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Sensory and analytical impacts of unmalted cereal adjunct usage in lager brewing

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Abstract

The use of unmalted cereal adjuncts (barley, wheat, rice and maize) is well established in the brewing industry, with typical incorporation rates of up to 30%. Unmalted cereal adjuncts are utilised in the production of light lager beers to provide cost and energy reductions and as a response to consumer demand for a lighter beer style. However, there is little comprehensive data concerning the sensory and analytical impacts of using high proportions of unmalted cereal adjuncts. This was the context to the main objectives of this PhD research: to better understand the sensory characteristics and consumer perception of beers produced with unmalted adjuncts and develop protocols to improve flavour matching to all-malt counterparts.

The sensorial and analytical profile of lager beers produced with relatively high concentrations (0, 30 and 60% of grist) of unmalted barley, wheat, rice and maize and an all-malt control were determined (Chapter 3). A trained sensory panel developed and defined an adjunct beer lexicon which was used to characterise the sensory properties of the beers. Results from the sensory evaluation showed that at 30% adjunct incorporation there was insignificant variation in the beer flavour profiles ($p>0.05$). Whereas, at 60% adjunct incorporation there were some significant sensory differences identified that were specific to particular adjunct materials. Furthermore, high adjunct incorporation significantly decreased wort free amino nitrogen (FAN) which impacted the fermentation volatile profile corresponding to specific characteristics in the sensory analysis.

In Chapter 4 two different approaches were investigated to enhance free amino nitrogen when brewing with high incorporations of unmalted cereal adjuncts: i) addition of exogenous thermostable proteolytic enzymes during mashing and ii) additions of yeast nutrients to the cold wort before fermentation. Beer samples produced with unmalted wheat and two types of unmalted barley at different grist proportions (15, 25, 35 and 45%) were supplemented by these two approaches to achieve a FAN concentration greater than 200 mg/L. Increasing the wort FAN concentration of adjunct beers was shown to improve the fermentation volatile profile consistency relative to an all-malt control; decreasing variation in ester and higher alcohol concentrations to less than 20% from control for all adjunct beers. The results of this work suggest that by balancing wort composition the analytical flavour profile of a full malt beer could

be more closely matched such that flavour may not be a limiting factor at higher levels of unmalted adjunct usage.

In Chapter 5, a sample set of 7 adjunct beers produced with unmalted cereal adjuncts up to 45% were assessed alongside an all-malt control lager by consumers (n=104). The consumers assessed beers for hedonic liking (to determine consumer acceptance) and purchase intent. Overall, adjunct beers produced with FAN boost supplementation were not significantly different in overall liking and purchase intent compared to the all-malt control ($p>0.05$). In terms of liking, three clusters of consumers were discovered with different patterns of preference, revealing that consumers are not consistent in their preference when considering adjunct based beers. Interestingly, it was found that one cluster (24% of consumers) had a preference for high adjunct beers. A Check-All-That-Apply (CATA) technique was used to determine consumer sensory perception of the beers. The combination of CATA and overall liking data provided key sensory liking and disliking drivers to guide future improvement of lager beers brewed with higher adjuncts rates. 'Malty', 'apple', 'sweet' attributes were all significant positive drivers for liking and 'lingering bitterness' was the only significant negative driver for liking ($p<0.05$). Consumers used terms such as 'refreshing', 'light' and 'weak flavour' to describe the beers which were not in the CATA lexicon determined by the trained panel, signifying the different language consumers use to define the product.

In Chapter 6, a non-targeted metabolomics approach was used to determine non-volatile metabolites which could contribute to flavour differences observed when brewing with unmalted barley. Ultra-high performance liquid chromatography (UHPLC) couple to a quadrupole time-of-flight mass spectrometer with an electro-spray ionisation source (ESI) was used to analyse three beer samples (100% malt and two 15% unmalted barley beers). The metabolomic profile of the three beers were compared using principal component analysis (PCA) to determine significantly different molecular features. Results found that the main differential molecular features were nitrogenous peptides and purine derivatives. This result can be attributed to the lack of malting process and thus lack of proteolytic enzyme activity being applied to the proportion of unmalted barley, reducing the protein and peptide breakdown of the unmalted barley. Several of the differential peptides identified had amino acid residues which are known for causing bitter and kokumi taste in

beer, which could attribute to the bitterness observed when brewing with unmalted barley.

Overall, this work has shown that the use of unmalted adjuncts at higher incorporation rates has fewer direct flavour impacts than was anticipated, provided that steps were taking to maintain the wort composition. The main sensory differences noted at high adjunct rates were lack of fullness and development of lingering bitterness and astringency. Interestingly, consumer data showed that there are consumers who actually prefer high adjunct beers. However, if the aim were to flavour match as closely as possible to an all-malt beer, the metabolomics study in Chapter 6 provides new metabolite targets and hypotheses which can be used to improve flavour matching.

Publications

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List of Abbreviations

ABV	alcohol by volume
ANOVA	analysis of variance
BCAAs	branched chained amino acids
BU	bitterness units
CA	correspondence analysis
CaSR	calcium sensing receptor
CATA	check-all-that-apply
CMC	carboxymethyl cellulose
CoA	coenzyme A
DMS	dimethyl sulphide
DMSO	dimethyl sulphoxide
EDTA	ethylenediaminetetraacetic acid
ESI	electro-spray ionisation source
FAN	free amino nitrogen
FID	flame ionisation detector
g	gram
GAX	glucuronoarabinoxylans
GC	gas chromatography
GGT	y-glutamyl transferase
HPAEC-PAD	high performance anion-exchange chromatography with pulsed amperometric detection
HPLC-UV-DAD	high performance liquid chromatography-ultraviolet-diode array detection
HS	headspace
HSD	honest significant difference
ICP-MS	inductively coupled plasma-mass spectroscopy
kWh	kilowatt hour
kg	kilogram
L	litre
LCMS	liquid chromatography-mass spectroscopy
mg	milligram
min	minute
ml	millilitre
mm	millimetre
MS	mass spectroscopy
NCR	nitrogen catabolite repression
nm	nanometre
NSP	non starch polysaccharides
OG	original gravity
PCA	principal component analysis
PFPD	pulsed flame photometric detector
PG	present gravity
POF	phenolic off flavour
ppm	parts per million
PROP	5-n-propylthioracial

PTS	prop taster status
PVPP	polyvinylpolypyrrolidone
QDA	quantitative descriptive analysis
QTOF	quadrupole time of flight
SMM	S-methyl methionine
SPME	solid phase microextraction
TCA	tricarboxylic acid
TOC-TN	total organic carbon-total nitrogen
TPA	total proanthocyanidin content
TPC	total polyphenol content
µg	microgram
UHPLC	ultra-high performance liquid chromatography
VDK	vicinal diketones

Chapter 1 Introduction

Malted barley is the favoured cereal grain to provide the main source of extract and essential nutrients required for the production of beer. However, the partial substitution of malted barley with cereal adjuncts such as wheat, maize and rice is well established in the brewing industry.

The use of adjuncts is typically economically driven, as adjuncts may provide a cheaper source of extract (depending on adjunct type and market conditions). Cost reductions can be driven by minimising the requirement for the malting process and its related costs (Kok *et al.*, 2019). Breweries residing in countries outside of the major malt exporting countries (European Union, Australia and Canada) can create a cost saving by replacing potentially expensive imported malts with cheaper native grains. The cultivation of barley is less optimal in tropical conditions therefore brewers are encouraged to use alternative tropical grains such as sorghum, rice and maize (Taylor *et al.*, 2013). Barley and corn are the most commonly used adjuncts in Europe and America, whilst rice usage is popular in Asia (Bogdan *et al.*, 2017).

Minimising malting requirements and supporting local agriculture not only provides breweries with an economic benefit but the overall sustainability of beer production can improve considerably. The malting process accounts for 217 kg CO_2eq/t, which is nearly half of the total carbon footprint incurred during malt production, with the agriculture of malting barley accounting for the additional 241 kg CO_2eq/t (Muntons Sustainability Statement, 2020). Therefore, utilising unmalted adjuncts can considerably reduce the carbon footprint of the malting and brewing chain. The use of local crops is less damaging to the environment because it lowers the CO_2 emissions associated with transportation of raw materials (Aastrup, 2010).

Legislation can also encourage or restrict the use of alternative raw materials in beer. Under the German purity law (Reinheitsgebot) beers can only be produced from malt, water, hops and yeast and other sources of starch and sugars are prohibited. In Japan, increasing beer tax started the development of adjunct based beer styles. Happoshu, a beer produced with a malt content between 25-67%, was launched in Japan to avoid high taxes and create cheaper beers (Mizuno, 2013). Likewise, in Nigeria in 1988, a government economic decision to ban the importation of malted barley forced local brewers

to turn to locally grown sorghum and maize crops (Goode *et al.*, 2005; Akinyoade *et al.*, 2016). The quality attributes of some of the world's leading global beer brands are based on adjuncts used in their recipes. Adjuncts are utilised by brewers both to modify beer quality (e.g., flavour, foam colloidal stability) and to enable production of new innovative products with specific desired features (Kok *et al.*, 2019).

Adjuncts are available to a brewer in a range of forms which are typically classified as either solid or liquid adjuncts. Solid adjuncts comprise cereals such as rice, maize, wheat and raw barley. Solid cereal adjuncts are available in a variety of forms and dependent on their starch gelatinisation temperatures, some adjuncts require pre-processing before being added to the brewing process, which is discussed further in Section 1.3.1. Adjuncts are also available in a liquid (syrup) form as a source of fermentable (glucose, maltose, maltotriose) and non-fermentable carbohydrates (higher oligosaccharides) suitable for brewing. Commonly used liquid adjuncts include malt extracts, sucrose-based syrups and hydrolysed cereal syrups.

The aim of this review is to discuss the known chemical composition of unmalted cereal adjuncts with a focus on two types of barley (barley (A) and barley (B)), wheat, maize and rice and their fate during the malting and brewing process. Thus, reviewing the positive impact malting has on the composition of cereal grains, in order to understand the issues that can arise when utilising unmalted adjuncts. Additionally, it will assess the impact differences in the chemical composition of adjuncts can have on the final beer quality and flavour and the sensory methods that can be applied to understand sensory perception and consumer acceptance of beers produced with unmalted cereal adjuncts.

1.1. Overview of the malting and brewing process

An outline of the malting and brewing process is depicted in Figure 1.1, showing the key brewing stages: malting, mashing, boiling, fermentation, conditioning and packaging.

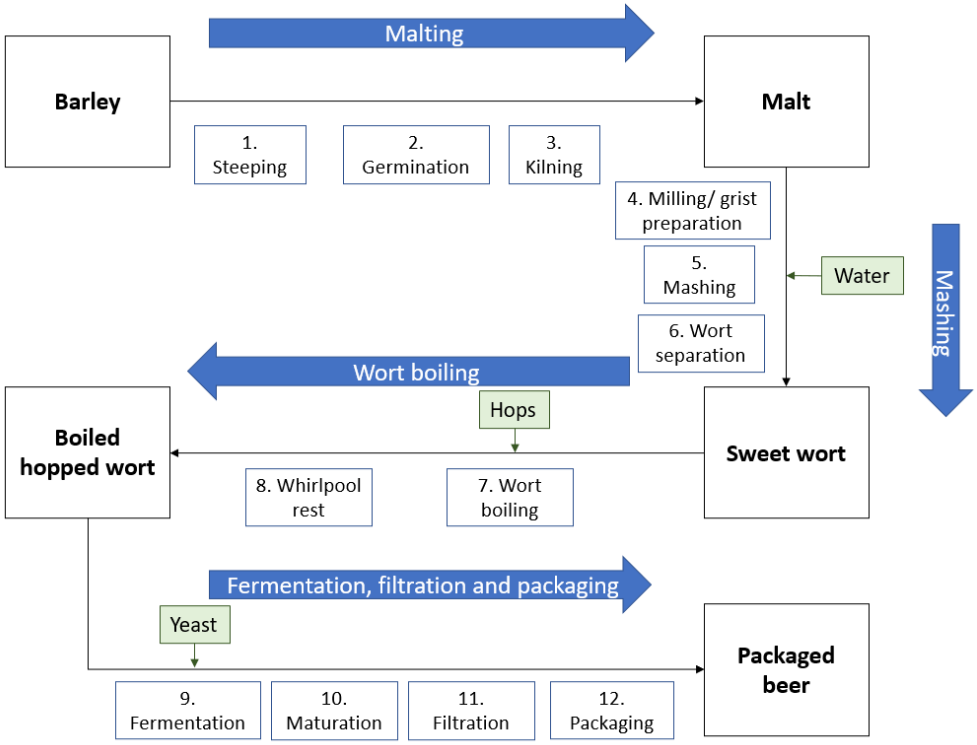


Figure 1.1. Overview of the malting and brewing process

The malting process consists of three main unit operations: steeping, germination and kilning. Steeping involves the hydration of barley seeds by steeping them in water to achieve a moisture content required to trigger germination (>32%). Controlled germination releases and activates endogenous enzymes resulting in the modification of the barley grain and degradation of the endosperm (Kok *et al.*, 2019). The extent of the processes that occur in germination are regulated by controlling the moisture and temperature of the grain during steeping and germination (Briggs *et al.*, 2004). Consequently, the starch and enzymes required are made available in the grain for the latter stages of beer production. The final stage of malting (kilning) circulates hot air to dry malt to a stable product (ca. 4-6% moisture) to stop germination and preserve enzymes, as well as generate aroma, flavour and

coloured compounds in the malt that help define the final beer character. Dependent on the thermal treatment applied during kilning (or roasting), different malt styles are produced. Pale malts are commonly dried at lower temperatures, whilst dark speciality malts are subjected to higher production temperatures, leading to more intense non-enzymatic browning (Maillard reactions) (Coghe *et al.*, 2006). The kilning process is energy intensive as industrial malting operations use approximately 800 kWh/t of energy to produce the heat required to kiln malt (Ferrari-John *et al.*, 2017). Minimising the need for kilning by utilising unmalted cereal adjuncts can improve the overall energy efficiency of the malting and brewing chain.

The aim of mashing is to extract the soluble content of the milled grist and facilitate the breakdown of the grain by enzymes which were generated through malting. Mashing involves combining milled grain with water which is subjected to a mashing schedule (time-temperature profile) to enable maximum enzymatic hydrolysis and extraction from the grain. The mash mixture is then transferred to a lauter tun to separate the spent grains from the liquid, containing the fermentable sugars and nutrients, also known as sweet wort.

The sweet wort is then transferred into another vessel (the 'kettle') and boiled with hops. The function of wort-boiling is to extract α-acids from the bittering hops which are isomerised to bitter tasting substances during the boil (iso-α-acids) (Malowicki *et al.*, 2006). The boiling step also precipitates some denatured proteins and sparingly soluble materials ('hot break') and sterilises the wort. At the end of boil, the wort is typically separated in a whirlpool rest from the trub and any residual hop debris.

The boiled wort is then quickly cooled through a plate heat exchanger, whereby a second separation of solids from the wort occurs ('cold break'), composed mostly of proteins and polyphenols and some associated lipids. Fermentation is then initiated by pitching the wort with a selected yeast strain. Lager (*S. pastorianus*) or ale (*S. cerevisiae*) yeast strains are used dependent on the beer style. The optimal fermentation temperature for lager yeast is between 6-15 °C and 12-25 °C for ale strains (Vidgren *et al.*, 2010). During fermentation the spectrum of fermentable carbohydrates present in the wort are converted to ethanol and carbon dioxide. A multitude of other by-products of yeast metabolism are formed during fermentation, many of these contribute to beer flavour and aroma (Briggs *et al.*, 2004). When the fermentation is complete the

green beer produced is held for a period of maturation to reduce off-flavours in the beer such as diacetyl reduction (Pires *et al.*, 2015). The matured beer is then chilled and filtered or centrifuged to remove residual yeast. The bright beers are carbonated and packaged into bottles, cans or kegs.

1.2. Cereal adjuncts

Cereals belong to the Gramineae (or Poaceae) family, one of the most important agricultural families (the 'grasses'). Globally, cereal grains are produced in higher quantities than any other type of crop for human consumption and feed production (Zartash *et al.*, 2020). The group includes common staples such as maize (*Zea mays* L.), common wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and rice (*Oryza sativa* L.). Cereal grains as whole grains are a rich source of carbohydrates, proteins, fats, minerals and vitamins.

1.2.1. The composition of cereal grains

The structural form of all cereal grains is similar enough that a generalised cereal grain structure can be depicted (Figure 1.2). Cereal grains comprise of the embryo, endosperm, nucellus, testa, and pericarp.

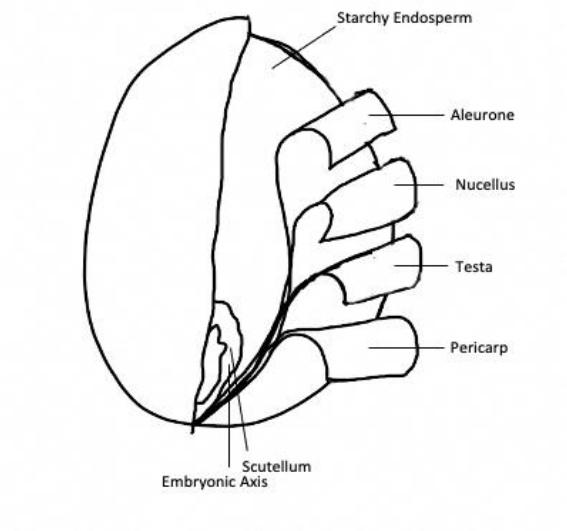


Figure 1.2. Cross section of generalised cereal grain structure reproduced from (Evers, 1999)

The embryo consists of the embryonic axis and the scutellum, during germination the embryo secretes hormones and enzymes whilst absorbing solubilised nutrients. The embryo has a high concentration of lipids and a high moisture content in the mature grain. An aspect of the cereal morphology that is uniform across the cereal grains is the large starchy endosperm, a central mass consisting of cells packed with starch and other nutrients (Evers *et al.*, 2002).

Surrounding the endosperm is the aleurone layer, consisting of layers of thick-walled cells and a nucleus. The aleurone plays a critical role in regulating the expression of endosperm-degrading enzymes during germination (Arendt *et al.*, 2013). The number of layers of aleurone cells walls present is dependent on the cereal species, barley has three layers whilst wheat, maize and rice have one layer (Evers, 1999). The pericarp is a multilayer structure which in some cereals is fused to the testa (seed coat) to form a layer that encompasses and protects the embryo, aleurone layer and endosperm. Finally, barley and rice are two of the four commercial species of grass that retains a husk after harvest. The husk plays an important role in protecting the germ from physical abrasion during handling and can be beneficial in the brewing process as a filtration aid (Fox, 2010).

Cereal grains consist of a high proportion of starch, moderate levels of proteins and a smaller proportion of lipids and minerals. Table 1.1 shows the gross chemical composition of four major cereal grains. At molecular levels, the cereal species are diverse which can influence the quality impacts of cereal adjuncts through malting and brewing which will be discussed throughout each section of this review.

Table 1.1. Average composition of cereal grains (dry basis)

	Starch (%)	Protein (%)	Fibre (%)	Lipids (%)	Minerals (%)
Barley	67.0	12.7	5.4	1.9	1.5-3.0
Wheat	69.8	13.2	2.6	1.9	<1.0
Maize	72.1	9.5	2.0	4.5	1.0-1.3
Rice	73.0	9.2	2.7	1.4	1.0-1.5

Data taken from (Arendt *et al.*, 2008; Agu *et al.*, 1998)

1.3. Starch, amylolytic enzymes and carbohydrates

1.3.1. Cereal starch

Starch is the most abundant compound in all cereal grains. Starch constitutes approximately 65-70% of the dry matter of the barley and wheat grain (Gupta *et al.*, 2010; Faltermaier *et al.*, 2014), with higher starch content in maize and rice grains (70-80%) (Yu *et al.*, 2018). In order for diastatic enzymes (α-amylase, β-amylase, limit dextrinase and α-glucosidase) to be able to efficiently solubilise starch during the mash, the starch structures need first to be gelatinised. Table 1.2 shows typical starch gelatinisation temperatures for cereal grains. The gelatinisation temperatures of barley and wheat fall in the optimum temperature range for amylase activity during mashing (62-75 °C) enabling simultaneous starch gelatinisation and saccharification of the grain to ensure conversion of starch to fermentable sugars (Parés Viader *et al.*, 2021). However, rice and maize have gelatinisation temperature higher than the optimum amylase activity (Table 1.2).

Table 1.2. The starch gelatinisation temperature for cereal grains

	Starch gelatinisation temperature (°C)
Barley[a]	55.8-66.7
Wheat[b]	58-64
Rice[c]	70.0
Maize[c]	72.7

Data taken from Langenaeken *et al.* (2019)[a], Faltermaier *et al.* (2014)[b] and Zobel (1988)[c]

Gelatinisation temperature is affected by several factors including the amylose content, amylopectin fraction and chain length, and the placement and content of starch granules in the grain (Taylor *et al.*, 2013). Chung *et al.* (2008) found that the approximate weight-average chain length of amylopectin in maize and rice are 30.4 and 30.2 compared with 26.8 and 27.9 for wheat and barley respectively. In this case, higher starch gelatinisation temperature in maize and rice relate to longer side chains of their amylopectin compared to wheat and

barley. The higher gelatinisation temperatures mean that pre-processing is required before maize and rice can be introduced into the mash. Pre-gelatinised maize and rice flakes are produced through steaming, rolling and drying and can be added directly to the mash. Cereal cookers are also used in the brewhouse to gelatinise and liquefy maize and rice. Small proportions of malt (10-15%) are typically added to the cereal cooker to increase liquefication before being added to the main mash (Sammartino, 2015; Hertrich, 2013).

1.3.2. Production of amylolytic enzymes during malting

Four main endogenous barley enzymes are involved in the conversion of starch into fermentable sugars during germination and mashing: α-amylase, β-amylase, limit dextrinase and α-glucosidase. α-amylase hydrolyses the interior (1,4)-α-D-glucosidic linkages of amylose and amylopectin chains. Limit dextrinase exclusively hydrolyses (1,6)-α-D-glucosidic linkages in amylopectin. These enzymes are synthesised *de novo* during the germination process of malting in the aleurone layer. Bera *et al.* (2018) found the malting process increased the α-amylase activity from 0.04-0.19 U/g to 150-224 U/g in the barley grain. Similar to unmalted barley, wheat, maize (2.9 U/g) and rice (11 U/g) contained minimal levels of α-amylase activity in the unmalted grain (Biazus *et al.*, 2009; Usansa *et al.*, 2011). Limit dextrinase activity is negligible in unmalted barley and does increase during malting but is not produced in the quantities associated with the other amylolytic enzymes (Ross *et al.*, 2003). Interestingly, unmalted rice has been found to exhibit levels of limit dextrinase activity higher than that of barley malt (Taylor *et al.*, 2013).

β-amylase is one of the most important enzymes as it releases maltose, the most prevalent fermentable sugar in brewing wort, by hydrolysing the penultimate (1,4)-α-glucan linkage at the non-reducing chain-end of amylose or amylopectin (Fox, 2018). Unmalted barley and wheat contain 'classical' endosperm β-amylase present in high amounts in the grain. Whereas maize and rice contain a different, tissue-'ubiquitous' form of the enzyme which is present in much lower amounts and activities (Ziegler, 1999). Unlike the other amylolytic enzymes, β-amylase is not synthesised during malting. Bera *et al.* (2018) found that the levels of β-amylase slightly reduced after malting from 14.0-18.3 to 12.6-16.0 U/g. This could be explained by the heat labile nature of β-amylase, a portion of which is destroyed during the kilning stage. Ultimately,

the removal of the malting process when utilising unmalted cereals will lead to a deficiency in essential amylolytic enzymes in the brewing process.

1.3.3. Action of amylolytic enzymes during mashing

The enzymatic activity in the mash is crucial for optimising starch hydrolysis. The saccharification step is typically held at a temperature where β-amylase and α-amylase are both active and barley starch gelatinisation can be completed (62-70 °C) (Parés Viader *et al.*, 2021). Stenholm *et al.* (1999) highlighted the importance of limit dextrinase as their study found a significant positive correlation between limit dextrinase activity in the mash and the fermentability of the corresponding wort.

When wort is produced with high rates of unmalted adjunct incorporation, there is a deficiency of amylolytic enzymes and large proportions of under modified grain which hinders enzyme action and leads to low extract yield. Lowe *et al.* (2004) found the incorporation of 50% unmalted barley caused a decrease in wort extract from 11.2 % to 9.6%. Similarly, Goode *et al.* (2005) found that a decrease in the proportion of malted barley used resulted in a linear decrease in extract recovery. This study found that worts produced with 100% unmalted barley mash had considerably lower α–amylase activity (5.44 Ceralpha units) compared to all malt mash (14,695 Ceralpha units). However, Goode *et al.* (2005) proved that this issue could be overcome with the use of exogenous enzymes. The addition of exogenous thermostable α–amylase was found to provide the best starch conversion and maximum extract recovery.

1.3.4. Impact of wort fermentable sugars on beer quality and flavour

The wort fermentable sugar (sucrose, fructose, glucose, maltose and maltotriose) composition depends on the grist materials utilised and the amylolytic enzyme activity in the mash. Several studies have found that there is a positive correlation between wort glucose content and the concentration of malt in the wort when brewing with differing proportions of unmalted adjuncts (Goode *et al.*, 2005; van Donkelaar *et al.*, 2016; Zhuang *et al.*, 2017; Yu *et al.*, 2018). Goode *et al.* (2005) found that a higher proportion of the total fermentable sugar was maltose in wort produced with 100% unmalted barley (83.9%) compared to wort produced with 100% malt (69.9%) which can be attributed to

increasing importance of the endogenous β–amylase activity in unmalted barley. High proportions of malt included in the mash increases the source of α-amylase which has a greater effect than β-amylase, consequently lowering maltose and increasing glucose and maltotriose concentrations in the wort.

For brewing yeast strains there is a generalised sugar uptake pattern through fermentation initiated with the assimilation of sucrose, then the simplest sugars (glucose and fructose), followed in increasing order of complexity (maltose and maltotriose). Different wort carbohydrate ratios have been shown to affect fermentation performance negatively. This has been observed in worts where glucose is the predominant carbohydrate which is usually associated with the incorporation of liquid adjuncts (Lodolo *et al.*, 2008).

The carbohydrate composition of wort and the manner in which it is utilised has a direct influence on yeast metabolism and the sensory profile of the final beer. Wort carbohydrates influence the production of higher alcohols and esters, which are well known flavour-active compounds produced by yeast which shape the flavour profile of beer. Higher alcohols in excess can have a negative impact on aroma and flavour as high concentrations can lead to strong, pungent alcoholic aromas in the beer. In moderate quantities, esters can add pleasant fruity or floral aromas to beer (Olaniran *et al.*, 2017). The production mechanism of higher alcohols and esters during yeast metabolism is discussed further in Section 1.5.4.

Increased wort sucrose levels have been reported to elevate production of esters and higher alcohols, while increased glucose or fructose has no significant effect on the esters and higher alcohols (Younis *et al.*, 1998; He *et al.*, 2014). Interestingly, increased maltose in wort results in lower levels of esters and higher alcohols. It is not completely understood why the metabolism of wort containing higher maltose concentrations produces different levels of volatiles. It has been previously suggested that maltose inhibits the transport of volatiles out of the yeast cell by altering plasma membrane structure (Younis *et al.*, 1998). An alternative theory is that the metabolism of maltose produces lower levels of acetyl-CoA, the main substrate for acetate ester synthesis (Stewart, 2006; Shindo *et al.*, 1992). Verstrepen *et al.* (2004) determined that higher wort glucose concentrations induced activation of the Ras/cAMP/PKA pathway which results in a stronger expression of the ester synthase gene *ATF1* increasing production of esters. Ultimately, this illustrates that the use of

unmalted adjuncts can influence the fermentable carbohydrate composition of wort and subsequently impact the aroma profile the beer.

Fermentable and non-fermentable carbohydrates remaining in the beer have a direct impact on beer flavour as the carbohydrates influence the sweetness of the beer. However, larger unfermentable carbohydrates such as dextrins can influence the mouthfeel of beer. Krebs *et al.* (2019) found that spiking dextrin into non-alcoholic beer did not significantly impact sweetness but did significantly increase palate fullness and mouthfeel attributes compared to the control. Li *et al.* (2020a) found the dextrin concentration of several commercial beers produced with adjuncts (rice and maize) were comparable to those of beers produced with barley malt which would suggest that mouthfeel differences observed when brewing with adjuncts may not be caused by dextrins.

1.4. Non-starch polysaccharides and cytolytic enzymes

1.4.1. Cereal non-starch polysaccharides

Cell walls of different cereals have some common components, but composition is not consistent across different species. The walls of the cereal aleurone and starchy endosperm consist predominantly of high molecular weight non-starch polysaccharides (NSP) such as arabinoxylans, (1,3)(1,4)-β-glucans with smaller amounts of cellulose and heteromannans (Evers, 1999). In barley grains, the cell walls consist of approximately 70% β-glucan, 25% arabinoxylan, 2% cellulose and 2% heteromannans (Fangel *et al.*, 2018). The endosperm cell walls of wheat are comprised mainly of arabinoxylans (66%) with a much lower β-glucan content compared to barley (Faltermaier *et al.*, 2014). Glucuronoarabinoxylans (GAX) are the predominant component of endosperm cell wall in maize. In maize, the ratio of heteroxylans to β-glucan is at least 8:1 (Huisman *et al.*, 2000). Rice is unique compared to other cereal grains in containing significant proportions of pectin and xyloglucan. Arabinoxylans and β-glucans still account for the major proportion (47-49%) of the endosperm cell wall of rice, there are substantial proportions of cellulose (23-28%) and pectic substances containing polygalacturonides (27%). In brewing, insufficient

breakdown of arabinoxylans and β-glucan in malting and mashing can provoke filtration problems, high wort viscosity and haze formation during beer storage.

1.4.2. Impact of NSP and cytolytic enzymes in malting and brewing

During malting, cytolytic enzymes (β-glucanases and xylanases) are important to ensure the degradation of the non-starch polysaccharide components of the endosperm cell walls in order to obtain sufficient modification of the grain for satisfactory brewing performance. Better malting performance is associated with lower levels of β-glucan in grains and higher levels of β-glucanase in malt. Wang *et al.* (2004) found that malting degraded approximately 80% of β-glucan present in the barley grain. The study also found that malting increased β-glucanase activity 8-fold. In the malting process, the strongest impact on β-glucan content is caused by germination temperature and time. The longer the germination period the more β-glucan is degraded (Steiner *et al.*, 2011a). There is negligible endo-β-glucanase and pentosanase activity in maize and rice and this does not increase with malting (Dziedzoave *et al.*, 2010). By using unmalted adjuncts there will be reduced cytolytic enzymes available for the mash and an increase in undesirable non-starch polysaccharides (Steiner *et al.*, 2012). The incomplete degradation of non-starch polysaccharides can also minimise starch granules exposure and reduce the degradation of the starch by the amylolytic enzymes in the mash (Hu *et al.*, 2014).

