.

7.3.2 Test with Solid-Phase Extraction (SPE)

The only terpenoid compounds to successfully bind to the SPE column and not wash out were β-linalool (**Figure 7.5**) and linalool oxide (**Figure 7.6**) as the vanillin assay results post-SPE were nearly identical to the vanillin assay results without SPE. All other terpene compounds had a limited reaction in the vanillin assay and most likely, did not bind to the column or were removed from the column during the washing steps of the procedure.

7.3.3 Test with Mixed Stocks

The GC/MS-SPME scan results of the mixed-stock solution demonstrated that many of the terpenes had indeed been washed from the column during the washing steps as concentrations of these terpenes were completely recovered (**Table 7.2**).

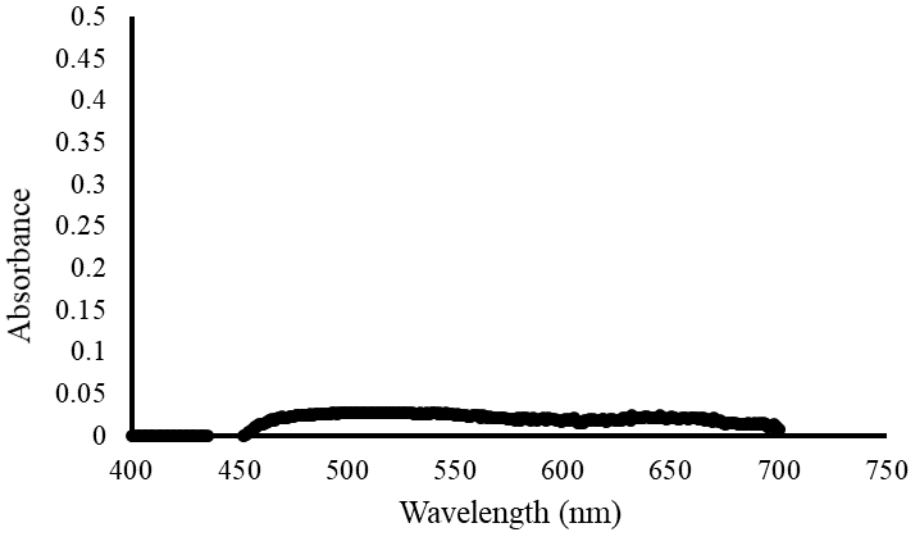
Table 7.2- GC/MS-SPME results of SPE wash water, pre-elution.

Compound	Calculated Concentration (µg/L)
β-myrcene	59.3
Linalool oxide	415.7
β-linalool	357.9
β-citronellol	36.9
trans-geraniol	79.6
β-caryophyllene	0.01
α-humulene	3.53
Caryophyllene oxide	85.9

7.3.4 Beer Test

In this study, the two-step SPE was not successful at sequestering terpenes from a commercially produced lager or India pale ale. There was no reaction in the vanillin assay, this was proved by the 300-700 nm spectrophotometer scan results which are presented in **Figure 7.13**.

A



B

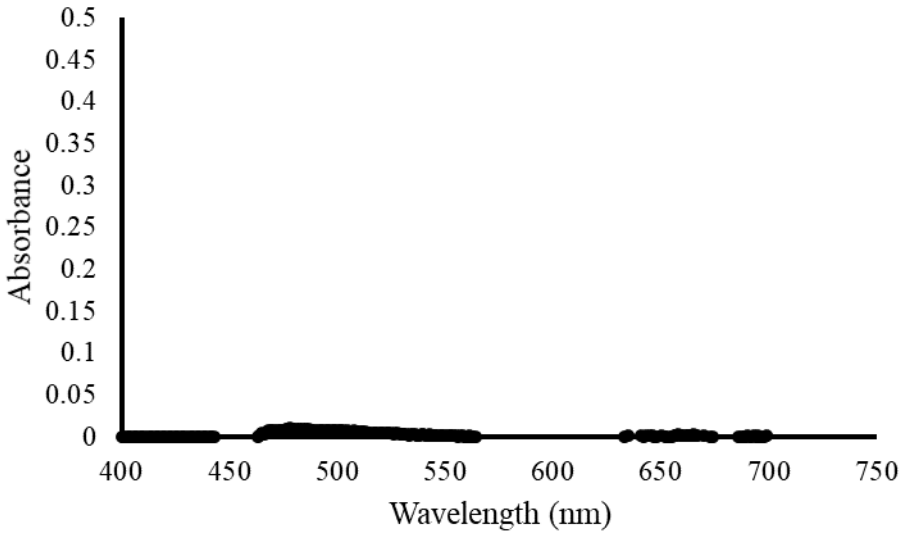


Figure 7.13- Vanillin assay results post-SPE with a commercial lager (A) and IPA (B).

7.4 Discussion

7.4.1 Terpene/Terpenoid Isolates

The vanillin assay was originally designed to assess the concentration of oxygenated terpene compounds found in citrus juices and in musk the wine industry (Attaway *et al.,* 1967; Dimitriadis *et al.,* 1984). The objective of the experimental work presented was to determine if the principles of detecting terpenes in fruit-juice extracts in the vanillin assay could be applied to identifying terpenes in beer. The assay works by utilising the intense reaction of oxygenated terpenes with a strong acid and vanillin (Attaway *et al.,* 1967). However, the assay was also used to measure condensed tannins in wine grapes and malt. Unfortunately, the results proved the chemical reaction of vanillin and sulphuric acid to be non-specific and was reacting with other chemical components in the sample (Price *et al.,* 1978). This could explain the disparity in the results between cyclic sesquiterpene hydrocarbons, terpene alcohols, and monoterpenes tested in the experiment (**Figure 7.5- Figure 7.12**).

It is speculated that the vanillin-sulphuric acid solution is also reactive to double bonds within the compounds. For example, α-humulene contains three double bonds and does demonstrate the highest signal of the three cyclic sesquiterpene hydrocarbons with the highest values recorded just above 0.6 absorbance units (**Figure 7.12**) with β-caryophyllene and caryophyllene oxide with two and one double bond, respectively, absorbing just above 0.2 absorbance units (**Figure 7.11 and Figure 7.9**). As each compound in the assay has a different sensory threshold and analytical level of detection, the concentration of the compounds tested in the assay may have been too low for measurement.

Cyclic sesquiterpene hydrocarbons β-caryophyllene (**Figure 7.11**), α-humulene (**Figure 7.12**), and caryophyllene oxide (**Figure 7.9**) had a limited reaction in the vanillin assay and did not adhere to the SPE column (**Figure 7.2**). It is suggested that cyclic sesquiterpene hydrocarbons do not react in the vanillin assay as their chemical structure is substantially different to that of monoterpenes or terpene alcohols. Other studies have only used monoterpenes and terpene alcohols in the vanillin assay (Attaway *et al.*, 1967; Dimitriadis *et al.*, 1984; Cacho *et al.*, 1990). It is plausible that the sulphuric acid-vanillin solution could not react with the double bonds within the ring structure of the cyclic sesquiterpene hydrocarbons.

Beta-linalool, linalool oxide, β-myrcene, and *trans*-geraniol reacted strongly with the 1.6% vanillin- sulphuric acid solution forming a colouration that was detectable in the spectrophotometer scans. Linalool oxide, β-linalool, and *trans*-geraniol each are oxygenated terpene alcohols and β-myrcene is a monoterpene- both proven to react well with vanillin and a strong acid (Attaway *et al.*, 1967; Dimitriadis *et al.*, 1984; Cacho *et al.*, 1990). Beta-citronellol still showed an absorbance in the vanillin assay at approximately 600 nm but did not form a gaussian peak like the other compounds. This could be due to the chemical structure of β-citronellol given that only one double bond exists between C2-C3 and only one -OH group located on C8 of the main chain.

7.4.2 Solid-Phase Extraction (SPE)

Solid phase extraction did not prove to be an effective method to isolate and enrich the volatile hop compounds examined in this study. There were several issues with the analysis including the solvent choice and the solubility of some of the compounds analysed. Sesquiterpene hydrocarbons should have bound to the column and eluted with absolute analytical grade ethanol (Praet *et al.*, 2014; Praet *et al.*, 2016b). However, if the compounds were weakly bound to the column material, the 70% ethanol solution may have prevented the cyclic sesquiterpenes from bonding to the column material.

Some of the terpenes, particularly caryophyllene oxide and β-myrcene are insoluble in water. In other methods, caryophyllene oxide and β-myrcene were dissolved in 1-propanol or methanol (Dimitriadis *et al.*, 1984; Cacho *et al.*, 1990). Ethanol was chosen for this experiment in an effort to keep the standards in the same solvent as the sample (beer). Based on observations, the concentration of the ethanol required for terpene fractions and for the vanillin assay was too high. The SPE method was not effective as the terpenoids with low binding affinity to the column immediately washed though the column (**Figure 7.9**, **Figure 7.11**, **Figure 7.12**). It is hypothesised that it would be appropriate to suspend the terpenes in an absolute ethanol stock and dilute the stock a lower working concentration (5% (v/v) ethanol) with water should the work be repeated. The low percentage of ethanol in the diluted terpene isolates would have a better opportunity to bond to the C18 column material. After the sample loading step, the compounds would then be eluted in absolute ethanol and diluted to a proper working concentration for the assay.

Figure 7.10 (B), Figure 7.11 (B), and **Figure 7.12 (B)** demonstrated negative absorbance. These were poor calibrations and the negative absorbance could be due to a fluorescent reaction of the column material and the 1.6% vanillin-sulphuric acid solution. Further testing and calibrations would be required to ensure that the fluorescent absorbance was due to column material.

Beta-linalool (**Figure 7.5**) and linalool oxide (**Figure 7.6**) were the only compounds to be extracted by SPE. This is, most likely, due to their structure, degree of conjugation, and affinity for the column material (**Figure 7.2**).

7.4.3 Beer Test

The two-column extraction technique utilised was not an effective method of extracting terpenes for the vanillin assay. It is possible that the concentration of the compounds eluted from the column were too low to detect in the vanillin assay (<100 mg/L). Various terpene hydrocarbons and sesquiterpenes have been enriched and isolated using SPE previously, however, the compounds were qualitatively analysed with GC/MS-SPME analysis (Van Opstaele *et al.*, 2012; Praet *et al.*, 2014). As GC/MS-SPME can detect terpene hydrocarbons and sesquiterpenes compounds in the µg/L concentration range, the concentration of these compounds may have been too low to detect after SPE (Andres-Iglesias *et al.*, 2014).

It is also possible that other non-polar beer constituents were competitively bonding with the column material, causing the targeted compounds to wash out of the column without chance of bonding to the material.

Another potential issue in using SPE for the vanillin assay is that a normal concentration of beer loaded onto the column is not heavily concentrated. Previous methods tested the vanillin assay with concentrated steam-distilled extracts that had been phase-separated with liquid-liquid extraction (Attaway *et al.*, 1967; Dimitriadis *et al.*, 1984; Cacho *et al.*, 1990). Concentrating beer by means of freeze-drying or passing several column volumes of beer through the SPE column is suggested as a potential way to concentrate the analytes targeted in this study.

Chapter 8- Conclusions and Future Work

"We can't solve problems by using the same kind of thinking we used when we created them." ~Albert Einstein

Beer stability and quality is a complex topic and subject to a great deal of research over the last 100 years. Breweries around the world work toward two main goals for beer that has left the brewery: aromatic stability and physical stability. Multiple variables affect beer stability such as light, oxygen, raw materials, process parameters, and storage temperatures to name a few. As all of these factors may help or hinder beer stability, for brewers and brewing scientists multiple questions arise. Can historical process data be used to identify issues in process leading to sporadic spikes in turbidity? How are unfilterable hazes formed and, what can be done to prevent their formation? How are hop aromas extracted into beer and, how stable are the compounds? Can sensory and analytical data correlate to trace beer flavour development? This thesis attempts to begin to answer some of these questions and explain the challenges identified.

8.1 Statistical analysis on Historical Process Data

Statistical analysis of historical process data was the foundational knowledge of the turbidity studies described in this thesis. The use of statistics is imperative to both reactive and proactive approaches to understanding and maintaining beer quality. By assessing the process holistically and intra-departmentally, a better snapshot of the brewing, fermentation, and packaging procedures were obtained.

The results of Spearman's rank-order correlation coefficient reported several significant values. The correlation coefficients concluded that the high sample numbers (n= 322) caused the correlation coefficients (ρ) to be artificially significant. Stepwise regression was also used to construct the most robust model possible with the factors listed in **Table 3.1**. Coincidentally, all of the values used to build the stepwise regression model were also correlated to increased turbidity levels with a high probability ($p< 0.05$) (**Table 3.1**). Despite this, none of the scatterplots in Appendix C.1-C.14 indicated that significant relationships existed between brewery parameters and increased turbidity. Additionally, a limitation that was unearthed during this study was that data management schemes were slightly different interdepartmentally. This

ultimately reduced some of the data that was available for analysis in the experiment, most notably, from the yeast management system.

Despite the lack of robust significant correlations, statistical analysis of historical process data may still be used as a diagnostic tool. It is imperative not only for quality checks, but to also identify inefficiencies in the process. If holistic brewery data is consistently checked, waste streams can be reduced and brewing procedures may be fine-tuned that will ultimately, end up in significant cost-savings.

8.2 Elucidation of Sporadic Haze Formation

The first experiment in the greater study utilised various enzymes to digest haze particulates in beer in an attempt to broadly identify the macromolecule that may be responsible for sporadic increases in turbidity. In all 'test' samples, pre and post-digestion differences were significant. When assessing pre and post-digestion values, pepsin was the only enzyme to reduce turbidity values at all light angles measured below the 'high turbidity' limit set by the experiment. Samples were classified as high haze or high turbidity samples if the turbidity values were 5.0 EBC or 20 NTU. Additionally, Ultraflo®Max reduced small particle sizes as the 90° angle of incidence saw values lower than the acceptance limit post-digestion in the high haze samples. It is possible that the particles may have been matter small enough to easily pass through the filter. As pepsin and Ultraflo®Max digest proteins and β-glucans, respectively, it was speculated that increases in turbidity could have been due to proteins and/or β-glucans. However, further studies were required to confirm these results and to rule out the influence of polyphenols as protein-polyphenol complexes are largely responsible for increases in beer turbidity (Siebert, Carrasaco, *et al.*, 1996; Siebert, Troukhanova, *et al.*, 1996; Siebert, 1999; Aron *et al.*, 2010).

Haze microscopy was utilised to independently confirm the enzyme digestion studies as a visual assessment of how particle morphology and abundance differed between high and low haze samples. Eosin Yellow dye, stained proteinaceous skins and flakes in the high haze samples, darkly staining an abundance of aggregates. While the low haze sample stained small particles a pale pink colour. Thionine stained dextrins and starches, large purple stained particulate matter were observed in the high haze samples. Thus, further confirming the results of the enzymatic digestion studies. Overall, high haze samples were determined to contain larger aggregates of all particles while low haze samples contained an abundance of small, fine particulate matter. It is

hypothesised that the particulate matter observed in high haze samples consisted of small particulate matter that had aggregated to form visible hazes. The particulate matter in the low haze beer samples was small enough not to cause visible turbidity. Future studies and further sample collection would be useful to confirm this hypothesis.

Wet-chemical analysis was used to quantify proteins, polyphenols, and β-glucans content as these compounds are the most likely culprits for sporadic increases in beer turbidity. The β-glucan concentration was the only macromolecule found at a higher concentration in high haze samples than in low haze samples. The protein concentration in high haze samples was only slightly below the high low/normal haze value of 5.34 g/L at 5.04 g/L. Two main conclusions were drawn from the experiment. Firstly, proteins in high haze samples have complexed with polyphenols, resulting in lower protein and polyphenol concentrations (**Table 4.7**). Secondly, the elevated β-glucan in high haze samples, when considering the results of the enzymatic digestion studies, are derived from yeast cell walls. It was hypothesised that yeast handling/fermentation procedures in this brewery, exposed the yeast to substantial stress causing the release of cell wall components, such as mannoproteins, as a stress response. The liberation of mannoproteins causing an increase in turbidity that was unfilterable as mannoproteins are smaller than 0.45 µm and able to pass through filter sheets used at the industry sponsor.

The hypothesis was confirmed by LC-QTOF-MS and protein fractionation following concentration utilising an ÄKTA Avant Liquid Chromatography system. The protein fractionation results confirmed that distinct differences in protein content existed between high haze and low haze beer samples. Two peaks were detected at A_{280} in the high haze sample and one peak was observed in the low haze sample (**Figure 4.14 and Figure 4.15**). To identify the differences in cell wall protein content between the samples, Targeted LC-QTOF-MS was used. The differences in protein content were not as robust as expected, however, differences were observed between high and low haze samples. The sequence coverage was higher in high haze samples, indicating that the targeted flocculation/mannoproteins were present in the sample. Finally, the D-mannose concentrations further confirmed that mannoproteins were a culprit of the increased turbidity as mannose levels were higher in the high haze samples measured (**Table 4.10**).

Over the course of the study, the brewery supporting this work altered yeast management and handling practices. As these changes were implemented, sporadic increases in turbidity decreased. When spikes in turbidity did occur, the values were lower (~6.0 EBC) compared to the values recorded at the beginning of the study (~13 EBC). With this knowledge and the results of this study, it is suggested that yeast components were the cause of sporadic increased turbidity in beer.

8.3 Terpene Studies in Packaged Beer

The overall conclusion from this part of the study was that sensory and analytical data could not be correlated. In some cases, sensory descriptors and analytical data did pair together, such as β-myrcene, which was found to share a relationship with the attributes 'earthy hop' or 'bitter' (**Figure 5.3**). This result was not surprising as earthy hop characteristics are often used to describe the flavour and aroma of β-myrcene (Zunkel, 2015). However, overall, it was found that common sensory descriptors could not be paired with their respective chemical compound in this study. This finding was unexpected as most of the sensory descriptors shared a relationship with the compounds measured in GC/MS-SPME analysis.

The evolution of aroma compounds over a set period of time might be of more interest to breweries as several trends relating days to either sensory descriptors or hop compounds were observed. For example, in the second and third sample sets, β-myrcene was always observed in fresh beer samples of less than 14 days old and the floral hop descriptor was apparent in beer aged 70 days or more (**Figure 5.4 and Figure 5.5**).

Finally, some sensory descriptors were found to contribute a stronger influence to the models than other descriptors. For example, the 'citrus hop' sensory descriptor had the greatest impact on all of the PCA plots. Therefore it is hypothesised that monitoring and plotting the change in 'citrus hop' characteristics over time could indicate the point at which beer loses its freshness.

Overall, it has been demonstrated that beer retains citrus, floral, and earthy hop aromas when stored at colder temperatures. These words are commonly used to describe 'fresh' beer flavours so it can be deduced that storing beer at a cold temperature retains freshness for a longer period of time. It is suggested that breweries should tighten guidelines as to how logistics departments and distributors control the environment beer

is stored and transported in. This ensures that products reach consumers in the best possible condition.

8.4 Dry-Hop Conditions Effect on Hop Oil and Terpene/Terpenoid Extraction

It can be assumed that, due the chemistry of the compounds, that not all terpenes/terpenoids share similar solubility characteristics. When examining the solubility and extraction of these compounds, it can be therefore assumed that the process is not is not a direct linear transfer of analytes from the vegetative plant material into beer. Ethanol affects the polarity of the solution, causing some non-polar compounds to be less soluble in solution, which ultimately effects terpene solubility in beer.

In the trial assessing the solubility of terpenes/terpenoids in Simcoe hops, differences in solubility and nebulous results were noted as the concentration were determined to be quite low (e.g. 0.77 mg/L β-myrcene) in comparison to Chinook (e.g. 5.08 mg/L β-myrcene) (**Figure 6.5**). Following on from this work, it is recommended that brewers should request information from their suppliers as to what each hop variety may chemically offer in terms of total oil content and terpene content. Armed with this knowledge, brewers will be able to make informed decisions on what to expect from the hop varieties utilised. The brewer must optimise their processes and systems depending on the impact desired from the dry-hopping process, on each individual product.