During mashing, an initial rest at lower temperatures (45-52 °C) can be used to support optimal activity of heat sensitive cytolytic and proteolytic enzymes to promote the degradation of NSPs and proteins. Li *et al.* (2005) found that a 30 minute rest at 49 °C when using 20% unmalted barley in the mash, decreased arabinoxylans levels by 39%, resulting in arabinoxylan concentrations comparable to those of all malt wort. This can be explained by endoxylanases supplied from the proportion of malt being able to hydrolyse arabinoxylans in the unmalted barley during the extended rest. However, Li *et al.* (2005) found that the hydrolysis of arabinoxylans in wort produced with 20% unmalted wheat included in the mash was not as intense as with 20% of unmalted barley due to the present of endoxylanase inhibitors in wheat.

Several studies have found that increasing the unmalted barley proportion in the grist led to a continuous increase in β-glucan content (Kunz *et al.*, 2012;

Goode *et al.*, 2005; Lowe *et al.*, 2004). β-glucans dissolved in the mash and wort can form viscous solutions and gels which can increase wort viscosity and inhibit wort separation in the lauter tun. Problems caused by NSPs in the brewhouse can be remedied with mashing regime alterations or by use of exogenous enzymes. Goode *et al.* (2005) found that including a commercial enzyme mixture (α-amylase, β-glucanase and protease) at addition rates between 0.025-0.2% gave an exponential increase in wort filtration rate and a resultant decrease in wort viscosity. Langenaeken *et al.* (2020) found that 31% of β-glucan present at the end of mashing was removed with the spent grain when the wort was filtered using a lauter tun.

The beer NSP content can have a major impact of beer filtration systems when filtering beer with filtration membranes or diatomaceous earth. Both the overall β-glucan content and, in particular, the amount of higher molecular weight fractions is important for filtration behaviour (Sadosky *et al.*, 2002; Kupetz *et al.*, 2015). When focusing on β-glucan content, corn, wheat and rice, have much lower concentrations compared to barley malt. Therefore, wort and beer produced with these adjuncts should not have significantly increased wort viscosity compared to wort produced with all malt (Bogdan *et al.*, 2017).

1.4.3. Non-starch polysaccharides impact on beer quality and flavour

The β-glucan content of beer can influence the sensorial perception, it is understood that β-glucans contribute to body and palate fullness (Langstaff *et al.*, 1993). In a study by Langstaff *et al.* (1991), the aftertaste sensory terms of ‘oily’, ‘mouthcoating’ and ‘stickiness’ were found to be positively correlated to β-glucan concentrations in commercial beers. In agreement with this study, Krebs *et al.* (2019) found that non-alcoholic beers spiked with β-glucan were described by ‘slimy’ and ‘mouthcoating’ terms.

β-glucan has previously been suggested to enhance beer foam stability (Lusk *et al.*, 2001). However, several studies found that there is not clear evidence for a positive relationship between β-glucan content in the beer and foam stability and any enhancement does not outweigh filterability issues (Evans *et al.*, 2002; Kordialik-Bogacka *et al.*, 2011; Lusk *et al.*, 2001). Thus, improvement of foam stability is typically more focused on the protein content of beer as beer foam is

stabilised by the interaction of beer proteins with isomerised hop α-acids and divalent metal ions (Steiner *et al.*, 2011b) .

1.5. Nitrogenous constituents

1.5.1. Protein and nitrogenous content in cereal grains

There is a natural diversity in cereal grain proteins, but all are composed of the same simple units, amino acids. Diversity in the proteins is caused by the 22 amino acids being arranged in different sequences and different sequence lengths. The storage proteins, prolamin and glutelin, account for approximately 50% of the total protein in cereal grains. With the exception of rice and oats, prolamins are the major endosperm storage protein of all cereal grains (Hill *et al.*, 2019). Prolamins are rich in the amino acids proline and glutamine (30-70% of the total) and are typically deficient in lysine, threonine and tryptophan (Hill *et al.*, 2019). The most abundant prolamins are assigned different names according to the cereal such as hordein (barley), gliadin (wheat) and zein (maize) (González-Pérez *et al.*, 2009). In contrast, glutelin-type storage proteins and globulin-type amino acid sequences are the major proteins in rice (Ombretta *et al.*, 2017; Taylor *et al.*, 2013).

The whole grain protein content is typically lower in maize and rice (9.5% and 9.2%) than for barley and wheat (12.7% and 13.2%). Although wheat has a similar protein content to barley, wheat does contain more higher-molecular-weight proteins (Faltermaier *et al.*, 2014; Mayer *et al.*, 2016). Higher-molecular-weight proteins such as wheat glycoproteins are important for foam quantity and stability and contributing to haze (Krstanović *et al.*, 2020). Processed cereal grains such as flaked grains have lower protein content than the unprocessed whole grain (Rocha dos Santos Mathias *et al.*, 2019).

Free amino nitrogen (FAN) is a measure of the nitrogen compounds that may be assimilated or metabolised by yeast during fermentation. These include α-amino acids, ammonia and small peptides (di and tri-peptides). Concerning FAN in the grain, whole grain maize (21.8 mg/100 g) and rice (35-53.9 mg/100g) have a lower FAN content than that of whole raw grain barley (73 mg/100g) (Sherman Kamara *et al.*, 2010; Kruger *et al.*, 2012; Goode *et al.*, 2005). Only 14-20% of wort FAN is in the ungerminated barley and 64-78% is produced as a result of proteolytic enzyme activity in germination during the malting process.

(Lekkas *et al.*, 2014; Jones *et al.*, 2005). Ejeta *et al.* (1987) found that the amino acid profile of whole cereal grains of maize, barley, wheat and rice were similar with rice containing slightly higher concentrations of aspartic acid, valine and arginine.

1.5.2. Proteolytic enzymes and proteins during malting

All unmalted cereals naturally contain low levels of the necessary proteolytic enzymes (endoproteases, carboxypeptidases and aminopeptidases) as they are produced *de novo* during germination. The endoproteases are diverse in the germinating barley grain, barley malt contains 42 different endoproteases: 27 cysteine, 8 serine, 4 aspartic and 3 metallo- proteases (Zhang *et al.*, 1995). An accumulation of endoproteases in the grain is vital to ensure sufficient protein hydrolysis in malting and mashing to produce sufficient FAN content in the wort. Malting is essential for the generation of yeast assimilable nitrogen, as up to 88% of assimilable nitrogen available in the wort is generated as a result of barley germination. Subsequently, the inclusion of any unmalted adjuncts can adversely influence the wort nitrogen quality (Lekkas *et al.*, 2014).

The soluble nitrogen, especially α–amino acids, formed during germination are also important in the production of colour and flavour-active compounds during kilning of the grain. The characteristic aromas and colours of dark malts are a result of the Maillard reaction that is initiated due to kilning temperatures and conditions. The Maillard reaction is a complex series of reactions starting from the reaction between amino compounds and reducing sugars. Many lower-molecular-weight Maillard reaction products have important flavour and aroma characteristics while high-molecular-weight melanoidins provide colour. For example, the Maillard reaction produces pyrazines, which provide aromas described as ‘roasted’, ‘toasted’, and ‘baked cereal’ to malt (van Boekel, 2006). Likewise it generates a range of furans present in malt which are associated with aroma descriptors ‘burnt’, ‘pungent’ and ‘caramel-like’ (van Boekel, 2006). The incorporation of unmalted adjuncts into the grist bill will dilute the concentration of Maillard reaction products that are created in the malt which can affect beer flavour, aroma and colour. Poreda *et al.* (2014) found that incorporation of unmalted maize at rates as low as 10-20% caused a significant decrease in the beer colour which was associated with a decreased amount of Maillard reaction products.

1.5.3. Action of proteolytic enzymes during mashing

During the mashing schedule, a protein stand at the optimum temperature for protease activity (44-55 °C) can be used to enhance protease activity and produce high wort FAN concentration. Rocha dos Santos Mathias *et al.* (2019) reported that adding two temperature stands, at 45 °C for 15 min and then 55 °C for 15 min, when brewing with 45% unmalted maize increased total nitrogen in the sweet wort by 27.5%. This result agrees with the findings of Kühbeck *et al.* (2005) who reported that increasing the protein stand when brewing with poorly modified barley increased the FAN concentration by up to 17%. However, increasing the length of mash schedules can be economically and environmentally costly, when brewing with high adjunct incorporation rates. An extended protein stand would not necessarily provide a sufficient increase in FAN concentration to outweigh the cost. Leiper *et al.* (2003) also determined that the inclusion of a protein rest can increase the haze-active proteins in beer, as the extra proteolysis may reduce the total soluble protein but can cause release of more haze active polypeptides.

The addition of exogenous proteolytic enzymes can facilitate better breakdown of the cereal endosperm storage proteins into soluble nitrogen when brewing with unmalted adjuncts. Lei *et al.* (2013b) found that three commercial exogenous proteases added to the mash greatly affected the fermentation performance of lager yeast. The exogenous proteases improved wort FAN concentrations and extract yield, thus improving fermentability and increasing flavour volatile formation.

1.5.4. Wort nitrogenous compounds: impact on beer flavour

The nitrogenous components of wort are diverse and include amino acids, peptides, polypeptides, protein, nucleic acids, and their degradation products. Wort nitrogen is typically composed of around 30% α-amino nitrogen, 20% high molecular proteins and 40% polypeptides (Ferreira *et al.*, 2018). The composition of the nitrogenous fraction affects the growth and metabolism of yeast cells, fermentation rate and the flavour formation in beer. Yeast cells are able to preferentially utilise different nitrogen sources or amino acids. During

the process, preferred amino acids repress the utilisation of non-preferred nitrogen sources. This regulatory behaviour is termed 'nitrogen catabolite repression' (NCR). Amino acids are utilised by yeast in a sequence as shown in Table 1.3. These groupings are typical, but some differences have been noted in the absorption pattern according to yeast strain (Stewart *et al.*, 2013; Gibson *et al.*, 2007).

Table 1.3. Classification of amino acids based on absorption rates from wort (Taken from Lodolo *et al.* (2008))

	Group A (Fast Absorption)	Group B (Intermediate Absorption)	Group C (Slow Absorption)	Group D (Little or No Absorption)
Amino Acids	Arginine Asparagine Aspartate Glutamate Glutamine Lysine Serine Threonine	Histidine Isoleucine Leucine Methionine Valine	Alanine Glycine Phenylalanine Tyrosine Tryptophan Ammonia	Proline

The inclusion of unmalted cereal adjuncts decreases FAN content which also impacts the amino acid content and composition of the wort when brewing with unmalted adjuncts. Schnitzenbaumer *et al.* (2014) found that brewing with 40% unmalted oats decreased all amino acid concentrations apart from asparagine in comparison to an all malt control. Similarly, Goode *et al.* (2005) found that with increasing proportions of unmalted barley the wort levels of all the individual amino acids decreased with the exception of cysteine. Another approach to overcoming amino acid deficiencies when incorporating unmalted adjuncts is to supplement the wort with specific key amino acids which can improve fermentation and flavour profile. Lei *et al.* (2013a) found that supplementing high-gravity wort with lysine and histidine improved the fermentation performance of lager yeast by improving wort fermentability and ethanol production.

A majority of the flavour volatiles present in beer are produced during fermentation and consist of metabolic intermediates or by-products of fermentation (Pires *et al.*, 2014). Yeast cells assimilate fermentable sugars (as

mentioned in Section 1.3.4), nitrogen compounds, minerals and other nutrients in the wort to produce compounds such as ethanol, higher alcohols, aldehydes, esters, vicinal diketones and sulphur compounds. An overview of yeast metabolism, with a focus on the production of key flavour metabolites, which are discussed below, is shown in Figure 1.3.

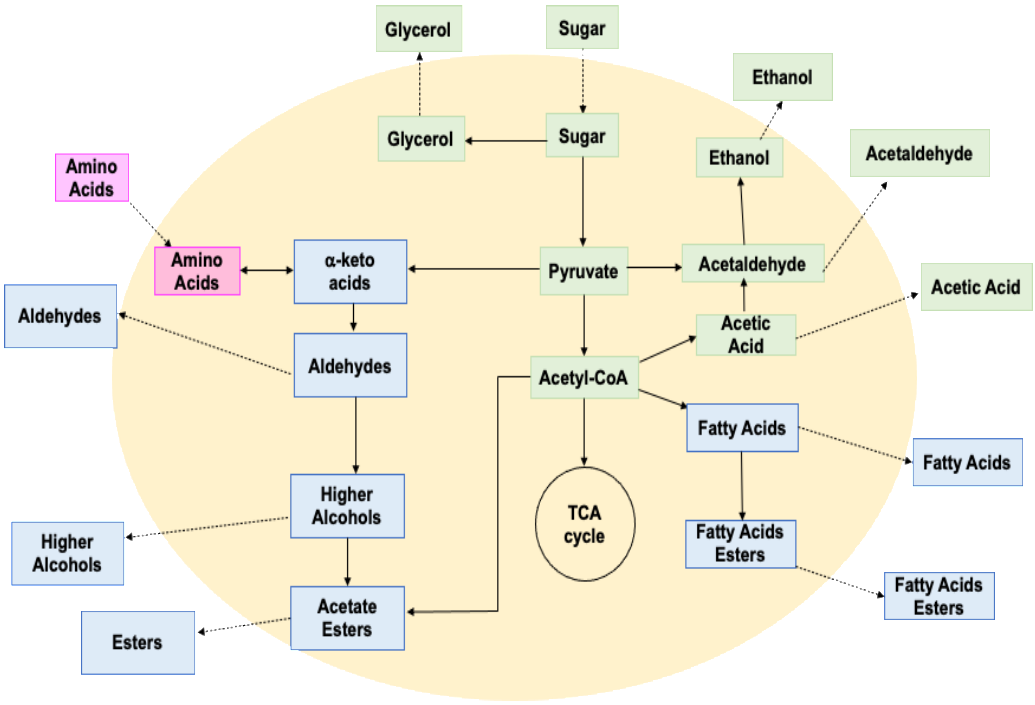


Figure 1.3. A schematic overview of the main metabolic routes in yeast metabolism
(Reproduced from Dzialo *et al.* (2017)

1.5.4.1. Higher alcohols

The major higher alcohols found in beer (n-propanol, isobutanol, isoamyl alcohol and phenethyl alcohol) can have either a positive or negative impact on the sensorial perception of beer (Olaniran *et al.*, 2017). Elevated concentrations of these compounds in beer may result in strong, pungent flavours that are out of balance with a particular beer style. Isoamyl alcohol can influence drinkability because the beer flavour becomes heavier if isoamyl alcohol concentration increases (Olaniran *et al.*, 2017).

Higher alcohols are the most abundant sensory compounds in beer. Higher alcohols can be produced by two pathways: the Ehrlich pathway from amino acid catabolism and the anabolic pathway involving the *de novo* synthesis of amino acids from glucose. In the catabolic Ehrlich pathway, amino acids are transformed into α-keto acids by branched-chain amino acids (BCAAs) transamination. In the second step, the keto acids are decarboxylated to form the respective aldehydes. After decarboxylation, the aldehydes are converted into their respective higher alcohols by the action of alcohol dehydrogenase (Pires *et al.*, 2014; Zhong *et al.*, 2020). In the anabolic pathway, α-keto acids are formed via the *de novo* biosynthesis of amino acids through carbohydrate metabolism.

Zhuang *et al.* (2017) found that beer brewed with 100% unmalted wheat and 100% unmalted barley had decreased propanol, isobutanol and isoamyl alcohol concentrations in comparison to an all malt beer. Whereas higher alcohols have been reported to be present in greater amounts in beers produced with maize and rice (Ombretta *et al.*, 2017; Szlavko, 1973). Szlavko (1973) found that the incorporation of 45% unmalted maize increased the higher alcohols (tryptophol, tyrosol and phenyl ethyl alcohol) from 23.99 mg/L to 40.45 mg/L. Changes in higher alcohol concentrations when using unmalted adjuncts are closely linked to changes in the wort nitrogen composition. Zhuang *et al.* (2017) found that brewing with 100% unmalted wheat and barley lowered wort FAN concentration by 42% and 60% respectively compared to the all malt control. A reduction in assimilable nitrogen will reduce the amount of amino acids that can be transformed into higher alcohols, reducing higher alcohol concentration. However, when nitrogen sources are too low, for example when using high amounts of maize or rice adjuncts, glucose metabolism is activated and forms large amounts of pyruvate and consequently higher alcohols (Zhong *et al.*, 2020).

1.5.4.2. Acetate and ethyl esters

Esters are amongst the most volatile flavour compounds in beer and hence affect beer aroma greatly. In moderate quantities esters can add pleasant fruity characters to the beer aroma (Verstrepen *et al.*, 2003). During yeast metabolism there are two potential routes for ester formation; a reaction between an alcohol or higher alcohol with a fatty acyl-CoA ester catalysed by alcohol acetyl

transferases (AATases) can be responsible for the formation of acetate esters (He *et al.*, 2014).

Volatile esters in beer can be divided into two major groups: the acetate esters and the medium-chain fatty acid ethyl esters. The acetate esters are formed biochemically with acetyl Co-A and ethanol or a higher alcohol. In the ethyl esters, ethanol is the alcohol and the carboxylic acid will be a medium-chain fatty acid. The key esters identified in beer and their sensory thresholds, flavour characteristics and typical concentrations found in beer are listed in Table 1.4.

Table 1.4. Sensory thresholds and flavour characteristics of key esters in lager beer (Data compiled from (Xu *et al.*, 2017; Pires *et al.*, 2014; Olaniran *et al.*, 2017)).

Esters	Flavour characteristics	Sensory threshold (mg/L)	Typical concentration found in lager beer (mg/L)
Ethyl acetate	Solvent, fruity, sweet	30	8-32
Ethyl butyrate	Papaya, pineapple, cream	0.4	0.1-0.2
Isoamyl acetate	Banana, apple, solvent	1.2	0.3-3.8
Isobutyl acetate	Solvent, fruity	1.0	0.03-1.2
Ethyl hexanoate	Sour apple	0.2	0.05-0.21
Ethyl octanoate	Sour apple	0.9	0.04-0.53
Phenyl ethyl acetate	Roses, honey, apple, sweet	3.8	0.1-0.78

The nitrogenous composition of wort can influence the acetate ester profile. Saerens *et al.* (2008) found that ethyl acetate and isoamyl acetate concentrations increased when more nitrogenous compounds were available from the fermenting medium. Similarly, Lei *et al.* (2013b) found increases in valine, isoleucine and leucine when supplementing wort with proteases induced the formation of isobutanol, amyl alcohol and isoamyl alcohol respectively. These higher alcohols are precursors for isobutyl acetate and isoamyl acetate, therefore an increase in higher alcohols increases the ester profile. In agreement, Zhuang *et al.* (2017) found that the concentration of a range of esters were lower in beers brewed with 100% unmalted cereals compared to malt beers. This was probably due to the role of higher alcohols as precursors for ester synthesis and the adjunct changing the amino acid profile of the wort.

1.5.4.3. Vicinal diketones (VDKs)

Vicinal diketones (VDKs) such as 2,3-butanedione (diacetyl) and 2,3-pentanedione, are bi-products of amino acid synthesis (valine and isoleucine respectively) that impart an undesirable 'buttery' or 'toffee' flavour to beer (Olaniran *et al.*, 2017). Diacetyl and 2,3-pentandione are formed indirectly as a result of valine and isoleucine anabolism, as they arise from the non-enzymatic oxidative decarboxylation of α-acetohydroxy acids that are intermediates in the valine and isoleucine biosynthesis pathway (Krogerus *et al.*, 2013a). Diacetyl production increases with increasing valine biosynthesis, which depends on the yeast's access to valine and other amino acids. Hence, if wort amino nitrogen is insufficient there can be an increase in diacetyl production. In agreement, Krogerus *et al.* (2013b) found that increased valine concentration (100 to 300 mg/L) resulted in decreased diacetyl concentrations at the end of fermentation (up to 33% lower). In the case of adjunct wort, Van *et al.* (2001) found that the quantity of vicinal diketones in beer produced with 40% rice was higher than in the all malt control or with nitrogen supplemented (protease, yeast extract) wort. This confirms that the amino acid composition differences caused by unmalted adjuncts may have a negative effect on the diacetyl concentration in the beer.

1.5.4.4. Sulphur compounds

Many sulphur-containing compounds found in beer derive directly from raw materials, malt and hops, but some are produced through yeast metabolism. However, sulphur compounds are synthesised in a different pathway to those of higher alcohols and vicinal diketones (Ferreira *et al.*, 2018). The concentration of sulphur containing amino acids influences the production of hydrogen sulphide and sulphur dioxide during fermentation. It is thought that these compounds are created by reduction of sulphate to sulphide, which is important for the biosynthesis of sulphur-containing amino acids (methionine and cysteine) (Duan *et al.*, 2004).

In their study, Duan *et al.* (2004) found that addition of cysteine to wort showed a marked increase in the production of hydrogen sulphide and sulphur dioxide compared to additions of methionine, serine and threonine. As mentioned previously, Goode *et al.* (2005) found that increasing the proportion of unmalted

barley in the wort increased cysteine concentrations. Thus, the inclusion of unmalted barley could increase the concentration of hydrogen sulphide and sulphur dioxide in the beer.

One of the most important sulphur flavours in beer is from dimethyl sulphide (DMS), which induces a cooked sweetcorn aroma in beer. There are two main routes for the formation of DMS; firstly the thermal degradation of the precursor S-methylmethionine (SMM) during kilning in malting and during wort boiling. Secondly, the reduction of dimethylsulfoxide (DMSO) during fermentation. Poreda *et al.* (2014) found that the incorporation of 20% unmalted maize in the wort reduced DMS concentration by 32%. This was because malted barley is the main source of DMS precursor so decreasing malt proportion resulted in lower levels of DMS. Additionally, maize is cereal cooked before being added to the mash and therefore higher heat load enables more effective DMS formation and removal.

1.6. Lipids

Beer quality and yeast metabolism of wort can be affected by the concentration of lipids in the cereal grain and wort. A number of long-chain fatty acids are reported as precursors for characteristic beer flavours, however, certain lipids may adversely affect flavour and foam stability (Gordon *et al.*, 2018).

1.6.1. Cereal lipids

Beer lipids originate in the brewing process from malt, adjunct material, hops or yeast. Whole maize grains have a considerably higher lipid content (4.5%), compared with barley (1.9%), wheat (1.9%) and rice (1.4%) (Agu *et al.*, 1998; Arendt *et al.*, 2008). Despite there being a substantially higher amount of lipids in maize grains, lipids are predominantly located in the germ and maize is typically degermed before brewing, reducing lipid content to approximately 1% and thus minimising the potential impacts on flavour and foam stability (Floridi *et al.*, 2009). The content and composition of key fatty acids (palmitic, oleic and linoleic acid) is somewhat similar between barley, maize and rice whole grains (Taylor *et al.*, 2013). Bravi *et al.* (2009) found that the inclusion of maize grits reduced palmitic and linoleic acids and increased oleic acid in the resultant beer, this can be attributed to the concentration of oleic acid being slightly higher in maize grits compared to barley.

1.6.2. Lipids in malting and brewing

The total lipid content during malting showed a significant decrease indicating a rapid degradation of lipids which can be attributed to lipolytic activity. During germination, the total lipid content decreases as the lipolytic potential (lipase, lipoxygenase and hydroperoxyl fatty acid catalysing enzymes) increases (Kaukovirta-Norja *et al.*, 1998; Gordon *et al.*, 2018). Consequently, the use of unmalted adjuncts may increase lipids in the wort and resulting beer.

The brewing process as a whole is extremely efficient at eliminating lipids. Lipids in wort and beer are reduced at every major brewing process step: mash separation (in the spent grain), wort boiling (by oxidation), trub removal, fermentation and filtration. Only a small proportion (<5%) of malt lipids exist in the final wort (Anniss *et al.*, 1985). Kaukovirta-Norja *et al.* (1998) found that between 12-43% of the lipid content of the malt was lost during the mash, depending on mashing conditions. There is strong evidence to suggest that these lipids are degraded, and it is likely that a proportion of the breakdown products are passed into the wort.

1.6.3. Impact of lipids on beer quality and flavour

The presence of lipids in brewing has long been considered to have adverse effects on beer quality. Flavours associated with beer aging have been attributed to oxidative degradation of lipids. One example of this is the presence of trans-2-nonenal, one of the more commonly recognised stale flavour compounds (‘cardboard/ damp parchment/ papery’) derived from lipid oxidation.

The foaming ability of the beer and stability of the foam is known to influence the perception of beer quality. Beer foam is created by bubbles formed at nucleation sites in the beer or on the surface of the glass which are surrounded by proteins with both hydrophilic and hydrophobic properties. Lipids are known for having a damaging effect on beer foam by binding to the foam supporting proteins at their lipophilic sites, making the foam unstable (Dickie *et al.*, 2001). On the other hand, lipids are necessary in fermenting wort for activation of yeast cell growth under anaerobic conditions and significantly affect the fermentation process (Bravi *et al.*, 2014).

The lipid content of wort can affect flavour by influencing the production of flavour-active esters. Saerens *et al.* (2008) found that a higher level of unsaturated fatty acids, oleic, linoleic and linolenic acids, in the fermenting wort resulted in a decrease in ethyl and acetate ester production with the highest impact on ethyl decanoate. This result is attributed to higher unsaturated fatty acids reducing alcohol acetyltransferase (AATase) activity, lowering ester production by repressing the *ATF1* gene, which is responsible for a major proportion of acetate ester synthesis (Fujii *et al.*, 1997; Saerens *et al.*, 2008).

The inclusion of unmalted barley, wheat and unprocessed maize and rice in the grist bill can increase lipid and fatty acid concentration in the wort and have a negative impact on the ester profile, foam quality and ageing. Evans *et al.* (2014) found that wort lipid content produced with unmalted barley was eight times higher than the all malt wort. However, this substantial increase was strongly influenced by the use of exogenous enzyme mixture Ondea Pro which contained lipase.

1.7. Mineral composition

It is important for the composition of wort to include adequate mineral elements as yeast requires inorganic ions for optimum growth and fermentation enabling accelerated growth and enhanced ethanol production. Inorganic ions in the wort can indirectly affect beer flavour and ions in the resultant beer can influence flavour and stability of the final product.

1.7.1. Cereal mineral composition

The mineral content of wort and beer is mainly influenced by the cereal raw materials, by brewing water source or deliberate addition by the brewer. The mineral contents of cereal grains are affected by genetic and environmental factors (soil quality, growing altitudes and climate). A comparable overall mineral content is found in barley (1.5%), wheat (<1.0%), maize (1.0-1.3%) and rice (1.0-1.5%) (Agu *et al.*, 1998; Arendt *et al.*, 2008). However, the mineral composition does vary between cereal grains. The most abundant macro-elements in cereal grains are magnesium, phosphorus and potassium. The magnesium contents of barley, wheat and maize are not substantially different

from each other. However, whole grain and flaked rice has a considerably lower magnesium content compared to other cereal grains (Omari *et al.*, 2020; Taylor *et al.*, 2013). Iron, manganese, zinc and copper are the main micro-elements in the grain (Arendt *et al.*, 2013). Poreda *et al.* (2015) found that the concentrations of microelements ($Mn^{2+,}$ Fe and Zn^{2+}) were significantly higher in wheat compared to barley. The zinc contents of maize and rice are similar; however, 50-90% of zinc can be lost when cereals are refined, owing to the removal of the mineral-rich pericarp.

1.7.2. Mineral ions during brewing

The wort metal ion concentrations represent only a fraction of the metal ions present in the grist, as most metal ions are retained in the spent grain or are removed in protein precipitation and wort clarification (Gibson, 2011). For example, zinc is extracted from malt in the mashing procedure. Some of the zinc remains soluble in the wort, but much of the zinc is removed during lautering or is precipitated with trub during the boil and wort cooling (Kreder, 1999). One of the main aims of wort boiling is the precipitation of macromolecules which form a trub of protein-polyphenol complexes and lipids. These complexes, owing to various functional groups, express a high affinity towards metal ions (Poreda *et al.*, 2015). The addition of hops during the boil can contribute more metal ions extracted into the boil. Hops, especially aroma hops, have a higher concentration of metal ions compared to cereal material. However, owing to lower dosages of hops used in the wort, it is estimated that only 1-3% of all ions present in the wort originated from the hop material (Poreda *et al.*, 2015; Porter *et al.*, 2016). Poreda *et al.* (2015) determined that the boiling process caused a substantial decrease in Mn and Fe with Mn content being reduced by 40% after boiling. The study also found that wort produced with 60% wheat increased the concentration of Mn ions determined in the trub compared to 100% malt (54.3 mg/kg and 29.6 mg/kg respectively). Increasing the proportion of wheat caused a synergistic effect on the absorption rate of manganese (Poreda *et al.*, 2015).

1.7.3. Wort mineral composition impact on fermentation

Zinc, magnesium, calcium, copper, manganese and potassium are elements involved in the regulation of metabolic activity during fermentation and promote good fermentation performance. Brewers may supplement wort with yeast nutrients including mineral ions to increase yeast nutrients for better fermentation.

Zinc acts as a structural or catalytic co-factor for a number of enzymes associated with glycolysis and alcohol synthesis. The optimum zinc concentration for a standard gravity (12 °P) wort is around 0.2 ppm. Rees *et al.* (1998) found that increasing wort zinc levels to as high as 327.5 ppm led to reduced attenuation time and increase production of ethanol by lager strains. Increasing wort zinc concentration can stimulate the breakdown of α-keto acids in yeast metabolism to increase the concentration of unfavourable higher alcohols in the beer (Quilter *et al.*, 2003). However, De Nicola *et al.* (2011) found that the volatile profile was only significantly changed when wort zinc levels were over 10 ppm. Like zinc, magnesium plays a role in regulating the activity of over 300 enzymes, including glycolytic enzymes and others involved in the synthesis of fatty acids. The metabolism of pyruvate by yeast (via pyruvate dehydrogenase or pyruvate decarboxylase pathways) also requires magnesium, thus magnesium is important in the formation of ethanol (Kordialik-Bogacka *et al.*, 2019). The concentration of magnesium in wort is typically in the range of 50-90 ppm (Gibson, 2011). Rees *et al.* (1999) found that supplementing wort with 500 ppm of magnesium resulted in increased ethanol production and enhanced vitality of ale and lager yeast strains. Conversely, supplementing with calcium resulted in detrimental effects on ethanol production and yeast vitality, these effects are attributed to calcium supressing magnesium-dependent enzymes. Calcium ions have a role in protecting cells against the toxic effects of ethanol, however, any positive effect from calcium is compromised by its antagonistic effect on magnesium uptake (Saltukoglu *et al.*, 1983).

1.7.4. Mineral composition and flavour stability

Iron, copper and manganese, even at low concentrations, can be detrimental to flavour stability. The formation of specific ageing compounds can be attributed to oxidative processes occurring in beer (Aron *et al.*, 2010). These compounds

are mainly formed by oxygen activation and the formation of reactive oxygen radicals (ROS) resulting from the Fenton and Haber-Weiss reactions (Figure 1.4).

Fenton's reaction:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^-$$

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + O_2^{\bullet -} + 2H^+$$

$$\text{Net: } 2H_2O_2 \xrightarrow{Fe} OH^{\bullet} + OH^- + O_2^{\bullet -} + 2H^+$$

Haber-Weiss Reaction:

$$Cu^{2+} + O_2^{\bullet -} \longrightarrow Cu^+ + O_2$$

$$Cu^+ + H_2O_2 \longrightarrow Cu^{2+} + OH^{\bullet} + OH^-$$

$$\text{Net: } H_2O_2 + O_2^- \xrightarrow{Cu} OH^{\bullet} + OH^- + O_2$$

Figure 1.4. Fenton and Haber-Weiss reaction leading to the formation of reactive oxygen species (ROS).

In the Fenton reaction, Fe^{2+} ions are oxidised to Fe^{3+} by hydrogen peroxide forming a hydroxyl radical and a hydroxyl ion. The Fe^{3+} ion reacts with a further molecule of hydrogen peroxide generating two protons and a superoxide radical. These superoxides react with Cu^{2+} ions to form Cu^+ and oxygen in the Haber-Weiss reaction. The Cu^+ ion generated can split a hydrogen peroxide molecule into a hydroxyl ion and a hydroxyl radical. The formed radicals from both Fenton and Haber-Weiss reactions are extremely reactive. The reactive oxygen containing species are crucial to the beer staling process because they can react with different organic compounds in beer including polyphenols, isohumulones, alcohols, fatty acids, iso-α-acids which can change the sensory profile of the beer (Pohl, 2008).

Manganese is also capable of catalysing reactions that produce ROS and may act synergistically along with iron and copper to catalyse oxidative staling reactions (Aron *et al.*, 2010). Zufall *et al.* (2008) found that copper and iron content was significantly reduced during wort boiling, trub removal and fermentation. However, manganese concentration was relatively constant throughout the brewing process, once extracted into wort. Increased manganese content in the beer accelerated flavour deterioration and a sherry aroma formed after four weeks of storage. Therefore, it is important to control metal ion concentrations throughout the brewing process to improve flavour stability in the finished beer. Gallotannins such as tannic acid are known as very strong chelators with 2- and 3- valent metals. Therefore, iron and copper can be removed by gallotannin treatment and filtration lowering the metal ion concentration in the beer (Briggs *et al.*, 2004; Withouck *et al.*, 2010).

1.8. Phenolic compounds

A diverse range of phenolic constituents are found in beer, belonging to classes of simple phenols, phenolic acids (hydroxybenzoic- and hydroxycinnamic- acid derivatives), coumarins, catechins, di-, tri- and oligomeric proanthocyanidins, chalcones and flavonoids. Phenolic compounds play an important role in beer quality as they are potential anti- or prooxidants and can influence beer flavour, colour and foam stability directly or by interacting with other beer constituents (e.g., metal ions and proteins) (Wannenmacher *et al.*, 2018).

1.8.1. Phenolic compounds in cereals

Around 70-80% of beer polyphenols are derived from cereal grains with the other 20% originating from hops. Phenolic compounds in cereals are predominantly found in the outer layer of the grain (husk, pericarp, testa and aleurone layer) while the concentration is noticeably lower in the endosperm. (Wannenmacher *et al.*, 2018).

Phenolic acids are the most abundant and well-characterised phenolic compounds in cereal grains. The phenolic acids occur in free, conjugated or insoluble bound forms, where approximately 85% of grain phenolic compounds are ester- or ether-linked to cell wall polysaccharides (Călinoiu *et al.*, 2018). Horvat *et al.* (2020) determined that maize had the highest total phenolic acid

content compared to barley and wheat (2213, 991 and 604 $\mu g/g_{dm}$ respectively). Similarly, Krstanović *et al.* (2020) found that the extracted total polyphenol contents (TPC) of malted barley and maize grits were higher than that of unmalted wheat (53.64, 41.33, and 18.66 mg/L respectively).The phenolic acid composition of maize, barley and corn were similar, with concentrations from highest to lowest of: ferulic acid, p-coumaric acid, vanillic, salicylic, syringic and p-hydroxybenzoic acid (Ndolo *et al.*, 2014). Although maize has a considerably higher phenolic acid content compared to barley, thermal processing of the grain before it is introduced to the mash reduces the phenolic content. Butts-Wilmsmeyer *et al.* (2018) found that the removal of bran and germ when dry milling maize reduced insoluble-bound ferulic acid by 50%.

In barley, proanthocyanidins are mainly located in the aleurone layer and composed of (+)-catechin and (+)-gallocatechin monomers. The most abundant dimeric proanthocyanidins in barley are procyanidin B3 and prodelphinidin B3 (Wannenmacher *et al.*, 2018). The amount of proanthocyanidins present in different cereals is highest in barley (0.7%) compared to wheat (0.4%), maize (0.4%) and rice (0.1%) (Evers, 1999).

1.8.2. Phenolic compound changes during malting

The malting process significantly influences the composition and content of phenolic compounds in barley and other cereal grains. Several studies have found that malt has a higher total phenolic content than the corresponding unmalted barley (Lu *et al.*, 2007; Qingming *et al.*, 2010; Leitao *et al.*, 2012). Lu *et al.* (2007) found that the steeping of the unmalted barley resulted in a 6% decrease in TPC in the barley grain. This result is in accordance with soluble phenolic compounds being leached into steep water. Lu *et al.* (2007) found that germination and kilning increased the TPC by 25% and 17.5% respectively. The increase can be attributed to a better extraction and higher release of phenolic compounds. Phenolic compounds bound to cellular structures are released due to activity of hydrolytic enzymes during germination. In addition, the friable nature of the malt as compared to barley allows a better extraction of phenolic compounds (Leitao *et al.*, 2012; Lu *et al.*, 2007). Leitao *et al.* (2012) found that the total phenolic content was 4-fold higher in malt compared to the corresponding unmalted barley.

Several studies have found that dimeric and trimeric proanthocyanidins remain almost unchanged in barley and malts, however, a significant decrease in catechin content is observed during the malting process (McMurrough *et al.*, 1983; Goupy *et al.*, 1999; Tomková-Drábková *et al.*, 2016). Goupy *et al.* (1999) found that over 80% of (+)-catechin present in barley was degraded during malting. The decrease in catechin content during malting can be ascribed to its partial conversion to epicatechin and catechin glycosylation resulting in an increase in catechin-7-O-glucoside during malting (Carvalho *et al.*, 2018).

1.8.3. Phenolic compounds in the wort production

Several steps during the brewing process (mashing, wort boiling, whirlpool and fermentation) have significant influences on the levels and variety of phenolic compounds in the wort and resulting beer.

During mashing, phenolic compounds, especially phenolic acids, are released and solubilised from the malt both by water extraction and enzyme activity. Barley enzymes, arabinofuranosidases and β-xylan endohydrolases, degrade and solubilise the arabinoxylan chains and allow phenolic acid extraction into the wort (Lentz, 2018). Pascoe *et al.* (2003) found that phenolic acids and (+)-catechin present in the wort significantly increased throughout the mashing profile. In agreement, Zhao *et al.* (2012) tracked the phenolic content throughout the mashing schedule and found that wort TPC increased by 8.8% as the mashing temperature increased from 45 to 78 °C. Vanbeneden *et al.* (2008) investigated the impacts of 50% flaked cereal adjunct usage (wheat, rice, maize and unmalted barley) on the release of ferulic acid and p-coumaric acid in the mash. The study found that all worts produced with cereal adjuncts had significantly lower free ferulic acid and p-coumaric acid, except the wort produced with rice flakes. During the production of the flakes, all enzymes are denatured. Therefore, the lack of cinnamoyl esterase and endoxylanase activity generally leads to reduced levels of hydroxycinnamic acids in the wort.

Pascoe *et al.* (2003) found that wort separation resulted in an increase in phenolic compounds, it is believed that phenolic compounds would be extracted into the wort from the husk filter bed enhancing TPC which can be due to their extraction from the spent grain. In contrast, Fumi *et al.* (2011) determined that the use of a mash filter and lauter tun both decreased TPC with a greater depletion when using a lauter tun compared to a mash filter (74% and 28%

respectively). However, it is not clear if these figures consider the addition of sparge water which would dilute the TPC concentration.

The wort boiling process can influence the phenolic content and composition due to hop addition. The total content of polyphenols in hops usually ranges between 2 and 6% (dry basis) depending on hop cultivar (Jurić *et al.*, 2015). During wort boiling, polyphenols are extracted from hops with a transfer rate of 50-70% of the TPC (Wannenmacher *et al.*, 2018). Fumi *et al.* (2011) noted an increase of TPC during wort boiling especially with regard to flavonoid content. There are many processes involved in wort boiling that can cause changes in the TPC including polyphenol-polyphenol and protein-polyphenol interactions, polymerised compound precipitation and formation of degradation compounds from protein and polyphenols (Fumi *et al.*, 2011). Proanthocyanidins are more active than monomeric phenolic compounds in forming complexes with proteins in the boil and consequently are significantly reduced in the wort with the settling of hot trub (Wannenmacher *et al.*, 2018). Worts produced with unmalted adjuncts differ in their polyphenol contents and nitrogen compounds and the polymerisation and precipitation process involved in the boil occur with different intensities causing dissimilarities in the final adjunct wort and all-malt wort (Fumi *et al.*, 2011). Thus, by lowering both the polyphenol and nitrogen content the incorporation of unmalted adjuncts can result in improved beer colloidal stability. Depraetere *et al.* (2004) found that the incorporation of 40% unmalted wheat decreased permanent haze formation compared to an all malt control.

During fermentation, the phenolic content reduces as phenolic compounds can bind to yeast mannoproteins and adhere to the yeast cells (León-González *et al.*, 2018). Similarly, beer filtration results in a significant decrease in the phenolic content of beer (Aron *et al.*, 2010). Downstream processes such as cold storage, filtration and stabilisation are used to reduce the polyphenol content of beer as a means to enhance colloidal stability. Techniques involving the use of adsorbents such as polyvinylpolypyrrolidine (PVPP) can be used for removal of haze-active polyphenols after fermentation. McMurrough *et al.* (1996) found that 48% of TPC were removed from beer treated with 100 g/hl PVPP.

1.8.4. Sensory properties of phenolic compounds in beer

Phenolic compounds are known for imparting astringency and bitterness to a range of beverages such as red wine, tea and beer (Soares *et al.*, 2017; Lesschaeve *et al.*, 2005). The oral sensation of astringency results from the precipitation of proline-rich proteins in saliva when forming insoluble complexes with polyphenols (Oladokun *et al.*, 2016b). In beer, phenolic acids, flavanols, flavonoids and stilbenes can be responsible for astringency (Aron *et al.*, 2010). Hydroxybenzoic and hydroxycinnamic acids induce sensory characteristics of bitterness and astringency, however, phenolic acids have high flavour thresholds in beer (20-50 mg/L) which is higher than the typical concentrations observed in beer (0.1-10 mg/L) (Callemien *et al.*, 2009; Habschied *et al.*, 2021). Several studies have found a significant effect of polyphenol content on the perceived character of bitterness. (Oladokun *et al.*, 2016b; McLaughlin *et al.*, 2008; Mikyška *et al.*, 2002). McLaughlin *et al.* (2008) investigated the quality of bitterness caused by polyphenolic compounds by adding a polyphenolic extract to beer consisting of a blend of (+)-catechin, (-)-epicatechin, (-)-epigallocatechin and (-)-epicatechin gallate at 0, 100 and 200 mg/L. A bitterness described as ‘harsh’, ‘medicinal’ and ‘metallic’ was detected in the samples with 200 mg/L that was not observed with 0 mg/L.

The substitution of malt with unmalted adjuncts typically leads to a decrease in polyphenol content in the finished beer. Depraetere *et al.* (2004) found that the use of 40% unmalted wheat in the grist decreased TPC by approximately 42%. Similarly, Kunz *et al.* (2012) found the use of 90% unmalted barley in the grist decreased TPC by 32%. Consequently, it is expected that the changes in phenolic content of beer brought about by using unmalted cereal adjuncts will influence the perception of beer flavour.

1.9. Sensory analysis of beer

In order to better understand the sensory properties and profiles of beer, several sensory techniques can be employed to characterise perception and the human response to the beer.

1.9.1. Descriptive analysis

In sensory evaluation, descriptive techniques are often used in the development of products as the technique can provide a complete sensory profile of the product, distinguishing the product's characteristics. Descriptive sensory tests involve the detection, discrimination and description of both the qualitative and quantitative sensory components of a product by a trained panel. The trained panel determine the qualitative aspect of the product including aroma, appearance, flavour, texture and aftertaste attributes to determine a qualitative profile of the products (Civille *et al.*, 2015). The determined sensory attributes are then quantified, typically using rating scales, in order to determine the magnitude of the differences of the perceived sensory attributes (Murray *et al.*, 2001). There are several different methods of descriptive analysis including Flavour Profile method, Quantitative Descriptive Analysis (QDA®), Spectrum™ and generic descriptive analysis.

Many studies have used quantitative descriptive analysis to evaluate the sensory profiles of beers with altered brewing parameters such as alcohol content (Juliana *et al.*, 2020), hop varieties used in dry hopping (Donaldson *et al.*, 2012) and barley malt sources (Bettenhausen *et al.*, 2018). However, there has to date been a lack of comprehensive studies applying robust descriptive analysis to determine the impacts of using unmalted cereal adjuncts in beer. Van *et al.* (2001) found that beer produced with 40% rice was described as being watery with a characterless mouthfeel and higher diacetyl flavour. The sensory evaluation in this study was performed according to method NF ISO 8587 (NFS ISO 8587, 1995). Sensory analysis was performed with 11 subjects using a ranking method of preference terms. The terms used to describe the beers were described by the panel using the international beer flavour terminology (Meilgaard *et al.*, 1979). However, the sensory evaluation was limited as the panel provided preference ranking instead of attribute intensity ratings which are normally determined in descriptive analysis methods. Steiner *et al.* (2012) found that beers produced with 100% unmalted barley had increased diacetyl flavour, less mouthfeel and better foam stability. Kunz *et al.* (2012) analysed the sensory profiles of varying (0, 25, 50, 75, 90%) amounts of unmalted barley included in beer and found that beer produced with a barley proportion of 90% showed a detectable change in bitterness, to a more astringent and abrasive bitterness. Both studies used the testing method of the German Deutsche Landwirtschafts Gesellschaft (DLG) with a panel of 10 expert

assessors (MEBAK, 1993). The DLG test method is a descriptive test with integrated assessment using a 5 point scale. A panel of 10 trained assessors were used for the sensory evaluation, however, it is not specified in these studies the extent of training the participants were provided before evaluation. The beers were evaluated on a 5 point scale from 0 (worst) to 5 (best), using broad descriptors ‘aroma’, ‘taste’, ‘mouthfeel’ and ‘bitterness’. The sensory analysis was performed in single evaluation and was averaged across the panel and detailed statistical analysis was not applied to the results. Therefore, the sensory methods used in these two studies did not provide comprehensive and reliable sensory evaluation on the impacts of adjunct usage.

In order to produce comprehensive sensory data, sensory professionals need to consider many factors when designing a sensory study which improve robustness of the data. For example, in sensory evaluation, assessors can be prone to psychological and physiological errors that lead to variation in the data. Psychological factors include expectation error, which can occur from the assessor knowing to much product information or bias from coding on samples (Stone *et al.*, 2004). The experimental design is important as the score assigned to a sample can be influenced by the sensory properties of the preceding product. This effect can be overcome by randomising or balancing the order of presentation of the samples. When using scales, central tendency error can occur as assessors tend to confine their ratings to the centre of the scale. It is important to use the right size of scale for discrimination and to train the assessors on the scale with a wide range of products. It is also important to consider physiological factors such as adaptation to the samples. If assessors are exposed to a stimulus extensively this results in a decrease in sensitivity to the stimulus. It is important to give assessors breaks and provide appropriate palate cleansers to avoid carry over effects from the samples (Kemp *et al.*, 2013). Appropriate statistical analysis techniques should be applied to the data to draw reliable and correct conclusions from the data obtained (Næs *et al.*, 2010).

Descriptive analysis can be combined with instrumental analysis to determine the relationship between the chemical composition of beer and the corresponding sensory attributes. Several studies have correlated the results of descriptive analysis techniques with instrumental analysis to determine key chemical drivers for sensory attributes (Lafontaine *et al.*, 2018; Oladokun *et al.*, 2017). Similarly, descriptive analysis has previously been combined with

consumer preference analysis to determine sensory drivers for consumer preference and acceptance (da Costa Jardim *et al.*, 2018; Ramsey *et al.*, 2021). Understanding consumer preference for the sensorial and analytical profiles of beers allows for product optimisation in brewing.

1.9.2. Consumer testing

Consumer testing uses techniques to determine subjective consumer sensory responses to products. Consumer testing techniques can be used to measure the personal reaction of consumers, such as acceptance or preference. In qualitative consumer research, consumers participate in discussion-based evaluation, where the focus is on gaining understating and resulting outputs are generally generated from focus groups and open-ended questions. Qualitative methods provide a deeper insight into consumers reactions to products and can determine critical sensory attributes from the consumer perspective. In quantitative consumer testing, respondents are asked specific questions to measure either preference or acceptance of the products and perception of associated attributes. The use of 9 point hedonic scales is the most popular for determining consumer acceptance and providing the magnitude of acceptance of attributes in the product (Civille *et al.*, 2012; Kemp *et al.*, 2013). The 9 point hedonic scale is categorical with limited choices making it easy for the study participants and researchers to use. The simplicity of the 9-point hedonic scale makes it suitable for use by a wide range of populations without extensive training (Lim, 2011). However, there is increasing use of rapid methodologies to gather information of the sensory characteristics of products with consumers. The application of Check-All-That-Apply (CATA) questions has been used as a method to gather information about consumer perceptions (Varela *et al.*, 2012). In this method, consumers are asked to try the product and to select all the terms that they consider appropriate for describing each of the samples from a list of pre-defined attributes. The pre-defined attribute list can be either pre-defined or consumer-defined. CATA questions have been reported to be a quick, simple and easy way to gather information (Varela *et al.*, 2012). Several studies have found that CATA with consumers provides similar information to descriptive analysis performed with a trained panel (Cadena *et al.*, 2014; Oliver *et al.*, 2018; Ares *et al.*, 2015a). For example, Cadena *et al.* (2014) compared descriptive analysis with trained panel to CATA, projective mapping and polarised sensory positioning with consumers. The study found that results from

CATA provide the most similar sample configurations to descriptive analysis (RV coefficient=0.93) compared to two other rapid profiling tests. Therefore, CATA can gather reliable information about the sensory characteristics of a product, directly from consumers without the need for extensive training. However, it is important to take into account that the frequency of term selection does not provide quantitative intensity information as consumers only evaluate if a sensory attribute is perceived and not the intensity of the attribute.

There is limited research available on the consumer perception of beers brewed using unmalted adjuncts. Zanetta *et al.* (2020) determined the difference in hedonics and acceptance of beers brewed with adjuncts compared to all malt. The study compared Brazilian 'common beer' (beer produced with up to 45% unmalted adjuncts) and 'pure malt' beer which by Brazilian law must only be made with 100% barley malt. In a blind consumer test, the acceptance of both samples presented were not significantly different. However, participants perceived differences in bitterness between samples and indicated higher scores for bitterness liking for the pure malt sample. Although this was the first study to compare consumer acceptance of beers incorporating different proportions of adjuncts, it is not clear which adjuncts were included in the common beer analysed. Thus, there are no prior studies investigating the consumer acceptance and perception of beers brewed with differing amounts of specified unmalted adjuncts.

1.10. Research aims and objectives

The utilisation of unmalted cereal adjuncts can minimise the need for the malting process, providing economic and environmental benefits. However, brewing with unmalted cereal adjuncts, particularly at higher concentrations, can be challenging and a more detailed understanding is required about the processing and sensorial factors which limit higher incorporation rates of unmalted adjunct materials. The sensory and analytical impacts on beer quality when brewing with high incorporations of unmalted adjuncts have not been systematically investigated. The aim of this research was thus to investigate the impacts of incorporation of high proportions of unmalted cereal adjuncts (two types of unmalted barley, rice, maize and wheat) on the sensory profile of lager beers. Furthermore, to evaluate the required modifications in the brewing process to achieve a closer flavour match to the desirable qualities of all-malt beers.

Overall this approach sought to ensure that the beer quality was maintained or even improved with the incorporation of unmalted cereal adjuncts.

In Chapter 3, quantitative descriptive analysis (QDA) with a trained panel was used to develop an unmalted adjunct beer sensory lexicon. The developed lexicon allowed the determination of the sensory profile of beers produced with unmalted adjuncts. The analytical profile of each beer was examined to develop an understanding of the sensory properties determined in the adjunct beers. In Chapter 4, two different approaches to increase soluble nitrogen: i) addition of yeast nutrients and ii) addition of exogenous thermostable proteolytic mash enzymes were investigated when brewing with unmalted adjuncts. This enabled us to identify whether increased soluble nitrogen levels could improve the flavour profile when brewing with adjuncts. In Chapter 5, the consumer sensory perception and preference of beers brewed with unmalted adjuncts were investigated. A sensory consumer study and corresponding analytical analysis was used to determine the key sensorial and analytical drivers of consumer liking. The research reported in Chapter 6 investigated the differences in metabolomic profiling between 100% malt beers and those produced with unmalted barley adjunct material with the aim to determine differential non-volatile metabolites which could contribute to flavour differences observed when brewing with unmalted barley.

Chapter 2 General material and methods

2.1. Brewing materials

The variety and source of each of the cereal grain grist materials (malted barley, unmalted barley, wheat, flake torrefied rice and flaked torrefied maize) used in each study is detailed in Table 2.1.

Table 2.1. The variety and source of the grist material used in each study

	Chapter 3	Chapter 4	Chapter 5	
	Variety	Variety	Variety	Source
Malt	Propino	Planet	Planet	Soufflet (Burton-on-Trent, UK)
Barley	Planet or Propino	Planet or Laureate	Planet or Venture	Frontier Agriculture (Lincolnshire, UK)
Wheat	Leeds or Revelation	Leeds or Bassett	Bassett or Barrell	Openfield (Lincolnshire, UK)
Rice	Flake Torrefied	-	-	Crispmalt (Ryburgh, UK)
Maize	Flake Torrefied	-	-	Crispmalt (Ryburgh, UK)

A mixture of varieties were used for barley and wheat for each chapter and the same variety was not used consistently throughout the research for the cereal grains. This was due to the grist being supplied by industry and therefore the composition of varieties were aligned with the composition of grains that were being used by industry at each time point. It is acknowledged that different varieties of grain will have different chemical compositions that will impact brewing measures such as extract, fermentability, β-glucan concentration and level of nitrogen. The nitrogen content differed across the different grist materials and across the varieties used, for example, the nitrogen content of malt ranged from 1.48-1.82% (Table A.1). Two types of unmalted barley are included throughout this research. The unmalted barley samples used were of the same variety and differed in terms of their processing; however, details of the related processing are proprietary and cannot be disclosed for commercial reasons. They will be referred to throughout as barley (A) and barley (B).

Zeus T90 hop pellets with 15.3% α-acid content and 30-40% co-humulone composition were sourced by Simply Hops (Tonbridge, UK). Saflager W34/70 dry lager yeast was sourced from Fermentis (France). FAN Boost, a liquid mashing protease (subtilisin, non-GMO, not modified using modern biotechnology) with an enzyme activity of 4 AU-A/g was sourced from

Novozyme (Copenhagen, Denmark). YeastLife Extra was sourced from AB Vickers (Burton-On-Trent, UK).

2.2. Reagents and chemicals

Reagents for the various assays were sourced as follows and were of analytical grade:

Total Polyphenolic Content: Ethylenediaminetetraacetic acid (EDTA), carboxymethylcellulose (CMC) were purchased from Fisher Scientific (Loughborough, UK). Ammonium hydroxide and ammonium iron citrate were purchased from VWR (Leicestershire, UK).

Bitterness Units: 2,2,4-Trimethylpentane (isooctane) and hydrochloric acid were purchased from Sigma-Aldrich (Dorset, UK).

Free Amino Nitrogen: Sodium phosphate dibasic dodecahydrate, potassium phosphate monobasic, ninhydrin, fructose, ethanol, potassium iodate and glycine were purchased from Sigma-Aldrich (Dorset, UK).

Total Proanthocyanidins Content (TPC): Butanol and hydrochloric acid were purchased from Sigma-Aldrich (Dorset, UK).

HPLC Analysis: Ethyl acetate, methanol, acetonitrile, orthophosphoric acid (85%), acetic acid (glacial), 4-hydroxybenzoic acid (99%), ferulic acid (99%), 4-hydroxyphenylacetic acid (98%), p-coumaric acid (98%), vanillic acid (97%) and catechin (99%) were purchased from Sigma-Aldrich (Dorset, UK). Syringic acid (98%), tryptophol (97%) and tyrosol (98%), were purchased from Fisher Scientific (Loughborough, UK).

GC Analysis: Acetaldehyde (99.5%), ethyl acetate (99.8%), 1-propanol (99.7%), isobutanol (99%), phenethyl alcohol (99%), isobutyl acetate (99%), isoamyl acetate (97%), phenethyl acetate (98%) isoamyl alcohol (98%), ethyl hexanoate (99%), ethyl octanoate (98%), ethyl butyrate (99%), ethyl propanoate (99%), ethyl isobutyrate (98%), ethyl pentanoate (98%), methyl octanoate (99%) ethyl heptanoate (99%), dimethyl sulphide (99%), ethyl methyl sulphide (96%) and 3-heptanone (98%) were purchased from Sigma- Aldrich (Dorset, UK). Ethyl-trans-4-decenoate (97%) and 3-methyl-2-pentanone (98%) were purchased from Fisher Scientific (Loughborough, UK).

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) Analysis: 2% PrimarPlus nitric acid (68%) (Fisher Scientific, UK).

Dionex Analysis: Sodium hydroxide solution (50%), D-(+)-glucose (99.5%), sucrose (99.5%), D-(+)-maltose (99%), D-(-)-fructose (99%) and maltotriose (90%) were purchased from Sigma-Aldrich (Dorset, UK).
Nitrogen Analysis: L-Aspartic acid (99%) was purchased from Sigma-Aldrich (Dorset, UK).

2.3. Wort and beer chemical analysis

2.3.1. Measurement of specific gravity and pH

Alcohol content (ABV), present gravity (PG) and original gravity (OG) were measured using an Alcolzyer Plus connected to a DMA 4500 densitometer (Anton Paar, Graz, Austria). The pH wort or beer samples was recorded using a 3510 pH meter (Jenway, Stafford, UK).

2.3.2. Determination of wort and beer colour

The wort and beer colour were determined using the Analytica EBC method (EBC 9.6). Samples were filtered through a 0.4 μm syringe filter before being transferred into a 10 mm cuvette and the absorbance was then measured using a spectrophotometer at 430 nm.

2.3.3. Determination of free amino nitrogen

Free amino nitrogen was determined using the Analytica EBC method (EBC 8.10.1). This method is based on the reaction of ninhydrin reacting with primary amino groups to form a purple dye. Wort and beer samples were diluted 1:100 with RO water. A 1 ml volume of a ninhydrin colour reagent was added to 2 ml of the diluted sample in a stoppered test tube. The samples were then heated for 16 minutes at 100 °C using a dry block incubator. These were then cooled for 20 min in a 20 °C water bath. 5 ml of a diluting solution was added to each sample before recording the absorbance at 570 nm with a spectrophotometer. Analysis was performed in triplicate and compared against a 2 mg/L glycine standard solution.

2.3.4. Determination of total polyphenol content

Total polyphenol content was determined using the American Society of Brewing Chemists (ASBC) method (Beer-35). This method is based on the measurement of the red colour produced when ferric iron reacts with polyphenols in an alkaline solution. A 10 ml volume of wort or beer was mixed with 8 mL a carboxymethylcellulose (CMC) and ethylenediaminetetraacetic acid (EDTA) reagent into a 25 mL volumetric flask. 0.5 mL of a 3.5% ammonium ferric citrate solution was added, then 0.5 ml of a 1:3 ammonium hydroxide solution before diluting to 25 mL with RO water. The sample was then left to stand for 10 min before measuring the absorbance at 600 nm with a spectrophotometer. Analysis was performed in triplicate.

2.3.5. Determination of total proanthocyanidins content

Total proanthocyanidins content was determined using a method previously reported in Bate-Smith (1973). This method is based on the red colour produced from the reaction of proanthocyanidins with butanol and hydrochloric acid to form cyanidine. A 3 ml volume of 5% hydrochloric acid in butanol reagent was added to 0.5 ml of the wort and beer samples in a test tube. The tubes were then heated for 100 °C for 2 hours on a dry block incubator. Samples were then cooled and the absorbance was measured at 550 nm. The total proanthocyanidins content was determined using Equation 2.1. Analysis was performed in triplicate.

Equation 2.1. The calculation for determining total proanthocyanidins content

$$\text{TPA (mg/L)} = \left(\frac{A}{\varepsilon \times l}\right) \times \text{dilution factor} \times M_r \times 1000$$

A= absorbance

ε= molar extinction coefficient (15000)

l= path length

M_r= molecular mass of cyanidine chloride (322.7)

Dilution factor= 7 (0.5 ml beer with 3 ml reagent)

2.3.6. Determination of beer bitterness units (BU)

The bitterness units of beers were determined using the ASBC method (Beer-23A). This method determines both iso-α-acids and other compounds that can

contribute to overall bitterness in the beer. A 5 mL volume of beer was acidified with 0.25 ml 6M HCl in a 50 ml centrifuge tube. Following that 10 ml of isooctane was added and the mixture was shaken on a mechanical shaker set to a speed of 8 for 15 min. The mixture was then centrifuged at 400 x *g* for 5 min periods until there was enough separation to obtain 5 mL in the clear upper isooctane layer. An aliquot of this upper layer was then measured with a spectrophotometer at 275 nm. Analysis was performed in triplicate.

2.4. Instrumental analysis

2.4.1. Nitrogen determination in cereal grains

A Thermo Flash Nitrogen Analyser (ThermoFisher Scientific, Waltham, Massachusetts, USA) was used to determine nitrogen content of the unmalted cereal grains and malted barley samples. The sample (50 mg) was sealed in a tin capsule and combusted at approximately 1800°C. Combustion gases were passed into a reduction reactor (at 680°C and containing reduced copper) where nitrogen oxides are converted to elemental nitrogen. Carbon dioxide, sulphur dioxide and water were removed via filters of soda lime, magnesium perchlorate and a molecular sieve. The effluent stream was passed through a nitrogen separation column (50°C) and into a thermal conductivity detector. Quantitation was achieved with Eager 300 software (ThermoFisher Scientific, Waltham, Massachusetts, USA) using an L-aspartic acid standard. Protein was determined using the N x 6.25 conversion factor. Analysis was performed in triplicate.

2.4.2. Wort amino acid analysis by Biochrom 20 Analyser

For amino acid analysis, 60 mg of 5-sulphosalicylic acid and internal standard norleucine were added to the wort samples. The samples were then allowed to stand at 4 °C for 1 hour. The samples were then centrifuged at 16000 for 15 min at 4 °C. The supernatant was then filtered through a 0.22 μm PVDF Millex-GC syringe filter. The prepared samples were than analysed using a Biochrom 20 amino acid analyser (Pharmacia LKB, Biochrom Ltd, Cambridge, UK) with ninhydrin detection. The concentration of the amino acids was then calculated using EZChrom Elite software (Scientific Software International Ltd, Duston, Northampton, UK). Samples were analysed once due to limited resources.