The preliminary work started here to understand terpene/terpenoid solubility is crucial to understanding how different factors such as alcoholic strength, affect the transfer of terpenes. Once this is understood, this could implicate procedures leading to an overall cost-savings for the brewery. If a large amount of essential oil remains in the spent hops, this represents a large loss of viable raw materials, incurring large costs for the brewery that cannot be recovered.

These results prove that general assumptions cannot be drawn from any dry-hopping technique and applied in multiple situations. As demonstrated previously by Vollmer and Shellhammer, (2016) the extraction of terpenes is not a linear process and simply adding more hops to a solution, does not imply that an increase of the desired hop aromas will result. The study presented here highlights the importance of understanding the composition of the hop varieties used and their potential impact. The

solubility of terpenes are dependent upon the extraction medium and may be more or less soluble depending on ethanol concentration.

8.5 The Vanillin Assay

Overall, the vanillin assay was not successful at quantifying terpene/terpenoid concentrations in beer in its current form. When assessing terpene/terpenoid isolates, the theory was proposed that the vanillin-sulphuric acid solution was more reactive to compounds with double bonds such as α-humulene, a compound with three double bonds, reported the highest absorbance value just above 0.6 absorbance units (**Figure 7.11**). Additionally, the concentration of terpene/terpenoid compounds present in beer samples are too low to be detected as the assay was designed for compounds in the mg/L range and not the µg/L range.

Solid-Phase Extraction (SPE) was a method used in place of liquid-liquid extraction to isolate and concentrate terpene/terpenoid compounds. The SPE method used was not successful at isolating terpenes/terpenoids. However, with the use of a different solvent and a larger sample volume passed through the column, the concentration step could be successful. In the SPE test, beer should be freeze-dried to concentrate the terpenes/terpenoids within solution. With an altered SPE method, it may be possible to successfully isolate volatile terpene hydrocarbons and sesquiterpenes in high enough concentrations to obtain meaningful results.

With further development, the method should only be used for targeted analysis of classes of terpenes. As monoterpene oxides generally had the strongest response in the Vanillin Assay, it would be beneficial to construct a concept such as a ‘monoterpene oxide equivalence value’. The ‘monoterpene oxide equivalence value’ would measure monoterpene oxides as a broad class and yield one, general concentration value. While a ‘monoterpene oxide equivalence value’ is not as robust as GC/MS-SPME analysis, it still provides brewers with a value of a particular class of aromatic compounds are present in the sample. From this, separate assays could be developed for sesquiterpenes, sesquiterpene oxides, monoterpenes, etc.

Finally, perhaps the largest limitation of the vanillin assay is the limit of detection. Hop terpene and terpenoid compounds present in beer exist at levels far below 100 mg/L and are more commonly found in concentrations of µg/L. For example, β-myrcene is commonly found above flavour threshold values in beer at the 30-100

µg/L while some monoterpene alcohols such as β-citronellol exist in even lower quantities (<10 µg/L) (Takoi *et al.*, 2014; Rettberg *et al.*, 2018). This issue could be addressed by utilising a solvent that produces less background noise, but further tests are required to prove this hypothesis.

8.6 Future Work

8.6.1 Statistical Analysis on Historical Process Data

Calculating correlation coefficients to search for relationships between brewery parameters and turbidity is a robust first step in statistical diagnostic studies. In future analysis, a principal component analysis (PCA) plot could be constructed and ellipses could be drawn to graphically determine if relationships exist between brewery/packaging parameters and increased turbidity. Alternatively, it could be advantageous to isolate 'high' turbidity data from 'low/normal' turbidity data, calculate the means and standard deviations of all brewery parameters and compare the 'low/normal' means to the 'high' means. This could provide a better representation of the true differences between sporadically high haze batches and the low haze/normal haze batches.

In future studies, it would be beneficial to include data from the yeast plant in statistical analysis. Information such as generation number and propagation conditions could unveil important relationships between increased turbidity and brewery processes. This could provide crucial information missed upon initial analyses in this study.

8.6.2 Elucidation of Sporadic Haze Formation

The experimental results have generated many questions that require future work. To determine if yeast-derived turbidity is indeed due to stress, it would be valuable to perform a confirmatory study by acid washing yeast and storing yeast at temperatures from 1°C to 15°C. Acid washing is a method of protecting brewer's yeast against beer-spoilage microorganisms. It is a common practice but, if done incorrectly, may have serious impacts on yeast health and quality and induce stress-responses (Cunningham *et al.*, 1998; Van Bergen *et al.*, 2004).

In future work, the suspension collected in the yeast-stress study and would be studied in tandem with high and low haze beer samples as positive/negative controls. First, the samples would be assessed utilising size-exclusion chromatography to determine the molecular weight of the compounds in suspension (Steiner *et al.*, 2010).

With this information, it is easier to identify classes of particles present or absent in suspension.

Glycogen would be beneficial to measure during wet-chemical analysis of the suspensions as the presence of glycogen in suspension implies problems with yeast management (Steiner *et al.*, 2010).

The LC-QTOF-MS would be repeated, but with slight alterations based on the preliminary studies completed in the thesis. It would be beneficial to concentrate a larger sample to obtain a stronger signal and more sequence coverage in the LC-QTOF-MS analysis. Additionally, samples should first be analysed by a tandem MS/MS search to determine what proteins are present within the sample. Following this, a targeted study of the proteins present in the sample, as well as mannoproteins and flocculation proteins should be assessed in this study. Proteins with internal repeats (Pir) proteins and glycosylphosphatidylinositol (GPI)-cell wall proteins (CWP) are important to measure in future research as some Pir protein phenotypes are expressed under heat stress, nitrogen starvation, or cell wall stress and GPI-CWP's are relevant to cell wall stress responses (Ecker *et al.*, 2006; Jung and Levin, 1999; Klis *et al.*, 2002; Ram *et al.*, 1998; Terashima *et al.*, and Kitada, 2000).

Finally, a more comprehensive sugars analysis should be completed in future work to determine what residual sugars exist, post-fermentation. The sugars analysis would ideally utilise HPLC incorporating D-mannose, D-fructose, and D-glucose into the suite of analysis. A sample preparation step should include deglycation. Deglycation is important in measuring hexose sugars as the compounds exist in their free-form following the deglycation process (Schulte *et al.*, 2016).

8.6.3 Terpene Studies in Packaged Beer

Future studies assessing the change of the citrus hop descriptor in beer over time could be useful in determining when beer 'freshness' is lost and when aged flavours become apparent. To trace the change in citrus hop aromas/flavours, beer would be spiked with a set concentration of limonene, β-linalool, or β-citronellol prior to packaging. The compounds would be measured over the course of ageing as well as the citrus hop sensory descriptor. PCA plots and Pearson correlation coefficients would be calculated to determine if the change or loss of the citrus hop character over time is significant.

It would be beneficial in future work to add additional sensory descriptors to more comprehensively describe the compounds analysed. By completing a true 'paired' study, sensory and analytical descriptors may correlate better and strong conclusions can be obtained from the study. Gas Chromatography-Olfactometry (GC-O) is a technique that would be beneficial in further studies. As compounds are eluted from the column, they travel by carrier gas to a 'sniff' port. Here, an analyst sniffs the elution and describes the smell. All analyst responses are recorded by software that can overlay the sensory descriptors and the analytical data. This is a common, but expensive technique to pair sensory and analytical data. However, its use in future experiments would be key to understanding where sensory and analytical data meet and diverge.

8.6.4 Dry-Hop Conditions Effect on Hop Oil and Terpene/Terpenoid Extraction

In future studies, it would be advantageous to test the experiment in a lager beer followed by expanding the experiments to include more beer styles. The solubility of individual terpenes in beer, chemically speaking, are based upon alcohol content and other analytes in solution. A limitation of the study was that it was solely studied in a simulated beer matrix and not in beer. Extraction efficiencies may differ between the two matrices and further studies are suggested to address this issue.

Previous studies have determined that dry-hopping in different beer styles result in different sensory characteristics (Kaltner *et al.*, 2013). In future work, it would first be beneficial to determine if terpene extraction in beer would be different to studies in a simulated beer matrix. Secondly, it would be valuable to determine how different beer styles might affect the solubility of terpenes/terpenoids.

In parallel with studying terpene/terpenoid extraction in different beer styles, the residual gravity would also be measured. It is possible that a high residual gravity will result in lower terpene/terpenoid extraction as the compounds cannot be easily extracted in a hyper-saturated solution. Finally, valuable data could be gained from repeating the studies outlined using multiple hop varieties. With this information, similarities and differences can be discussed between new world/old world hop varieties effectiveness in the dry-hopping process.

It would also be interesting to collect the spent hop material from these samples and extract the remaining hop oil from the spent hops utilising methanol. The

extractions would then be studied via GC/MS-SPME or GC-FID to determine if relevant aromatic compounds remain in hops post-extraction.

8.6.5 The Vanillin Assay

If the Vanillin Assay were to be developed further it is suggested that beer samples should be freeze-dried prior to resuspending the material in methanol, and performing SPE on the beer/methanol solution. The concentrated beer solution should contain a higher concentration of terpenes could be extracted via SPE and detected in the vanillin assay.

Additionally, a 'monoterpene oxide equivalence value' would be a more suitable option to obtain significant results from the assay. With this, the concentrations of total monoterpene oxides in the solution can be determined and used by breweries of all sizes. Other methods require development to quantify other terpenoid groups such as sesquiterpenoids, sesquiterpenes, aldehydes, ketones, etc.

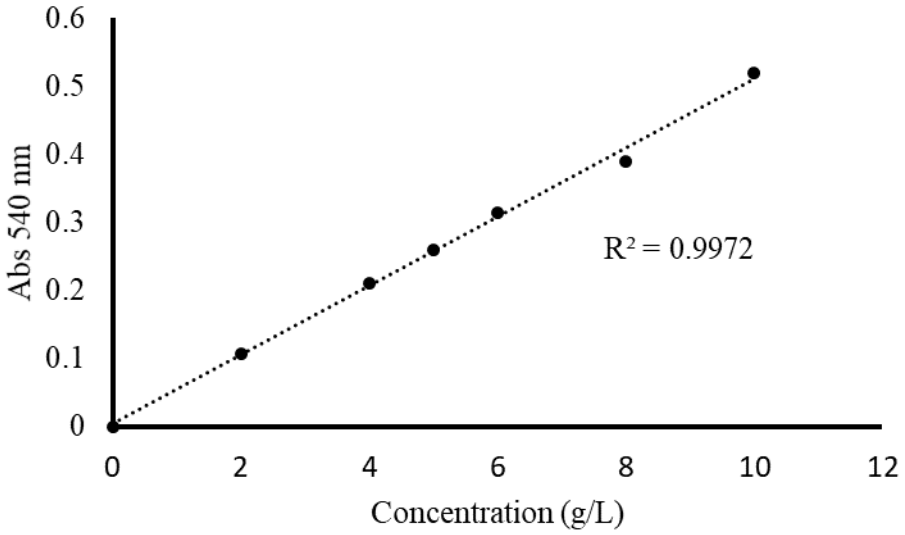
8.7 Conclusions

Monitoring and using insightful knowledge of how the quality of *all* raw materials affect physical-chemical stability of beer is important in producing a high quality product. High quality products, brand dependant, should contain very low turbidity with stable flavours. Yeast, the soul of beer, converts fermentable sugars into alcohol, esters, and aldehydes and should not contribute to turbidity. If yeast is not stored cold and in low-stress inducing environments, sporadic increases in turbidity may be observed as a result (Chapter 3 and 4).

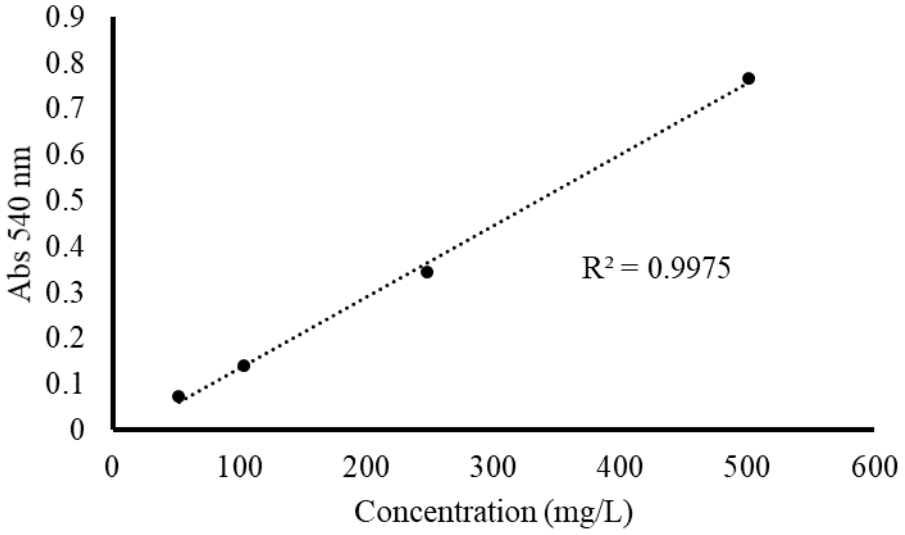
Flavour extraction must first be understood before attempting to understand flavour stability. Overall, it is imperative for brewers to understand what terpenes/terpenoids are contained within the essential oil of each hop variety used. This further informs hop selection as the solubility of each terpene/terpenoid is based upon the polarity of the compound. Additionally, changes in flavour/aroma post-packaging is imminent but may not always correlate to sensory data. If the pairing of sensory and analytical data is desired, it is crucial to train panellists on the flavour/aroma of specific compounds in beer. This way, a better snapshot of the change in beer aroma over time can be determined. Finally, it is possible to develop an assay to determine the concentration of varying terpene classes in beer. However, further method development is required to achieve this goal.

Appendix A

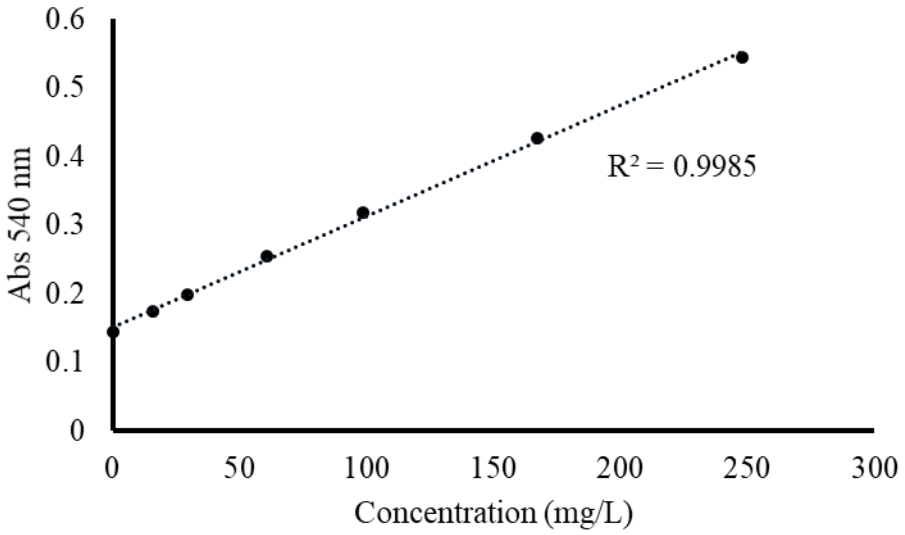
Appendix A.1. Total Protein Calibration Curve using the Gallery™ Plus Beermaster Automated Photometric Analyser.



Appendix A.2. Total Polyphenol Calibration Curve using the Gallery™ Plus Beermaster Automated Photometric Analyser.



Appendix A.3. Total β-glucan Calibration Curve using the Gallery™ Plus Beermaster Automated Photometric Analyser.



Appendix B

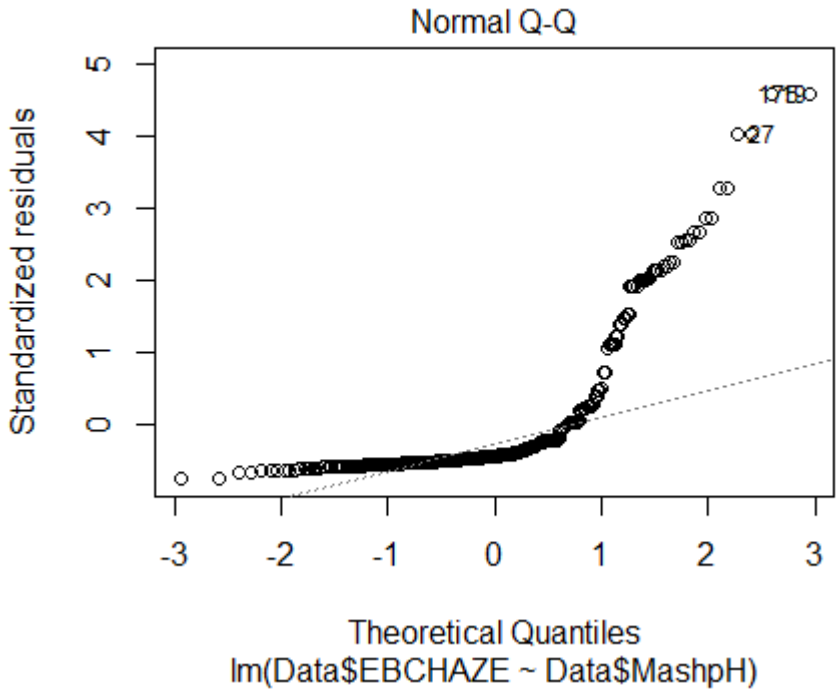


Figure B.1- Quantile- Quantile (Q-Q) plot assessing normality of mash pH and EBC haze data. As the data points are skewed right, the data set is not normally distributed.

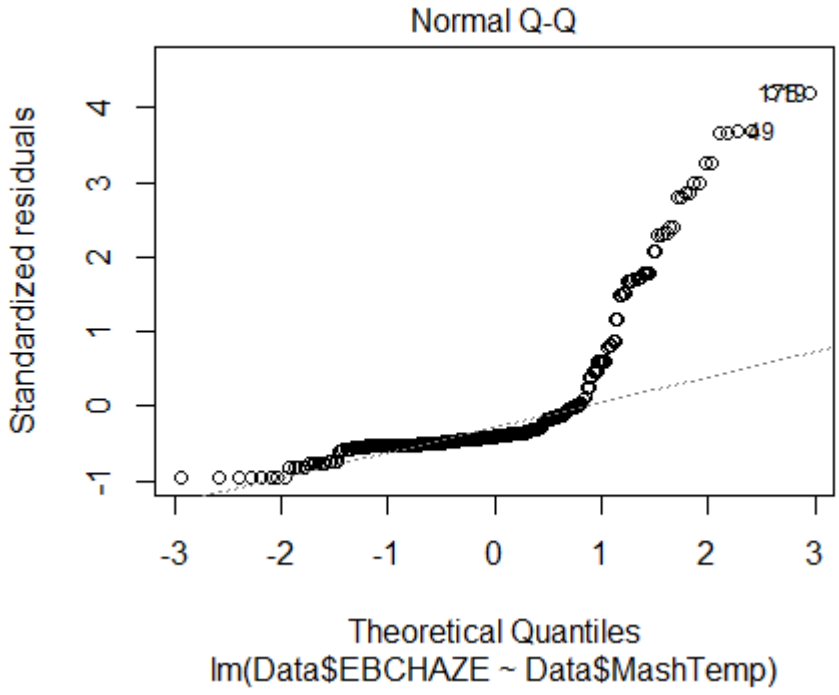


Figure B.2- Quantile- Quantile (Q-Q) plot assessing normality of mash temperature (°C) and EBC haze data. As the data points are skewed right, the data set is not normally distributed.