2.4.3. Determination of fermentable sugar concentration by HPAEC-PAD

The concentrations of fermentable sugars (glucose, sucrose, fructose, maltose and maltotriose) in wort and beer samples were quantified using an HPAEC-PAD Dionex ICS6000 (Thermo Fisher Scientific, Loughborough, UK). Wort and beer samples were filtered through a 0.45 µm PVDF Millex-GC syringe filter and diluted in 10 mM NaOH (1 in 1000 for wort samples; 1 in 100 for beer samples). A carbopac PA20 IC column (3 x 150 mm) and a carbopac PA20 guard column (ThermoScientific, Loughborough, UK) were used. The mobile phase was 10mM NaOH with a flow rate of 0.5 mL per min. Data produced were processed using Chromeleon 7.2 software (ThermoFisher Scientific, Waltham, Massachusetts, USA). Authentic standards of the monosaccharides with concentrations within ranges of 0.3125 to 30 mg/L were used to produce six-point calibration curves used for monosaccharide quantification. Analysis was performed in four replicates per sample.

2.4.4. Elemental analysis determined by ICP-MS

Concentrations of Ag, Al, As, B, Ba, Be, Cd, Ca, Co, Cr, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, U, V and Zn in wort and beer samples were determined using a Thermo-Fisher iCAP-Q ICP-MS (Thermo-Fisher Scientific, UK). Samples were filtered through a 0.45 µm PVDF Millex-GC syringe filter then diluted 1 in 10 with 2% PrimarPlus nitric acid (68%) (Fisher Scientific, UK). Samples were introduced via a covered autosampler (Cetac ASX-520) through a concentric glass ventrui nebuliser (Thermo-Fisher Scientific, UK). The instrument has a flatopole collision cell (charged with helium gas) upstream of the analytical quadrupole to reduce polyatomic interferences. External calibration standards were used in the range of 0-100 µg/L. Data produced were processed using Qtegra software (Thermo-Fisher Scientific, UK). Analysis was performed in triplicate.

2.4.5. Total nitrogen content analysis by TOC-TN

Total nitrogen (TN) contents of wort and beer samples were determined using a total organic carbon analyser TOC-V CPH/CPN (Shimadzu, Milton Keynes,

UK) coupled with a total nitrogen measuring unit TNM-1 (Shimadzu, Milton Keynes, UK). Samples were filtered through a 0.45 µm PVDF Millex-GC syringe filter then diluted 1 in 100 with Milli Q water. A calibration curve was created using potassium nitrate solution in the range of 1-10 mg/L. Analysis was performed in triplicate.

2.4.6. HPLC determination of phenolic compounds

Analysis of phenolic compounds was performed on a Waters Alliance 2695 HPLC coupled with a Waters 2996 diode array UV detector. A Purospher STAR rp-18 end-capped column coupled with a C18 guard cartridge was used for separation of the phenolic compounds. The polyphenolic fraction was extracted and analysed according to the chromatographic method reported previously (Oladokun *et al.*, 2017). Degassed beer (5 mL) was acidified with orthophosphoric acid (0.25 mL) in a 50 mL falcon tube. Ethyl acetate (10 mL) was added and the mixture was extracted on a roller bed for 30 min. The residual beer from the bilayer mixture was discarded and reverse osmosis (RO) water (5 mL) was added to the ethyl acetate extract and further extracted for 15 min on the roller bed. The water layer was then removed and discarded. The ethyl acetate extract was evaporated to dryness using a controlled flow of nitrogen. The residue was reconstituted in 2 mL of methanol and analysed by HPLC. The mobile phase solvent system used for this method consisted of (A) 1.25% acetic acid and (B) 0.1% orthophosphoric acid in acetonitrile. The gradient elution protocol was as follows: 0-25 min: 98% A, 2% B; 25-30 min: 76% A, 24% B; 35-40 min; 55% A, 45% B; 45 min; 15% A, 85% B; 50 min: 0% A, 100% B; 55-65 min: 98% A, 2% B. All data produced was processed using Empower 2 HPLC software (Waters, MA, USA). Five-point calibration curves (0.5-20 mg/L) generated from external standards of syringic acid, vanillic acid, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, ferulic acid, p-coumaric acid and catechin were used to determine the concentration of compounds.

2.4.7. Gas chromatography analysis of fermentation volatiles by HS-GC-FID

Samples for analysis were prepared by adding 3.5 g of sodium chloride and 50 µL of 1-butanol (internal standard) to 10 mL of degassed beer sample in 20 mL headspace vials. Headspace (500 µL) was injected (split ratio 1:20) into a Bruker 400-GC equipped with a 60 m × 0.25 mm × 0.5 µm ZB-Wax column

(Phenomenex, Macclesfield, UK) and Flame Ionisation Detector (at 250 °C). Volatile compounds were separated according to a modified version of EBC Analytica method 9.39. Helium was used as the carrier gas at a constant pressure of 15 psi and the injector temperature was 150 °C. The oven temperature profile started at 85 °C for 10 min hold and was then increased by 25 °C/min and held at 110 °C for 13 min, then increased at 8 °C/min to 200 °C (13.25 min hold). Six-point calibration curves generated from external standards of acetaldehyde, propanol, isobutanol, isoamyl alcohol, ethyl acetate, isoamyl acetate, ethyl hexanoate, ethyl octanoate and ethyl butyrate were used to determine the concentrations of volatile compounds.

2.4.8. Gas chromatographic analysis of fermentation volatiles by SPME-GC-MS

Samples were prepared for analysis by adding 3.5 g sodium chloride and 100 µg/L of 3-heptanone (internal standard) to 5 mL of beer sample in 20 mL headspace vials. Volatile compounds were absorbed on to a multiphase SPME fibre (PDMS/DVB; 60 µm, Supelco, Dorset, UK; extraction for 10 min at 35 °C with agitation) and subsequently desorbed in the injector port (250 °C) of a TRACE TM 1300 Gas Chromatograph (Thermo Scientific Inc., Waltham, MA, USA) fitted with a Zebron 30 m x 0.25 mm x 0.25 µm ZB-WAX column (Phenomenex, Macclesfield, UK) and coupled to a single quadrupole EI-MS (ISQ-QD, Thermo Scientific Inc. Waltham, MA, USA) scanning the mass range *m/z* 35-230. The carrier gas was helium (1 mL/min) and split ratio 1:10. The oven temperature profile was 40 °C for 2 min and then programmed to reach 240 °C at a rate of 8 °C/min and held for 1 min. Transfer line temperature was 250 °C. Five-point calibration curves generated from external standards were used to determine the concentrations of volatile compounds. The following selected ions were used for quantification: isobutyl acetate (*m/z* 73), phenethyl acetate (*m/z* 91), ethyl hexanoate, ethyl octanoate, ethyl butyrate, ethyl propanoate, ethyl isobutyrate, ethyl pentanoate, ethyl heptanoate (*m/z* 88), 3-methyl-2-pentanoate (*m/z* 85) and phenethyl alcohol (*m/z* 91).

2.4.9. Gas chromatographic analysis of dimethyl sulphide by SPME-GC-PFPD

Samples were prepared by adding 3.5 g sodium chloride and 50 ul of ethyl methyl sulphide to 10 mL of beer sample in 20 mL screw-top headspace vials. Volatile compounds were absorbed on to a multiphase SPME fibre (DVB/Carboxen/PDMS; 50/30 µm, Supelco, Dorset, UK) and subsequently desorbed in the injector port (250 °C) of a Bruker 400-GC fitted with a 60 m x 0.25 mm x 1 µm ZB-1MS column (Phenomenex, Macclesfield, UK). Carrier gas was helium (1 mL/min) and split ratio 1:10. The oven temperature was 40 °C (held for 7 min) and then programmed to reach 110 °C at a rate of 7 C/min, then increased to 190 °C at a rate of 11 °C/min to then 235 °C at a rate of 22 °C/min. A sulphur-specific Pulsed Flame Photometric Detector (PFPD) at 210 °C recorded the elution of sulphur containing molecules from each sample. A six-point calibration curve was generated from an external standard to determine the concentration of dimethyl sulphide.

Chapter 3 Brewing with unmalted cereal adjuncts: Sensory and analytical impacts on beer quality

3.1. Introduction

Brewing with unmalted cereal adjuncts, particularly at higher concentrations, can be challenging. The main disadvantage in terms of processability when including unmalted adjuncts is the decrease in enzymatic activities in the grist (discussed in Section 1.3). The varied biochemical composition of cereal materials will have impacts both on the brewing process performance and on finished beer quality. A deficiency of enzyme activity and variations in composition of unmalted adjuncts can consequently impact the flavour profile of adjunct beers (Bogdan *et al.*, 2017; Buiatti *et al.*, 2018). However, such impacts to the aroma and flavour of the finished beer are still not fully understood.

Sensory analysis of beer provides a comprehensive evaluation of the beer quality aspects including appearance aroma, flavour, foam etc. Descriptive sensory techniques such as quantitative descriptive analysis are commonly used in determining the sensory characteristics of beer (Donadini *et al.*, 2011; Donaldson *et al.*, 2012; Liu *et al.*, 2012), as discussed in Section 1.9. There have been several studies investigating the production of beer by partial or complete substitution with adjuncts, with basic sensory evaluation of the adjunct based beers. Kunz *et al.* (2012) determined that the incorporation of unmalted barley at up to 50% of grist produced a beer with comparable preference ratings in odour and taste in comparison to an all-malt beer. However, as the barley incorporation was increased to 90% an increase in astringency was detected. Whilst Steiner *et al.* (2012) reported that the use of 100% unmalted barley produced beer with less body and mouthfeel.

The use of rice in brewing is considered to produce neutral, clean and dry sensory characteristics in the beer whilst the addition of maize can induce a fuller mouthfeel (Bogdan *et al.*, 2017). These prior studies have suggested how adjuncts can influence the sensory profile of beer. However, there is little comprehensive sensory and analytical data concerning the flavour profile induced by adjuncts, and a lack of comprehensive sensory studies comparing the impacts of using high grist ratios of a range of unmalted cereals.

The aims of the study reported in this chapter was to firstly produce adjunct based lager beers with varying incorporation rates of barley (A), barley (B), rice, maize and wheat adjuncts. Two incorporation rates were used in the study- the first aligned with typical current industry usage (30%) and then a higher and more challenging incorporation rate (60%). Using quantitative descriptive analysis (QDA) with a trained panel, an adjunct beer sensory lexicon was developed and allowed for the determination of the sensory properties for the adjunct beers and an all malt control. Furthermore, the chemical composition of each beer was examined to develop understanding of the underlying basis for the sensory properties of adjunct beers.

3.2. Materials and methods

The brewing materials and analytical methods used in this study are described in Chapter 2.

3.2.1. Brewing process for adjunct and control beers

Adjunct based lager beers with five different unmalted cereal adjuncts (rice, maize, wheat, barley (A) and barley (B)) at differing adjunct concentrations (0% (all malt control), 30% and 60%) were brewed, producing in total eleven different beers. Flaked torrefied maize and rice were used in this study to remove the requirement of pre-processing the cereal in the brewery and to enable a standardised brewing recipe across each adjunct. Thermal processing techniques ('cereal cooking') are required when using unmalted rice and maize as a result of higher gelatinisation temperatures in tropical grains as previously discussed in Section 1.3.1.

All grains were milled with a Roppi-250 roller mill (Robix, Veszprém, Hungary) at a gap setting of 0.85 mm. The portion of each adjunct material included in each adjunct brew was measured by the percentage of grist as is. Brewing was performed in a 25 L Grainfather system (The GrainFather, Auckland, New Zealand).). For each brew, a liquor to grist ratio of 3.6:1 was used. Mash-in temperature was 55 °C and held for 20 min, followed with a 90 min stand at 65 °C, then a 10 min stand at 72 °C with final mash-off at 78 °C for 5 min. Temperature increases between stands were ramped at a rate of between 1.4–1.5 °C/min. . Sparge water (17L) was passed through the grain bed at a 4.25L/kg sparge water to grist ratio. and the resultant wort was boiled with 20 g of T90

hop pellets (variety: Zeus) for 60 min with a target to produce beers with a final bitterness of 20 BU. The wort was cooled to 20 °C and fermented with SafLager W34/70 yeast (11.5 g to 25 L wort) in a FastFerment 30 L plastic conical fermenter for 7 days, followed by maturation at 4 °C for 4 days. The wort was fermented at a marginally higher temperature than the recommended temperature for W34/70 yeast (12-18 °C). This was due to the scale of the production and unavailability of lower temperature controlled equipment for fermenting eleven samples in a short period of time to enable freshness for sensory analysis. Beers were packaged into 500 mL PET brown bottles, bottle conditioned (3 g of dextrose monohydrate (The Homebrew shop, Hampshire, UK) per bottle) at 20 °C for 3 weeks and subsequently stored at 4 °C prior to sensory evaluation.

3.2.2. Protocol for laboratory wort production

Laboratory mash bath wort samples were prepared in triplicate for each adjunct (rice, maize, wheat, barley and barley (B)) at differing concentrations (0, 30% and 60%) using an R8 mash bath (1-Cube, Havlickuv Brod, Czech Republic). All grist materials were milled using a laboratory DFLU disc mill (Buehler Miag, Uzwil, Switzerland). For each wort sample, a liquor to grist ratio of 3.6:1 was attained by mixing 180 mL of reverse osmosis (RO) water at 55 °C to 50 g of the mixed grist weighted into a metal mashing beaker. The mash was stirred throughout the mash profile and the same mash profile used for the brewing trial (55 °C for 20 min, 65 °C for 90 min, 72 °C for 10 min, 78 °C for 5 min). Wort samples were then cooled to 20 °C and adjusted with RO water and filtered through pleated filter paper (Whatman, 2555 ½, 320 mm, Sigma-Aldrich, Dorset, UK). The specific gravity and free amino nitrogen content of each wort sample was determined according to the method described in Section 2.2.3.

3.2.3. Sensory analysis

Before conducting this study, ethics approval was requested and accepted by the Faculty of Medicine & Health Sciences Research Ethics Committee at the University of Nottingham (Ethics Reference No. 269-1803). All panellists provided consent to partake and were provided a disturbance allowance for their

participation. The sensory evaluation of the adjunct beers was conducted following a modified Quantitative Descriptive Analysis methodology (Stone *et al.*, 2012).

3.2.3.1. Preparation of samples and reference solutions

For all sessions, 20 mL of freshly opened sample was measured into 60 mL vials secured with a lid no more than one hour before the session. The final 150 mL of the 500 mL sample bottles were discarded to minimising the introduction of potential residue from bottle conditioning. Prepared samples were stored at 4 ± 2 °C until required for evaluation. Samples were presented in amber vials labelled with random three-digit codes throughout the study to minimise expectation error and to reduce bias from appearance. Attribute references were developed over several sessions and placed into labelled amber vials. Acetaldehyde and dimethyl sulphide flavour reference standards were purchased from Aroxa (Cara Technology, Surrey, UK). Isoamyl acetate was purchased from Sigma-Aldrich (Dorset, UK) and all compounds used were food grade. All samples and reference solutions were prepared in a food safe environment.

3.2.3.2. Sensory panel recruitment

Panellists who had previously been screened for their general sensory acuity were additionally screened to evaluate their discrimination and descriptive ability of the adjunct beer samples. The main aim of this screening session was to determine if the prospective panellists could recognise and clearly communicate any sensory descriptors identified in the beers, individually and in a group discussion setting. After reviewing the data, eight experienced assessors (4 males and 4 females) were invited to take part.

3.2.3.3. Attribute generation and panel training

After recruitment, the attribute lexicon was developed. Panellists were individually presented with all eleven beer samples (according to Section 3.2.3.1) over two 120-min sessions and were asked to generate terms to

describe the sensations elicited in each sample under four categories: aroma, flavour, taste and mouthfeel. This generated a list of 206 attributes that panellists had identified individually. Two sessions were then held to consolidate the initial attribute list by means of Check-All-That-Apply (CATA) technique (Ares *et al.*, 2015b) and group discussions. These sessions helped remove ambiguous and overlapping descriptors whilst highlighting the most applied and discriminating descriptors.

Fifteen 120-min training sessions were dedicated to further consolidating the attribute list and creating definitions for each attribute. Reference materials were provided to the panellists to establish references for the lexicon and aid understanding of the particular attribute. Training sessions included replicate rank rating tests, to determine discrimination ability. Replicate samples were included to ensure reproducibility and group discussions were used to address any inconsistencies in panel understanding of the attributes or method of assessment. Samples modified with additional flavour compounds including acetaldehyde and dimethyl sulphide were included in training sessions to ensure the panel could accurately discriminate intense samples. Panellists were trained on the use of a 10 cm line scale for evaluating attributes. Throughout the training sessions, the panel developed a protocol for tasting, smelling and palate cleansing to ensure optimum identification and performance in the final evaluation of the samples. An assessment order for the attributes was determined by the panel which corresponded to the order of perceived sensations and when fresh samples would be provided. Panel performance was monitored throughout training. The purpose of monitoring panel performance was to examine the reproducibility and discriminative ability of the panel individually and as a group. Prior to the final evaluation, a mock evaluation was conducted to establish panel performance and highlight any panel inconsistencies that needed to be resolved before the final evaluation.

3.2.3.4. Sample evaluation

Panellists assessed the all malt control and eleven adjunct beers individually in triplicate over six 120-min sessions in sensory booths. Each session involved the evaluation of six samples to ensure less than 1 UK alcohol unit was consumed per session for ethical compliance. The samples were labelled with a 3-digit random code and presented in a completely randomised order. All

samples were served in amber vials at 4 ± 2 °C. In the final evaluation, attributes were divided into three sections and fresh 20 mL aliquots were provided for each section to ensure freshness and consistent temperature throughout assessment. The intensity of each attribute was rated on a 10 cm unstructured line scale which was displayed on a computer screen along with each attribute description. Panellists were given three-minute breaks between each sample to avoid palate fatigue with a 10 min comfort break at the midpoint of evaluation (after 3 samples). During breaks, panellists used water (Evian, Danone, Paris, France) and unsalted crackers (Rakusens, Leeds, UK) to cleanse the palate and to minimise sample carry-over. Data were collected using Compusense Cloud (Compusense, Guelph, ON, Canada).

3.2.4. Statistical analysis

Statistical analysis was carried out using XLSTAT. Three-way mixed model ANOVA (sample, replicate, panellist) was conducted on all sensory attributes and the corresponding interactions between factors were used to determine the effects of the panel performance. Tukey's Honest Significant Difference (HSD) test was applied to evaluate significant differences between samples for each attribute. Dunnett's test was used to compare adjunct samples to the 100% malt control for the sensory attributes and chemical compounds. Principal Component Analysis (PCA) analysis was conducted on average scores were produced to provide a visual depiction of the relationship of the adjunct beers to the sensory and analytical characteristics highlighted. Significance was derived at $p = 0.05$.

3.3. Results and Discussion

3.3.1. Sensory evaluation of adjunct and control beers

3.3.1.1. Attribute generation

In the initial sessions, panellists generated over 200 attributes describing the adjunct and control beers, which were then consolidated over several sessions by means of Check-All-That-Apply (CATA) tests and group training sessions. The final 18 attributes with their description and reference training materials are presented in Table 3.1.

The lexicon comprised of three basic tastes, seven aroma, six flavour, one trigeminal sensation (astringent) and one temporal character of bitterness (lingering bitterness). Four terms (green apple, banana, malty and grainy) were assessed both for aroma (orthonasal assessment) and flavour (retronasal assessment).

Table 3.1. The adjunct beer attribute lexicon, description and reference standards developed in the study

Modality	Attribute	Definition	Reference Material
Aroma	Green Apple/Emulsion Paint	The skin of a green apple and the aroma of emulsion paint	15 mg/L of acetaldehyde in water (Aroxa)
	Banana/Pear Drops	Artificial banana and pear drop sweets	3.5 mg/L of isoamyl acetate (Aroxa) in water
	Sweetcorn/Cooked Vegetable	Tinned sweetcorn and cooked vegetables	150 µg/L of dimethyl sulphide (Aroxa) in water
	Smoky	Stale acrid smoke such as stale cigarette smoke or faint burnt rubber	
	Malty	Sweet processed or cooked malt grains	20 g of malt loaf, 20 g of barley malt extract
	Grainy	Dry light barley malt grains	50 g of dry light barley malt
	Plum Fruit	Cooked plum fruit (plums, damsons and prunes)	
Flavour	Green Apple/Emulsion Paint	The skin of a green apple and emulsion paint	15 mg/L of acetaldehyde in water (Aroxa)
	Banana/Pear Drops	Artificial banana and pear drop sweets	3.5 mg/L of isoamyl acetate (Aroxa) in water
	Apple	Bruised sweet red apple	
	Malty	Sweet processed or cooked malt grains	20 g of malt loaf, 20 g of barley malt extract
	Grainy	Dry light barley malt grains	50 g of dry light barley malt
	Plum Fruit	Cooked plum fruit (plums, damsons and prunes)	
Taste	Sweet	Intensity of sweetness after swallowing	
	Sour	Intensity of sourness after swallowing	
	Bitter	Peak intensity of bitterness after swallowing	
	Lingering Bitterness	Intensity of bitterness 20 s after swallowing	
Mouthfeel	Astringent	Drying sensation in the mouth after swallowing	

3.3.1.2. Panel performance

In order to determine panel performance and any variation in the panel evaluation, three-way ANOVA (sample, panellist and replicate) with interaction was applied to the attribute scores in the final evaluation as shown in Table 3.2.

Table 3.2. Mixed model Analysis of Variance (ANOVA) p-values for sensory attributes rated for adjunct beers

Modality	Attribute	Sample	Replicate	Panellist	Sample * Replicate	Sample * Panellist	Replicate * Panellist
Aroma	Green Apple/Emulsion Paint	0.324	0.622	<0.001 ***	0.920	0.708	0.306
	Sweetcorn/Cooked Veg.	0.002 **	0.373	<0.001 ***	0.979	0.796	0.269
	Banana/Pear Drops	0.041 *	0.089	<0.001 ***	0.046 *	0.035 *	0.346
	Smoky	0.038 *	0.177	<0.001 ***	0.676	0.020 **	0.789
	Malty	0.078	0.454	<0.001 ***	0.788	0.309	0.005 **
	Grainy	0.077	0.535	<0.001 ***	0.881	0.233	0.545
	Plum Fruit	0.166	0.021 *	<0.001 ***	0.992	0.026 *	0.147
Flavour	Green Apple/Emulsion Paint	0.012 *	0.238	<0.001 ***	0.187	0.496	0.628
	Apple	0.066	0.581	<0.001 ***	0.139	0.400	0.571
	Banana/Pear Drops	0.025 *	0.162	<0.001 ***	0.365	0.037 *	0.829
	Malty	0.101	0.846	<0.001 ***	0.170	0.001 **	0.011 *
	Grainy	0.052	0.395	<0.001 ***	0.178	0.015 **	0.323
	Plum Fruit	0.110	0.146	<0.001 ***	0.988	0.009 **	0.139
Taste	Sweet	0.336	0.257	<0.001 ***	0.601	0.155	0.104
	Sour	<0.001 ***	0.999	<0.001 ***	0.963	0.290	0.432
	Bitter	0.468	0.394	<0.001 ***	0.710	0.864	0.481
Mouthfeel	Astringent	0.326	0.005 **	<0.001 ***	0.727	0.038 **	0.235
	Lingering Bitterness	<0.001 ***	0.326	<0.001 ***	0.748	0.007 **	0.181

* ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

All attributes showed significant effect of panellist ($p < 0.05$) which indicates that the panel varied in their use of the scale. However, further analysis of the raw data showed that this was due to minor variations across the panel that were considered acceptable (Stone *et al.*, 2012). Furthermore, for the majority of attributes there were no significant effects for replication ($p > 0.05$) (except plum fruit aroma and astringent mouthfeel) indicating intra-panellist consistency. Nine attributes showed significant ($p < 0.05$) sample * panellist interaction indicating that there was not a strong agreement between panellists on the intensity order across the samples for these attributes. However, interrogation of the interaction plots showed that the majority of these interactions were minor.

Altogether, only 7 out of the 18 attributes ($p < 0.05$) were found to discriminate between the samples (Table 3.2). This lack of discrimination could indicate low levels of variance between the samples or high levels of variance in the panel. Extensive screening and training with the panel aimed to maximise performance resulting in very little variation across replicates. Therefore, it is proposed here that the lack of discrimination is due to low levels of perceivable differences (variance) between the samples.

3.3.1.3. Descriptive analysis of adjunct beers

Table 3.3 shows the mean rating scores for each attribute and the resultant groupings of the Tukey's HSD post-hoc test for significant attributes (in bold).

Significant differences ($p < 0.05$) were reported for three aroma attributes (sweetcorn, banana and smoky), two flavour (green apple, banana) and two taste (sour and lingering bitterness) attributes. No significant differences ($p > 0.05$) were observed for the remaining 11 attributes indicating that the panel could not significantly discriminate between the samples for these attributes. However, apple and grainy flavour attributes were borderline significant ($p = 0.066$ and $p = 0.052$). The main trends observed for these attributes were: a relatively low score for apple flavour in the 60% barley (B) adjunct beer and a trend towards higher scores for grainy flavour in the 60% maize and 60% rice adjunct beers.

Table 3.3. Mean rating scores and Tukey's Honest Significant Difference (HSD) test for adjunct-based beers and all malt control

Modality	Attribute	100% Malt Control	Barley (A)		Barley (B)		Wheat		Rice		Maize	
			30%	60%	30%	60%	30%	60%	30%	60%	30%	60%
Aroma	Green Apple/Emulsion Paint	2.22 ^{a}	2.43 ^{a}	2.86 ^{a}	2.42 ^{a}	1.95 ^{a}	2.67 ^{a}	2.78 ^{a}	2.61 ^{a}	3.86 ^{a*}	2.87 ^{a}	2.36 ^{a}
	Sweetcorn/Cooked Veg.	**3.36** ^{ab}	**3.23** ^{ab}	**1.44** ^{b}	**3.69** ^{ab}	**3.47** ^{ab}	**1.71** ^{ab}	**1.36** ^{b}	**4.33** ^{a}	**3.60** ^{ab}	**3.16** ^{ab}	**3.14** ^{ab}
	Banana/Pear Drops	**2.29** ^{ab}	**2.27** ^{ab}	**2.18** ^{ab}	**2.50** ^{ab}	**2.47** ^{ab}	**2.45** ^{ab}	**3.34** ^{a}	**1.80** ^{b}	**2.06** ^{b}	**1.93** ^{ab}	**1.80** ^{b}
	Smoky	**2.30** ^{abc}	**2.59** ^{ab}	**1.70** ^{abc}	**2.08** ^{abc}	**1.03** ^{bc}	**0.64** ^{c}	**3.04** ^{a}	**1.60** ^{abc}	**1.45** ^{abc}	**0.55** ^{c}	**0.78** ^{bc}
	Malty	4.50 ^{a}	4.00 ^{a}	3.72 ^{a}	4.02 ^{a}	3.48 ^{a}	3.56 ^{a}	2.86 ^{a}	3.81 ^{a}	2.95 ^{a}	3.86 ^{a}	3.09 ^{a}
	Grainy	3.0 ^{a}	2.51 ^{a}	2.32 ^{a}	2.18 ^{a}	2.10 ^{a}	2.38 ^{a}	1.49 ^{a}	3.44 ^{a}	3.52 ^{a}	3.25 ^{a}	3.01 ^{a}
	Plum Fruit	3.17 ^{a}	2.00 ^{a}	2.29 ^{a}	3.20 ^{a}	2.50 ^{a}	3.14 ^{a}	2.96 ^{a}	2.43 ^{a}	1.86 ^{a}	3.16 ^{a}	2.29 ^{a}
Flavour	**Green Apple/Emulsion Paint**	**2.74** ^{b}	**3.43** ^{ab}	**3.92** ^{ab}	**3.83** ^{ab}	**2.79** ^{b}	**3.57** ^{ab}	**3.80** ^{ab}	**4.01** ^{ab}	**5.52** ^{a*}	**3.22** ^{b}	**3.68** ^{ab}
	Apple	2.19 ^{a}	2.03 ^{a}	1.37 ^{a}	1.84 ^{a}	1.71 ^{a}	2.73 ^{a}	2.45 ^{a}	1.70 ^{a}	2.30 ^{a}	2.15 ^{a}	2.38 ^{a}
	Banana/Pear Drops	**2.8** ^{ab}	**1.92** ^{ab}	**1.63** ^{b}	**2.40** ^{ab}	**2.46** ^{ab}	**2.78** ^{ab}	**3.21** ^{a}	**2.30** ^{ab}	**2.35** ^{ab}	**2.86** ^{ab}	**1.63** ^{ab}
	Malty	4.30 ^{a}	3.74 ^{a}	3.00 ^{a}	3.55 ^{a}	3.33 ^{a}	3.68 ^{a}	4.53 ^{a}	4.23 ^{a}	3.06 ^{a}	3.75 ^{a}	3.41 ^{a}
	Grainy	2.53 ^{a}	4.05 ^{a}	3.89 ^{a}	3.83 ^{a}	3.02 ^{a}	3.81 ^{a}	2.40 ^{a}	3.83 ^{a}	4.21 ^{a}	3.68 ^{a}	4.30 ^{a}
	Plum Fruit	3.11 ^{a}	2.95 ^{a}	2.48 ^{a}	2.50 ^{a}	1.92 ^{a}	3.04 ^{a}	3.90 ^{a}	3.06 ^{a}	2.92 ^{a}	2.52 ^{a}	2.99 ^{a}
Taste	Sweet	3.46 ^{a}	2.59 ^{a}	2.64 ^{a}	2.84 ^{a}	3.07 ^{a}	2.50 ^{a}	3.18 ^{a}	3.12 ^{a}	3.41 ^{a}	3.38 ^{a}	2.35 ^{a}
	Sour	**2.35** ^{c}	**3.56** ^{abc}	**4.15** ^{ab}	**3.38** ^{abc}	**3.74** ^{abc}	**2.90** ^{bc}	**2.94** ^{bc}	**3.72** ^{abc}	**3.57** ^{abc}	**3.66** ^{abc}	**4.81** ^{a*}
	Bitter	4.35 ^{a}	4.96 ^{a}	5.08 ^{a}	4.18 ^{a}	4.20 ^{a}	5.08 ^{a}	4.75 ^{a}	4.52 ^{a}	4.44 ^{a}	4.14 ^{a}	4.75 ^{a}
Mouthfeel	Astringent	4.43 ^{a}	5.09 ^{a}	5.47 ^{a}	5.19 ^{a}	4.39 ^{a}	4.62 ^{a}	4.81 ^{a}	5.18 ^{a}	4.34 ^{a}	4.73 ^{a}	5.09 ^{a}
	Lingering Bitterness	**4.17** ^{ab}	**4.89** ^{ab}	**5.54** ^{a}	**4.38** ^{ab}	**3.25** ^{b}	**4.38** ^{ab}	**4.94** ^{ab}	**4.68** ^{ab}	**3.45** ^{b}	**3.69** ^{b}	**4.82** ^{ab}

Bold font: attributes were rated significantly differently across the beers ($p < 0.05$). Superscript letters indicate post-hoc groupings of beers according to Tukey's HSD test. Asterisks denote samples that were significantly different compared to the all malt control according to Dunnett's post hoc analysis ($p < 0.05$).