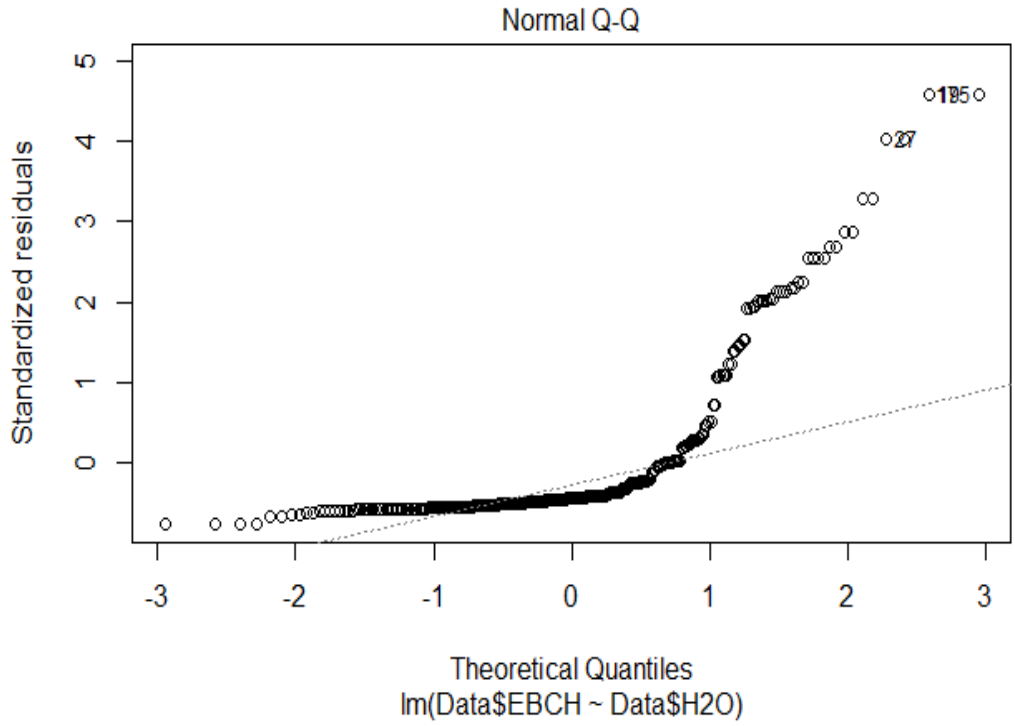


Figure B.3- Quantile- Quantile (Q-Q) plot assessing normality of strike water volume (hL) and EBC haze data. As the data points are skewed right, the data set is not normally distributed.

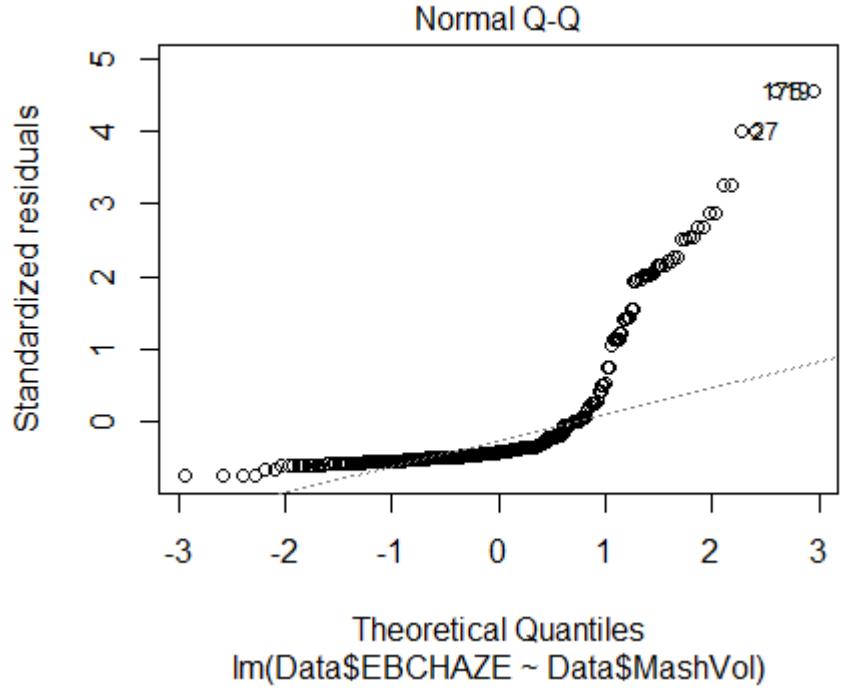


Figure B.4- Quantile- Quantile (Q-Q) plot assessing normality of mash in volume (hL) and EBC haze data. As the data points are skewed right, the data set is not normally distributed.

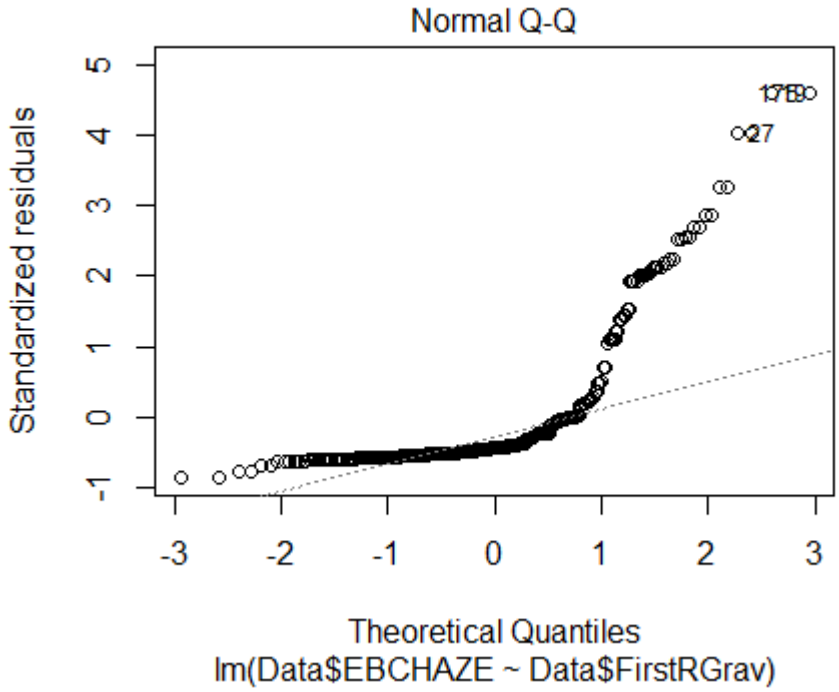


Figure B.5- Quantile- Quantile (Q-Q) plot assessing normality of first runnings gravity and EBC haze data. As the data points are skewed right, the data set is not normally distributed.

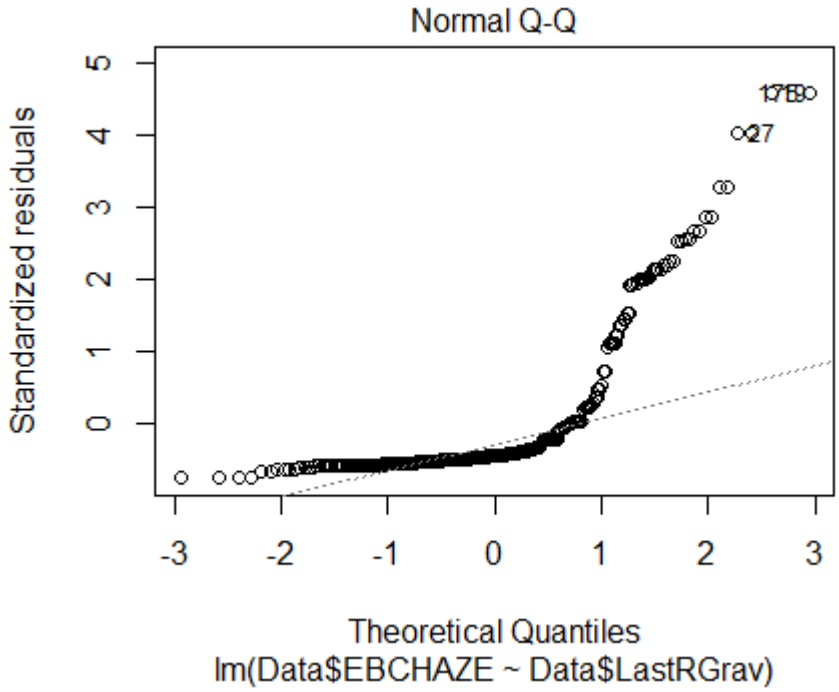


Figure B.6- Quantile-Quantile (Q-Q) plot assessing normality of last runnings gravity and EBC haze data. As the data points are skewed right, the data set is not normally distributed.

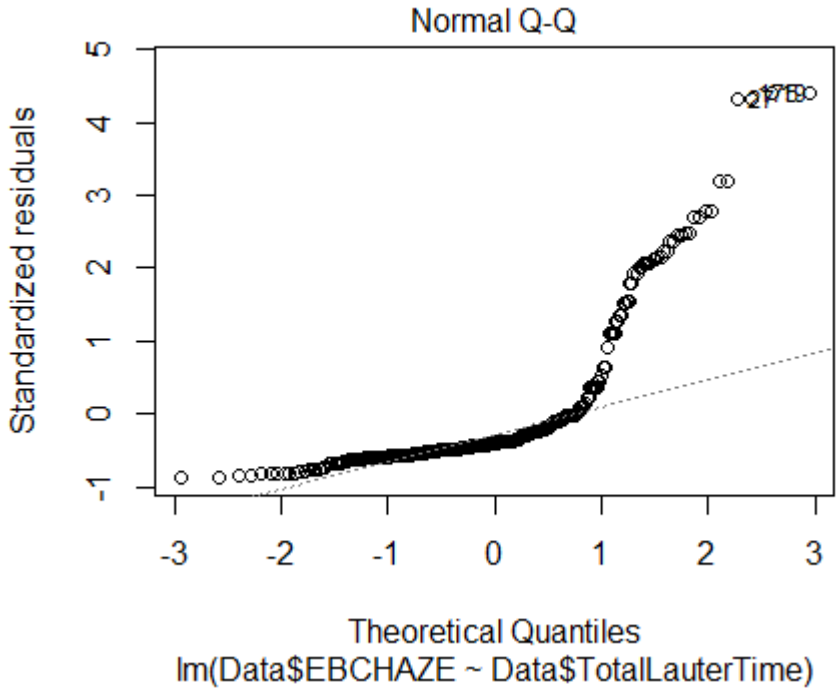


Figure B.7- Quantile- Quantile (Q-Q) plot assessing normality of total lauter time (min) and EBC haze data. As the data points are skewed right, the data set is not normally distributed.

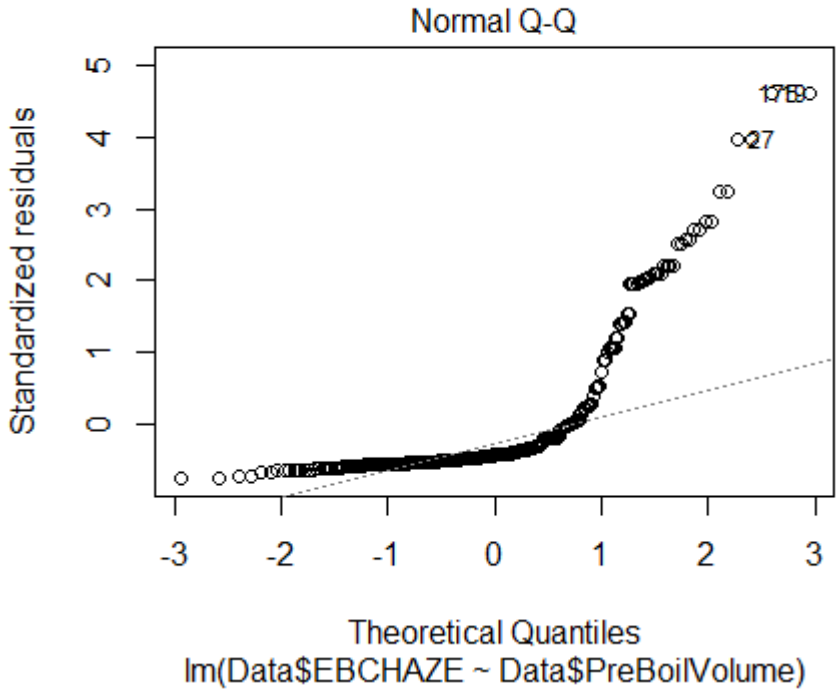


Figure B.8- Quantile- Quantile (Q-Q) plot assessing normality of pre-boil volume (hL) and EBC haze data. As the data points are skewed right, the data set is not normally distributed.

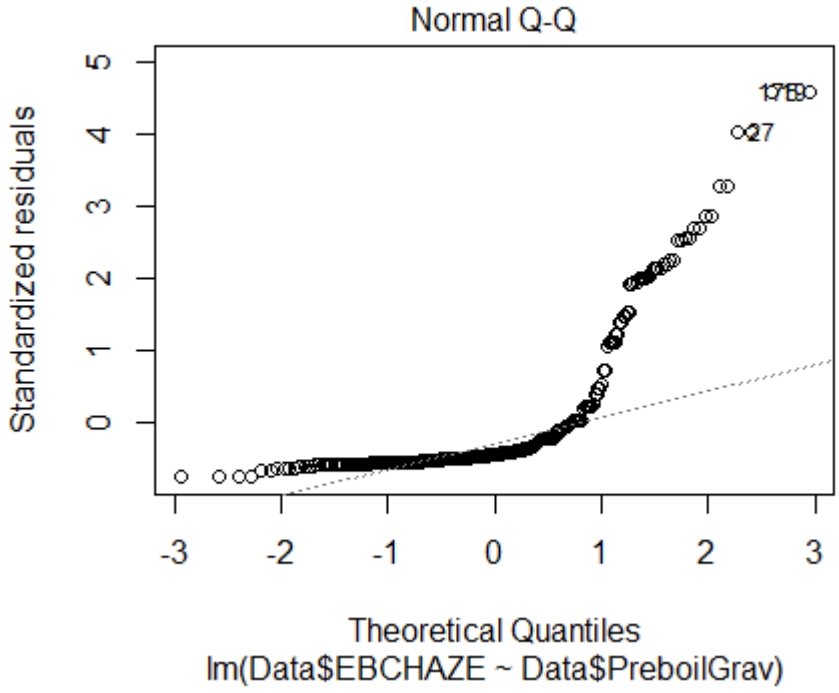


Figure B.9- Quantile- Quantile (Q-Q) plot assessing normality of pre-boil gravity and EBC haze data. As the data points are skewed right, the data set is not normally distributed.

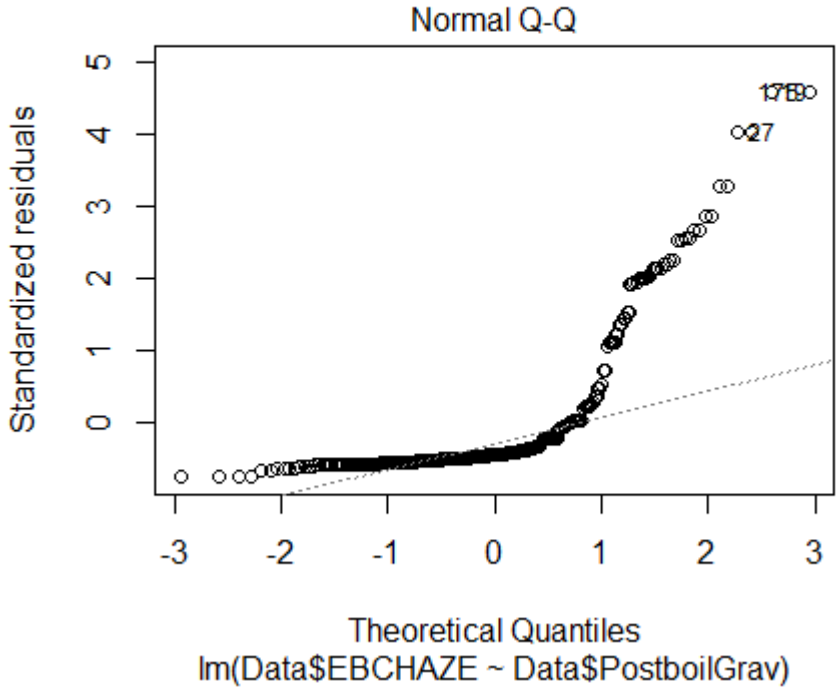


Figure B.10- Quantile- Quantile (Q-Q) plot assessing normality of post-boil gravity and EBC haze data. As the data points are skewed right, the data set is not normally distributed.

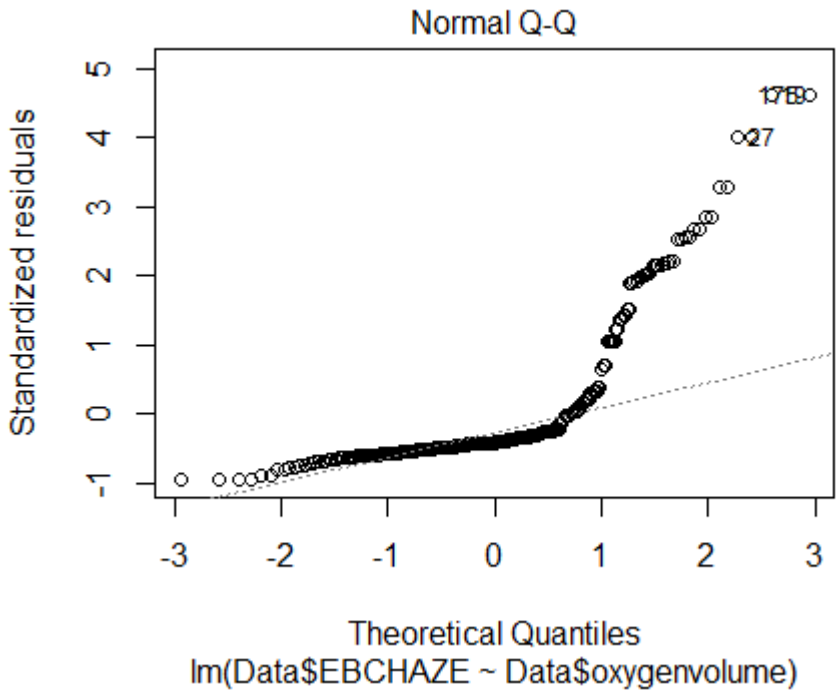


Figure B.11- Quantile- Quantile (Q-Q) plot assessing normality of oxygen volume (L) and EBC haze data. As the data points are skewed right, the data set is not normally distributed.

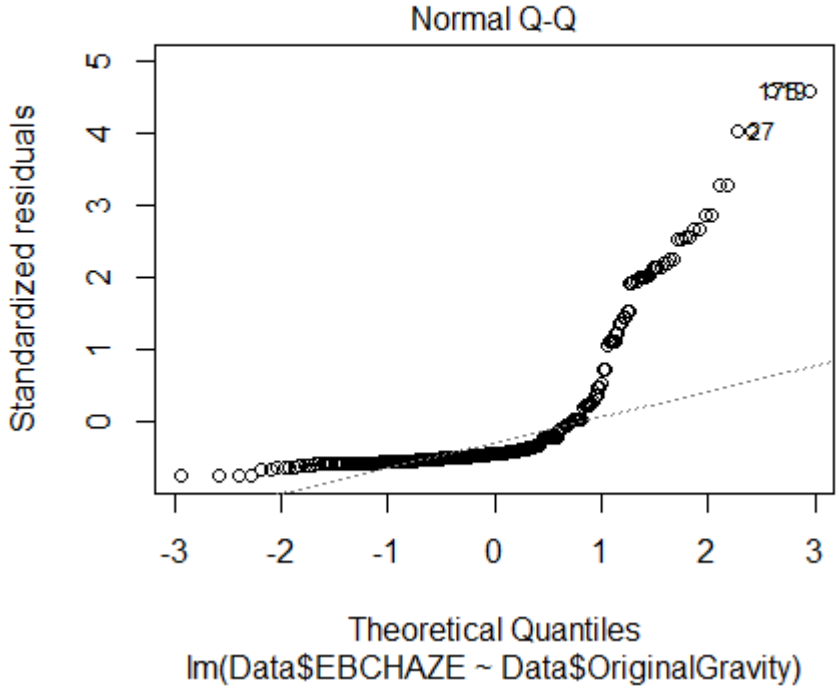


Figure B.12- Quantile- Quantile (Q-Q) plot assessing normality of original gravity and EBC haze data. As the data points are skewed right, the data set is not normally distributed.