The incorporation of 60% barley (A) produced significantly higher attribute scores for the lingering bitterness attribute and although not statistically significant, the incorporation of barley (A) produced the highest mean scores for bitter and astringent characteristics. This is in accordance with prior literature, where the incorporation of unmalted barley at high proportions has been reported to increase the perception of bitterness and astringency to an undesirable level (Steiner *et al.*, 2012; Buiatti *et al.*, 2018; Yano *et al.*, 2008). Notably, the incorporation of barley (A) influenced the temporal quality of bitterness perceived as the sample showed the highest intensity of lingering bitterness. Longer durations of perceived bitterness at higher intensities could lead to an unpleasant organoleptic experience. Interestingly, the increase in bitterness profile was not observed with the incorporation of barley (B). In contrast, the use of 60% barley (B) significantly decreased the lingering bitterness mean score (relative to that for barley(A)) and reduced the bitterness and astringency scores to lower than those for the 100% malt control. The sensory profiles of 60% barley (A), 60% barley (B) and the 100% malt control are visualised as radar plots in Figure 3.1.

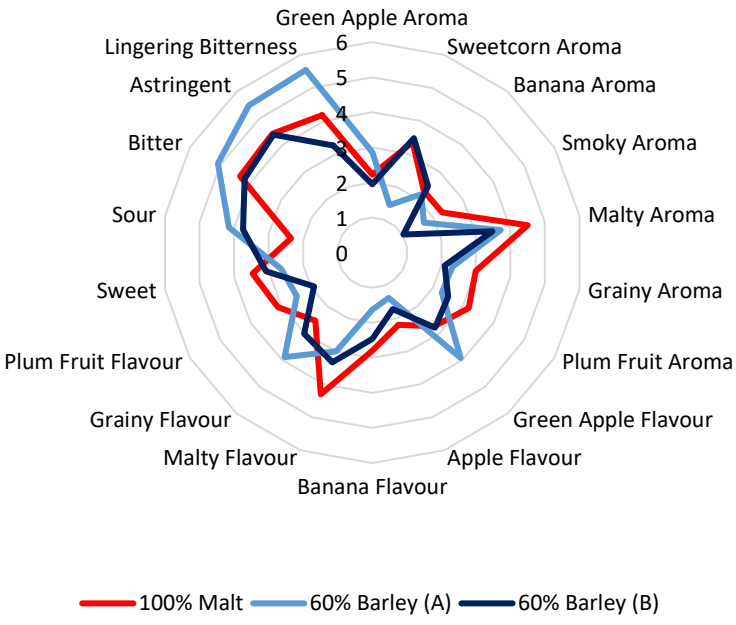


Figure 3.1. Radar plots of the mean rating scores from 60% barley, 60% barley (B) and 100% malt control beers

The incorporation of 60% barley (B) gave a closer match to the profile of the all malt control beer, although it was on average rated a little more sour, but less malty, smoky and plum-fruit flavoured. Whereas, in comparison use of 60% barley (A) led to a beer which was rated on average as more sour, bitter and astringent, which had a more lingering bitterness. This demonstrates that processing applied to the barley grain can diminish unfavourable sensory attributes that are associated with high incorporation rates of unmalted barley.

Use of 60% unmalted wheat adjunct led to beers that were characterised by the highest perception of banana aroma and flavour and the highest perception of smoky aroma. Strong phenolic and some smoke character are characteristic of Belgian white beers (Witbier) made with unmalted wheat; however, this is a result of the phenolic off-flavour positive (POF+) yeast strains used when brewing Belgian beers. The use of unmalted wheat in lager has been described as creating a 'neutral type of beer' with a specific, refreshing taste (Krstanović *et al.*, 2020). However, this is the first study that has identified specific flavour characteristics relating to the use of high incorporations of unmalted wheat in lager.

The incorporation of 60% torrefied rice was characterised by significantly higher attribute scores for green apple flavour, and the highest rating score for the green apple aroma attribute, although this attribute was not statistically significant. The incorporation of 30% rice caused a significant increase in the sweetcorn/cooked vegetable aroma detected. The change in perception of these attributes did not linearly increase with the proportion of rice in the grist, highlighting that these flavour differences are not a direct flavour imparted by the rice, rather they are most likely associated with changes in fermentation vigour as a result of adjunct-linked reductions in soluble nitrogen. In the brewing literature, beers produced with all rice have been described as having low concentrations of flavour attributes and relatively flat or neutral character compared to an all malt beer (Mayer *et al.*, 2016; Ombretta *et al.*, 2017).

Use of 60% torrefied maize resulted in beer with the highest mean score for sourness which was significantly more sour than the all malt control and the wheat adjunct beers. A moderate sour taste is necessary to balance the flavour of beer. Nevertheless, too strong a sour taste will destroy the harmony of the beer flavour (Li *et al.*, 2015). A prominent sour taste can be perceived with

adjunct beers in which worts have weak buffering capacity and a resultant change in pH of the beer (Li *et al.*, 2015).

Dunnett's post-hoc analysis was also performed on attribute scores for all eighteen attributes creating a comparison of each sample with the 100% malt sample as a control. Two samples had significantly different sensory attribute scores compared to the control ($p < 0.05$). The beer produced with 60% maize was significantly more sour and 60% rice had significantly more green apple flavour compared to the 100% malt beer. Samples of 30% barley (A), barley (B), wheat and maize were not described by any specific sensory attribute as indicated by the low attribute scores and lack of attributes discriminating them. It is important to note that the majority of the sensory attributes were not found to discriminate between the beers. This is important because even at incorporation rates of up to 60%, sensory impacts of the unmalted adjunct incorporation were subtle especially when comparing the samples against an all malt control.

All beers in the study were subjected to a range of standard beer quality analyses as well as chromatographic flavour analysis, in an attempt to identify factors which might correlate with the noted changes in sensory attributes.

3.3.2. Analytical evaluation of adjunct worts and beers versus an all malt control

3.3.2.1. Free amino nitrogen content of worts

Free amino nitrogen (FAN) is a measure of yeast assimilable nitrogen which consists of amino acids, ammonium ions and peptides. The level and composition of wort FAN is important for yeast growth and efficient fermentation (Hill *et al.*, 2019; Olaniran *et al.*, 2017). More importantly, wort FAN has a significant impact on the aroma profile of beer as previously discussed in Section 1.5.4. Wort samples were produced in laboratory mash bath trials using the same mash conditions and raw materials used for the final beer production, to determine the impact of adjunct incorporation on wort FAN concentrations. The incorporation of all five adjuncts decreased wort FAN levels compared to the all-malt control (Table 3.4).

Table 3.4. Free amino nitrogen of adjunct wort samples

	Malt	Barley (A)		Barley (B)		Maize		Rice		Wheat	
	100%	**30%**	**60%**	**30%**	**60%**	**30%**	**60%**	**30%**	**60%**	**30%**	**60%**
Wort FAN * (mg/L)	243 ± 26	**184** ± 1	**127** ± 6	**140** ± 2	**85** ± 4	**114** ± 10	**58** ± 5	**95** ± 15	**61** ± 2	**141** ± 11	**79** ± 9

* Free amino nitrogen concentration was standardised to 10 °P. ANOVA with Dunnett's post hoc analysis was used to compare the wort FAN concentration. **Bold font** denotes the samples that were significantly different compared to the all malt control ($p < 0.01$).

It has been reported that the wort FAN concentration should be above 150 mg/L to achieve a healthy fermentation with normal gravity (10–12 °P) (Hill *et al.*, 2019). In this case, all adjunct worts had FAN concentrations lower than this, particularly those produced with torrefied maize and rice adjuncts. Lower wort FAN content can be partially attributed to the lower nitrogen content in the maize and rice grains (1.22% and 1.16% on dry basis) compared to malted barley grains (1.48% on dry basis). However, the absence of malting is the main reason that unmalted adjuncts produce lower wort FAN concentrations. As previously discussed in Section 1.5.2, substantial breakdown of proteins occurs through malting leading to increased soluble nitrogen. Unmalted maize and rice are almost devoid of proteolytic enzymes which leads to a dilution of the FAN content of the wort (Taylor *et al.*, 2013). This identifies a complication to brewing at higher adjunct rates which could impact on finished beer flavour profile.

3.3.2.2. Finished beer quality parameters

The specifications of the finished beers used in this study are reported in Table 3.5.

Extract and final gravity principally indicate the sugar content of the wort and beer, providing information on the efficiency of fermentation and the potential alcohol by volume (ABV) in the finished beer. In this study, original extract decreased relative to control with incorporation of all adjuncts, especially with 60% torrefied maize (Table 3.5). This is in agreement with prior published data where the incorporation of 20% maize yielded lower extract despite the fact that the starch extract yield potential of maize is greater than that of barley (Agu, 2002; Poreda *et al.*, 2014). This result is also expected as exogenous amylase enzyme were not added to the mash when producing adjunct beers to increase saccharification. The addition of exogenous enzymes could increase the

original extract of adjunct beers. At the end of fermentation, maize and rice beers were characterised by lower extract compared with the all-malt beer, in agreement with previous studies with maize adjunct (Poreda *et al.*, 2014). A slightly higher apparent attenuation was observed for the maize and rice beers.

Table 3.5. Physiochemical analysis of adjunct beers

	Original Extract (°P)	Final Gravity (°P)	Apparent Attenuation (%)	ABV (%)	pH	BU	Colour (EBC)	Total Polyphenol Content (mg/L)
100% Malt	11	1.8	84	4.8	4.48	17	10	170 ± 10
30% Barley (A)	9.4	1.8	81	4	4.45	19	8	137 ± 5
60% Barley (A)	10	2.6	75	3.8	4.49	17	8	160 ± 3
30% Barley (B)	9.1	1.9	80	3.7	4.40	16	6	160 ± 5
60% Barley (B)	9.7	2.6	74	3.7	4.47	15	5	181 ± 7
30% Wheat	9	1.8	81	3.7	4.38	17	9	117 ± 4
60% Wheat	10.3	2.4	77	4.1	4.41	16	8	100 ± 2
30% Maize	9.4	1.9	80	3.9	4.25	14	8	103 ± 3
60% Maize	8.8	1.4	85	3.8	4.01	15	5	68 ± 4
30% Rice	9.1	1.4	85	3.9	4.24	14	6	119 ± 6
60% Rice	9.7	1.3	87	4.1	4.06	16	5	84 ± 1

ABV = Alcohol by Volume; BU = bitterness units.

The beer pH ranged from 4.01 to 4.49 which is within the expected pH range for lager beers (Diakabana *et al.*, 2013). The pH of beer is important when considering the sensory profile, it has been previously reported that low pH beers become more ‘sharp’ and have an increased sour taste (Li *et al.*, 2015; Bamforth, 2001). The intensity of sour taste is related to the total concentration of free and undissociated hydrogen ions (Li *et al.*, 2015). This could provide an explanation for the significant increase in sourness observed by the panel with the 60% maize sample, as the 60% maize beer had the lowest pH (4.01). Nitrogenous compounds in wort and beer have buffering capacity and are important in establishing the pH. Adjuncts such as rice and maize contain low levels of nitrogenous compounds and phytase, the enzyme responsible for the breakdown of phytic acid to release phosphates. Nitrogenous compounds such as peptides play a substantial role as buffers in wort and beer, therefore low nitrogen adjuncts lessen the buffer capacity of the wort. If the buffering capacity is reduced, then assuming other factors that impact beer pH are kept consistent

during fermentation, the increase in hydrogen ion concentration would give rise to an adjunct beer with lower pH (Coote *et al.*, 1976). Therefore, adjuncts lessen the buffering capacity of wort and beer, leading to lower wort and beer pH values (Bamforth, 2001). In this study, the mash pH and wort pH was not measured, however this would be a useful measure in determining if the nitrogenous composition of the adjunct wort impacted the wort pH and subsequent beer pH. Increasing nitrogenous compounds in the wort or reducing the amount of free hydrogen ions when brewing with adjunct material could potentially minimise this off flavour obtained in the 60% maize sample.

The beers produced with unmalted adjuncts presented a lighter colour in comparison to the all-malt beer. Brewing with torrefied rice and maize generated beers of lighter colour relative to the all malt control. It has been previously reported that maize causes a decrease in beer colour proportional to the ratio of its addition, owing to low soluble nitrogen content and therefore less Maillard products which are the main source of beer colour (Mayer *et al.*, 2016; Ombretta *et al.*, 2017).Therefore, balancing soluble nitrogen in wort and beer could potentially increase beer colour to values comparable to an all malt beer.

The hop addition during production was targeted at a final bitterness of 20 BU. However, in practice there was slight variation as analytical bitterness ranged from 14 to 19 BU across the sample set. The determination of bitterness units is a simplistic analytical measurement of iso-α-acids and bittering compounds that can contribute to bitterness. However, bitterness characteristics of beer can differ due to other chemical compounds not determined in this analysis (Oladokun *et al.*, 2016b). Therefore, it is unlikely that the slight variation in bitterness units across the sample set would have a significant effect on the sensory perception of the beers.

Phenolic compounds are important for the sensory experience of beer as polyphenols contribute directly to flavour, astringency, haze, body and fullness of beer (Section 1.8.4). It could be hypothesised that the changes in bitterness intensity and character observed in the sensory analysis of the adjunct beers (Figure 3.1) when including barley could be a result of changes in polyphenolic character in the beer. Previous sensory studies have determined a change in bitterness character and determined longer harsher bitterness characteristics when polyphenolic content increased (Oladokun *et al.*, 2016b; McLaughlin *et al.*, 2008). The polyphenol content of beer is largely related to the raw materials

used, as approximately 75% of polyphenols originate from malted barley, dependent to some extent on hopping strategy and levels (Aron *et al.*, 2010). Consequently, malt substitutes typically decrease the concentration of phenolic compounds. The incorporation of torrefied maize significantly decreased the total polyphenol content (TPC) at 30% adjunct and even more so at 60% adjunct. This finding is in agreement with the work of Fumi *et al.* (2011) where including maize at 86% was reported to decrease the total polyphenol content. The highest TPC was found in the beers produced with malt and barley-based adjuncts (Table 3.5). Similarly to previous investigations the incorporation of unmalted barley reduced the TPC of beer. (Kunz *et al.*, 2012; Yano *et al.*, 2008; van Donkelaar *et al.*, 2016). However, the incorporation of barley (B) increased TPC marginally, indicating that the process applied to the barley has affected the extraction of polyphenols from the grist into wort and subsequent transfer into beer. A limitation to the determination of total polyphenol content is that the method is based on the chelation of phenols with iron and multiple complexing sites are required to produce a reaction (Callemien *et al.*, 2009). Phenolic acids are not measured in this assay as they do not cause an interaction. The polyphenolic fraction of the trial beers was thus further characterised using HPLC-UV-DAD analysis to quantify phenolic acids and polyphenol monomers.

3.3.2.3. Phenolic acid analysis by HPLC-UV-DAD

Hydroxybenzoic acids and hydroxycinnamic acids can provide bitterness and astringency to the organoleptic characteristics of beer. Similarly, (+)-catechin can provide bitterness to beer. Concentrations of the principal phenolic acids and (+)-catechin are summarised for the trial beers in Table 3.6.

Higher concentrations of catechin were observed in the barley-based beers and a significantly higher concentration was identified in beers brewed with barley (B) relative to the all malt control. Although catechin is known to introduce bitterness to a beer, the concentrations obtained in the adjunct beers are lower than the reported flavour threshold of 20 mg/L in beer (Dadic *et al.*, 1973).

Phenolic acid concentrations were typically significantly reduced by the incorporation of adjuncts, or did not significantly change compared to the all-malt control. However, the concentration of vanillic acid significantly increased with 60% barley (A) and 60% wheat incorporation. The analysed phenolic acid concentrations were below the flavour thresholds reported in beer. However,

the presence of multiple polyphenolic compounds can enhance or reduce the intensity of bitterness perception even at sub-threshold levels (Baert *et al.*, 2012; Guadagni *et al.*, 1963). If the polyphenolic compounds behave in a synergistic or antagonistic manner, the perceived intensity can be higher or lower than the sum of the individual intensities. Therefore, a combination of polyphenolic compounds could still influence the bitterness and astringency characteristics determined in the sensory analysis.

Table 3.6. Phenolic acid analysis of adjunct and control beers (mg/L)

	4-HPA	Syringic Acid	Vanillic Acid	4-HBA	Ferulic Acid	p-Coumaric Acid	Catechin
100% Malt	2.30	0.52	1.41	0.59	3.52	2.79	2.60
30% Barley (A)	1.58 **	0.32 **	1.56	0.52	2.55 *	1.60 **	2.01
60% Barley (A)	2.21	0.42	2.07 **	0.70	3.05	2.22 **	2.92
30% Barley (B)	1.33 **	0.21 **	1.11	0.47	1.83 **	1.75 **	3.71 **
60% Barley (B)	1.32 **	0.16 **	1.53	0.48	2.67 **	1.62 **	3.43 **
30% Wheat	1.62 **	0.40	1.48	0.63	2.73 **	1.37 **	0.97 **
60% Wheat	1.56 **	0.56	1.84 **	0.52	3.49	1.11 **	0.80 **
30% Maize	1.66 **	0.31 **	1.06	0.45 **	1.76 **	2.50	0.87 **
60% Maize	1.00 **	0.23 **	0.35 **	0.52	1.94 **	3.11	0.75 **
30% Rice	1.16 **	0.28 **	0.97 **	0.49	2.46 **	1.85 **	1.25 **
60% Rice	2.39	0.32 **	0.70 **	0.85 **	2.11 **	1.57 **	0.94 **

ANOVA with Dunnett's post hoc analysis was used to compare the phenolic acid concentrations. Asterisks indicate samples that were significantly different compared to the all malt control (* p <0.05, ** p < 0.01). Data are the mean of four replicate measurements. 4-HBA = 4-hydroxybenzoic acid; 4-HPA = 4-hydroxyphenylacetic acid

3.3.2.4. *Analysis of flavour-active fermentation volatiles*

The majority of key flavour-active compounds in beer are yeast secondary metabolites produced during fermentation and are composed of aldehydes, esters, higher alcohols and sulphur compounds (Section 1.5.4). The trial beers were analysed using gas chromatography to identify and quantify these flavour active compounds (Table 3.7).

The acetaldehyde concentration was considerably higher in 60% rice beer compared to the sample set. This finding is in agreement with the work of Mayer

et al. (2016) who reported that beer produced with an all rice grist had acetaldehyde concentrations three times over the flavour threshold (10 mg/L). Acetaldehyde is known for imparting a green apple/emulsion paint character as reflected in the attribute description used in the sensory lexicon (Table 3.1). Therefore, the significant increase of green apple flavour identified in the 60% rice sample is likely due to the elevated acetaldehyde concentrations.

Acetaldehyde is produced as a result of decarboxylation of pyruvate and is an intermediate in the formation of ethanol during glycolysis (Stewart, 2017), as discussed in Section 1.5.4. Acetaldehyde concentration can be increased with wort gravity, oxygen concentration, fermentation temperatures and yeast stress (Stewart, 2017). Since most of these conditions were standardised across the trial beers, this could have been related to yeast stress or sluggish fermentation as a result of the low FAN level in the 60% torrefied rice brew (Table 3.4) However, since other adjunct brews, e.g., 60% maize adjunct, were equally FAN constrained but did not result in such high acetaldehyde levels, there were clearly other yeast nutrition factors involved.

Higher alcohols such as n-propanol, isoamyl alcohol and isobutanol can create strong, pungent aromas in beer at high concentrations (>300 mg/L) and can contribute to beer flavour by intensifying alcoholic perception and imparting a warm mouthfeel (Olaniran *et al.*, 2017; He *et al.*, 2014). Aromatic alcohols such as tryptophol and tyrosol are considered to impart undesirable flavours to beer (Pires *et al.*, 2014). The majority of the higher alcohols identified significantly increased with 60% maize and rice incorporation, whilst the concentration of these higher alcohols significantly decreased with the use of barley (A), barley (B) and wheat (Table 3.7). Higher alcohols are biosynthesised by yeast during fermentation by two pathways; the Ehrlich pathway for amino acid catabolism and by means of the anabolic pathway for amino acid synthesis (Liu, 2015). Higher alcohol production is closely related to wort nitrogen composition. When nitrogen content is sufficient, amino acids are transformed into higher alcohols in reactions involving branched-chain amino acid aminotransferase (BCAT) and ethanol dehydrogenase (Zhong *et al.*, 2020). However, when nitrogen sources are low this activates glucose metabolism and forms large amounts of pyruvate and consequently higher alcohols (Zhong *et al.*, 2020).Therefore, the increase in higher alcohols in 60% adjunct beers made from torrefied rice or maize could be explained by the very low wort FAN concentrations (Table 3.4).

Table 3.7. Fermentation volatile composition of adjunct beers versus an all malt control

Compound	100% Malt Control	Barley (A)		Barley (B)		Wheat		Maize		Rice	
		30%	60%	30%	60%	30%	60%	30%	60%	30%	60%
Acetaldehyde (mg/L)	2.99	2.52	1.89	2.17	3.72	3.32	7.11 **	6.73 **	4.11 *	3.71	22.96 *
Propanol (mg/L)	11.47	9.32 **	9.89 **	8.92 **	8.65 **	10.21	10.96	10.68	8.82 **	9.22 **	9.23 **
Isobutanol (mg/L)	13.76	11.9	13.92	12.11	13.6	12.11	16.19 **	13.75	21.43 **	13.68	20.00 **
Isoamyl Alcohol (mg/L)	63.24	52.65 **	56.47 **	53.36 **	55.81 **	53.72 **	62.89	57.86 **	72.83 **	57.21 **	75.29 **
Phenethyl Alcohol (mg/L)	35.73	23.74	31.69	28.3	32.99	32.95	33.68	34.67	37.83	34.35	35.28
Tyrosol (mg/L)	19.31	12.68 **	13.36 **	12.41 **	13.18 **	14.36 **	18.64	15.32	31.64 **	14.03 **	32.92 **
Tryptophol (mg/L)	3.71	1.64	6.51 **	2.25	3.18	2.18	7.06 **	2.75	8.09 **	2.66	8.60 **
Ethyl Acetate (mg/L)	19.59	12.19 **	12.03 **	11.87 **	11.64 **	13.56 **	15.92	13.55 **	11.45 **	10.83 **	12.22 **
Isoamyl Acetate (mg/L)	1.17	0.64 **	0.65 **	0.57 **	0.78 **	0.71 **	0.97	0.83 **	0.58 **	0.76 **	0.67 **
Isobutyl Acetate (mg/L)	0.15	0.10 **	0.09 **	0.09	0.12 **	0.09 **	0.13	0.12	0.11 **	0.10 **	0.11 **
Phenethyl Acetate (mg/L)	0.57	0.27 **	0.33 **	0.31 **	0.44	0.38 **	0.59	0.45	0.34 **	0.43	0.42
Ethyl Hexanoate (mg/L)	0.11	0.06 **	0.08 **	0.06 **	0.07 **	0.08 **	0.1	0.08 **	0.09	0.1	0.11
Ethyl Octanoate (mg/L)	0.26	0.18 **	0.19 **	0.19 **	0.19 **	0.19 **	0.21	0.19 **	0.19 **	0.19 **	0.19 **
Ethyl Butyrate (mg/L)	0.07	0.04 **	0.04 **	0.03 **	0.03 **	0.05 **	0.06	0.05 **	0.03 **	0.04 **	0.04 **
Ethyl Propanoate (mg/L)	0.013	0.009 **	0.009 **	0.010 **	0.009 **	0.009 **	0.012	0.009 **	0.009 **	0.008 **	0.009 **
Ethyl Isobutyrate (µg/L)	0.83	0.61 *	0.76	0.7	0.61 *	0.62 *	0.69	0.68	0.72	0.66	0.74
Ethyl Pentanoate (µg/L)	0.08	0.08	0.12 **	0.07	0.06	0.09	0.13 **	0.07	0.08	0.17 **	0.21 **
Ethyl Heptanoate (µg/L)	2.07	1.92	1.94	1.36 **	1.42 **	1.52 **	1.87	1.51 **	1.31 **	2.25 **	2.52 **
3-methyl-2-pentanone (µg/L)	2.63	1.9	2.51	1.8	1.3	2.08	2.2	3.08	3.18	2.15	1.8

ANOVA with Dunnett's post hoc analysis was used to compare the fermentation volatile concentrations. Asterisks indicate samples that were significantly different compared to the all malt control (* p <0.05, ** p < 0.01). Results are means of four replicate runs.

Esters are one of the most important groups of flavour compounds produced by yeast (Pires *et al.*, 2014). Ester production is affected by many fermentation parameters such as yeast strain, fermentation temperatures and changes in fermentation medium. An important parameter is the carbon-to-nitrogen ratio of the fermentation medium. The data in Table 3.7 indicate that all identified acetate and ethyl esters reduced in concentration with the addition of any unmalted adjuncts. Previous studies have determined that decreasing FAN concentration decreases the concentration of acetate esters and ethyl esters such as ethyl octanoate and decanoate (Saerens *et al.*, 2008). Similar patterns were observed in this study, as ester concentrations decreased in the beers with corresponding decreases in FAN concentration. Improving the nitrogen composition and concentration could minimise significant variation in the volatile profile observed with adjunct use. Interestingly, the 60% unmalted wheat beer had the most similar volatile profile to the 100% malt control, with significantly different concentrations only observed for acetaldehyde, tryptophol, isobutanol and ethyl pentanoate.

To explore the reasons for significant sensory differences in sweetcorn/cooked vegetable aroma amongst trial beers (Table 3.2), their sulphur volatile profiles were determined using GC-PFPD (Table 3.8). A range of sulphur compounds (e.g., sulphides, thiols, thioesters) are known for adding cooked vegetable and sweetcorn characters to beer. Sulphur volatiles have low flavour thresholds and can make a sensory impact at low concentrations. Sulphur compounds can originate from the raw materials, Maillard browning reaction and yeast metabolism during fermentation (Miracle *et al.*, 2005).

Table 3.8. Selected sulphur compound concentrations in adjunct and control beers

Compound	100% Malt Control	Barley (A) 30%	Barley (A) 60%	Barley (B) 30%	Barley (B) 60%	Wheat 30%	Wheat 60%	Maize 30%	Maize 60%	Rice 30%	Rice 60%
Dimethyl Sulphide (µg/L)	31.76	20.83 **	15.27 **	17.56 **	16.09 **	15.25 **	18.28 **	22.33 **	15.26 **	13.04 **	8.85 **
Unknown Sulphur (As/Ais)	0.7	0.86	0.74	0.63	0.74	0.58	0.66	0.74	0.66	1.28 **	0.68

As/Ais denotes the peak area of the sample standardised to the peak area of the internal standard. ANOVA with Dunnett's post hoc analysis was used to compare the sulphur compound concentrations. Asterisks indicate samples that were significantly different compared to the all malt control (** $p < 0.01$). Results are means of four replicate runs.

Dimethyl sulphide (DMS), which imparts a tinned sweetcorn aroma to beer, was identified in all beers with the highest concentration obtained in the all-malt beer. The decrease in DMS is expected with the use of unmalted adjuncts and is due to their reduced contents of DMS precursor, S-methylmelthionine (SMM). SMM is developed during germination of the grain and is then broken down to DMS during thermal processes such as kilning (Bamforth, 2014). In the present data set, DMS concentrations did not directly correlate to the trend of sweetcorn/cooked vegetable aroma in the sensory evaluation. In part this may be because concentrations in all beers were below typical threshold concentrations in beer (30 µg/L). The 30% rice beer was rated by the panel on average as highest in the sweetcorn/cooked vegetable attribute. Consequently, the sensory attribute scored by the panel is not believed to be simply due to the DMS content of the beer samples. Another unidentified sulphur compound was present in the beers. The concentration of this unknown compound was significantly higher in the 30% rice adjunct beer which scored most highly for the sweetcorn/cooked vegetable attribute. The unknown compound (Table 3.8) had a retention index of 858 on the ZB1 column. Authentic standards of diethyl sulfide, ethanethiol, ethylthioacetate, S-methylthioacetate, thiazole, methional, methionol and dimethyl trisulfide were run under identical conditions but did not show chromatographic similarity to the unknown compound. Further work is thus required to identify this sulphur compound and understand the cause for a higher concentration present in the 30% rice beer.

3.3.3. Principal component analysis of beer sensory and analytical data

Principal component analysis was performed to visualise the relationship between the samples, sensory attributes and chemical composition. Figure 3.2 shows a biplot of PC1 and PC2 which accounted for 51.70% of the variance in the data set. PC1 explained 29.62% of the variation. A positive loading on PC1 corresponds with high concentrations of sweet and fruity sensory attributes specifically banana aroma and flavour exemplified by the 100% malt and 60% unmalted wheat beers. These sensory attributes can be assigned to high concentrations of isoamyl acetate and isobutyl acetate that are known to impart banana aroma (Olaniran *et al.*, 2017), as well as ethyl propanoate and ethyl butyrate imparting fruity and sweet notes (Pires *et al.*, 2014).

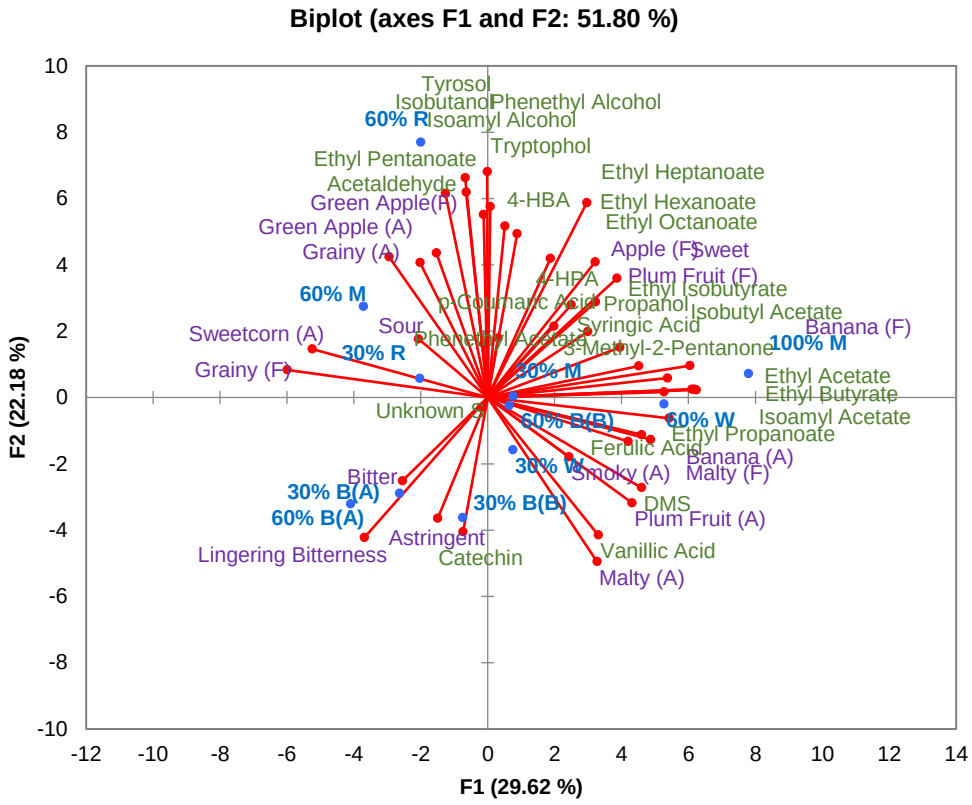


Figure 3.2. Principal Component Analysis (PCA) of normalised sensory and analytical profiles on principal components 1 and 2.