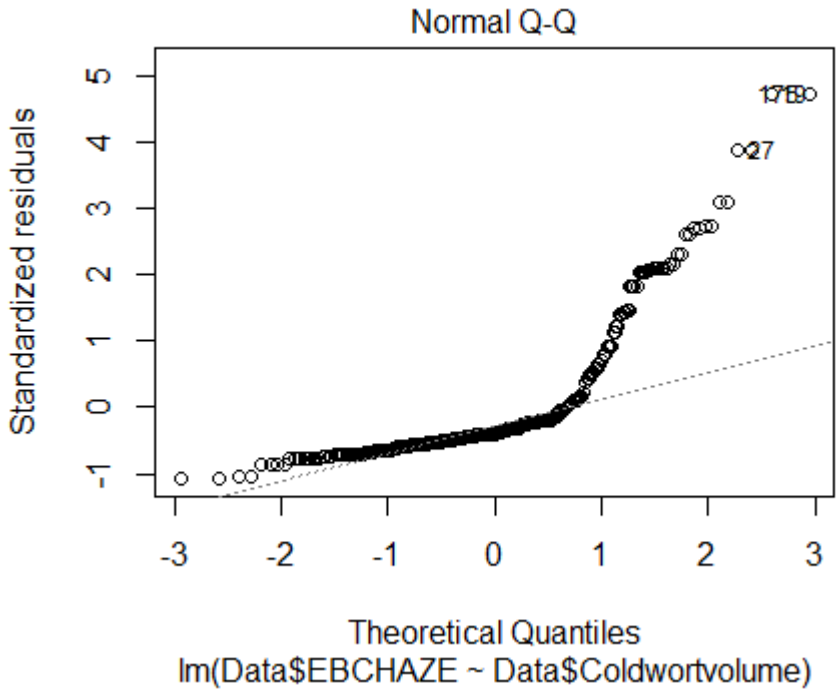


Figure B.13- Quantile- Quantile (Q-Q) plot assessing normality of final wort volume (hL) and EBC haze data. As the data points are skewed right, the data set is not normally distributed.

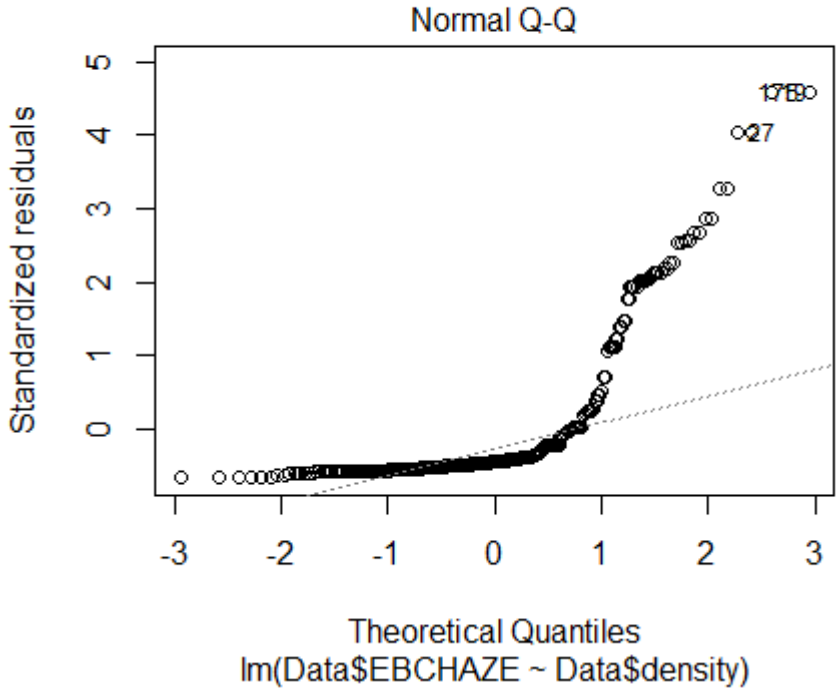


Figure B.14- Quantile- Quantile (Q-Q) plot assessing normality of beer density and EBC haze data. As the data points are skewed right, the data set is not normally distributed.

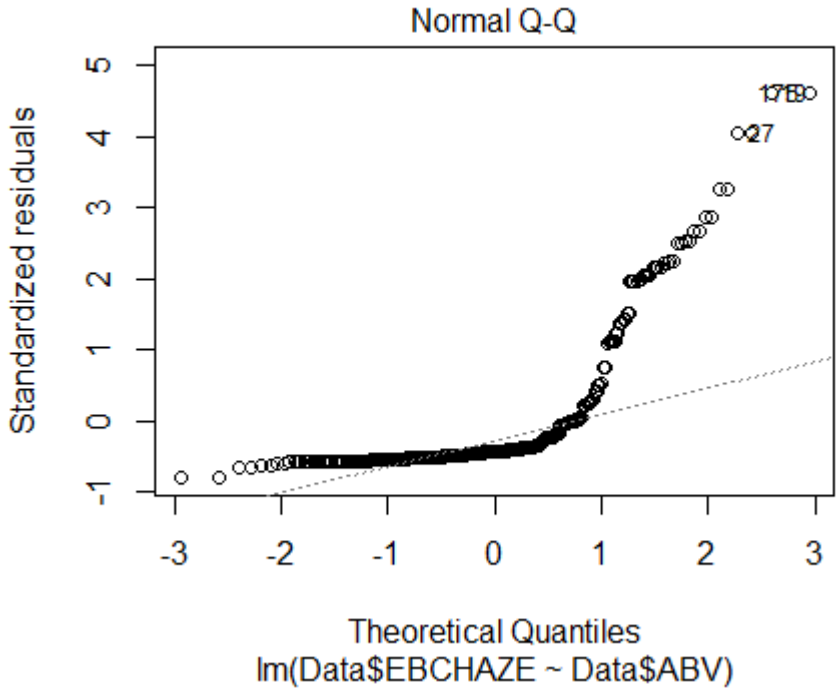


Figure B.15- Quantile- Quantile (Q-Q) plot assessing normality of alcohol by volume and EBC haze data. As the data points are skewed right, the data set is not normally distributed.

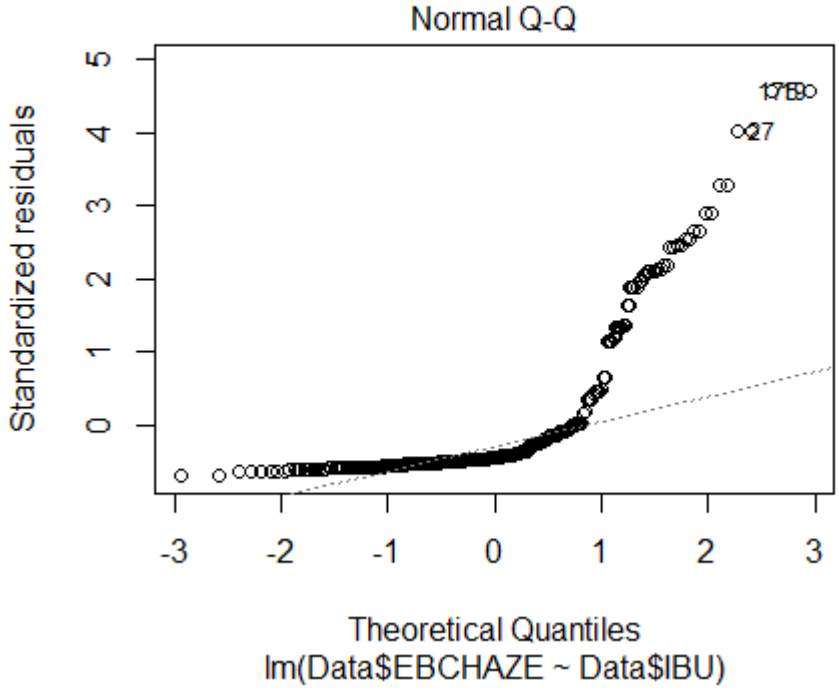


Figure B.16- Quantile- Quantile (Q-Q) plot assessing normality of International Bitterness Units (IBU) and EBC haze data. As the data points are skewed right, the data set is not normally distributed.

Appendix C

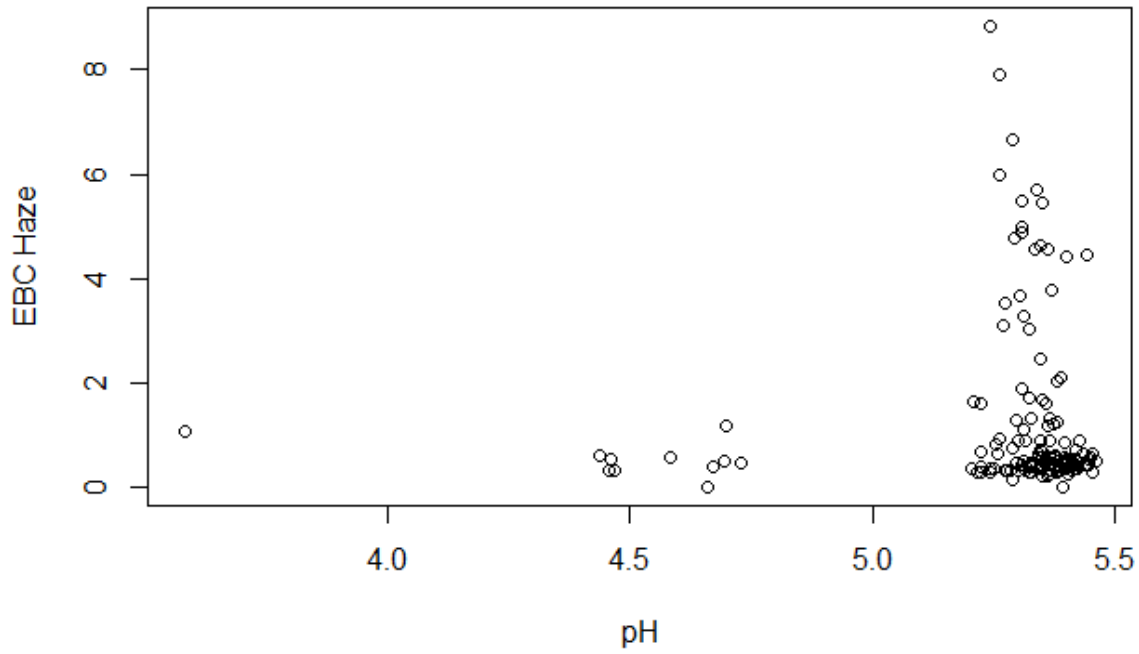


Figure C.1- Scatter plot displaying relationship of mash pH and EBC haze.

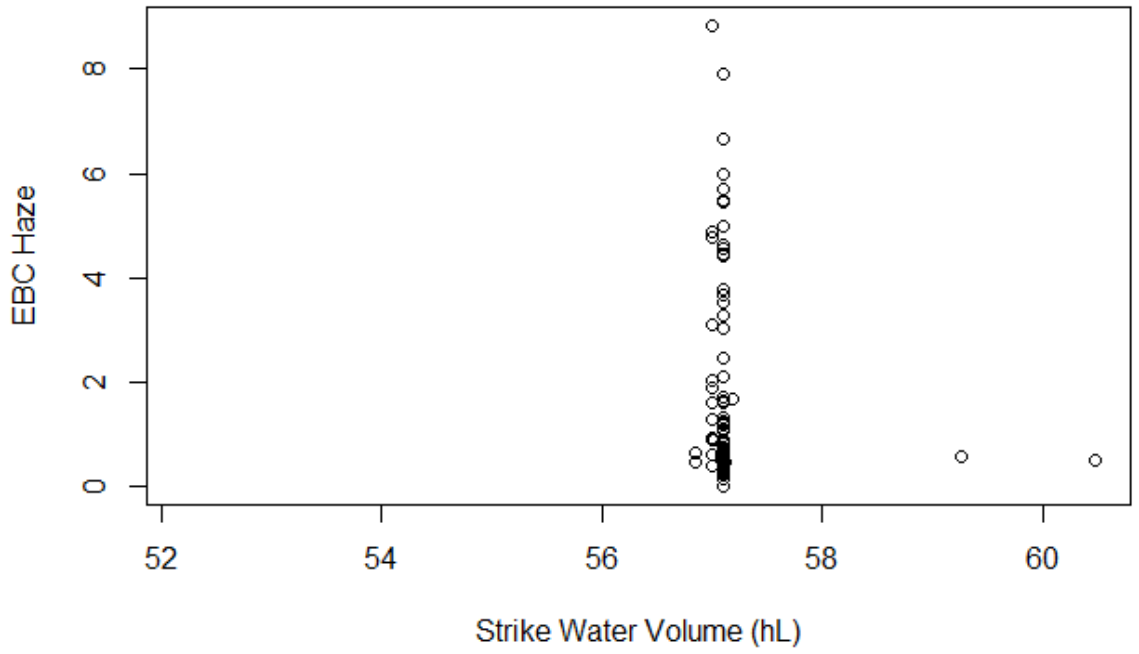


Figure C.2- Scatter plot displaying relationship of strike water volume (hL) and EBC haze.

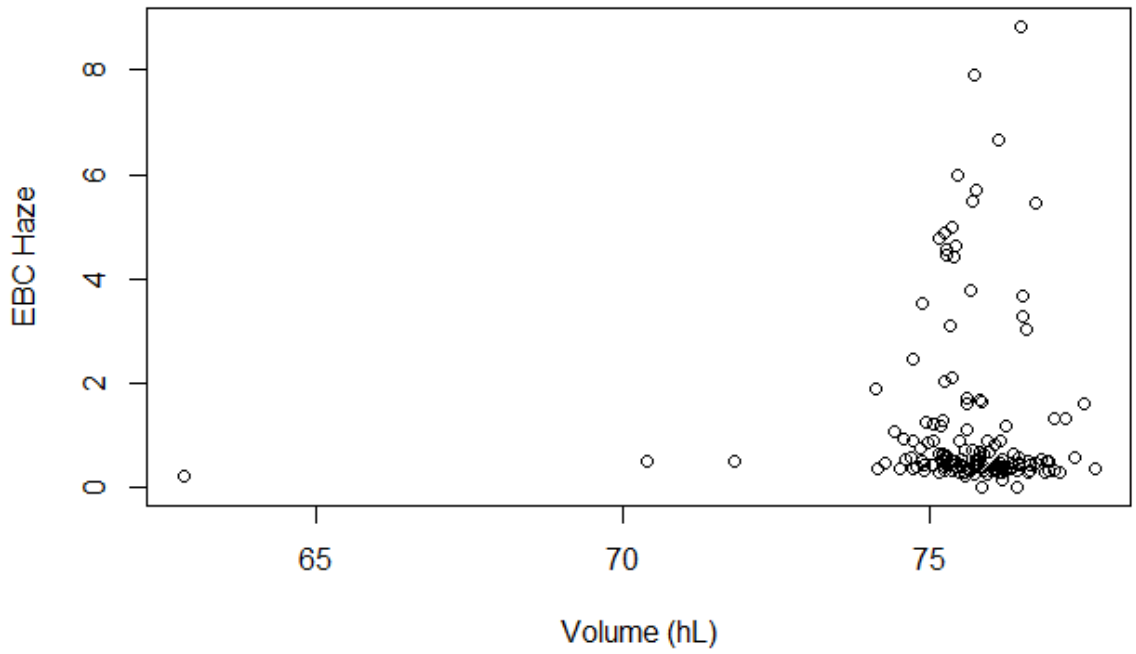


Figure C.3- Scatter plot displaying relationship of strike mash in volume (hL) and EBC haze.

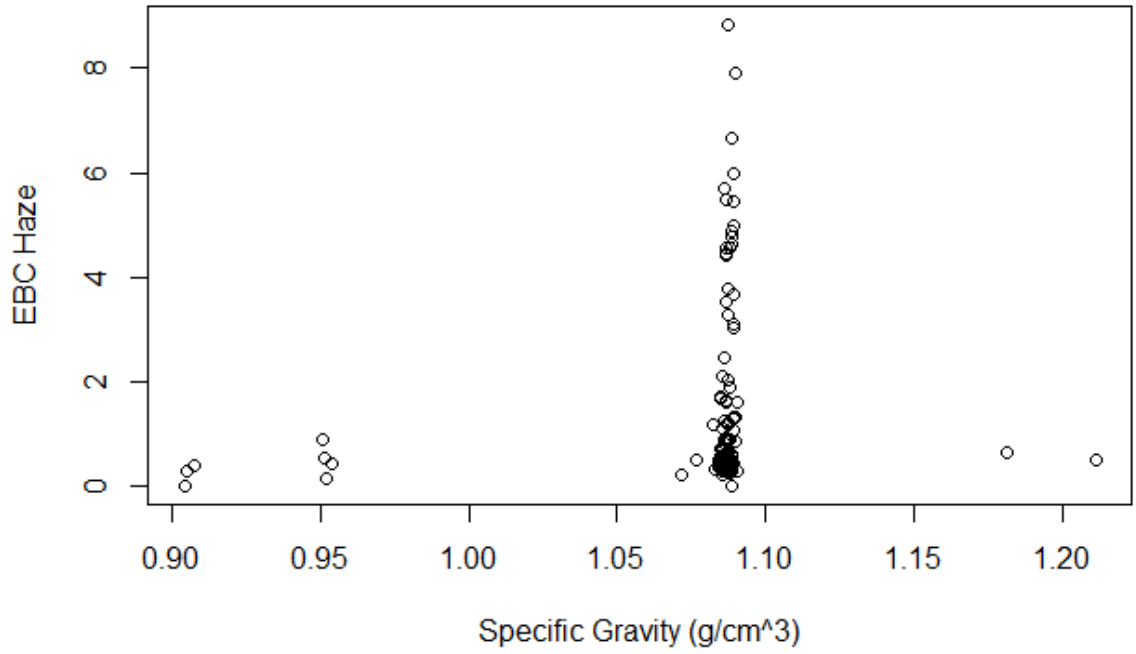


Figure C.4- Scatter plot displaying relationship of first runnings gravity (g/cm^3) and EBC haze.