Samples are in blue, chemical compounds in green and sensory attributes in purple font. Sample Codes: M (maize); R (rice); W (wheat); B(A) (barley(A)); B(B) (barley (B)). Sensory attributes: (A) aroma; (F) flavour.

On the upper right quadrant of the biplot, ethyl heptanoate, ethyl hexanoate and ethyl octanoate are closely located together. These esters correspond on the biplot with apple (ethyl hexanoate) and plum fruit (ethyl octanoate) flavour sensory attributes. Negative loadings on PC1 correspond with high perception of grainy flavour and sweetcorn aroma exemplified by the 30% torrefied rice and 60% torrefied maize beers. In the lower left quadrant of the biplot, the bitterness and astringent attributes are co-located with the 60% barley beers and the catechin concentration. This suggests that catechin may have contributed to the perception of bitterness and astringency in this study, since its concentration co-varied with their sensory ratings across the sample set.

The positive loading of PC2 corresponds with the characteristics of 60% rice adjunct beers and the impacts of low wort FAN on fermentation such as high acetaldehyde and higher alcohol concentrations. Although no sensory attributes were found to directly associate with the higher alcohols, the positive loading of PC2 is associated with green apple aroma and flavour corresponding to high

concentrations of ethyl pentanoate and acetaldehyde, both of which are described sensorially as imparting crisp apple aroma.

The samples 30% maize, 30% wheat and 60% barley (B) plotted close to the centre of the biplot because these samples could not be described by any key attributes or compounds. The biplot highlights that the sensory properties of the 60% unmalted wheat beer came closest to matching the sensory and analytical profile of the 100% malt control.

3.4. Conclusions

This study showed that the use of five different unmalted cereal adjuncts influenced the wort physiochemical properties and consequently the finished beer specifications. However, sensory and analytical analysis showed that even at incorporation rates of up to 60%, the sensory flavour impacts of unmalted adjuncts were more subtle and nuanced than was expected. This suggests that, in many cases, unmalted adjunct incorporation rates could be pushed higher, from a flavour perspective alone. Furthermore, the present study adopted a strategy of looking at the direct flavour impacts of using high levels of unmalted adjunct materials, without balancing the C:N ratio of wort, or micronutrient levels. Such strategies would enable fermentation-related flavour imbalance to be improved. Overall the results presented are positive in terms of future prospects for unmalted adjunct usage in brewing. In generic terms, 30% unmalted adjunct usage produced beers that were characterised more by a lack of malty or sweet, fruity character, than by the presence of any negative attributes. Whether this is regarded as positive or negative depends principally on the beer style being produced and the target market. At 60% adjunct level unmalted barley (A) introduced increased bitter, astringent and lingering bitterness characteristics, although these sensations were less pronounced in the processed sample unmalted barley (B). Beers containing 60% torrefied maize were characterised by becoming more sour, grainy and with sweetcorn aroma, whilst 60% torrefied rice beers were largely characterised by ‘defects’ of low nitrogen fermentation—i.e., high levels of higher alcohols and acetaldehyde—as well as the absence of sweet malty character. This is consistent with prior knowledge that rice itself imparts neutral clean flavour characteristics. It is recognised that torrefied adjuncts are relatively expensive grist materials and were used in the current investigations solely to facilitate processing without an additional cereal cooking step. Interestingly, this study

identified that use of unmalted wheat at 60% of grist came closest to the sensory profile of an all malt control beer. Although there are many other factors to consider when formulating beers, such as colloidal stability for example, this study suggests that using unmalted wheat in conjunction with other unmalted cereals might help to retain some 100% malt beer flavour characteristics, if that is desired.

This study advances knowledge of the sensory impacts of unmalted adjunct usage and identified PCA correlations between sensory attribute ratings of adjunct beers and their underlying chemical flavour composition (Figure 3.2). However, it should be borne in mind that correlation does not prove causality and there are likely still to be unknown compounds which contributed significantly to the sensory experience of adjunct beers in this study. Investigating the flavour impacts of high levels of unmalted adjuncts when balancing C:N ratio of wort and micronutrient levels could determine if flavour ‘defects’ caused by low FAN could be eliminated. It would be interesting to investigate further the classes of compound responsible for the lingering bitterness and astringency associated with high levels of unmalted barley.

Chapter 4 A ‘balanced FAN’ approach to flavour matching when using unmalted cereal adjuncts

4.1. Introduction

Nitrogen compounds in wort are fundamental for effective fermentation and beer quality and stability. They play an important role in beer flavour and the formation of flavour active compounds. The levels of soluble nitrogen compounds in wort and beer are strongly affected by raw materials and the use of unmalted cereal adjuncts can adversely affect both the content and composition of nitrogenous compounds. Unmalted adjuncts have low levels of enzymes with key brewing functionality (amylolytic, cytolytic and proteolytic enzymes) as previously discussed in Section 1.3.4. Subsequently, the incorporation of unmalted adjuncts typically dilutes the soluble nitrogen content, adversely influencing the wort quality.

Free amino nitrogen (FAN) is a measurement of nitrogenous compounds that can be assimilated and metabolised by yeast during fermentation, consisting of ammonium nitrogen, free amino acids and small peptides (Kok *et al.*, 2019). FAN content can be used as an indicator of the potential for healthy yeast growth and can determine fermentation efficiency and viability; low FAN levels can lead to incomplete or sluggish fermentations (Dlamini *et al.*, 2015). The minimum wort FAN concentration is generally agreed to be around 130 mg/L with a minimum varying between 100 mg/L to 140 mg/L for adequate fermentation of normal gravity wort (10-12 ºP) (Hill *et al.*, 2019). For adequate attenuation of high-gravity wort (16 ºP), increased levels of FAN are required, with FAN wort concentration recommended over 160 mg/L (Lekkas *et al.*, 2007; McCaig *et al.*, 1992). FAN is also an important index for final beer quality as the wort nitrogen content and composition influences the biosynthesis of fermentation volatiles that affect the flavour and stability of beer (Lekkas *et al.*, 2014).

When brewing with unmalted grains at high incorporation rates, there are a few approaches that can be used to enhance soluble nitrogen. The specification of the base malt can influence the soluble nitrogen composition. Higher nitrogen and well-modified malts can be used in an infusion mashing system to balance the dilution of nitrogen due to adjunct usage. Higher nitrogen barleys are more likely to develop higher levels of soluble nitrogen during malting and mashing, thus producing higher levels of wort FAN (Agu, 2003; Sá *et al.*, 2005). Balancing

nitrogen dilution with selection of a well modified malt can be effective at lower incorporation rates of adjuncts but becomes less viable at high adjunct percentages as mixing unmalted barley with malt can have an inhibitory effect on malt endopeptidases, resulting in a decrease in wort FAN (Hill *et al.*, 2019). Unmalted barley has been shown to contain inhibitors of the cysteine proteases and metalloproteases which are necessary to perform proteolysis during mashing (Jones, 2005).

Another approach to increasing soluble nitrogen when including unmalted adjuncts is to alter the mashing schedule. It is considered that under modified malts and raw grains can benefit from a protein rest at lower temperatures at the start of a mash profile. Barley malt proteases have optimal activity at a temperature range of 45-55 °C, these enzymes are heat sensitive and are deactivated at 70-75 °C (Sammartino, 2015; Kok *et al.*, 2019). Including a proteolytic rest in the mashing schedule may be relevant when wort FAN content is a concern. However, malt protease activities are greatly diminished by kilning and therefore a proteolytic rest will only have a small effect (10%) on the wort FAN concentration (Sammartino, 2015).

There have been several studies that utilised exogenous enzymes added into the mash when brewing with raw grains such as barley and sorghum. The addition of exogenous enzymes can facilitate optimal biochemical changes to occur during mashing (Steiner *et al.*, 2012). Supplementing proteolytic enzymes in the mash can be used to intensify the proteolysis during the mash leading to increased assimilable nitrogen (Le *et al.*, 2001). Schnitzenbaumer *et al.* (2014) applied the enzyme cocktail Ondea Pro to a mash consisting of 40% unmalted oats. The application of Ondea Pro, which contains endoproteases, significantly increased the wort FAN concentration by 55.9% when used in a 40% unmalted oat brew, demonstrating that exogenous enzymes can be used to successfully increase FAN concentrations in adjunct brews.

Another approach to increasing assimilable nitrogen in wort is to supplement the cold wort at the start of fermentation. Yeast extracts (typically water soluble autolysates derived from spent yeast) can provide a rich source of nitrogen whilst commercial yeast foods are available which typically consist of ammonium ions together with other inorganic nutrients which can improve fermentation performance and increase soluble nitrogen (Gibson, 2011; Hill *et al.*, 2019). Le et al. (2001) found that supplementing rice adjunct wort with yeast extracts improved fermentation and created beer with better sensory properties.

The present study investigated brewing with unmalted wheat, barley (A) and barley (B) at different grist proportions (15, 25, 35 and 45%) whilst using two different approaches to enhance soluble nitrogen. The first strategy was to supplement proteolytic enzymes in the mash to increase proteolysis during mashing and the second strategy was to supplement the cold wort with a commercially available yeast nutrient before the start of fermentation.

Firstly, supplementation treatment rates and mash conditions required to achieve target FAN levels were determined through laboratory mashing trials. Then by developing protocols to achieve target wort FAN, an in-depth analysis of the wort and finished beer could be performed to obtain a more objective impression of the direct flavour impacts of each unmalted adjunct. Thus, we could address the question as to whether successfully increasing FAN levels could improve flavour 'defects' of low nitrogen fermentation- i.e., high levels of higher alcohols and acetaldehyde, which were reported in Chapter 3 when brewing with 60% incorporation of unmalted adjuncts (Section 3.3.2.4).

4.2. Materials and methods

The brewing materials and analytical methods applied to the wort and beer samples used in this study are described in Chapter 2.

4.2.1. Procedure for laboratory mashing to adjust wort temperature profile

Wort samples were produced with three different unmalted cereal adjuncts (barley (A), barley (B) and wheat) at differing adjunct concentration (0, 15, 25, 35 and 45%) using an R8 mash bath (1-Cube, Havlickuy Brod, Czech Republic). All grist materials were milled using a laboratory DFLU disc mill (Buehler Miag, Uzwil, Switzerland). For each wort sample, a liquor to grist ratio of 2.5:1 was used by mixing 250 ml of reverse osmosis (RO) water at 65 °C to 100 g of the mixed grist weighed into a metal mashing beaker. The specific gravity target for the wort produced was 16 °P. Three temperature profiles were compared to determine a suitable mash profile that could be used in the pilot scale production of beer (Figure 4.1). The longest mash scheme, as used in Chapter 3 (Section 3.2.1), was as follows: 55 °C for 20 min, 65 °C for 90 min, 72 °C for 10 min and 78 °C for 5 min (140 min). The second reduced mashing scheme was as follows; 65 °C for 90 min, 72 °C for 10 min and 78 °C for 5 min (115 min). The third mashing schedule was reduced further to 65 °C for 60 min, 72 °C for 10 min

and 78 °C for 5 min (85 min). Temperature increases between stands were ramped at a rate of between 1.4–1.5 °C/min. Wort samples were then cooled to 20 °C and adjusted to 500 g with RO water and filtered using filter paper (Whatman, 2555 ½, 320 mm, Sigma-Aldrich, UK). The specific gravity and free amino nitrogen content of each wort sample was determined according to the method described in Section 2.5.3.

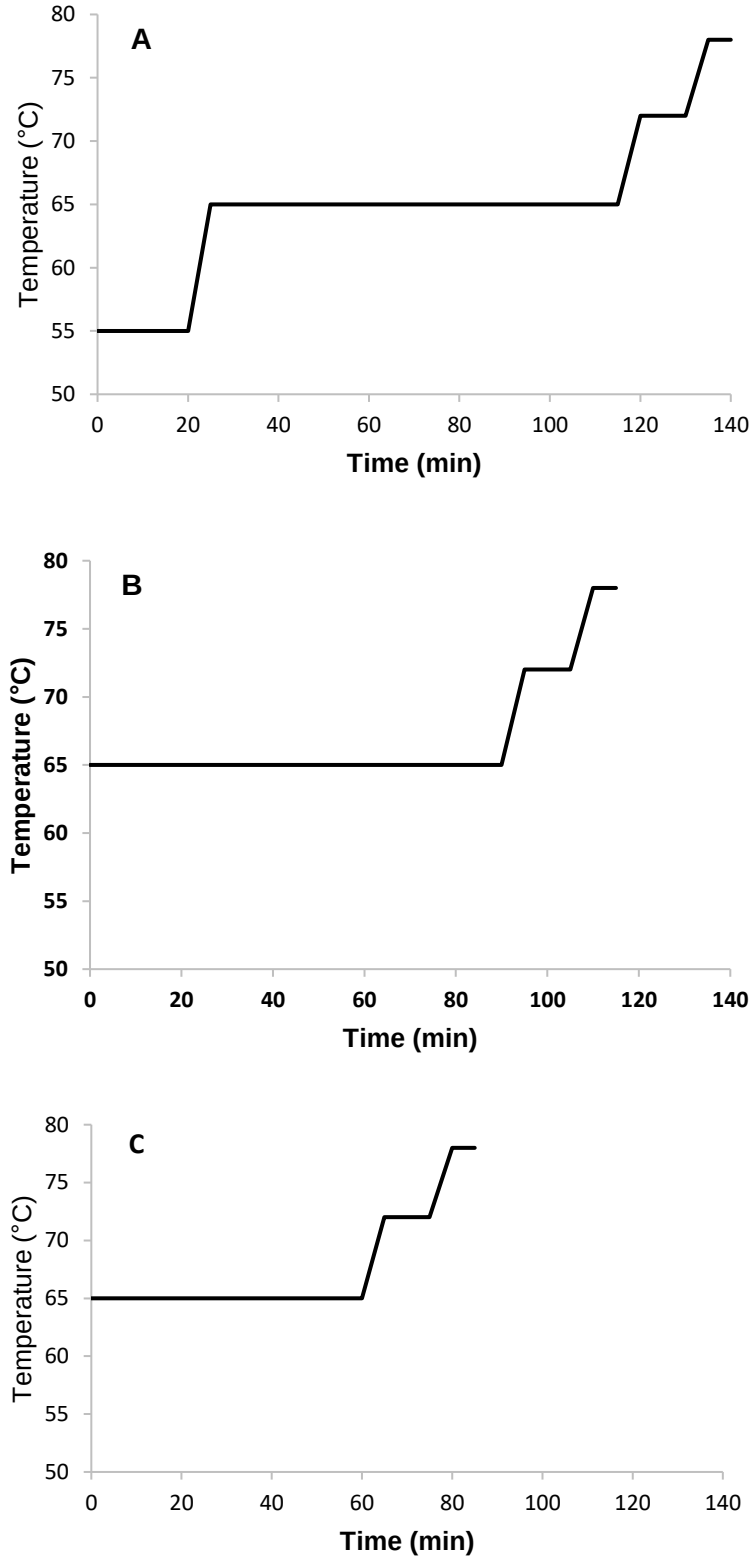


Figure 4.1. The mashing profiles used to investigate impacts of mash schedule on wort FAN

A) 140 min extended profile B) 115 min with removal of protein rest C) 85 min short mash schedule

4.2.2. Procedure for upscaling mash schedule adjustment to pilot scale

In order to investigate the effect of reducing the mashing scheme described in Section 4.2.1 on a larger scale than laboratory mashing, two comparative wort samples were produced using a 40 L pilot scale Briggs brewline at the University of Nottingham. A grist incorporating 25% barley (B) was used for both brews. The wort samples were produced in the same manner with only a change in the mash schedule. One brew consisted of the longest mash scheme (140 min) and the other used the shortest mash scheme (85 min) as shown in Figure 4.1. The grist composition was weighed and mixed before being milled with a Roppi-250 roller mill (Robix, Hungary) at a gap setting of 0.4 mm. For each brew, a liquor to grist ratio of 2.5:1 was used to directly compare the laboratory mashing scheme. Samples were then taken to measure the FAN concentration after lautering and before the boil to evaluate this at a comparable time point to the laboratory mashing trial. The specific gravity and free amino nitrogen content of each wort sample was determined according to the method described in section 2.5.3.

4.2.3. Determining YeastLife and FAN Boost treatment rates required for adjunct worts

Wort samples were produced using different incorporation rates of barley, barley (B) and wheat (Table 4.1) according to the method described in Section 4.2.1 using an 85 min mash profile (65 °C for 90 min, 72 °C for 10 min and 78 °C for 5 min).

Table 4.1. Grist composition used for determining YeastLife and FAN Boost treatment rates

	FAN supplementation	Malt (%)	Wheat (%)	Barley (A) (%)	Barley (B) (%)
35% B(A)	YL	65	0	35	0
45% B(A)	YL	55	0	45	0
35% B(B)	YL	65	0	0	35
45% B(B)	YL	55	0	0	45
35% W	YL	55	35	0	0
45% W	YL	65	45	0	0
35% B(B)	FB	65	0	0	35
45% B(B)	FB	55	0	0	45

B(A)= barley (A), B(B)= barley (B), W= wheat, YL= YeastLife, FB= Fan Boost

In order to determine the amount of YeastLife required to increase FAN concentration over 200 mg/L, YeastLife was added to the cool wort samples to produce dosing concentrations of 0.5, 0.65, 0.75, 0.85 and 1 g/L. FAN boost was added at mash-in, immediately before the mash profile was started at treatment rates of 0, 0.3, 0.5, 0.6, 0.9, 1, 1.2, 1.5, 1.8, 2 and 3 g/kg. All YeastLife and FAN boost treatments were performed in triplicate. The specific gravity and free amino nitrogen content of each wort sample was determined according to the method described in Section 2.5.3.

4.2.4. Pilot scale procedure for the production of FAN supplemented adjunct beers and an all-malt control brew

Twenty three adjunct based beers were produced using a 40 L Briggs pilot scale brewline at the University of Nottingham. A set of fifteen beers were brewed using unmalted wheat, barley (A) and barley (B) at different grist compositions (one replicate for 15, 25, 35 and duplicate 45%) and two all-malt controls, subsequently producing seventeen wort and beer samples (Table 4.2).

This set of beers included the addition of YeastLife to worts produced with adjunct incorporation rates of 25, 35 and 45% to achieve a target wort FAN concentration over 200 mg/L. The second set of beers followed the same brewing recipe and methodology; however, FAN boost was used to achieve a target wort FAN concentration over 200 mg/L. An all-malt control and four barley (B) beers with FAN boost additions (25, 35 and 45%) were produced. A repeated brew of 45% barley (B) with YeastLife addition was produced in this trial to observe the consistencies across the two trials (Table 4.2). The grist materials were weighed and mixed before being milled with a Roppi-250 roller mill (Robix, Hungary) at a gap setting of 0.4 mm. The grist to liquor ratio used in the mash was 2.5:1 and the mash-in temperature was 65 °C with addition of 90 mg/L calcium chloride. When FAN boost was included in the recipe, a laboratory pre-determined dosage was added at the point of mash-in to the wet mash. This was followed by an 85 min mash profile (65 °C for 90 min, 72 °C for 10 min and 78 °C for 5 min) selected for the study in Section 4.2.1 and Section 4.2.2. The mash was separated using a lauter tun vessel and 8 L of sparge water was passed through the grain bed. The resulting wort was boiled with T90 pellets (variety: Zeus) for 60 min with a target final bitterness of 20 BU. The wort was adjusted with brewing liquor to 16 °P at the end of boil in the kettle. When YeastLife was included in the sample, the determined amount required from the

FAN calculation was added after dilution in the kettle. The wort was then cooled and transferred into a stainless-steel conical fermenter (The Grainfather, New Zealand) and fermented with SafLager W34/70 dried yeast (1 g/L addition rate) at 15 °C for 10-12 days in line with the recommended fermentation temperature for W34/70 yeast (12-18 °C), followed by maturation at 4 °C for 4 days.
Wort samples were taken from the kettle before YeastLife was added and then final wort samples were taken from the cold wort at the point of transfer to fermenter. Samples of green beer after fermentation were taken for analysis and the beer was also packaged in 500 ml PET brown bottles, bottle conditioned (3 g of dextrose monohydrate (The Homebrew shop, Hampshire, UK) per bottle) at 20 ˚C for 3 weeks and subsequently stored at 4 ˚C.

Table 4.2. Grist composition and FAN supplementation treatments for the production of adjunct beers and control

	Malt (kg)	Barley (A) (kg)	Barley (B) (kg)	Wheat (kg)	YeastLife Treatment (g/L)	FAN Boost Treatment (g/kg)
100% Malt (1)	7.00	-	-	-	-	-
100% Malt (2)	7.00	-	-	-	-	-
15% B(B)	5.95	-	1.05	-	-	-
25% B(B)YL	5.25	-	1.75	-	0.50	-
35% B(B)YL	4.55	-	2.45	-	0.80	-
45% B(B)YL (1)	3.85	-	3.15	-	1.00	-
45% B(B)YL (2)	3.85	-	3.15	-	0.90	-
15% B(A)	5.95	1.05	-	-	-	-
25% B(A)YL	5.25	1.75	-	-	0.30	-
35% B(A)YL	4.55	2.45	-	-	0.30	-
45% B(A)YL (1)	3.85	3.15	-	-	0.40	-
45% B(A)YL (2)	3.85	3.15	-	-	1.00	-
15% W	5.95	-	-	1.05	-	-
25% WYL	5.25	-	-	1.75	0.30	-
35% WYL	4.55	-	-	2.45	0.60	-
45% WYL (1)	3.85	-	-	3.15	1.20	-
45% WYL (2)	3.85	-	-	3.15	1.00	-
100% Malt (3)	7.00	-	-	-	-	-
25% B(B)FB (1)	5.25	-	1.75	-	-	0.60
25% B(B)FB (2)	5.25	-	1.75	-	-	0.60
35% B(B)FB	4.55	-	2.45	-	-	0.70
45% B(B)FB	3.85	-	3.15	-	-	1.33
45% B(B)YL (3)	3.85	-	3.15	-	1.00	-

B(A)= barley (A), B(B)= barley (B), W= wheat, YL= YeastLife, FB= FAN Boost

The wort and beer pH, total polyphenol content, total proanthocyanidin content, fermentable carbohydrates, total nitrogen and mineral composition by ICP-MS were measured according to the methods described in Section 2.4. The beer samples were analysed for bitterness units, volatile fermentation compounds by SPME-GC-MS/MS and HS-GC-FID and phenolic compounds by HPLC according to methods described in Section 2.4.

4.2.5. Statistical analysis

Statistical analysis was performed using XLSTAT. One-way ANOVA with post hoc multiple comparisons Dunnett's test were used to compare adjunct wort and beer samples to the all-malt control for each of the analytical parameters determined. Analysis of variance (ANOVA) with a mixed model was used to evaluate significant effects of adjunct type, adjunct incorporation rate and FAN supplementation methods. Principal Component Analysis (PCA) biplots were produced to provide a visual depiction of the variance between the adjunct wort and beer samples in terms of the analytical measurements made on them. Significance was derived at $p = 0.05$.

4.3. Results and Discussion

4.3.1. Preliminary laboratory wort trials

4.3.1.1. Selection of mash schedule for use in the pilot scale brewing study

Before brewing with adjuncts using the pilot scale set up, it was necessary to establish a suitable mashing schedule. In Chapter 3, a 140 min multi-step mash profile (55 °C for 20 min, 65 °C for 90 min, 72 °C for 10 min and 78 °C for 5 min) was used (Section 3.2.1). The multi-step mash includes a 20 min proteolytic rest at 55 °C and an extended saccharification rest of 90 min at 65 °C. This extended mash profile demands time as well as generating higher energy costs. Shortening the mash profile and removing the proteolytic step is common practice when aiming to minimise energy and production costs.

Three different mash profiles were investigated are shown in Figure 4.1 (Section 4.2.2). The FAN concentrations were determined for wort samples with different barley (B) concentrations (25, 35 and 45%) with three different mash profiles as shown in Figure 4.2.

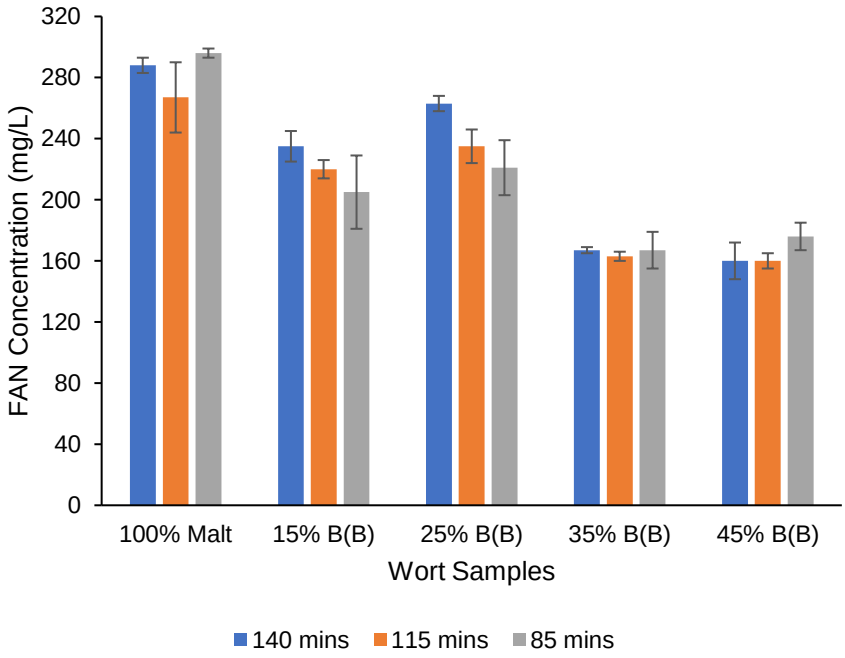


Figure 4.2. Wort FAN concentration with differing adjunct usage and total mash schedule time

Data are the mean of three replicate FAN measurements.

The FAN concentration as shown in Figure 4.2 only varied by a maximum of 20% with changing mash profile. A decrease in FAN with shortening mash stand time was observed at 15% and 25% barley (B) incorporation rates whereas at 35% and 45% barley (B) incorporation FAN levels were considerably reduced but there was no impact of stand time. To understand the implications of the mashing schedule on a larger (40 L) scale, the FAN concentration of two comparative 25% barley (B) wort samples produced with the longest and shortest mash schedule were measured. The FAN concentrations differed by 10% when comparing the 140 min (258 mg/L) and 85 min (221 mg/L) even at this larger scale. This result agrees with the findings of Kühbeck et al. (2005) who reported that increasing mashing time only increased the FAN concentration by up to 17%. Therefore, proceeding with a long mash schedule does not provide the FAN compensation that is required at high adjunct incorporation and the FAN concentration gain would not outweigh the economic and environmental benefit that occurs from shortening the mash profile. For this reason, the following mash profile of 85 min (65 °C for 60 min, 72 °C for 10 min and 78 °C for 5 min) was used for the pilot study brewing trials throughout this chapter.

4.3.1.2. Optimisation of YeastLife and FAN Boost dose rates for pilot scale trials

In preparation for the production of adjunct based beers, laboratory trials were conducted to determine the YeastLife dosing required to increase FAN concentration over the targeted 200 mg/L. YeastLife was added to the cool, clarified wort samples at different concentrations and the resultant FAN concentration was determined. Figure 4.3 shows the increase in FAN concentration when adding YeastLife to wort samples consisting of 35% and 45% barley (A), barley (B) or wheat. The addition of YeastLife successfully increased wort FAN concentration, however, the adjunct type and incorporation rate impacted the effectiveness of YeastLife, as shown by the fact that wheat worts required less YeastLife to increase the FAN concentration compared to barley worts. This result was unexpected as the yeast nutrient is simply adding nitrogen to the wort, thus it would be expected the addition of YeastLife should increase FAN consistently with different types of adjuncts incorporated. However, this data enabled accurate additions to be calculated for each adjunct wort produced in the nanobrewery trial.

Similarly, laboratory trials were conducted to determine the FAN boost dose required to increase FAN concentration. Figure 4.4 shows the increase in FAN concentration when adding FAN boost to mash samples produced with AB. The dose rates of FAN Boost were only determined for barley (B) based wort samples as FAN Boost was only used in the barley (B) adjunct brews in this study (Table 4.2). Similarly to YeastLife, the adjunct grist percentage influenced the effectiveness of FAN boost as the 45% barley (B) samples required higher FAN boost dosage to achieve target FAN compared to 25% barley (B).

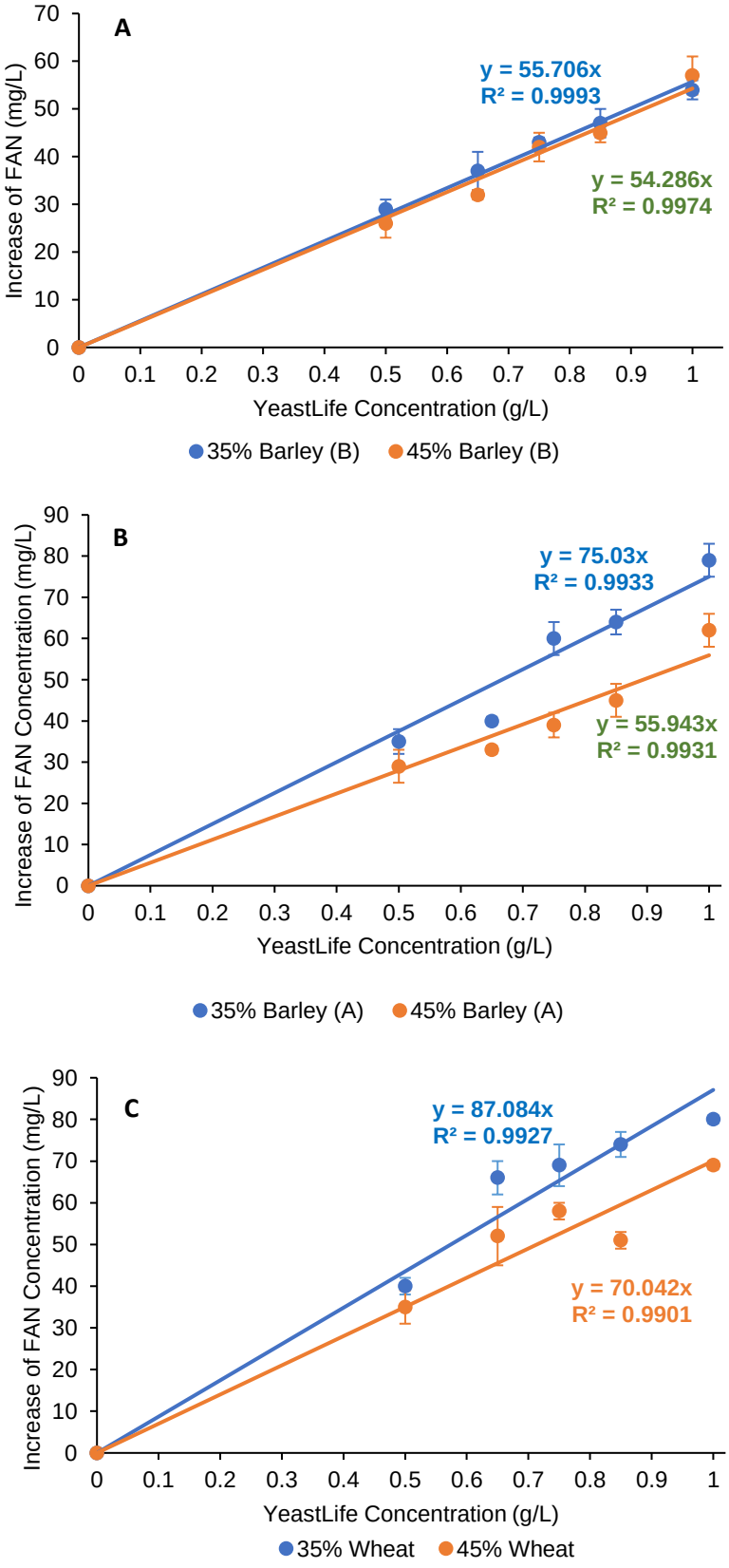


Figure 4.3. The impact of different YeastLife addition rates on the increase in FAN concentration

A) Barley (B), B) Barley (A) and C) wheat. Results are the mean of triplicate wort samples.