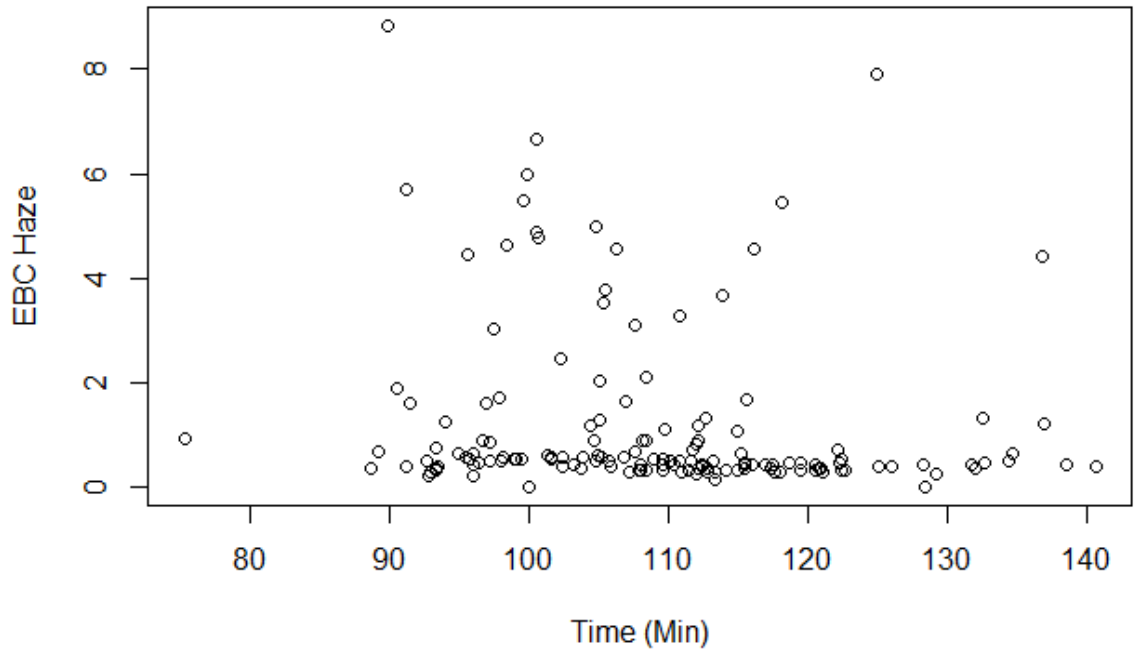


Figure C.5- Scatter plot displaying relationship of total lauter time (minutes) and EBC haze.

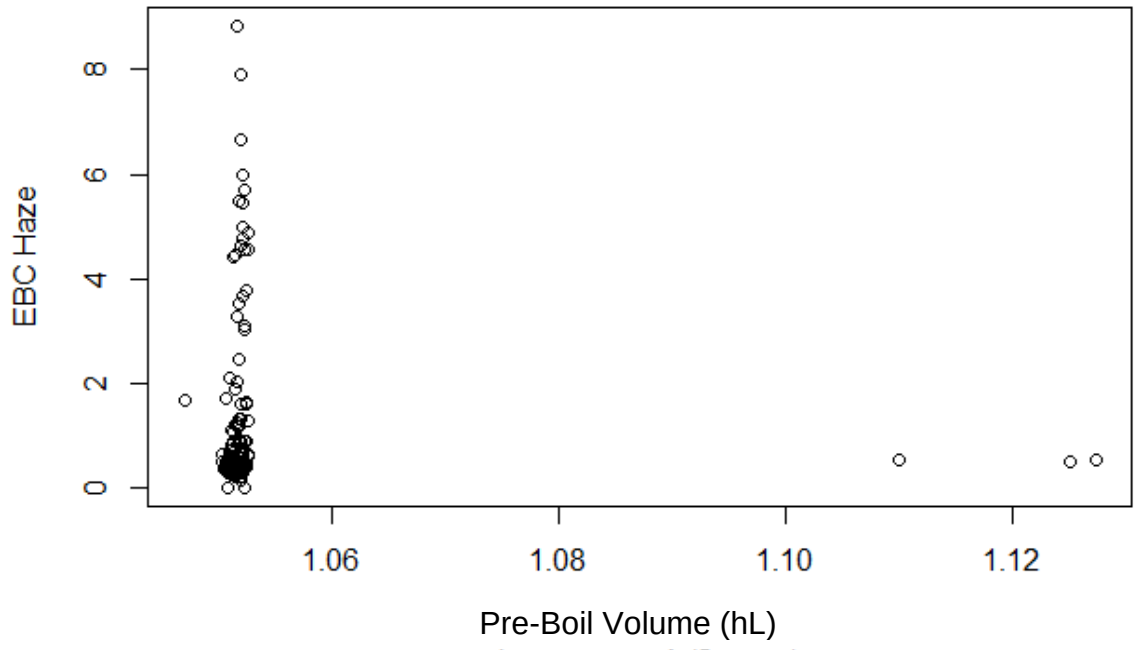


Figure C.6- Scatter plot displaying relationship of pre-boil volume (hL) and EBC haze.

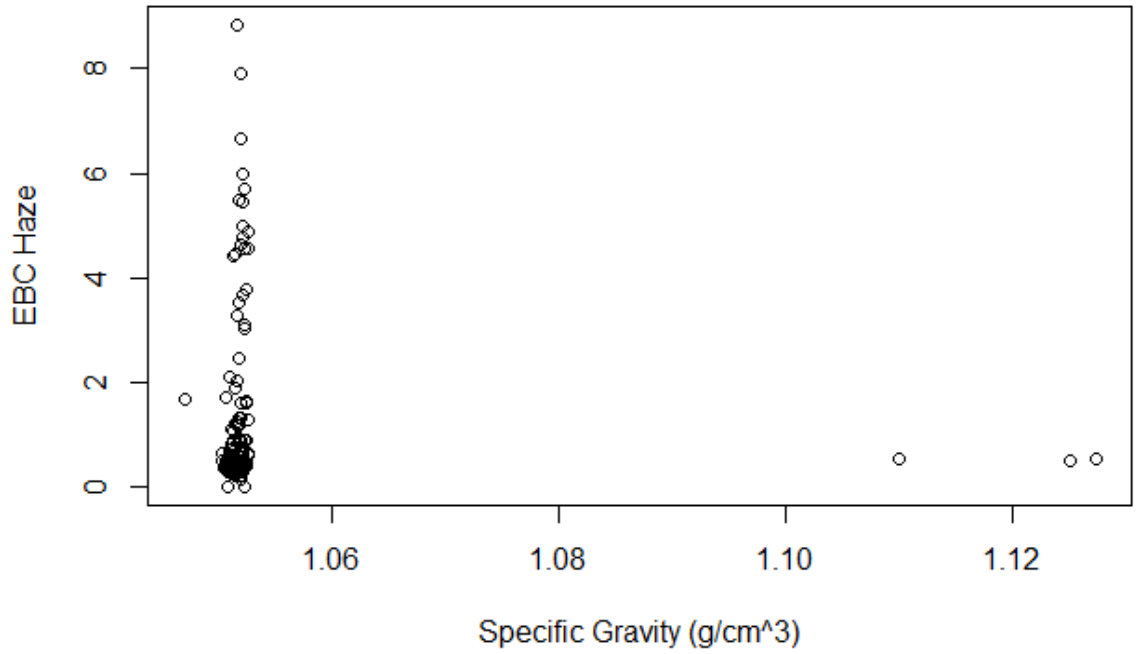


Figure C.7- Scatter plot displaying relationship of pre-boil gravity (g/cm^3) and EBC haze.

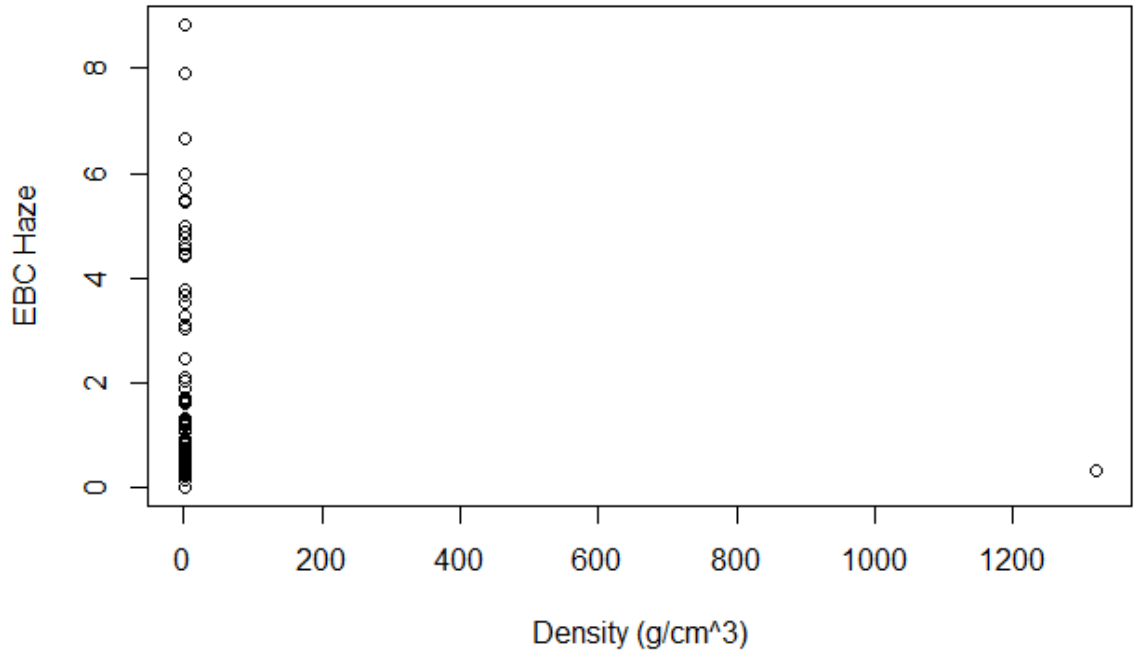


Figure C.8- Scatter plot displaying relationship of post-boil volume (g/cm^3) and EBC haze.

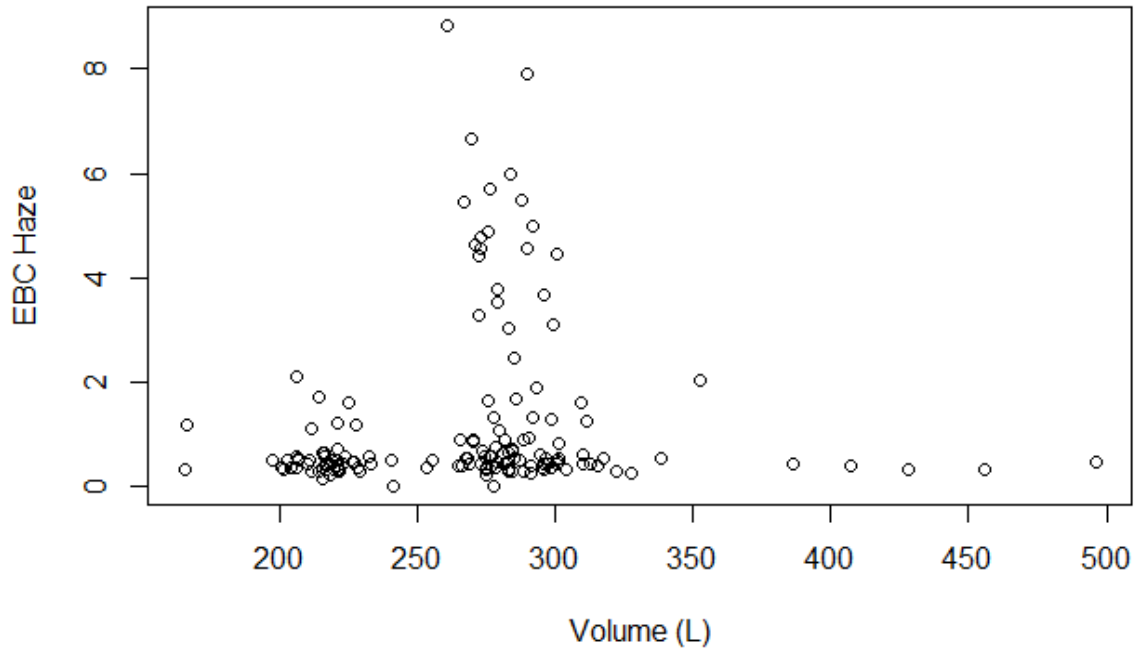


Figure C.9- Scatter plot displaying relationship of oxygen volume (L) and EBC haze.

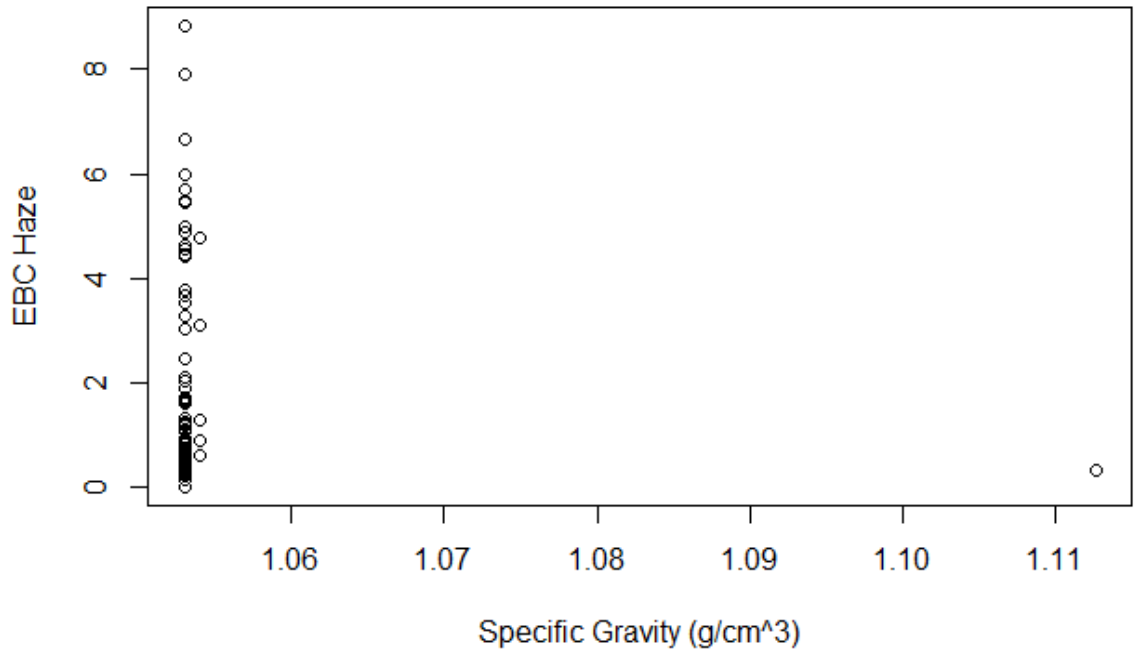


Figure C.10- Scatter plot displaying relationship of original gravity (g/cm^3) and EBC haze.

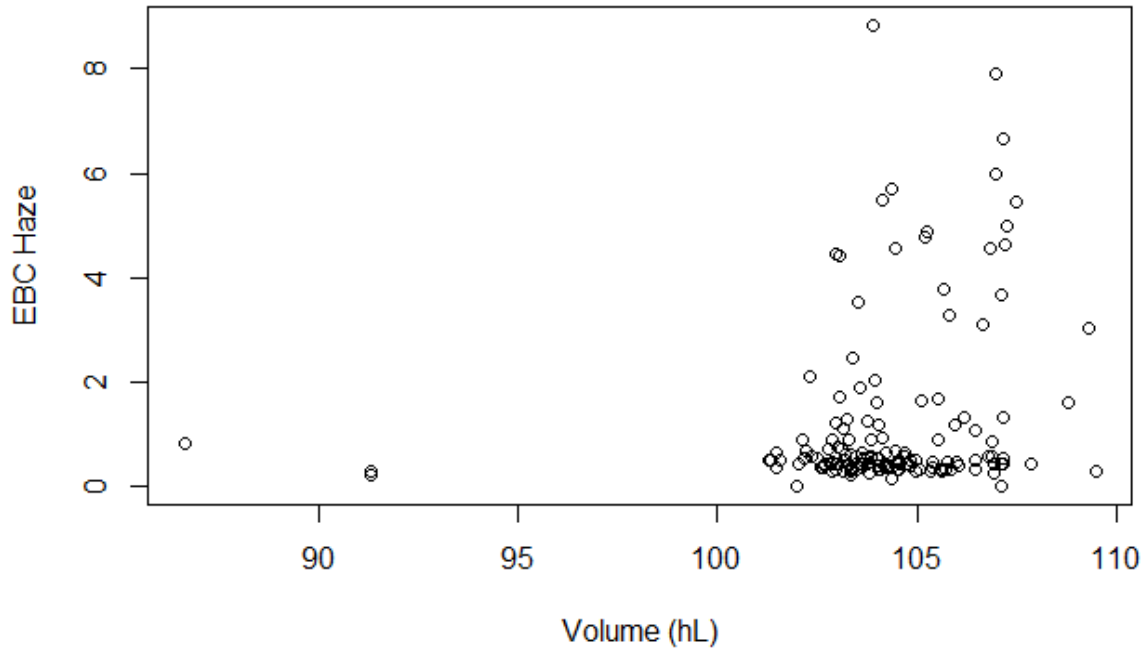


Figure C.11- Scatter plot displaying relationship of final wort volume (hL) and EBC haze.

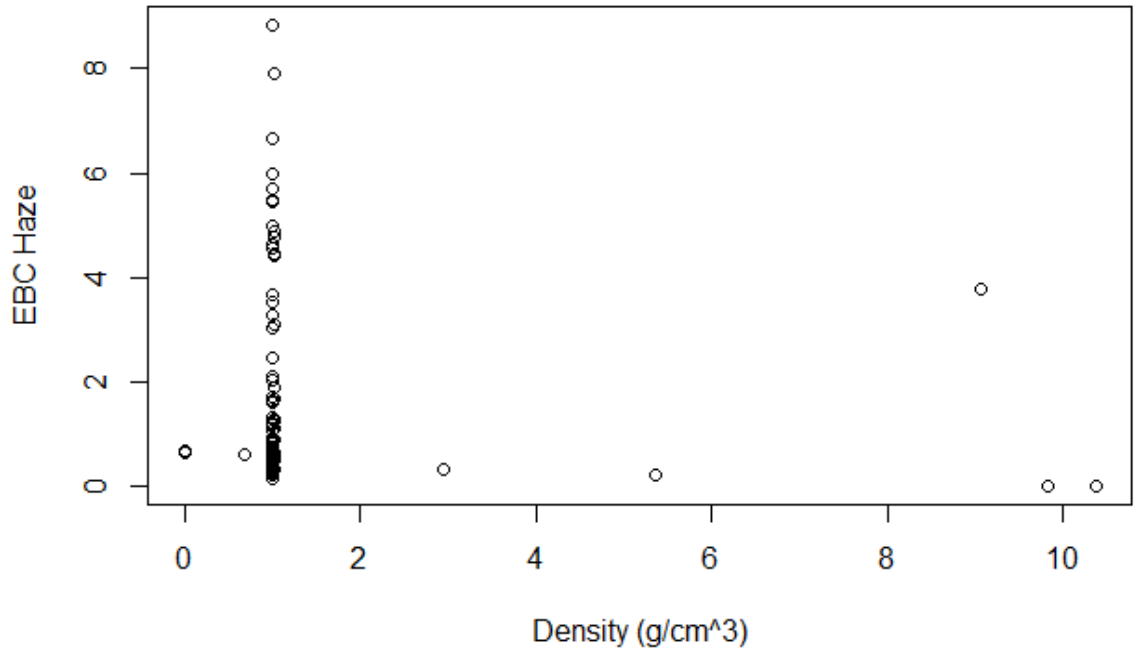


Figure C.12- Scatter plot displaying relationship of density (g/cm^3) and EBC haze.

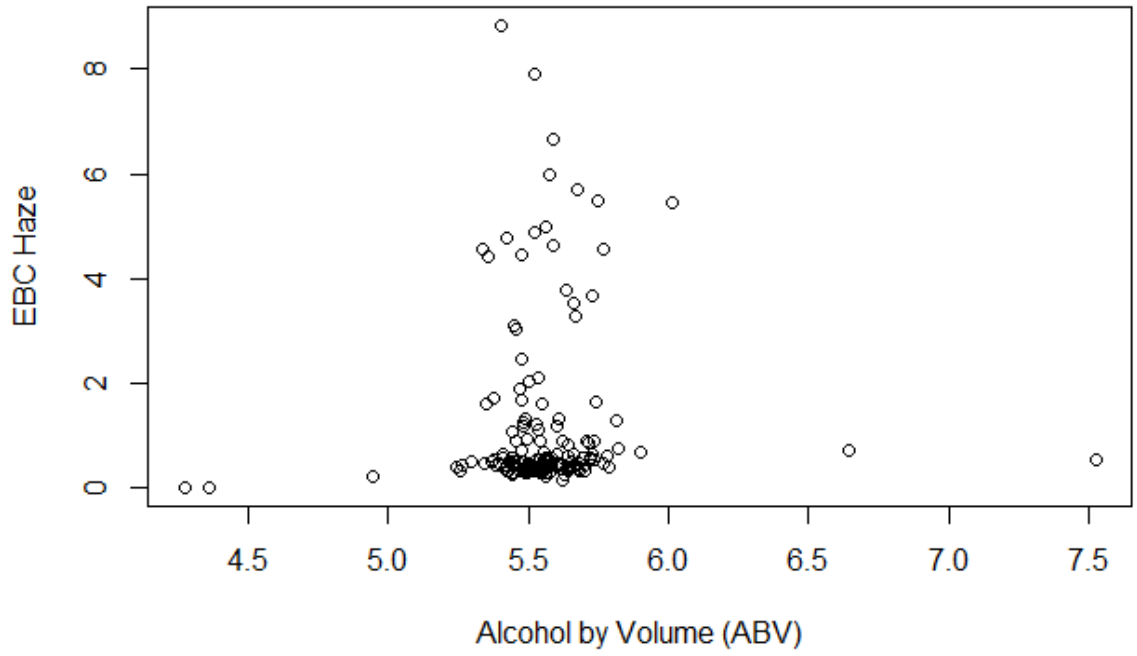


Figure C.13- Scatter plot displaying relationship of alcohol by volume (ABV) and EBC haze.

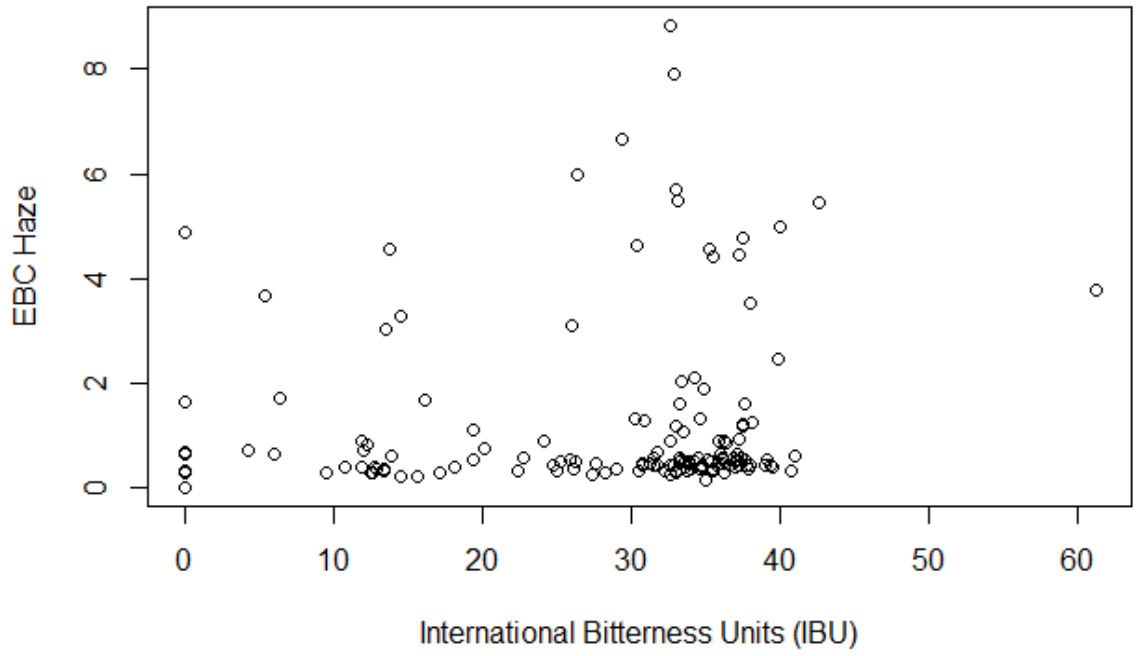


Figure C.14- Scatter plot displaying relationship of international bitterness units (IBU) and EBC haze.

Figure C.15- RStudio Script of Backward Elimination of Brewhouse Data.

```
    PostboilGrav + Dilutionwater + extraboiltime + Coldworttotal +
    TotalMinCasting + oxygenvolume + density + ABV + PG + OG +
    PH + EBCCOLOUR + IBU

                  Df Sum of Sq    RSS    AIC
- water            1     0.172 635.38 268.47
- PH               1     0.317 635.52 268.54
- IBU              1     0.357 635.56 268.56
- PostboilGrav     1     0.672 635.88 268.71
- PG               1     0.691 635.90 268.72
- Time             1     0.741 635.95 268.75
- ABV              1     1.118 636.32 268.93
- OG               1     1.230 636.43 268.99
- MashVol          1     1.315 636.52 269.03
- oxygenvolume     1     1.512 636.72 269.12
- PreboilGrav      1     1.954 637.16 269.34
- extraboiltime    1     2.442 637.65 269.57
- FirstRGrav       1     2.608 637.81 269.66
- LastRGrav        1     2.751 637.96 269.73
- EBCCOLOUR        1     3.408 638.61 270.05
<none>                       635.20 270.39
- PreBoilVolume    1     5.260 640.46 270.94
- MashpH           1     5.740 640.94 271.18
- density          1     7.241 642.45 271.90
- Dilutionwater    1    10.880 646.08 273.65
- MashTemp         1    18.202 653.41 277.14
- TotalLauterTime  1    27.746 662.95 281.64
- TotalMinCasting  1    37.736 672.94 286.28
- Coldworttotal    1    47.433 682.64 290.71

Step:  AIC=268.47
EBCHAZE ~ Time + MashTemp + MashpH + MashVol + FirstRGrav + LastRGrav
+
    TotalLauterTime + PreboilGrav + PreBoilVolume + PostboilGrav +
    Dilutionwater + extraboiltime + Coldworttotal + TotalMinCasting +
    oxygenvolume + density + ABV + PG + OG + PH + EBCCOLOUR +
    IBU

                  Df Sum of Sq    RSS    AIC
- PH               1     0.275 635.65 266.61
- IBU              1     0.391 635.77 266.66
- PG               1     0.614 635.99 266.77
- PostboilGrav     1     0.684 636.06 266.80
- Time             1     0.793 636.17 266.86
- MashVol          1     1.194 636.57 267.05
- OG               1     1.240 636.62 267.07
- ABV              1     1.282 636.66 267.10
- oxygenvolume     1     1.483 636.86 267.19
- PreboilGrav      1     2.020 637.40 267.45
- extraboiltime    1     2.436 637.81 267.66
- FirstRGrav       1     2.639 638.02 267.75
- LastRGrav        1     2.780 638.16 267.82
- EBCCOLOUR        1     3.470 638.85 268.16
<none>                       635.38 268.47
- PreBoilVolume    1     5.256 640.63 269.02
- MashpH           1     5.870 641.25 269.32
- density          1     7.145 642.52 269.94
- Dilutionwater    1    10.850 646.23 271.72
```

```
- MashTemp         1    18.211 653.59 275.23
- TotalLauterTime  1    27.625 663.00 279.66
- TotalMinCasting  1    37.620 673.00 284.30
- Coldworttotal    1    47.294 682.67 288.73

Step:  AIC=266.6
EBCHAZE ~ Time + MashTemp + MashpH + MashVol + FirstRGrav + LastRGrav
+
    TotalLauterTime + PreboilGrav + PreBoilVolume + PostboilGrav +
    Dilutionwater + extraboiltime + Coldworttotal + TotalMinCasting +
    oxygenvolume + density + ABV + PG + OG + EBCCOLOUR + IBU

                  Df Sum of Sq    RSS    AIC
- IBU              1     0.358 636.01 264.78
- PostboilGrav     1     0.633 636.29 264.91
- Time             1     0.880 636.53 265.03
- MashVol          1     1.090 636.74 265.13
- ABV              1     1.119 636.77 265.15
- PG               1     1.458 637.11 265.31
- OG               1     1.543 637.20 265.36
- oxygenvolume     1     1.637 637.29 265.40
- PreboilGrav      1     1.921 637.57 265.54
- FirstRGrav       1     2.440 638.09 265.79
- extraboiltime    1     2.462 638.11 265.80
- LastRGrav        1     2.733 638.38 265.93
- EBCCOLOUR        1     3.376 639.03 266.25
<none>                       635.65 266.61
- PreBoilVolume    1     5.257 640.91 267.16
- MashpH           1     5.680 641.33 267.36
- density          1     7.865 643.52 268.42
- Dilutionwater    1    10.575 646.23 269.72
- MashTemp         1    18.310 653.96 273.41
- TotalLauterTime  1    27.402 663.05 277.69
- TotalMinCasting  1    37.468 673.12 282.36
- Coldworttotal    1    47.070 682.72 286.75

Step:  AIC=264.78
EBCHAZE ~ Time + MashTemp + MashpH + MashVol + FirstRGrav + LastRGrav
+
    TotalLauterTime + PreboilGrav + PreBoilVolume + PostboilGrav +
    Dilutionwater + extraboiltime + Coldworttotal + TotalMinCasting +
    oxygenvolume + density + ABV + PG + OG + EBCCOLOUR

                  Df Sum of Sq    RSS    AIC
- PostboilGrav     1     0.617 636.63 263.08
- Time             1     0.868 636.88 263.20
- MashVol          1     1.082 637.09 263.31
- ABV              1     1.194 637.20 263.36
- oxygenvolume     1     1.540 637.55 263.53
- PG               1     1.724 637.73 263.62
- PreboilGrav      1     1.988 638.00 263.75
- OG               1     2.001 638.01 263.75
- FirstRGrav       1     2.374 638.38 263.93
- extraboiltime    1     2.482 638.49 263.99
- LastRGrav        1     2.768 638.78 264.12
- EBCCOLOUR        1     3.762 639.77 264.61
<none>                       636.01 264.78
- PreBoilVolume    1     5.130 641.14 265.27
- MashpH           1     5.512 641.52 265.45
- Dilutionwater    1    10.475 646.49 267.84
- density          1    14.429 650.44 269.73
- MashTemp         1    18.041 654.05 271.45
```

```
- TotalLauterTime  1    27.051 663.06 275.69
- TotalMinCasting  1    37.111 673.12 280.36
- Coldworttotal    1    46.714 682.72 284.75

Step:  AIC=263.08
EBCHAZE ~ Time + MashTemp + MashpH + MashVol + FirstRGrav + LastRGrav
+
    TotalLauterTime + PreboilGrav + PreBoilVolume + Dilutionwater +
    extraboiltime + Coldworttotal + TotalMinCasting + oxygenvolume +
    density + ABV + PG + OG + EBCCOLOUR

                  Df Sum of Sq    RSS    AIC
- Time             1     0.834 637.46 261.49
- MashVol          1     1.146 637.77 261.64
- ABV              1     1.198 637.82 261.66
- oxygenvolume     1     1.724 638.35 261.92
- PG               1     1.756 638.38 261.93
- PreboilGrav      1     1.959 638.59 262.03
- OG               1     1.979 638.61 262.04
- FirstRGrav       1     2.367 638.99 262.23
- extraboiltime    1     2.476 639.10 262.28
- LastRGrav        1     2.719 639.35 262.40
- EBCCOLOUR        1     3.903 640.53 262.97
<none>                       636.63 263.08
- PreBoilVolume    1     5.147 641.77 263.57
- MashpH           1     5.433 642.06 263.71
- Dilutionwater    1    10.640 647.27 266.22
- density          1    14.395 651.02 268.01
- MashTemp         1    18.083 654.71 269.76
- TotalLauterTime  1    27.104 663.73 274.00
- TotalMinCasting  1    37.255 673.88 278.71
- Coldworttotal    1    46.921 683.55 283.12

Step:  AIC=261.49
EBCHAZE ~ MashTemp + MashpH + MashVol + FirstRGrav + LastRGrav +
    TotalLauterTime + PreboilGrav + PreBoilVolume + Dilutionwater +
    extraboiltime + Coldworttotal + TotalMinCasting + oxygenvolume +
    density + ABV + PG + OG + EBCCOLOUR

                  Df Sum of Sq    RSS    AIC
- ABV              1     1.078 638.54 260.01
- MashVol          1     1.169 638.63 260.05
- OG               1     1.834 639.30 260.38
- oxygenvolume     1     1.875 639.34 260.40
- PG               1     1.961 639.42 260.44
- PreboilGrav      1     2.028 639.49 260.47
- FirstRGrav       1     2.347 639.81 260.62
- extraboiltime    1     2.381 639.84 260.64
- LastRGrav        1     2.624 640.09 260.76
<none>                       637.46 261.49
- EBCCOLOUR        1     4.208 641.67 261.52
- PreBoilVolume    1     5.105 642.57 261.96
- MashpH           1     5.375 642.84 262.09
- Dilutionwater    1    10.126 647.59 264.37
- density          1    14.472 651.93 266.44
- MashTemp         1    18.194 655.65 268.21
- TotalLauterTime  1    26.920 664.38 272.31
- TotalMinCasting  1    37.365 674.83 277.14
- Coldworttotal    1    48.112 685.57 282.04

Step:  AIC=260.01
EBCHAZE ~ MashTemp + MashpH + MashVol + FirstRGrav + LastRGrav +
```

```
    TotalLauterTime + PreboilGrav + PreBoilVolume + Dilutionwater +
    extraboiltime + Coldworttotal + TotalMinCasting + oxygenvolume +
    density + PG + OG + EBCCOLOUR

                  Df Sum of Sq    RSS    AIC
- OG               1     0.859 639.40 258.43
- MashVol          1     0.952 639.49 258.47
- oxygenvolume     1     1.808 640.35 258.88
- FirstRGrav       1     2.198 640.74 259.07
- extraboiltime    1     2.372 640.91 259.16
- LastRGrav        1     2.527 641.07 259.23
- EBCCOLOUR        1     3.717 642.26 259.81
<none>                       638.54 260.01
- PreboilGrav      1     4.609 643.15 260.24
- PreBoilVolume    1     4.786 643.32 260.32
- MashpH           1     6.975 645.51 261.38
- PG               1     9.632 648.17 262.65
- Dilutionwater    1    10.245 648.78 262.94
- density          1    13.396 651.93 264.44
- MashTemp         1    17.117 655.66 266.21
- TotalLauterTime  1    26.534 665.07 270.63
- TotalMinCasting  1    36.981 675.52 275.46
- Coldworttotal    1    47.580 686.12 280.29

Step:  AIC=258.43
EBCHAZE ~ MashTemp + MashpH + MashVol + FirstRGrav + LastRGrav +
    TotalLauterTime + PreboilGrav + PreBoilVolume + Dilutionwater +
    extraboiltime + Coldworttotal + TotalMinCasting + oxygenvolume +
    density + PG + EBCCOLOUR

                  Df Sum of Sq    RSS    AIC
- MashVol          1     1.035 640.43 256.93
- FirstRGrav       1     2.204 641.60 257.49
- extraboiltime    1     2.494 641.89 257.63
- LastRGrav        1     2.514 641.91 257.64
- oxygenvolume     1     2.709 642.11 257.74
- PreboilGrav      1     3.969 643.37 258.34
<none>                       639.40 258.43
- PreBoilVolume    1     4.751 644.15 258.72
- EBCCOLOUR        1     5.140 644.54 258.91
- MashpH           1     7.046 646.44 259.82
- Dilutionwater    1    10.559 649.96 261.50
- density          1    14.907 654.30 263.57
- MashTemp         1    16.277 655.67 264.22
- PG               1    20.967 660.36 266.43
- TotalLauterTime  1    28.177 667.57 269.80
- TotalMinCasting  1    36.589 675.99 273.68
- Coldworttotal    1    48.786 688.18 279.22

Step:  AIC=256.93
EBCHAZE ~ MashTemp + MashpH + FirstRGrav + LastRGrav + TotalLauterTime
+
    PreboilGrav + PreBoilVolume + Dilutionwater + extraboiltime +
    Coldworttotal + TotalMinCasting + oxygenvolume + density +
    PG + EBCCOLOUR

                  Df Sum of Sq    RSS    AIC
- FirstRGrav       1     2.143 642.58 255.96
- extraboiltime    1     2.290 642.72 256.03
- LastRGrav        1     2.646 643.08 256.20
- oxygenvolume     1     2.721 643.15 256.24
<none>                       640.43 256.93
```

```
- PreboilGrav      1     4.381 644.81 257.04
- PreBoilVolume    1     4.493 644.93 257.10
- EBCCOLOUR        1     5.247 645.68 257.46
- MashpH           1     6.940 647.37 258.27
- Dilutionwater    1    10.435 650.87 259.94
- density          1    14.758 655.19 261.99
- MashTemp         1    16.040 656.47 262.60
- PG               1    20.483 660.92 264.69
- TotalLauterTime  1    27.367 667.80 267.90
- TotalMinCasting  1    36.996 677.43 272.34
- Coldworttotal    1    52.538 692.97 279.37

Step:  AIC=255.96
EBCHAZE ~ MashTemp + MashpH + LastRGrav + TotalLauterTime +
PreboilGrav +
    PreBoilVolume + Dilutionwater + extraboiltime + Coldworttotal +
    TotalMinCasting + oxygenvolume + density + PG + EBCCOLOUR

                 Df Sum of Sq    RSS    AIC
- extraboiltime   1     2.334 644.91 255.09
- LastRGrav       1     2.728 645.30 255.28
- oxygenvolume    1     2.762 645.34 255.29
<none>                       642.58 255.96
- PreBoilVolume   1     4.354 646.93 256.06
- PreboilGrav     1     4.533 647.11 256.14
- EBCCOLOUR       1     5.389 647.96 256.55
- MashpH          1     6.711 649.29 257.18
- Dilutionwater   1     9.503 652.08 258.51
- density         1    14.711 657.29 260.98
- MashTemp        1    15.822 658.40 261.50
- PG              1    24.295 666.87 265.47
- TotalLauterTime 1    31.826 674.40 268.95
- TotalMinCasting 1    36.662 679.24 271.16
- Coldworttotal   1    51.016 693.59 277.65

Step:  AIC=255.09
EBCHAZE ~ MashTemp + MashpH + LastRGrav + TotalLauterTime +
PreboilGrav +
    PreBoilVolume + Dilutionwater + Coldworttotal + TotalMinCasting +
    oxygenvolume + density + PG + EBCCOLOUR

                 Df Sum of Sq    RSS    AIC
- LastRGrav       1     2.645 647.55 254.36
- oxygenvolume    1     2.831 647.74 254.44
- PreBoilVolume   1     3.995 648.91 255.00
<none>                       644.91 255.09
- PreboilGrav     1     4.337 649.25 255.16
- EBCCOLOUR       1     5.136 650.05 255.55
- MashpH          1     6.149 651.06 256.03
- Dilutionwater   1     7.905 652.82 256.86
- MashTemp        1    13.939 658.85 259.72
- density         1    15.094 660.00 260.26
- PG              1    24.525 669.44 264.66
- TotalLauterTime 1    31.362 676.27 267.81
- TotalMinCasting 1    36.223 681.13 270.03
- Coldworttotal   1    49.082 693.99 275.82

Step:  AIC=254.36
EBCHAZE ~ MashTemp + MashpH + TotalLauterTime + PreboilGrav +
    PreBoilVolume + Dilutionwater + Coldworttotal + TotalMinCasting +
    oxygenvolume + density + PG + EBCCOLOUR
```

```
                  Df Sum of Sq    RSS    AIC
- oxygenvolume     1     3.378 650.93 253.97
- PreBoilVolume    1     4.023 651.58 254.28
<none>                         647.55 254.36
- PreboilGrav      1     4.226 651.78 254.37
- EBCCOLOUR        1     5.498 653.05 254.98
- MashpH           1     6.305 653.86 255.36
- Dilutionwater    1     7.846 655.40 256.09
- MashTemp         1    14.467 662.02 259.20
- density          1    15.209 662.76 259.55
- PG               1    24.759 672.31 263.99
- TotalLauterTime  1    29.469 677.02 266.15
- TotalMinCasting  1    35.723 683.28 269.00
- Coldworttotal    1    48.504 696.06 274.75

Step:  AIC=253.97
EBCHAZE ~ MashTemp + MashpH + TotalLauterTime + PreboilGrav +
    PreBoilVolume + Dilutionwater + Coldworttotal + TotalMinCasting +
    density + PG + EBCCOLOUR

                  Df Sum of Sq    RSS    AIC
- PreboilGrav      1     4.008 654.94 253.87
<none>                         650.93 253.97
- PreBoilVolume    1     5.022 655.96 254.35
- EBCCOLOUR        1     5.754 656.69 254.70
- MashpH           1     5.869 656.80 254.75
- Dilutionwater    1     8.054 658.99 255.78
- density          1    13.346 664.28 258.26
- MashTemp         1    17.967 668.90 260.41
- PG               1    22.397 673.33 262.45
- TotalLauterTime  1    27.517 678.45 264.80
- TotalMinCasting  1    35.914 686.85 268.62
- Coldworttotal    1    53.577 704.51 276.49

Step:  AIC=253.87
EBCHAZE ~ MashTemp + MashpH + TotalLauterTime + PreBoilVolume +
    Dilutionwater + Coldworttotal + TotalMinCasting + density +
    PG + EBCCOLOUR

                  Df Sum of Sq    RSS    AIC
<none>                         654.94 253.87
- PreBoilVolume    1     4.835 659.78 254.15
- EBCCOLOUR        1     5.437 660.38 254.43
- Dilutionwater    1     6.929 661.87 255.13
- MashpH           1     7.310 662.25 255.31
- density          1    11.835 666.78 257.42
- PG               1    19.595 674.54 261.01
- MashTemp         1    20.538 675.48 261.44
- TotalLauterTime  1    26.697 681.64 264.26
- TotalMinCasting  1    36.902 691.84 268.86
- Coldworttotal    1    53.879 708.82 276.38

Call:
lm(formula = EBCHAZE ~ MashTemp + MashpH + TotalLauterTime +
    PreBoilVolume + Dilutionwater + Coldworttotal + TotalMinCasting +
    density + PG + EBCCOLOUR, data = Data)

Coefficients:
    (Intercept)         MashTemp           MashpH  TotalLauterTime
PreBoilVolume
      -29.13484          0.08602          0.62228         -0.02599
0.02738
```

```
  Dilutionwater    Coldworttotal  TotalMinCasting          density
PG
       -0.18640          0.17011          0.01829          0.22944
0.31822
      EBCCOLOUR
       -0.03188

> summary(FitAll)

Call:
lm(formula = EBCHAZE ~ ., data = Data)

Residuals:
    Min      1Q  Median      3Q     Max
-2.1499 -0.9034 -0.3578  0.3383  6.1331

Coefficients: (1 not defined because of singularities)
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)     -2.679e+01  2.958e+01  -0.906 0.365778
Time            -3.371e-06  5.855e-06  -0.576 0.565206
MashTemp         9.195e-02  3.219e-02   2.856 0.004600 **
MashpH           5.808e-01  3.623e-01   1.603 0.110092
water            9.349e-02  3.364e-01   0.278 0.781283
MashVol          5.186e-02  6.755e-02   0.768 0.443223
FirstRGrav       2.847e+00  2.634e+00   1.081 0.280665
LastRGrav       -9.535e-04  8.585e-04  -1.111 0.267656
TotalLauterTime -2.813e-02  8.039e-03  -3.499 0.000541 ***
PreboilGrav     -1.232e+01  1.317e+01  -0.936 0.350171
PreBoilVolume    2.901e-02  1.888e-02   1.536 0.125608
PostboilGrav    -4.455e-04  8.117e-04  -0.549 0.583532
Dilutionwater   -2.481e-01  1.123e-01  -2.209 0.027968 *
extraboiltime   -1.292e-01  1.235e-01  -1.046 0.296511
Coldworttotal    1.686e-01  3.664e-02   4.602 6.30e-06 ***
TotalMinCasting  1.868e-02  4.547e-03   4.108 5.23e-05 ***
oxygenvolume     1.609e-03  1.954e-03   0.823 0.411082
OriginalGravity -7.727e-02  2.254e+01  -0.003 0.997267
Coldwortvolume           NA         NA      NA       NA
density          2.897e-01  1.608e-01   1.801 0.072711 .
ABV             -9.170e-01  1.555e+00  -0.590 0.555782
PG               1.356e-01  2.741e-01   0.495 0.621333
OG               1.180e-01  1.976e-01   0.597 0.550917
PH              -3.066e-01  9.710e-01  -0.316 0.752409
EBCCOLOUR       -3.613e-02  2.923e-02  -1.236 0.217422
IBU              4.977e-03  1.244e-02   0.400 0.689477
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 1.493 on 285 degrees of freedom
  (12 observations deleted due to missingness)
Multiple R-squared:  0.2517,   Adjusted R-squared:  0.1887
F-statistic: 3.994 on 24 and 285 DF,  p-value: 7.271e-09
```