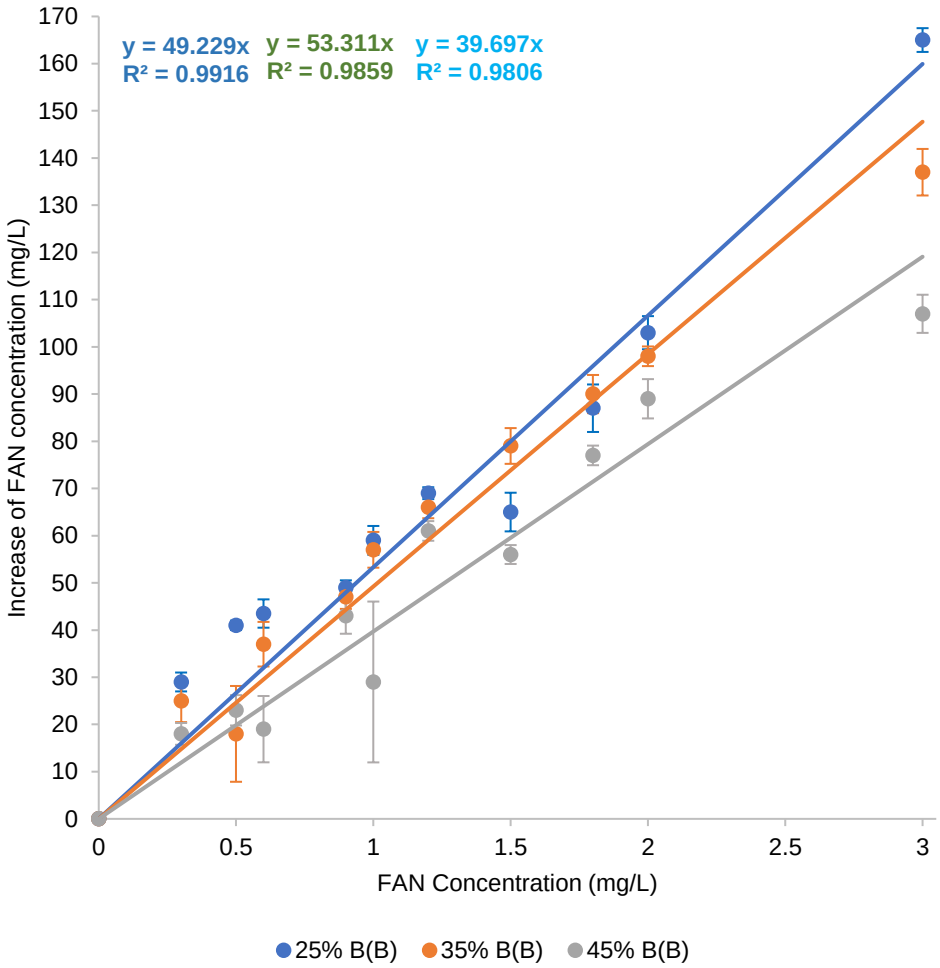


Figure 4.4. The impact of FAN Boost addition rate on wort FAN concentration at three grist levels of barley (B)

Results are the mean of triplicate wort samples.

4.3.2. Pilot brewing trials (40 L)

4.3.2.1. Extract, Carbohydrates and Fermentation

Extract is one of the most important measurable wort attributes as it gives an indication of the sugar content of the wort which has an impact on the potential alcohol by volume of the resulting beer.

In this study, the wort was adjusted with brewing liquor after the boil for each wort sample to yield a target final extract of 16 °P. In practice this resulted in wort samples with an extract content between 15.1-16.8 °P, the variation in the wort extract shown in Table 4.3 is not owing to differing raw materials and is a result of sources of error during dilution in the brewhouse. This was due to the difficulty of accurately measuring the volume of wort in the pilot brewery kettle, which made accurate calculations for dilution somewhat challenging.

Table 4.3. Specific gravity and fermentation parameters for each brew

	FAN Supplementation	Extract (°P)	Final Gravity (°P)	Concentrated ABV (%)	Apparent Attenuation (%)
100% Malt	-	15.9 ± 0.5	2.4 ± 0.3	7.39 ± 0.23	85
15% B(A)	-	15.6	2.8	6.83	82
25% B(A)	YL	15.7	3.6	6.63	77
35% B(A)	YL	16.8	3.6	7.40	79
45% B(A)	YL	15.6 ± 0.5	4.2 ± 0.1	6.32 ± 0.04	73
15% B(B)	-	16.6	3.2	7.43	81
25% B(B)	YL	16.2	3.6	6.96	78
35% B(B)	YL	16.3	4.0	6.81	75
45% B(B)	YL	15.5 ± 0.4	4.2 ± 0.1	6.34 ± 0.06	73
25% B(B)	FB	16.0 ± 0.6	3.2 ±	6.92 ±	80
35% B(B)	FB	15.8	3.8	6.6	76
45% B(B)	FB	16.2	4.3	6.42	73
15% W	-	15.6	3	7.02	81
25% W	YL	16.1	3	6.98	81
35% W	YL	16.3	3.5	7.06	79
45% W	YL	16.1 ± 0	3.8 ± 0.2	6.89 ± 0.22	76

B(A)= barley, B(B)= barley (B), W= wheat, YL= YeastLife, FB= FAN Boost

Data are the mean of two replicate brews for 100% malt, 45% B(A) YL, 45% B(B) YL, 45% W YL and 25% B(B) FB. Other data relate to individual brews.

The contents of wort fermentable carbohydrates were analysed to determine if unmalted adjuncts impact the carbohydrate profile (Table A.1). ANOVA with Dunnett's post hoc analysis determined that the wort concentrations of sucrose, fructose, maltose and maltotriose were not significantly different ($p > 0.05$) across the whole sample set. This shows that modifying the grist with adjuncts up to 45% incorporation rates did not have a significant effect on essential fermentable sugar concentrations. Figure 4.5 shows that glucose levels decreased with increasing amounts of all three adjuncts. ANOVA and Dunnett's post hoc analysis identified that at the 45% level for all adjuncts the wort glucose concentrations were significantly lower than for the all malt control ($p = 0.036$ for adjunct incorporation rate). This result agrees with a study conducted by van Donkelaar *et al.* (2016) which found that wort glucose concentration decreased with increasing incorporation of raw barley. Also in agreement with the present study, Zhuang *et al.* (2017) found that brewing with unmalted barley and wheat produced maltose and maltotriose levels comparable to that of an all malt control.

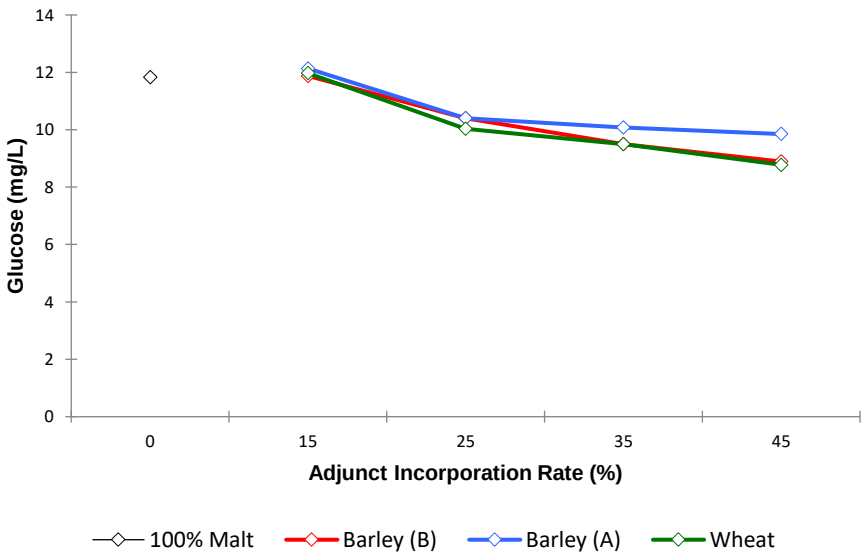


Figure 4.5. The impact of adjunct incorporation rate (%) on wort glucose concentrations for different adjunct materials

Results are averaged across all samples at a specific incorporation rate, including both YeastLife and FAN Boost trials.

For all fermentations, the wort gravities decreased in a broadly similar fashion (Figure 4.6). However, the length of fermentation, final gravities and attenuation

differed somewhat with adjunct incorporation rate and FAN supplementation (Table 4.3). The all-malt control reached the lowest final gravity of 2.4 °P and highest attenuation of 85%. For all adjunct types there was an increase in final gravity and consequently a decrease in attenuation. The greatest impact was observed with 45% barley (A) or barley (B) incorporation rate where the final gravity was 4.3 °P with a subsequent attenuation of 73%. This decrease in attenuation observed when increasing the usage of unmalted barley is similar to published findings where worts produced with 100% unmalted barley had a 33.6% lower fermentability compared to a 100% malt wort (Goode *et al.*, 2005). The fermentation of wort supplemented with FAN boost attenuated 48 h earlier (based on 24 h sampling intervals) than the control and any YeastLife supplemented wort. This finding is in agreement with previous results which found that when worts were supplemented with an exogenous protease, a reduction in lag phase and an increase in specific growth rate was observed (Piddocke *et al.*, 2011). Their study also found that additions of ammonium ions to wort resulted in the highest amount of residual sugars. As previously discussed (Section 1.5.4), the presence of higher concentrations of ammonia and glutamine in the wort can cause repression of the enzymes required for the uptake of key amino acids, a phenomenon known as nitrogen catabolite repression (NCR). This is a regulatory mechanism that inhibits expressions of the genes required for the uptake and degradation of alternative nitrogen sources when ammonia and glutamine are provided (Jiménez-Martí *et al.*, 2008). Similarly, Lei *et al.* (2013b) found that the use of Protamex (a serine protease) in wort, increased glutamine concentrations to 126.7 mg/L compared to 58.1 mg/L in the control wort. It could be possible that the supplementation of FAN boost could also increase glutamine concentration in the wort. Therefore, the increase of ammonia caused by YeastLife and increased glutamine with FAN Boost supplementation could cause NCR leading to higher residual sugars.

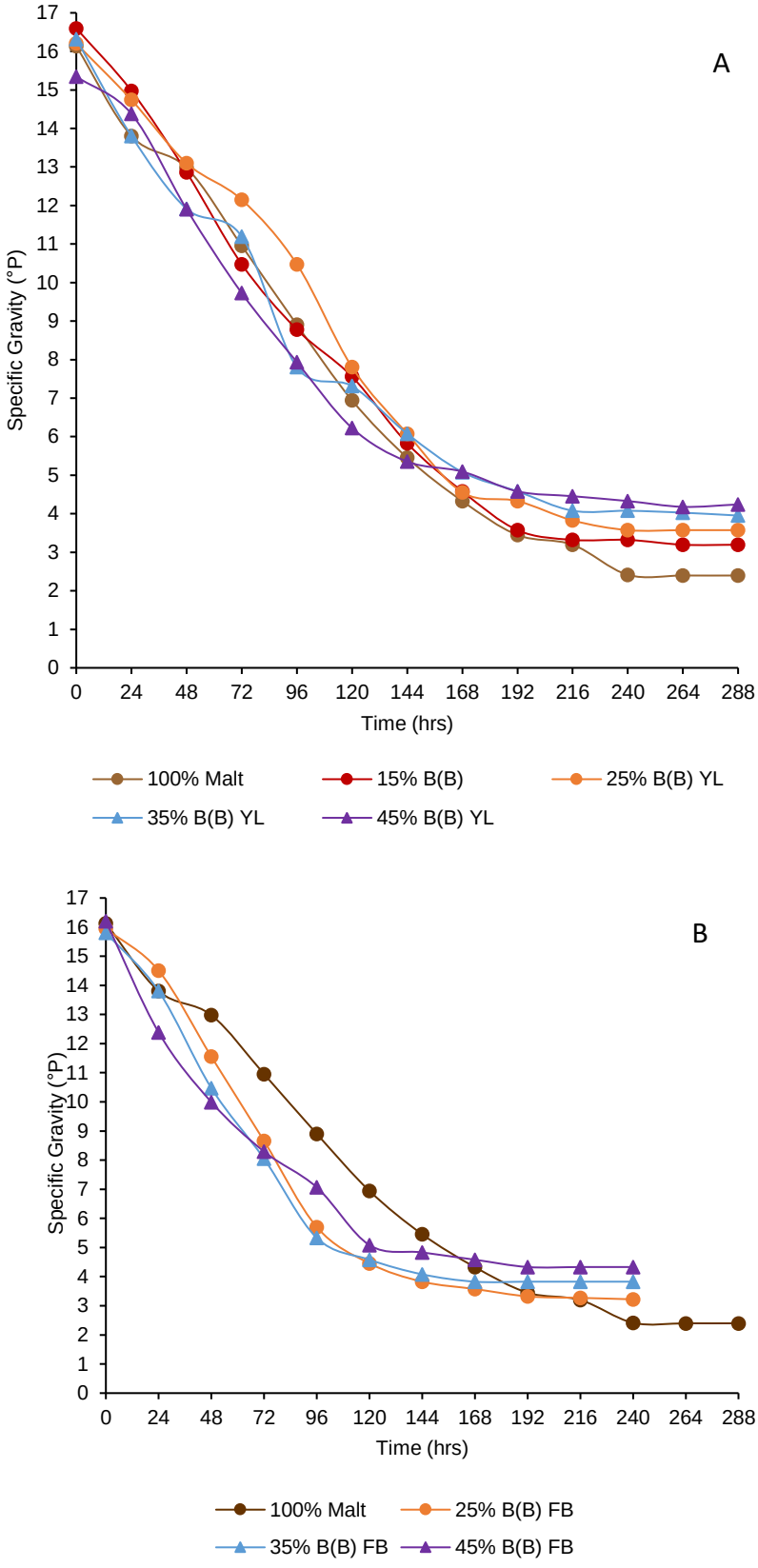


Figure 4.6. Fermentation profiles of wort produced with different adjunct material

A) Fermentation of B(B) wort with FAN Boost. B) Fermentation of B(B) wort with YeastLife.

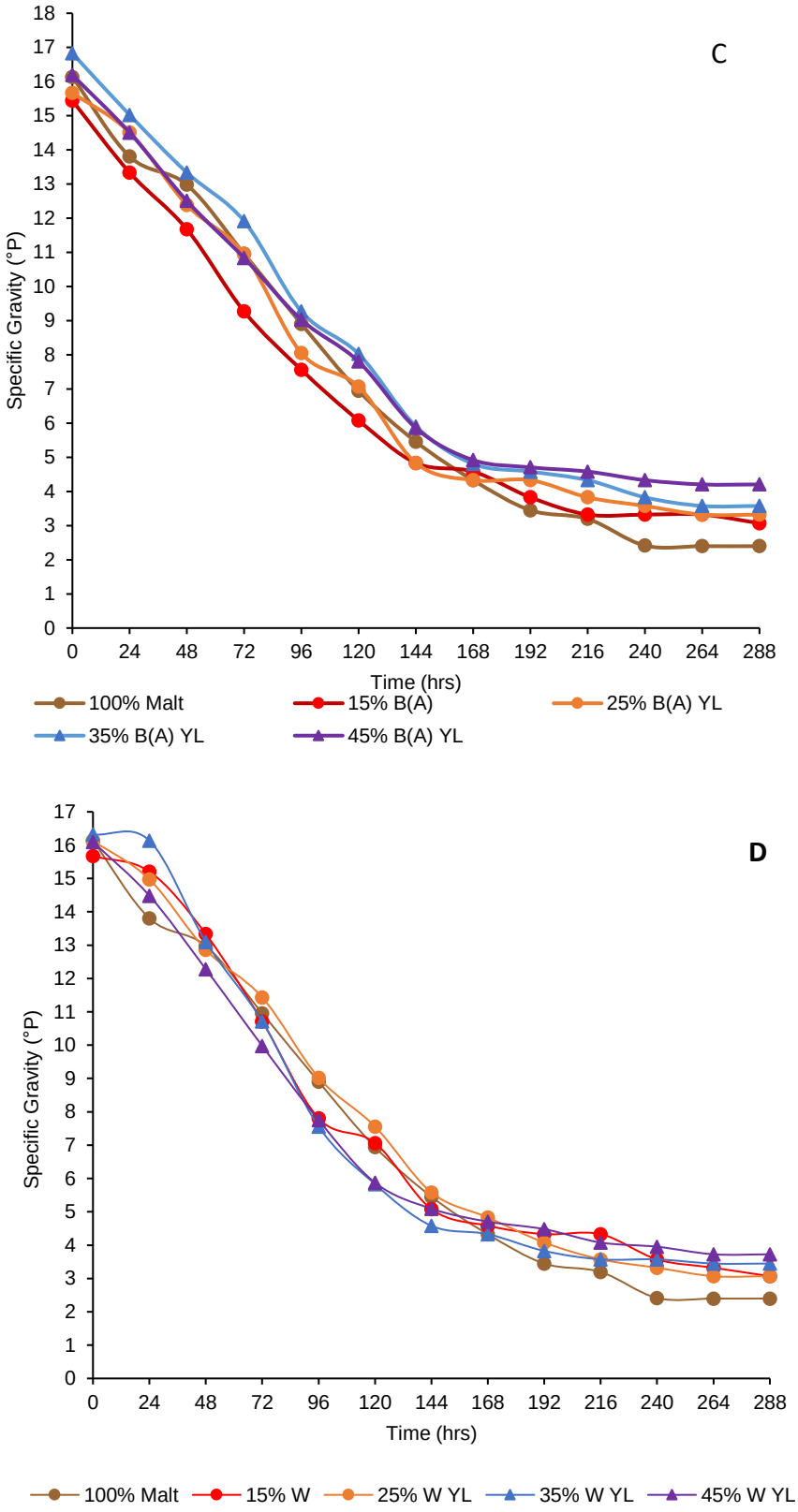


Figure 4.6. (continued) Fermentation profiles of wort produced with different adjunct material.

C) Fermentation of B(A) wort with YeastLife. D) Fermentation of wheat wort with YeastLife.

Table 4.4 Fermentable sugars concentrations in the green beer, standardised to 4% ABV.

Table 4.4. Fermentable carbohydrate content of trial beers (g/L)

	FAN Supplementation	Glucose	Sucrose	Maltose	Maltotriose	Total Sugars
100% Malt	-	0.067	0.098	1.304	0.315	1.785
15% B(B)	-	0.063	0.088	**0.738****	0.425	1.314
25% B(B)	FB	0.061	0.083	1.337	0.374	1.856
35% B(B)	FB	0.062	**0.071****	0.850	0.340	1.324
45% B(B)	FB	0.059	**0.072****	0.759	0.376	**1.266****
25% B(B)	YL	**0.094****	0.085	1.476	**0.466****	2.123
35% B(B)	YL	0.081	**0.074****	**0.401****	0.379	**0.935****
45% B(B)	YL	0.076	**0.076****	**0.714****	0.386	1.252
15% B(A)	-	0.064	0.092	**0.772****	0.374	1.303
25% B(A)	YL	**0.128****	**0.079****	1.392	**0.487****	2.092
35% B(A)	YL	0.052	0.081	**0.512****	0.362	**1.008****
45% B(A)	YL	**0.118****	0.077**	0.954	0.443	1.594
15% W	-	0.108	0.091	1.036	0.400	1.635
25% W	YL	0.122	0.082	1.505	**0.526****	2.237
35% W	YL	0.080	0.082	1.239	0.461	1.843
45% W	YL	**0.119****	**0.077****	0.878	0.355	1.429
Mixed model analysis of variance						
Incorporation rate (%)		**0.001**	**0.004**	<0.0001	0.234	**<0.0001**
Adjunct		**0.004**	0.629	**0.002**	0.817	**0.003**
Supplementation		**0.036**	0.489	**0.320**	**0.001**	0.726

B(A)= barley (A), B(B)= barley (B), W= wheat, YL= YeastLife, FB= FAN Boost

One-way ANOVA with Dunnett's post hoc analysis was used to compare the fermentable carbohydrates. Asterisks indicate samples that were significantly different in the samples compared to the all-malt control (**p<0.01).

ANOVA with Dunnett's post hoc analysis determined that there were significant differences in concentration for all fermentable sugars across the sample set ($p<0.01$). The concentrations of glucose and maltotriose typically increased in samples supplemented with YeastLife compared to the all-malt sample. However, residual sucrose and maltose concentrations tended to decrease at the higher adjunct incorporation rates (35 and 45%) and both sources of nitrogen supplementation. Thus, the higher residual sugars expected from the fermentation profiles could be caused by other carbohydrates such as malto-oligosaccharides, dextrins and non-starch polysaccharides (NSP). In agreement with this, Li *et al.* (2020a) determined that malto-oligosaccharides, dextrins and NSP content were consistent with extract content and monosaccharides were only present in very small proportions in finished beer. Their study found that not only did dextrin and NSP content correlate well with extract concentration but also depended on the grist material used in the brewing process.

4.3.2.2. Mash, wort and beer pH

The pH was measured throughout the brewing process, Table 4.5 shows the mash, wort and beer pH values for the adjunct and control samples.

Table 4.5. Variations in pH across the process for trial beers

	FAN Supplementation	Mash pH	Wort pH	Beer pH
100% Malt	-	5.44 ± 0.10	5.39 ± 0.05	4.45 ± 0.09
15% B(A)	-	5.36	5.34	4.28
25% B(A)	YL	5.36	5.53	4.33
35% B(A)	YL	5.35	5.50	4.47
45% B(A)	YL	5.48 ± 0.03	5.59 ± 0.02	4.64 ± 0.11
15% B(B)	-	5.37	5.34	4.27
25% B(B)	YL	5.33	5.58	4.40
35% B(B)	YL	5.33	5.94	4.40
45% B(B)	YL	5.56 ± 0.03	5.88 ± 0.05	4.61 ± 0.01
25% B(B)	FB	5.57 ± 0.04	5.36 ± 0.03	4.52
35% B(B)	FB	5.50	5.31	4.55
45% B(B)	FB	5.52	5.32	4.53
15% W	-	5.39	5.34	4.39
25% W	YL	5.34	5.54	4.35
35% W	YL	5.51	5.80	4.46
45% W	YL	5.52 ± 0.01	6.17 ± 0.04	4.63 ± 0.01

B(A)= barley (A), B(B)= barley (B), W= wheat, YL= YeastLife, FB= FAN Boost

Data are from two replicate brews for 100% malt, 45% B(A) YL, 45% B(B) YL, 45% W YL and 25% B(B) FB. Other data points represent individual brews.

Table 4.5 shows that the mash pH values obtained in this study align with the optimal pH range for amylase activity (5.2-5.8) (Bamforth, 2001). Samples supplemented with FAN boost at the start of the mashing scheme had a mash pH over 5.5. The higher mash pH observed for these samples would be beneficial as the supplier states that there should be sufficient protease activity at mashing pH 5.6-5.7. Decreasing mash pH to between 4.7-5.2 is potentially another approach for increasing total soluble nitrogen. However, most enzymes display considerable activity to either side of their optimum and other factors such as heat tolerance are more significant (Bamforth, 2001).

Wort pH was significantly increased by higher doses of YeastLife (Table 4.5). For example, the addition of 1.2 g/L of YeastLife into the 45% wheat wort increased the pH to 6.17 compared to 5.39 for the control. The beer pH for these samples ranged from 4.27-4.64 (Table 4.5). An acceptable range for beer pH is 4-4.4, therefore some of the beers produced in this study have slightly

higher beer pH than recommended (Bamforth, 2001). The beer samples with high YeastLife supplementation had correspondingly higher beer pH values. Studies have found that beers with a pH >4.4 have been described as having soapy and caustic notes, while at even higher pH values, differences in mouthfeel were reported (Bamforth, 2001).Therefore, higher pH observed throughout the process when using YeastLife could cause flavour issues in the beer. Acidification of the wort could be used to counteract this issue to minimise additional flavour and stability defects.

4.3.2.3. Nitrogenous compounds

The wort FAN and total nitrogen content was measured before YeastLife addition, in the cold wort samples and in the resultant beer (Table 4.6).

Table 4.6. FAN and total nitrogen analysis of adjunct wort and beer

	FAN Supp.	Wort FAN (mg/L)[a]		Beer FAN (mg/L)	FAN Utilisation (%)	Wort Total Nitrogen (mg/L)	Beer Total Nitrogen (mg/L)
		Before YL addition	After YL addition				
100% Malt	-	260 ± 2	261 ± 2	110 ± 10	58 ± 11	1137 ± 90	615 ± 6
15% B(A)	-	218	218	104	52.3	845	671
25% B(A)	YL	205	225	112	50.2	872	701
35% B(A)	YL	191	213	120	43.7	911	558
45% B(A)	YL	163 ± 0	207 ± 30	99 ± 9	52 ± 2	641 ± 43	530 ± 11
15% B(B)	-	210	210	109	48.1	1054	651
25% B(B)	YL	182	210	113	46.2	1117	651
35% B(B)	YL	178	213	110	48.4	1198	660
45% B(B)	YL	158 ± 5	208 ± 4	89 ± 0	57 ± 1	775 ± 15	616 ±
25% B(B)	FB	-	236 ± 0	79	66.5	1316	617
35% B(B)	FB	-	218	89	59.2	1345	582
45% B(B)	FB	-	228 ± 3	86 ± 1	62.3 ± 1	1372 ± 20	639 ± 20
15% W	-	218	218	106	51.4	1020	653
25% W	YL	180	202	108	46.5	924	671
35% W	YL	172	206	125	39.3	892	649
45% W	YL	140 ± 5	213 ± 8	84 ± 19	60 ± 8	655 ± 16	636 ± 14

B(A)= barley (A), B(B)= barley (B), W= wheat, Fan Supp.= Fan Supplementation, YL= YeastLife, FB= FAN Boost

[a]Wort free amino nitrogen concentration was standardised to 16 °P. Data is from two replicate brews for 100% malt, 45% B(A) YL, 45% B(B) YL, 45% W YL and 25% B(B) FB. Other data points represent individual brews.

As previously observed in Chapter 3 (Section 3.3.2.1), FAN concentration decreased with increasing adjunct incorporate rates, particularly with the incorporation of wheat at 45% which showed the lowest wort FAN concentration of 140 mg/L. The different dose rates previously calculated from the laboratory mash bath study (Figure 4.3 and 4.4) successfully increased wort FAN

concentrations over the target 200 mg/L. However, a large dose rate of up to 1.2 g/L of YeastLife was required to increase the FAN concentration to this degree, which is over 10 times the recommended dosing rate provided by the supplier. YeastLife predominantly consists of ammonium salts and much less amino acid and peptides than we had expected. Therefore, the yeast nutrient added nitrogen to the wort by ammonium salts instead of increasing amino acid and peptides which is expected with the use of proteases. The addition of FAN boost increased the wort FAN successfully to above 218 mg/L in the adjunct worts. The ninhydrin test used to determine FAN content is based on a reaction with primary amino groups and other amino groups to form chromophores that can be detected. This is a simplistic indicator for the nitrogenous profile of the wort; therefore, the total nitrogen content and amino acid composition were also determined.

The two approaches used to correct assimilable nitrogen improved the wort total nitrogen to similar concentrations as for the all-malt control, with the exception of the 45% adjunct YeastLife wort samples (Table 4.6). This indicates a limit in the extent to which YeastLife can improve the nitrogen composition at high adjunct incorporation rates. Figure 4.7 shows the influence of adjunct incorporation rate and nitrogen supplementation method on the wort total nitrogen.

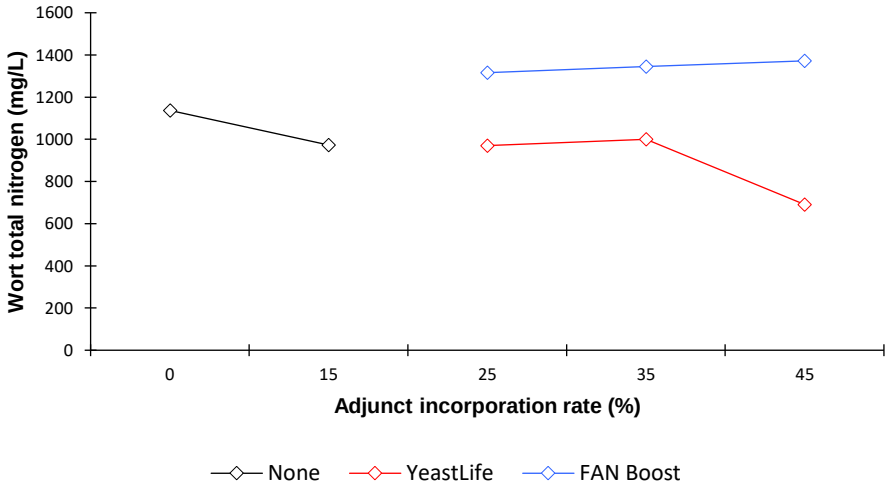


Figure 4.7.The influence of adjunct incorporation rate and nitrogen supplementation method on the wort total nitrogen

Results are averaged across all adjunct types (barley (A), barley (B) and wheat) at each incorporation rates

Adjunct worts supplemented with FAN boost showed an increase in total nitrogen compared to the all malt control and the YeastLife samples (Figure 4.7). The addition of an exogenous serine protease in the mash causes hydrolysis of the cereal proteins liberating polypeptides and peptides during mashing thus increasing total nitrogen content. Fermentation performance and the production of sensorially important flavour compounds depend on the concentration and the nature of the assimiable nitrogen as discussed in detail in Section 1.5.4.