Figure C.16- RStudio Script of Forward Selection of Brewhouse Data

```
Start:  AIC=314.27
EBCHAZE ~ 1

                  Df Sum of Sq    RSS    AIC
+ MashTemp         1    54.881 793.97 295.55
+ Coldworttotal    1    45.854 803.00 299.05
+ Coldwortvolume   1    45.854 803.00 299.05
+ PG               1    35.011 813.85 303.21
+ TotalLauterTime  1    21.842 827.01 308.19
+ TotalMinCasting  1    17.094 831.76 309.96
+ OG               1    12.827 836.03 311.55
+ PH               1    10.269 838.59 312.50
+ FirstRGrav       1     9.342 839.51 312.84
+ PreBoilVolume    1     8.954 839.90 312.98
+ oxygenvolume     1     7.142 841.71 313.65
+ IBU              1     5.728 843.13 314.17
<none>                         848.86 314.27
+ extraboiltime    1     4.069 844.79 314.78
+ water            1     3.903 844.95 314.84
+ Time             1     3.628 845.23 314.94
+ Dilutionwater    1     2.583 846.27 315.32
+ EBCCOLOUR        1     2.481 846.38 315.36
+ PostboilGrav     1     1.725 847.13 315.64
+ LastRGrav        1     1.611 847.25 315.68
+ PreboilGrav      1     1.597 847.26 315.69
+ MashpH           1     1.525 847.33 315.71
+ ABV              1     1.380 847.48 315.76
+ OriginalGravity  1     1.237 847.62 315.82
+ MashVol          1     0.855 848.00 315.96
+ density          1     0.381 848.48 316.13

Step:  AIC=295.55
EBCHAZE ~ MashTemp

                  Df Sum of Sq    RSS    AIC
+ Coldworttotal    1    42.434 751.54 280.52
+ Coldwortvolume   1    42.434 751.54 280.52
+ TotalMinCasting  1    22.539 771.44 288.62
+ PG               1    14.157 779.82 291.97
+ TotalLauterTime  1    11.629 782.35 292.98
+ OG               1     9.031 784.94 294.00
+ PH               1     8.632 785.34 294.16
+ PreBoilVolume    1     7.608 786.37 294.56
+ FirstRGrav       1     5.715 788.26 295.31
<none>                         793.97 295.55
+ oxygenvolume     1     3.017 790.96 296.37
+ Time             1     2.858 791.12 296.43
+ MashpH           1     2.667 791.31 296.50
+ ABV              1     2.183 791.79 296.69
+ IBU              1     2.133 791.84 296.71
+ MashVol          1     2.127 791.85 296.72
+ water            1     1.655 792.32 296.90
+ OriginalGravity  1     1.431 792.54 296.99
+ PostboilGrav     1     1.386 792.59 297.01
+ LastRGrav        1     1.207 792.77 297.08
+ PreboilGrav      1     1.054 792.92 297.14
+ EBCCOLOUR        1     0.587 793.39 297.32
+ Dilutionwater    1     0.256 793.72 297.45
+ density          1     0.028 793.95 297.54
```

```
+ extraboiltime    1     0.000 793.97 297.55

Step:  AIC=280.52
EBCHAZE ~ MashTemp + Coldworttotal

                  Df Sum of Sq    RSS    AIC
+ TotalMinCasting  1   29.3049 722.24 270.19
+ TotalLauterTime  1   13.7925 737.75 276.78
+ PG               1    8.2939 743.25 279.08
+ FirstRGrav       1    6.5930 744.95 279.79
+ Dilutionwater    1    6.4206 745.12 279.86
+ MashpH           1    5.6653 745.88 280.18
<none>                         751.54 280.52
+ PH               1    4.7693 746.77 280.55
+ OG               1    4.4596 747.08 280.68
+ PreBoilVolume    1    3.9389 747.60 280.89
+ EBCCOLOUR        1    2.1692 749.37 281.63
+ LastRGrav        1    1.3329 750.21 281.97
+ PostboilGrav     1    1.0505 750.49 282.09
+ PreboilGrav      1    1.0368 750.50 282.09
+ IBU              1    0.9266 750.61 282.14
+ OriginalGravity  1    0.9245 750.62 282.14
+ Time             1    0.6906 750.85 282.24
+ oxygenvolume     1    0.5588 750.98 282.29
+ water            1    0.4566 751.08 282.33
+ ABV              1    0.4494 751.09 282.34
+ MashVol          1    0.1236 751.42 282.47
+ extraboiltime    1    0.1107 751.43 282.48
+ density          1    0.1030 751.44 282.48

Step:  AIC=270.19
EBCHAZE ~ MashTemp + Coldworttotal + TotalMinCasting

                  Df Sum of Sq    RSS    AIC
+ TotalLauterTime  1   25.0060 697.23 261.27
+ Dilutionwater    1   11.6313 710.60 267.16
+ FirstRGrav       1    6.8211 715.42 269.25
+ PG               1    4.9601 717.28 270.06
+ MashpH           1    4.8038 717.43 270.12
<none>                         722.24 270.19
+ PH               1    3.6339 718.60 270.63
+ OG               1    3.4225 718.81 270.72
+ PreBoilVolume    1    3.2611 718.98 270.79
+ EBCCOLOUR        1    2.8033 719.43 270.99
+ LastRGrav        1    1.2698 720.97 271.65
+ PostboilGrav     1    0.9340 721.30 271.79
+ IBU              1    0.9334 721.30 271.79
+ OriginalGravity  1    0.6971 721.54 271.89
+ ABV              1    0.6640 721.57 271.91
+ PreboilGrav      1    0.6190 721.62 271.93
+ oxygenvolume     1    0.4649 721.77 271.99
+ Time             1    0.3943 721.84 272.02
+ density          1    0.3730 721.86 272.03
+ water            1    0.1567 722.08 272.12
+ extraboiltime    1    0.0712 722.16 272.16
+ MashVol          1    0.0094 722.23 272.19

Step:  AIC=261.27
EBCHAZE ~ MashTemp + Coldworttotal + TotalMinCasting + TotalLauterTime

                  Df Sum of Sq    RSS    AIC
+ Dilutionwater    1    9.9448 687.29 258.81
```

```
+ PG               1    5.5934 691.64 260.77
+ MashpH           1    5.1873 692.04 260.95
<none>                         697.23 261.27
+ PreBoilVolume    1    3.6201 693.61 261.65
+ LastRGrav        1    3.5353 693.69 261.69
+ FirstRGrav       1    3.0321 694.20 261.92
+ PH               1    2.7309 694.50 262.05
+ OG               1    2.5138 694.72 262.15
+ EBCCOLOUR        1    2.1855 695.04 262.30
+ IBU              1    2.1481 695.08 262.31
+ oxygenvolume     1    1.4349 695.80 262.63
+ PostboilGrav     1    0.9691 696.26 262.84
+ PreboilGrav      1    0.9331 696.30 262.85
+ density          1    0.5970 696.63 263.00
+ Time             1    0.5961 696.63 263.00
+ MashVol          1    0.4319 696.80 263.08
+ extraboiltime    1    0.2141 697.02 263.17
+ OriginalGravity  1    0.2062 697.02 263.18
+ water            1    0.1102 697.12 263.22
+ ABV              1    0.0633 697.17 263.24

Step:  AIC=258.81
EBCHAZE ~ MashTemp + Coldworttotal + TotalMinCasting + TotalLauterTime
+
    Dilutionwater

                  Df Sum of Sq    RSS    AIC
+ MashpH           1    5.1615 682.12 258.48
+ PG               1    4.5488 682.74 258.76
<none>                         687.29 258.81
+ FirstRGrav       1    4.1835 683.10 258.92
+ PreBoilVolume    1    4.0242 683.26 258.99
+ LastRGrav        1    3.5188 683.77 259.22
+ IBU              1    2.2832 685.00 259.78
+ PreboilGrav      1    1.9042 685.38 259.95
+ PH               1    1.7940 685.49 260.00
+ OG               1    1.6861 685.60 260.05
+ EBCCOLOUR        1    1.5477 685.74 260.12
+ extraboiltime    1    1.4310 685.85 260.17
+ oxygenvolume     1    1.3712 685.91 260.20
+ Time             1    1.1451 686.14 260.30
+ PostboilGrav     1    0.7427 686.54 260.48
+ density          1    0.7309 686.55 260.49
+ MashVol          1    0.5047 686.78 260.59
+ OriginalGravity  1    0.3253 686.96 260.67
+ water            1    0.2511 687.03 260.70
+ ABV              1    0.0032 687.28 260.81

Step:  AIC=258.48
EBCHAZE ~ MashTemp + Coldworttotal + TotalMinCasting + TotalLauterTime
+
    Dilutionwater + MashpH

                  Df Sum of Sq    RSS    AIC
<none>                         682.12 258.48
+ FirstRGrav       1    4.3832 677.74 258.48
+ PG               1    4.3524 677.77 258.49
+ PreBoilVolume    1    4.2932 677.83 258.52
+ LastRGrav        1    3.4363 678.69 258.91
+ IBU              1    2.5631 679.56 259.31
+ extraboiltime    1    1.9333 680.19 259.60
+ EBCCOLOUR        1    1.7987 680.33 259.66
```

```
+ oxygenvolume     1    1.6209 680.50 259.74
+ OG               1    1.5180 680.61 259.79
+ Time             1    1.3389 680.78 259.87
+ PreboilGrav      1    1.1633 680.96 259.95
+ PH               1    1.1391 680.98 259.96
+ density          1    1.0870 681.04 259.98
+ PostboilGrav     1    0.8632 681.26 260.08
+ MashVol          1    0.5052 681.62 260.25
+ OriginalGravity  1    0.3838 681.74 260.30
+ water            1    0.3692 681.75 260.31
+ ABV              1    0.0310 682.09 260.46

Call:
lm(formula = EBCHAZE ~ MashTemp + Coldworttotal + TotalMinCasting +
    TotalLauterTime + Dilutionwater + MashpH, data = Data)

Coefficients:
    (Intercept)         MashTemp    Coldworttotal  TotalMinCasting
TotalLauterTime
      -25.31543          0.10506          0.18515          0.01990
-0.02438
  Dilutionwater           MashpH
       -0.22087          0.51989
```

Figure C.17- RStudio Script of Stepwise Regression of Brewhouse Data.

```
#Combining Forward and Backward Selection- Stepwise Regression
> FitStart<- lm(EBCHAZE~1, data = Data)
> summary(FitStart)

Call:
lm(formula = EBCHAZE ~ 1, data = Data)

Residuals:
    Min      1Q  Median      3Q     Max
-1.2356 -0.8631 -0.7290 -0.0606  7.5969

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  1.23564    0.09414   13.13   <2e-16 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 1.657 on 309 degrees of freedom
  (12 observations deleted due to missingness)

> step(FitStart, direction="both",scope=formula(FitAll))
Start:  AIC=314.27
EBCHAZE ~ 1

                  Df Sum of Sq    RSS    AIC
+ MashTemp         1    54.881 793.97 295.55
+ Coldworttotal    1    45.854 803.00 299.05
+ Coldwortvolume   1    45.854 803.00 299.05
+ PG               1    35.011 813.85 303.21
+ TotalLauterTime  1    21.842 827.01 308.19
+ TotalMinCasting  1    17.094 831.76 309.96
+ OG               1    12.827 836.03 311.55
+ PH               1    10.269 838.59 312.50
+ FirstRGrav       1     9.342 839.51 312.84
+ PreBoilVolume    1     8.954 839.90 312.98
+ oxygenvolume     1     7.142 841.71 313.65
+ IBU              1     5.728 843.13 314.17
<none>                         848.86 314.27
+ extraboiltime    1     4.069 844.79 314.78
+ water            1     3.903 844.95 314.84
+ Time             1     3.628 845.23 314.94
+ Dilutionwater    1     2.583 846.27 315.32
+ EBCCOLOUR        1     2.481 846.38 315.36
+ PostboilGrav     1     1.725 847.13 315.64
+ LastRGrav        1     1.611 847.25 315.68
+ PreboilGrav      1     1.597 847.26 315.69
+ MashpH           1     1.525 847.33 315.71
+ ABV              1     1.380 847.48 315.76
+ OriginalGravity  1     1.237 847.62 315.82
+ MashVol          1     0.855 848.00 315.96
+ density          1     0.381 848.48 316.13

Step:  AIC=295.55
EBCHAZE ~ MashTemp

                  Df Sum of Sq    RSS    AIC
+ Coldworttotal    1    42.434 751.54 280.52
+ Coldwortvolume   1    42.434 751.54 280.52
+ TotalMinCasting  1    22.539 771.44 288.62
```

```
+ PG               1    14.157 779.82 291.97
+ TotalLauterTime  1    11.629 782.35 292.98
+ OG               1     9.031 784.94 294.00
+ PH               1     8.632 785.34 294.16
+ PreBoilVolume    1     7.608 786.37 294.56
+ FirstRGrav       1     5.715 788.26 295.31
<none>                         793.97 295.55
+ oxygenvolume     1     3.017 790.96 296.37
+ Time             1     2.858 791.12 296.43
+ MashpH           1     2.667 791.31 296.50
+ ABV              1     2.183 791.79 296.69
+ IBU              1     2.133 791.84 296.71
+ MashVol          1     2.127 791.85 296.72
+ water            1     1.655 792.32 296.90
+ OriginalGravity  1     1.431 792.54 296.99
+ PostboilGrav     1     1.386 792.59 297.01
+ LastRGrav        1     1.207 792.77 297.08
+ PreboilGrav      1     1.054 792.92 297.14
+ EBCCOLOUR        1     0.587 793.39 297.32
+ Dilutionwater    1     0.256 793.72 297.45
+ density          1     0.028 793.95 297.54
+ extraboiltime    1     0.000 793.97 297.55
- MashTemp         1    54.881 848.86 314.27

Step:  AIC=280.52
EBCHAZE ~ MashTemp + Coldworttotal

                  Df Sum of Sq    RSS    AIC
+ TotalMinCasting  1    29.305 722.24 270.19
+ TotalLauterTime  1    13.793 737.75 276.78
+ PG               1     8.294 743.25 279.08
+ FirstRGrav       1     6.593 744.95 279.79
+ Dilutionwater    1     6.421 745.12 279.86
+ MashpH           1     5.665 745.88 280.18
<none>                         751.54 280.52
+ PH               1     4.769 746.77 280.55
+ OG               1     4.460 747.08 280.68
+ PreBoilVolume    1     3.939 747.60 280.89
+ EBCCOLOUR        1     2.169 749.37 281.63
+ LastRGrav        1     1.333 750.21 281.97
+ PostboilGrav     1     1.050 750.49 282.09
+ PreboilGrav      1     1.037 750.50 282.09
+ IBU              1     0.927 750.61 282.14
+ OriginalGravity  1     0.924 750.62 282.14
+ Time             1     0.691 750.85 282.24
+ oxygenvolume     1     0.559 750.98 282.29
+ water            1     0.457 751.08 282.33
+ ABV              1     0.449 751.09 282.34
+ MashVol          1     0.124 751.42 282.47
+ extraboiltime    1     0.111 751.43 282.48
+ density          1     0.103 751.44 282.48
- Coldworttotal    1    42.434 793.97 295.55
- MashTemp         1    51.461 803.00 299.05

Step:  AIC=270.19
EBCHAZE ~ MashTemp + Coldworttotal + TotalMinCasting

                  Df Sum of Sq    RSS    AIC
+ TotalLauterTime  1    25.006 697.23 261.27
+ Dilutionwater    1    11.631 710.60 267.16
+ FirstRGrav       1     6.821 715.42 269.25
+ PG               1     4.960 717.28 270.06
```

```
+ MashpH           1     4.804 717.43 270.12
<none>                        722.24 270.19
+ PH               1     3.634 718.60 270.63
+ OG               1     3.423 718.81 270.72
+ PreBoilVolume    1     3.261 718.98 270.79
+ EBCCOLOUR        1     2.803 719.43 270.99
+ LastRGrav        1     1.270 720.97 271.65
+ PostboilGrav     1     0.934 721.30 271.79
+ IBU              1     0.933 721.30 271.79
+ OriginalGravity  1     0.697 721.54 271.89
+ ABV              1     0.664 721.57 271.91
+ PreboilGrav      1     0.619 721.62 271.93
+ oxygenvolume     1     0.465 721.77 271.99
+ Time             1     0.394 721.84 272.02
+ density          1     0.373 721.86 272.03
+ water            1     0.157 722.08 272.12
+ extraboiltime    1     0.071 722.16 272.16
+ MashVol          1     0.009 722.23 272.19
- TotalMinCasting  1    29.305 751.54 280.52
- Coldworttotal    1    49.200 771.44 288.62
- MashTemp         1    57.333 779.57 291.87

Step:  AIC=261.27
EBCHAZE ~ MashTemp + Coldworttotal + TotalMinCasting + TotalLauterTime

                  Df Sum of Sq    RSS    AIC
+ Dilutionwater    1     9.945 687.29 258.81
+ PG               1     5.593 691.64 260.77
+ MashpH           1     5.187 692.04 260.95
<none>                        697.23 261.27
+ PreBoilVolume    1     3.620 693.61 261.65
+ LastRGrav        1     3.535 693.69 261.69
+ FirstRGrav       1     3.032 694.20 261.92
+ PH               1     2.731 694.50 262.05
+ OG               1     2.514 694.72 262.15
+ EBCCOLOUR        1     2.186 695.04 262.30
+ IBU              1     2.148 695.08 262.31
+ oxygenvolume     1     1.435 695.80 262.63
+ PostboilGrav     1     0.969 696.26 262.84
+ PreboilGrav      1     0.933 696.30 262.85
+ density          1     0.597 696.63 263.00
+ Time             1     0.596 696.63 263.00
+ MashVol          1     0.432 696.80 263.08
+ extraboiltime    1     0.214 697.02 263.17
+ OriginalGravity  1     0.206 697.02 263.18
+ water            1     0.110 697.12 263.22
+ ABV              1     0.063 697.17 263.24
- TotalLauterTime  1    25.006 722.24 270.19
- TotalMinCasting  1    40.518 737.75 276.78
- MashTemp         1    43.979 741.21 278.23
- Coldworttotal    1    53.881 751.11 282.34

Step:  AIC=258.81
EBCHAZE ~ MashTemp + Coldworttotal + TotalMinCasting + TotalLauterTime
+
    Dilutionwater

                  Df Sum of Sq    RSS    AIC
+ MashpH           1     5.162 682.12 258.48
+ PG               1     4.549 682.74 258.76
<none>                        687.29 258.81
+ FirstRGrav       1     4.184 683.10 258.92
```

```
+ PreBoilVolume    1     4.024 683.26 258.99
+ LastRGrav        1     3.519 683.77 259.22
+ IBU               1     2.283 685.00 259.78
+ PreboilGrav      1     1.904 685.38 259.95
+ PH                1     1.794 685.49 260.00
+ OG                1     1.686 685.60 260.05
+ EBCCOLOUR         1     1.548 685.74 260.12
+ extraboiltime    1     1.431 685.85 260.17
+ oxygenvolume     1     1.371 685.91 260.20
+ Time              1     1.145 686.14 260.30
+ PostboilGrav     1     0.743 686.54 260.48
+ density           1     0.731 686.55 260.49
+ MashVol           1     0.505 686.78 260.59
+ OriginalGravity  1     0.325 686.96 260.67
+ water             1     0.251 687.03 260.70
+ ABV               1     0.003 687.28 260.81
- Dilutionwater    1     9.945 697.23 261.27
- TotalLauterTime  1    23.319 710.60 267.16
- MashTemp          1    37.212 724.50 273.16
- TotalMinCasting  1    45.487 732.77 276.68
- Coldworttotal    1    63.254 750.54 284.11

Step:  AIC=258.48
EBCHAZE ~ MashTemp + Coldworttotal + TotalMinCasting + TotalLauterTime
+
    Dilutionwater + MashpH

                  Df Sum of Sq    RSS    AIC
<none>                          682.12 258.48
+ FirstRGrav       1     4.383 677.74 258.48
+ PG               1     4.352 677.77 258.49
+ PreBoilVolume    1     4.293 677.83 258.52
- MashpH           1     5.162 687.29 258.81
+ LastRGrav        1     3.436 678.69 258.91
+ IBU               1     2.563 679.56 259.31
+ extraboiltime    1     1.933 680.19 259.60
+ EBCCOLOUR         1     1.799 680.33 259.66
+ oxygenvolume     1     1.621 680.50 259.74
+ OG                1     1.518 680.61 259.79
+ Time              1     1.339 680.78 259.87
+ PreboilGrav      1     1.163 680.96 259.95
+ PH                1     1.139 680.98 259.96
+ density           1     1.087 681.04 259.98
+ PostboilGrav     1     0.863 681.26 260.08
+ MashVol           1     0.505 681.62 260.25
+ OriginalGravity  1     0.384 681.74 260.30
+ water             1     0.369 681.75 260.31
+ ABV               1     0.031 682.09 260.46
- Dilutionwater    1     9.919 692.04 260.95
- TotalLauterTime  1    23.691 705.81 267.06
- MashTemp          1    38.349 720.47 273.43
- TotalMinCasting  1    44.515 726.64 276.08
- Coldworttotal    1    66.357 748.48 285.26

Call:
lm(formula = EBCHAZE ~ MashTemp + Coldworttotal + TotalMinCasting +
    TotalLauterTime + Dilutionwater + MashpH, data = Data)

Coefficients:
    (Intercept)         MashTemp    Coldworttotal  TotalMinCasting
TotalLauterTime
```

```
      -25.31543          0.10506          0.18515          0.01990
-0.02438
  Dilutionwater           MashpH
       -0.22087          0.51989

> lm(formula = EBCHAZE ~ MashTemp + Coldworttotal + TotalMinCasting +
+      TotalLauterTime + Dilutionwater + MashpH, data = Data)

Call:
lm(formula = EBCHAZE ~ MashTemp + Coldworttotal + TotalMinCasting +
    TotalLauterTime + Dilutionwater + MashpH, data = Data)

Coefficients:
    (Intercept)         MashTemp    Coldworttotal  TotalMinCasting
TotalLauterTime
      -25.31543          0.10506          0.18515          0.01990
-0.02438
  Dilutionwater           MashpH
       -0.22087          0.51989

> BestModel<-lm(formula = EBCHAZE ~ MashTemp + Coldworttotal +
TotalMinCasting +
+      TotalLauterTime + Dilutionwater + MashpH, data = Data)
> summary(BestModel)

Call:
lm(formula = EBCHAZE ~ MashTemp + Coldworttotal + TotalMinCasting +
    TotalLauterTime + Dilutionwater + MashpH, data = Data)

Residuals:
    Min      1Q  Median      3Q     Max
-1.9493 -0.9274 -0.3727  0.0937  6.2606

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)     -25.315434   4.423625  -5.723 2.52e-08 ***
MashTemp          0.105060   0.025455   4.127 4.75e-05 ***
Coldworttotal     0.185150   0.034103   5.429 1.16e-07 ***
TotalMinCasting   0.019899   0.004475   4.447 1.23e-05 ***
TotalLauterTime  -0.024376   0.007514  -3.244  0.00131 **
Dilutionwater    -0.220875   0.105225  -2.099  0.03664 *
MashpH            0.519891   0.343346   1.514  0.13102
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Appendix D

Table D.1- ANOVA adjusted P-values for comparison of each enzyme treatment at each light angle measured to each sample group (high, medium, normal),. P-values for high vs control, normal vs control, and normal vs high are highlighted in yellow.

Enzyme Treatments and Light Angles	High-Control	Normal-Control	Normal-High
Amyloglucosidase EBC	<0.05	0.46	<0.05
Amyloglucosidase 25°	<0.05	0.43	<0.05
Amyloglucosidase 90°	<0.05	0.73	<0.05
Pepsin EBC	<0.05	0.08	<0.05
Pepsin 25°	<0.05	0.13	<0.05
Pepsin 90°	<0.05	0.26	<0.05
Ultraflo®Max EBC	<0.05	0.86	<0.05
Ultraflo®Max 25°	<0.05	0.57	<0.05
Ultraflo®Max 90°	<0.05	0.87	<0.05

Appendix E

Table E.1- Comparison of pre and post digestion values of each enzyme treatment at each light angle measured. Significant P-values (p= <0.05) are listed and non-significant p-values are bolded.

Enzyme Treatments and Light Angles	Normal	High	Control
Amyloglucosidase EBC	<0.05	<0.05	<0.05
Amyloglucosidase 25°	<0.05	<0.05	<0.05
Amyloglucosidase 90°	<0.05	<0.05	<0.05
Pepsin EBC	<0.05	<0.05	<0.05
Pepsin 25°	<0.05	<0.05	<0.05
Pepsin 90°	<0.05	<0.05	<0.05
Ultraflo®Max EBC	<0.05	<0.05	<0.05
Ultraflo®Max 25°	<0.05	<0.05	**0.09**
Ultraflo®Max 90°	<0.05	<0.05	<0.05

Appendix F

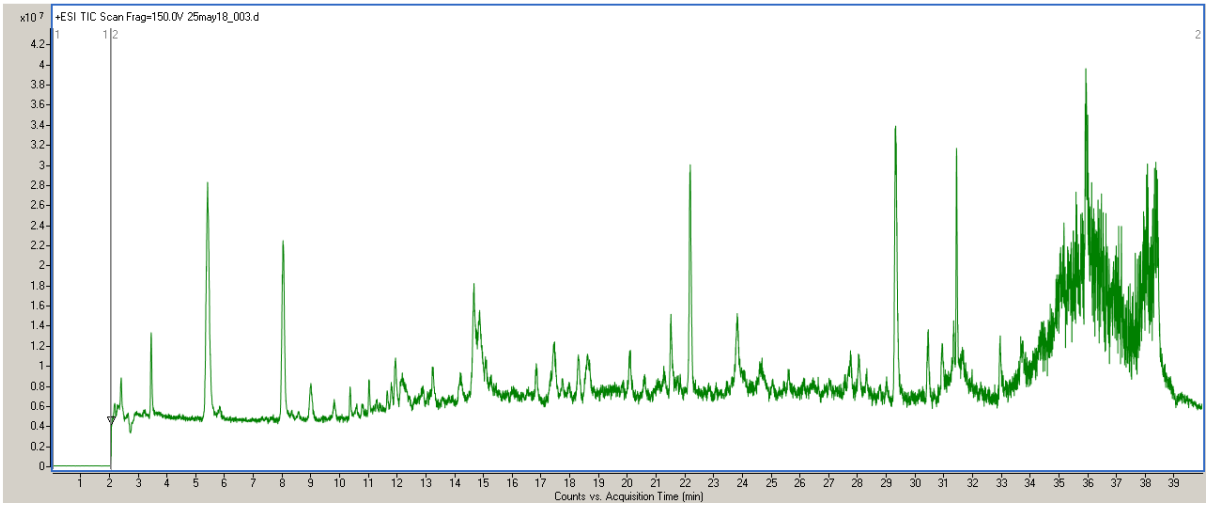


Figure F.1- Total ion chromatogram obtained from EC/ESI-QTOF-MS for low haze, intact protein beer sample.

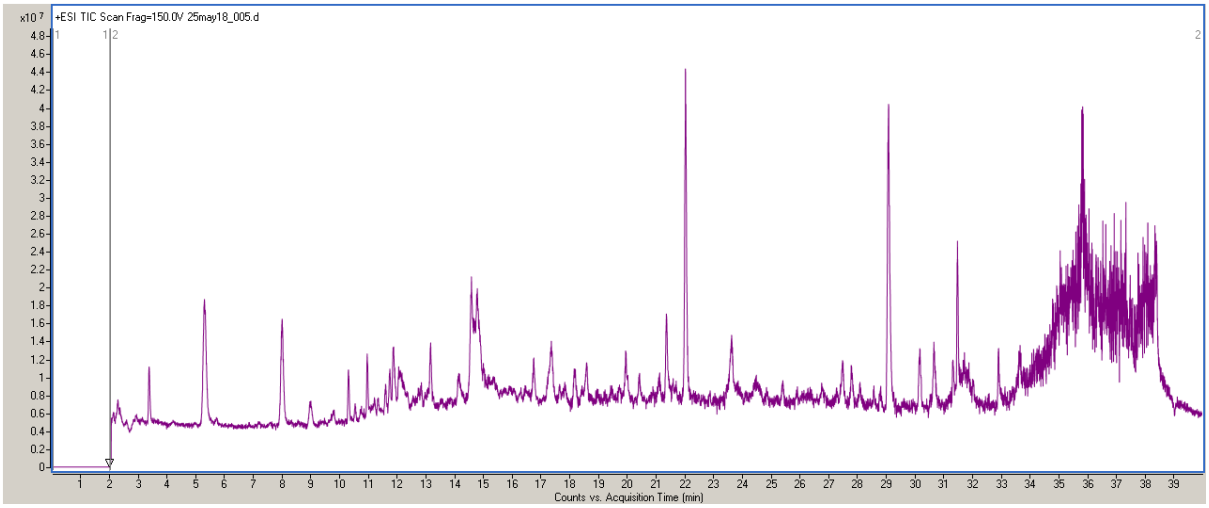


Figure F.2- Total ion chromatogram obtained from EC/ESI-QTOF-MS for high haze, intact protein sample.

Appendix G

Table G.1- Standard deviations of collated sensory data from all assessment points of sample set one at 4°C. Standard deviations for day 14 and 28 could not be calculated as only one set of samples were assessed. Panellist attendance ranged from n=6 to n=15.

Days	Astringent	Bitter	Catty Hop	Citrus Hop	Damascenone	Earthy Hop	Floral Hop	Freshly Cut Grass	Isovaleric	Passionfruit Hop	Peach Hop	Pine Hop	Raw Hop
0	±0.35	±0.42	±0.07	±0.14	±0.07	±0.07	±0.35	±0.14	±0.35	±0.21	±0.28	±0.07	±0.21
4	±0.07	±0.21	±0.07	±0.14	±0.28	±0.42	±0.49	±0.07	±0.07	±0.07	±0.28	±0.00	±0.28
7	±0.00	±0.14	±0.21	±0.57	±0.21	±0.07	±0.07	±0.00	±0.14	±0.35	±0.07	±0.21	±0.07
14	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
28	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
42	±0.42	±0.07	±0.64	±0.14	±1.20	±0.07	±0.49	±0.21	±0.07	±0.07	±0.21	±0.07	±0.00
56	±0.07	±1.06	±0.28	±0.99	±1.06	±0.00	±0.28	±0.00	±0.28	±0.21	±0.07	±0.21	±0.28
70	±0.21	±0.92	±0.42	±0.99	±1.70	±0.64	±0.14	±0.28	±1.20	±0.07	±0.14	±0.00	±0.28
84	±0.28	±0.07	±0.64	±1.06	±0.71	±0.35	±0.42	±0.21	±0.14	±0.57	±0.35	±0.00	±0.07
98	±0.00	±0.28	±0.07	±0.49	±0.00	±0.14	±0.07	±0.21	±0.07	±0.00	±0.07	±0.07	±0.00
112	±0.21	±0.21	±0.21	±0.14	±0.78	±0.14	±0.07	±0.14	±0.21	±0.00	±0.00	±0.35	±0.14

Table G.2- Standard deviations of collated sensory data from all assessment points of sample set one at 20°C. Panellist attendance ranged from n=6 to n=15.

Days	Astringent	Bitter	Catty Hop	Citrus Hop	Damascenone	Earthy Hop	Floral Hop	Freshly Cut Grass	Isovaleric	Passionfruit Hop	Peach Hop	Pine Hop	Raw Hop
0	±0.07	±0.28	±0.14	±0.28	±0.07	±0.07	±0.07	±0.14	±0.21	±0.14	±0.21	±0.14	±0.14
4	±0.07	±0.14	±0.00	±0.64	±0.14	±0.00	±0.00	±0.35	±0.07	±0.35	±0.49	±0.07	±0.07
7	±0.07	±0.21	±0.42	±0.07	±0.07	±0.00	±0.21	±0.35	±0.00	±0.14	±0.07	±0.07	±0.07
14	±0.21	±0.71	±0.14	±0.78	±0.42	±0.14	±0.49	±0.64	±0.35	±0.07	±0.00	±0.07	±0.71
28	±0.07	±0.35	±0.07	±0.00	±0.07	±0.00	±0.14	±0.07	±0.07	±0.07	±0.14	±0.07	±0.07
42	±0.28	±0.71	±0.42	±0.99	±1.98	±0.14	±0.35	±0.78	±0.21	±0.21	±0.14	±0.21	±0.14
56	±0.00	±1.27	±0.42	±1.27	±1.84	±0.21	±0.57	±0.35	±0.64	±0.21	±0.07	±0.35	±0.78
70	±0.28	±0.28	±0.14	±0.28	±0.64	±0.21	±0.21	±0.78	±0.07	±0.14	±0.21	±0.00	±0.21
84	±0.28	±0.21	±0.14	±0.14	±0.49	±0.07	±0.00	±0.07	±0.28	±0.21	±0.07	±0.21	±0.00
98	±0.00	±0.42	±0.35	±0.00	±0.35	±0.14	±0.28	±0.21	±0.28	±0.07	±0.14	±0.07	±0.07
112	±0.35	±0.35	±0.28	±0.14	±0.28	±0.07	±0.64	±0.14	±0.00	±0.14	±0.07	±0.28	±0.14

Appendix H

Table H.1-Initial concentrations of terpenes utilised in the Vanillin Assay.

Compounds	Level 6 (µg/L)	Level 5 (µg/L)	Level 4 (µg/L)	Level 3 (µg/L)	Level 2 (µg/L)	Level 1 (µg/L)
B-myrcene	153.72	76.86	34.16	12.81	3.66	0.73
Linalool oxide	912.04	456.02	202.68	76.00	21.72	4.34
Linalool	475.74	237.87	105.72	39.64	11.33	2.27
β-citronellol	862.90	431.45	191.76	71.91	20.55	4.11
Trans-geraniol	464.59	232.30	103.24	38.72	11.06	2.21
β-caryophyllene	494.60	247.30	109.91	41.22	11.78	2.36
α-humulene	158.86	79.43	35.30	13.24	3.78	0.76
(-)-caryophyllene oxide	146.58	73.29	32.57	12.21	3.49	0.70

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