According to the YeastLife specification, YeastLife contains a range of amino acids (alanine, aspartic acid, glutamic acid, glycine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, serine, tyrosine and valine) with a total concentration of approximately 60 mg/g. The amino acid composition was analysed before and after YeastLife addition at the 35% incorporation rate for barley (A), barley (B) and wheat to determine the change in amino acid composition (Table 4.7). The addition of YeastLife in all three circumstances did not significantly change the individual amino acid concentrations and for most amino acids there was a slight decrease in concentration. This unexpected result could be due to the point of sampling as the 'before' samples were taken from the kettle and the 'after' samples were taken from the cold wort before fermentation, therefore some of the amino acids may be been removed in the trub. A signficant increase in ammonium chloride was observed on the addition of YeastLife, indicating that the yeast nutrient is primarily functioning to add ammonium ions through the addition of ammonium chloride.

The grist material used in the brewing process has an impact on the amino acid composition. The malting process is important for the enzymatic degradation of barley proteins and consequently an increased concentration of amino acids exist in malted barley compared to unmalted cereal grains (Vandecan *et al.*, 2011). Any incorporation of unmalted adjunct in the grist material will dilute the amino acid content as well as the dilution of proteolytic activity in the mash, leading to overall lower levels of amino acids in the wort. When comparing data for the 35% adjunct level in Table 4.7 there is a decrease in concentration in an order of barley (A) > barley (B) > wheat for most amino acids with the exception of glutamic acid and tryptophan. The increased concentration of tryptophan when using wheat as an adjunct can be attributed to the proteins in wheat being rich in tryptophan, therefore this increase originates from the grain composition (Landry *et al.*, 1993). The processing applied to the barley grain has affected

the protein content and this could be a reason why the wort amino acid concentrations were lower with barley (B) than barley (A).

Table 4.7. Amino acid (and ammonium chloride) composition of 35% adjunct worts (mg/L) and an all-malt control: impacts of YeastLife addition

	100% Malt	35% B(B)[a]	35% B(B)[b]	35% W[a]	35% W[b]	35% B(A)[a]	35% B(A)[b]
Alanine	149.9 ± 5.3	113.6	112.9	99.9	103	129.8	129.8
Ammonium Chloride	156.2 ± 2.3	113.3	318.4	137.5	253.5	150.3	239.7
Arginine	17.61 ± 5.7	137.9	132.4	123.5	123.9	151.7	149.7
Asparagine	144.9 ± 5.5	140.5	135.3	114.7	114.9	146.6	144.6
Aspartic Acid	95.8 ± 4.0	87.2	85.1	79.9	81.2	94.8	93.5
Citrulline	5.7 ± 0.4	3.9	3.7	2.9	2.9	4.0	3.9
Ethanolamine	9.1 ± 0.8	7.6	7.3	7.8	8.1	9.1	9.2
Glutamic Acid	148.7 ± 14.9	119.1	119.9	121.7	126.6	150.2	150.2
Glutamine	64.8 ± 11.9	59.1	50.1	38.8	33.3	43.4	38.7
Glycine	41.3 ± 1.0	32.1	31.9	27.4	28.4	36.2	36.5
Histidine	64.9 ± 2.4	49.5	45.5	44.9	44.3	54.4	55.8
Isoleucine	96.65 ± 2.8	69.1	68.2	62.2	64	79	78.8
Leucine	219.9 ± 5.4	158.2	154.7	143.5	146.1	178.4	177.6
L-Ornithine	1.3 ± 0.1	0.9	1.5	0.9	1.2	1.2	1.4
Lysine	116.1 ± 3.8	86.8	84.0	73.0	74.1	97.7	97.1
Methionine	49.2 ± 1.2	33.7	33.1	29.6	30.5	37.9	38.3
Phenylalanine	184.0 ± 4.3	132.2	128.6	119.2	120.9	150.1	149.9
Proline	726.4 ± 16.1	522.1	485.5	471.5	469.6	559.8	571.0
Serine	98.4 ± 3.0	72.3	71.1	65.3	67.4	82.6	82.5
Threonine	84.1 ± 2.7	61.9	62.3	54	56.7	69.9	70.6
Tryptophan	30.3 ± 0.9	22.7	24.3	35.1	35.8	28.6	26.7
Tyrosine	132.8 ± 3.2	94.3	91.5	82.6	84.2	107.2	107.4
Valine	159.1 ± 8.8	116.9	114.1	104.7	107.4	133.2	128.2

[a] denotes the sample is taken before YeastLife addition, [b] denotes the sample was taken after YeastLife addition. 100% malt results are the average of two wort replicates. Amino acid analysis was performed in triplicate.

4.3.2.4. Polyphenol analysis

Polyphenols are important as they influence beer quality characteristics such as flavour, colour, colloidal and flavour stability (which is discussed in detail in Section 1.8.4).

Figure 4.8 displays the influence of adjunct incorporation rate on the total polyphenol content (TPC) of wort and beer.

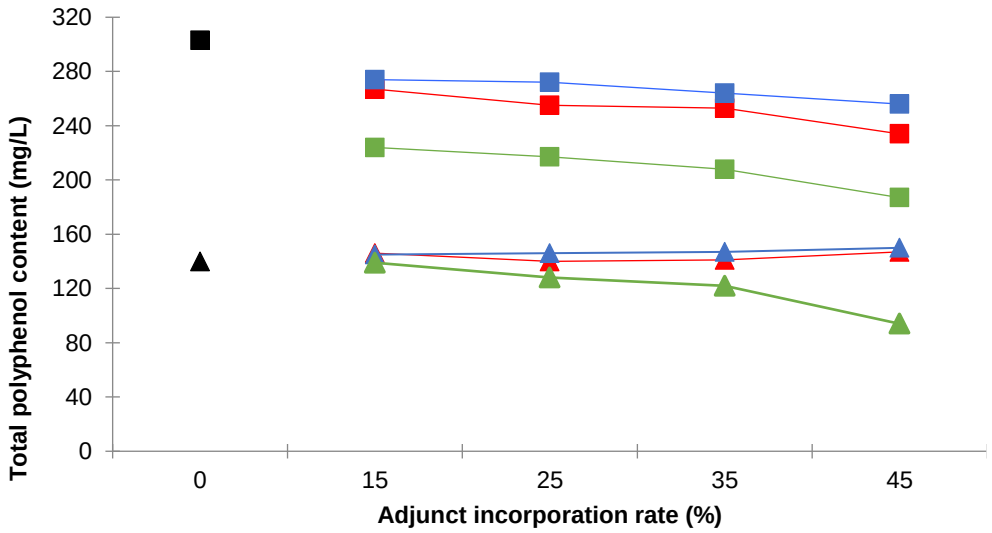


Figure 4.8 The influence of adjunct incorporation rate on the total polyphenol content of wort and beer

Results are averaged across all samples at a specific incorporation rate, including both YeastLife and FAN Boost trials. Square markers indicate wort samples, triangle markers indicate beer samples. Blue markers are barley (A) samples, red markers are for barley (B) samples and green markers are for wheat samples.

ANOVA analysis showed that the adjunct type ($p<0.0001$) and the adjunct incorporation rate ($p<0.00001$) significantly decreased the wort TPC. Several studies have found that malt has a higher total phenolic content than the corresponding unmalted barley (Lu *et al.*, 2007; Qingming *et al.*, 2010; Leitao *et al.*, 2012). Therefore, it is expected that the incorporation of any unmalted adjuncts will decrease wort TPC. In this study, the use of unmalted wheat caused the most significant decrease in TPC relative to the all-malt control brew. This is a result of raw wheat grains having a significantly lower polyphenol content compared to malted barley. Krstanović et al (2020) found that the

extracted total polyphenol concentration of malted barley was 53.54 mg/L compared to 18.86 mg/L in unmalted wheat. The dilution of wort TPC observed when utilising wheat was also carried through to the finished beers and a significant decrease in beer TPC was observed in resultant beer compared to the all-malt control (Figure 4.8, Table 4.8).

Table 4.8. Total polyphenol and proanthocyanidin contents of adjunct wort and beers

	FAN Supplementation	Wort TPC (mg/L)	Beer TPC (mg/L)	Wort TPA (mg/L)	Beer TPA (mg/L)
100% Malt	-	303 ± 23	140 ± 12	100 ± 4	12 ± 2
15% B(A)	-	274	145	104	10
25% B(A)	YL	272	146	93	10
35% B(A)	YL	**264****	147	91	12
45% B(A)	YL	**256 ± 9****	150 ± 1	91 ± 2	12 ± 1
15% B(B)	-	267	146	98	10
25% B(B)	YL	**248****	144	97	10
35% B(B)	YL	**209****	143	86	**9****
45% B(B)	YL	**234 ± 12****	155 ± 2	84 ± 8	12 ± 1
25% B(B)	FB	**264 ± 4****	135 ± 2	96 ± 2	10 ± 1
35% B(B)	FB	**253****	139	94	10
45% B(B)	FB	**234****	138	91	11
15% W	-	**224****	139	**79****	10
25% W	YL	**217****	**128***	**79****	**9****
35% W	YL	**208****	**122****	**75****	**9****
45% W	YL	**187 ± 5****	**94 ± 0****	**61 ± 5****	**9 ± 1****

TPC denotes total polyphenol content, TPA denotes total proanthocyanidin content. One-way ANOVA with Dunnett's post hoc analysis was used to compare the TPC and TPA analysis. Asterisks indicate samples that were signifcantly different compared to the all malt control (**p<0.01). Data is from two replicate brews for 100% malt, 45% B(A) YL, 45% B(B) YL, 45% W YL and 25% B(A) FB. Other data points represent individual brews.

A significant decrease was observed with barley (A) and barley (B) in the wort TPC but not in the finished beer (Table 4.8). Polyphenolic substances typically decrease from wort to beer as the polyphenolic substances adsorb to yeast cells during fermentation and can be reduced during warm rests and maturation (Fumi *et al.*, 2011). It could be hypothesised that the significant decreases in wort TPC with increasing barley (A) and barley (B) additions were not observed in the final beer due to the impacts of the fermentation and maturation stages. Downstream processes including beer filtration can also decrease TPC, however the beers produced in this study were not filtered.

The assay used to determine TPC is based on chelation of phenols with iron under alkaline conditions causing a red colour change (λmax= 600 nm)

(Callemien *et al.*, 2009). It has been found that vanillic, syringic, gentisic and ferulic acid do not cause an interaction in the Bishop assay used for this analysis (Callemien *et al.*, 2009). Therefore, the content of a range of phenolic acids were determined by HPLC-UV-DAD and discussed in Section 4.3.2.5.

Proanthocyanidins are also important to consider in brewing due to their protein-precipitating activity and involvement in haze formation. The data in Table 4.8 show that wort TPA decreased slightly when using barley (A) and barley (B), relative to the all malt control. This agrees with van Donkeelar's (2016) study which found that wort anthocyanogens decreased with increasing raw barley content. Similarly to the results for TPC, the wort TPAs reduced significantly with increasing wheat incorporation. This can be attributed to wheat having a lower content of extractable PAs as compared to barley (McCallum *et al.*, 1990). It is apparent that the type of FAN supplementation did not have an impact on the wort TPC (p=0.454), wort PAs (0.439) or beer TPC (p=0.079), therefore it can be concluded that the changes in the adjunct beers were a result of changing the adjunct material. The decrease in PAs when using unmalted wheat is also apparent in the finished beer (Table 4.8), where use of 25% or more wheat significantly decreased the beer PAs compared to the all-malt control.

4.3.2.5. Simple phenolic acids and monomeric polyphenols analysed by HPLC-UV-DAD

Table 4.9 shows the individual concentrations of phenolic compounds identified by HPLC-UV-DAD in the adjunct beer and all malt control. The use of barley (A) and barley (B) in this study increased catechin levels at high adjunct incorporation rates (Table 4.9). This result is most likely attributed to the lack of malting process being applied. As discussed previously in Section 1.8.2, the amount of oligomeric flavanols remained constant during malting, however a marked decrease was observed in catechin concentration (Dvorakova *et al.*, 2008; McMurrough *et al.*, 1983). The incorporation of wheat and the method of FAN supplementation method applied did not significantly affect the catechin concentration in the beer samples.

Table 4.9. Polyphenolic acid composition of adjunct beers

Sample	FAN Supplementation	4-HBA	4-HPA	Ferulic Acid	Vanillic Acid	Syringic Acid	Catechin	p-Coumaric Acid
100% Malt	-	0.25	1.31	1.53	0.97	0.12	1.80	0.90
15% B(B)	-	0.22	1.16	1.60	1.12	0.11	1.90	0.72
25% B(B)	FAN Boost	0.22	1.24	1.16	0.75	0.07	1.82	**0.69****
35% B(B)	FAN Boost	0.23	1.44	1.81	0.80	0.14	1.83	**0.68****
45% B(B)	FAN Boost	0.23	1.54	1.23	0.87	0.1	**2.62****	**0.69****
25% B(B)	YeastLife	0.23	1.14	1.87	1.10	0.12	1.57	0.86
35% B(B)	YeastLife	0.23	1.16	1.81	1.13	**0.21****	**2.27****	0.89
45% B(B)	YeastLife	0.29	1.2	1.32	**1.24****	**0.23****	**2.44****	0.84
15% B(A)	-	0.22	1.22	1.65	**1.24****	0.14	1.86	1.04
25% B(A)	YeastLife	0.23	1.16	**2.00***	**1.47****	**0.3****	2.06	**1.11****
35% B(A)	YeastLife	0.25	**0.86****	1.81	**1.5****	**0.23****	1.23	0.98
45% B(A)	YeastLife	0.30	1.29	**2.00***	**1.55****	**0.34****	**4.21****	**1.13****
15% W	-	0.31	1.17	1.64	**1.4****	0.19	1.85	0.75
25% W	YeastLife	0.23	**0.93****	1.68	1.18	**0.25****	1.21	0.83
35% W	YeastLife	0.23	1.32	1.61	1.10	**0.23****	1.79	**0.61****
45% W	YeastLife	0.27	1.29	1.64	1.16	**0.27****	1.69	**0.56****
Mixed model analysis of variance								
Adjunct type		0.11	**0.001**	0.219	**0.032**	**0.001**	**< 0.0001**	**0.011**
Incorporation rate (%)		0.176	0.843	**0.027**	**<0.0001**	**<0.0001**	**0.000**	**<0.0001**
FAN supplementation method		0.514	**0.001**	0.400	**<0.0001**	**<0.0001**	0.994	**<0.0001**

One-way ANOVA with Dunnett's post hoc analysis was used to compare the phenolic acid concentrations. Asterisks indicate samples that were significantly different in the samples compared to the all malt control (*p<0.01). Data is from two replicate brews for 100% malt, 45% B(A) YL, 45% B(B) YL, 45% W YL and 25% B(B) FB. Data are the mean of four replicate measurements. 4-HBA = 4-hydroxybenzoic acid; 4-HPA = 4-hydroxyphenylacetic acid.

Dvořáková *et al.* (2008) determined the concentration of a range of free phenolic acids in barley grain samples before and after malting. Their study found that the malting process significantly decreased free phenolic acids including vanillic acid, syringic acid, p-coumaric acid and ferulic acid when investigating several barley varieties This result is in accordance with our study, as the incorporation of unmalted barley typically caused an increase in these phenolic acids in the beers (Table 4.9). An increase in these three phenolic acids was not observed to the same extent with barley (B). This demonstrates that the processing applied to the barley grain can reduce the phenolic acid content. Interestingly, the incorporation of wheat only significantly increased the concentration of syringic acid whilst the other phenolic acids analysed had comparable or lower concentrations in wheat adjunct beers relative to the 100% malt control. This can be attributed to wheat typically having a slightly higher concentration of syringic acid in the grain compared to malt (Hefni *et al.*, 2019).

ANOVA analysis identified that the type of FAN supplementation caused a significant effect for vanillic, syringic and p-coumaric acid (Table 4.9). It can be seen that when FAN boost is used there is a slight decrease in these three phenolic acids compared to when YeastLife is used. It could be hypothesised that adding a protease to the mash would lead to an accumulation of polypeptides that could complex with phenolic acids in the boil and then be removed in the trub, thus decreasing the phenolic acid content of resultant beers.

4.3.2.6. Mineral composition from grain to beer

Metal ions play an important role in brewing and yeast performance. As previously discussed in Section 1.7, metal ion content in wort and beer can be influenced by cereal raw material or deliberate addition by the brewer. The mineral composition was determined for the grist materials that were used to produce the adjunct and control wort and beer samples (Table 4.10).

Table 4.10. Mineral ion composition of grist materials used in the study (mg/kg)

	Na	Mg	K	Ca	Mn	Fe	Cu	Zn
Malt	66.1[a]	1008.1[a]	4539.9[a]	438.5[b]	9.9[b]	42.5[a]	4.3[a]	28.5[a]
Barley (A)	63.2[a]	988.2[a]	3566.6[b]	555.5[a]	9.9[b]	33.5[b]	4.1[a]	24.3[b]
Barley (B)	53.4[b]	846.3[b]	3676.3[b]	291.8[c]	8.1[c]	29.6[b]	4.3[a]	24.8[b]
Wheat	9.2[c]	971.8[a]	4520.0[a]	277.2[c]	20.6[a]	29.5[b]	3.2[b]	15.4[c]

One-way ANOVA with Tukey's HSD post hoc analysis was used to compare the mineral ion concentrations in the cereal grains. Superscript letters indicate post-hoc groupings of samples according to Tukey's HSD test. Results are means of three replicate runs.

The data in Table 4.10 shows that the unmalted adjuncts in this study contained lower concentrations of mineral ions compared to malted barley; this was especially the case for unmalted wheat. The wheat sample had significantly lower concentrations of sodium, calcium, iron and zinc as compared to malt and unmalted barley. Interestingly, however, the concentration of manganese was nearly twice that present in malt. A previous study also identified that wheat had a significantly higher manganese content compared to malt (Poreda *et al.*, 2015). It can be hypothesised from the grain analysis results that malt substitution with unmalted wheat would have the most impact on the mineral composition of the wort and resulting beer. A small decrease in the content of mineral ions was observed in barley (B), indicating that the processing applied to the barley grain decreases the mineral composition.

In order to understand the flavour composition of adjunct beers and the effects of FAN supplementation, it is important to evaluate the mineral composition of the wort which influences the fermentation and consequently the beer flavour. Table 4.11 shows the mineral ion composition of the adjunct and all malt control wort samples. Wort produced with higher proportions (35% and 45%) of unmalted wheat contained significantly less sodium relative to the all-malt control ($p < 0.01$). This was expected based on the raw materials analysis (Table 4.10).

Table 4.11. Mineral ion composition of adjunct wort samples (mg/L)

	FAN Supplementation	Na	Mg	K	Ca	Mn	Fe	Cu	Zn
100% Malt	-	16.59	100.86	663.90	36.11	0.17	0.086	0.104	0.13
15% B(B)	-	15.26	92.91	647.84	29.72	0.16	**0.045****	**0.070****	0.10
25% B(B)	FB	**18.50****	**122.96****	**760.51****	**53.49****	**0.21****	0.065	0.126	0.15
35% B(B)	FB	**19.82****	**127.92****	**781.20****	**57.14****	**0.25****	0.062	0.126	0.17
45% B(B)	FB	**21.17****	**131.93****	**822.52****	**58.56****	**0.27****	0.054	0.129	0.18
25% B(B)	YL	16.74	97.50	644.88	36.11	0.15	**0.039****	**0.063****	0.39
35% B(B)	YL	**18.52****	104.50	**748.67****	34.42	0.16	**0.027****	**0.062****	**0.63****
45% B(B)	YL	**20.56****	113.76	**770.66****	40.28	0.17	**0.029****	**0.059****	**0.96****
15% B(A)	-	**19.16****	104.81	**765.46****	37.76	0.20	**0.048****	**0.069****	0.13
25% B(A)	YL	**18.74****	100.00	**756.32****	42.08	0.17	**0.032****	**0.063****	**0.51***
35% B(A)	YL	**19.50****	110.44	**844.73****	37.11	0.19	**0.042****	**0.053****	0.34
45% B(A)	YL	**19.97****	103.73	**814.46****	30.82	0.17	**0.030****	**0.074****	**0.51***
15% W	-	17.21	99.93	**804.41****	30.27	**0.22****	0.075	0.079	0.16
25% W	YL	14.35	89.44	726.06	27.78	0.19	**0.046****	**0.063****	0.36
35% W	YL	**12.61****	**76.75****	668.26	**16.78****	0.16	**0.024****	**0.049****	0.44
45% W	YL	**12.34****	**70.83****	686.44	**12.15****	0.15	**0.033****	**0.057****	**0.79****
Mixed model analysis of variance on the wort mineral ions									
Incorporation rate (%)		0.081	0.657	0.266	0.359	**0.042**	0.234	0.363	**<0.0001**
Adjunct type		**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0.068	0.245	0.365	**0.002**
FAN supplementation method		0.067	**<0.0001**	**0.001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

One-way ANOVA with Dunnett's post hoc analysis was used to compare the mineral ion concentrations. Asterisks indicate samples that were signifcantly different compared to the all malt control (**p<0.01). Results are means of three replicate runs.

Magnesium is important for yeast growth and is an important cofactor for a number of enzymes. Calcium is also important as magnesium-dependent enzymes can be suppressed by calcium, exerting an antagonistic action (Gibson, 2011). The magnesium to calcium ratio is an important measure when considering a healthy fermentation, therefore although the incorporation of FAN boost significantly increased magnesium and calcium concentrations in wort compared to the all-malt control, the magnesium to calcium ratio stayed comparable to that of the all malt and YeastLife samples (between 2.3-3.3). Interestingly, the use of unmalted wheat at 45% of grist significantly increased the magnesium to calcium ratio to 5.8. This change could be attributable to raw wheat having a lower concentration of calcium in the grain compared to barley malt (Table 4.10).

Zinc is essential for yeast growth and metabolism, as zinc is a cofactor in several enzyme classes, including alcohol dehydrogenase, the terminal enzyme of the fermentation pathway (De Nicola *et al.*, 2011) (Section 1.7.3). Wort zinc concentrations below 0.1 ppm can lead to sluggish fermentations and brewers are recommended to supplement wort with zinc to increase wort zinc levels over 0.25 ppm to improve fermentation performance (De Nicola *et al.*, 2011). Table 4.11 shows that all wort samples had zinc levels above 0.1 ppm. A considerable increase in wort zinc concentration was caused by YeastLife supplementation, confirming that YeastLife included a high concentration of zinc. Studies have been conducted on the effect of zinc on production of flavour congeners. Studies by Quilter *et al.* (2003) reported that higher alcohols were elevated with increasing zinc concentrations. Their study proposed that zinc addition resulted in increased production of higher alcohols by stimulating the breakdown of α-keto acids to their corresponding higher alcohols. However, De Nicola *et al.* (2011) found that flavour congener profile was only significantly changed when zinc levels of 10 ppm were used. The fermentation volatile composition of the adjunct beer is discussed further in Section 4.3.2.7.

Iron and copper are involved in the regulation of structure and metabolic activity of the cells during growth and fermentation (Philpott *et al.*, 2008). In the present study, the addition of YeastLife caused a significant decrease in iron and copper ions which was not observed when FAN boost was used (Table 4.11). The reduction in iron observed in the adjunct wort could be a result of changes in grist material as the unmalted adjuncts had lower concentrations of iron compared to malt. The addition of FAN Boost increased wort copper levels whereas the wort samples supplemented with YeastLife had wort copper levels

lower or closer to that of the 15% adjunct incorporation wort. The increased peptides and soluble nitrogen produced by the addition of FAN Boost may have bound to the copper ions in the mash. These nitrogen and copper complexes could have passed through the brewing process into the wort as opposed to the copper being removed by co-precipitation into the spent grain.

Manganese is important as an intracellular regulator of key enzyme activities and acts as a catalytic centre for several enzymes (Stewart, 2006). Interestingly, only a small insignificant fluctuation of manganese content was observed when including wheat in wort and beer. This was not expected, as the concentration of manganese in raw wheat was almost double that of malt (Table 4.10). A previous study determined that when utilising wheat in the grist, there is also a rising amount of manganese in the spent grain, explaining that despite a higher input of manganese in the raw material, there is a comparable amount in the wort and finished beer (Poreda *et al.*, 2015). This highlights that the ionic composition of wort is strongly dependent on the removal rate of ions from the wort during lautering and hot trub separation, rather than being wholly dependent on the amount present in the raw material (Poreda *et al.*, 2015).

Figure 4.9 shows the influence of adjunct incorporation rate and the method of FAN supplementation on the beer and wort manganese concentration. This shows that the addition of a protease increases the manganese content in both the wort and beer, compared to YeastLife which decreases the manganese content. Manganese is important for yeast nutrition but at higher concentration there is a risk of flavour deterioration in the beer (Poreda *et al.*, 2015). Zufall *et al.* (2008) found that beer manganese concentration as small as 90 ppb can shorten the ‘lag-time’ in chemiluminescence measurement indicating an early increase of radical reactions in the beer which can cause flavour deterioration (Section 1.7.4).

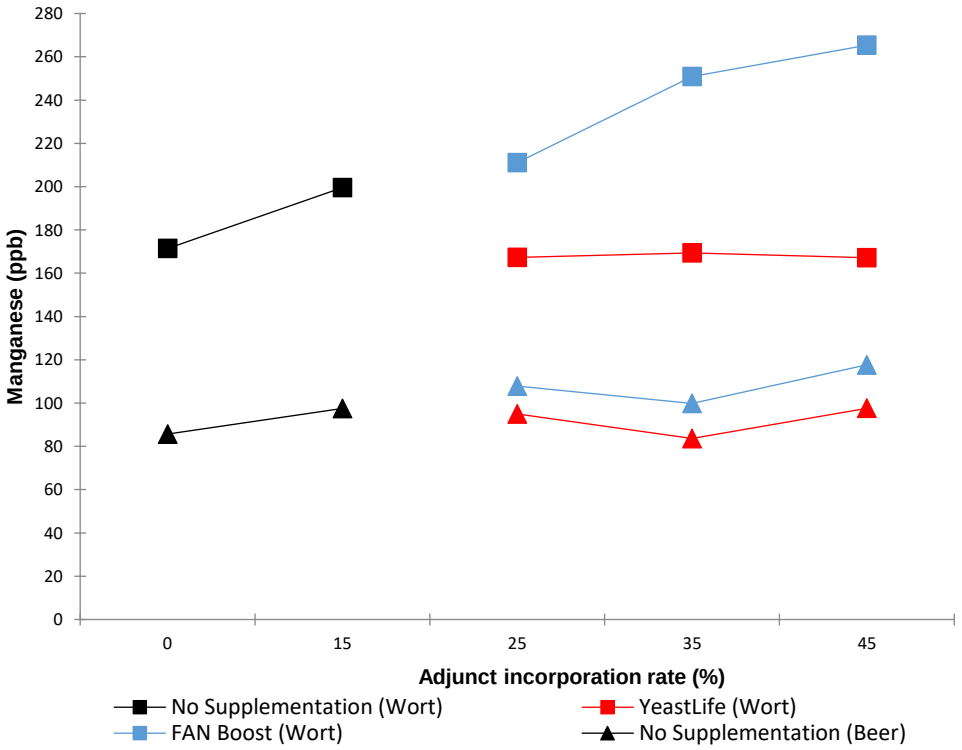


Figure 4.9. The influence of adjunct incorporation rate and FAN supplementation on the wort and beer manganese concentration

Results are averaged across all adjunct types (barley (A), barley (B) and wheat) at each incorporation rate.

The mineral content throughout the brewing process influences beer flavour directly, by the direct flavour impacts of specific ions on the palate of beer, and indirectly through their influence on the formation of flavour active compounds during fermentation. Table 4.12 shows the concentration of key mineral ions in the adjunct beer samples.

Sodium does not have a significant influence on the brewing process, although at higher concentrations in beer, sodium is responsible for salty flavours. However, the concentrations observed in the adjunct beers are much lower than typically reported (Gibson, 2011).

High concentrations of magnesium in beer can produce a sour or bitter flavour of the beer, although there was some variation in magnesium concentrations amongst the adjunct beers, much greater concentrations are required to contribute directly to flavour. Zinc was nearly fully utilised in all fermentations except for the 45% adjunct YeastLife samples, where there was residual zinc in the beer. This is because of the high amount of zinc contributed by YeastLife at the highest dose rate required at 45% adjunct usage.

Table 4.12. Mineral ion composition of adjunct beer samples (mg/L)

	FAN Supplementation	Na	Mg	Ca	Mn	Fe	Cu	Zn
100% Malt	-	11.4	60.67	18.82	0.086	0.013	0.045	0.003
15% B(B)	-	**10.24***	60.1	16.44	0.077	**0.005****	0.037	0.003
35% B(B)	FB	**12.01***	66.99	**23.39****	0.100	0.007	0.045	0.005
25% B(B)	FB	12.05	65.71	**22.35****	**0.108****	0.009	**0.032****	0.003
45% B(B)	FB	**13.36****	**71.67****	**24.24****	**0.118****	0.012	**0.033****	0.002
25% B(B)	YL	11.76	63.83	21.92	0.090	**0.004****	**0.033****	0.003
35% B(B)	YL	12.13	60.71	20.02	0.864	**0.005****	**0.028****	0.010
45% B(B)	YL	**15.26****	**69.55****	**23.75****	0.104	0.007	**0.023****	**0.055****
15% B(A)	-	11.75	63.03	19.2	0.097	0.014	0.036	0.003
25% B(A)	YL	**13.01****	61.61	**24.61****	0.097	**0.004****	0.038	0.004
35% B(A)	YL	11.86	62.27	20.25	0.090	**0.005****	**0.023****	0.003
45% B(A)	YL	**13.74****	61.93	20.77	0.100	0.012	0.036	**0.070****
15% W	-	11.36	62.24	17.39	**0.119****	0.007	0.043	0.002
25% W	YL	**9.96****	60.19	16.2	0.098	**0.006***	**0.034****	0.003
35% W	YL	**8.07****	**47.28****	**9.94****	0.074	**0.005****	**0.024****	0.005
45% W	YL	**9.89****	**50.47****	**8.35****	0.084	0.007	**0.034****	**0.053****
				Mixed model analysis of variance				
Incorporation rate (%)		**<0.001**	**0.019**	0.057	**0.000**	**0.000**	**<0.0001**	**<0.0001**
Adjunct type		**<0.001**	**<0.0001**	**<0.0001**	0.560	**0.000**	**<0.0001**	0.843
FAN supplementation method		0.144	0.096	**0.032**	**0.000**	**0.000**	**<0.0001**	0.189

One-way ANOVA with Dunnett's post hoc analysis was used to compare the mineral ion concentrations. Asterisks indicate samples that were signifcantly different compared to the all malt control (**p<0.01)

The transition metals iron and copper are of particular concern to brewers because they play an important role in flavour stability. The formation of specific ageing compounds can be attributed to oxidative processes catalysed by iron and copper in beer (Section 1.7.4). In this study, the iron and copper concentrations strongly diminished after fermentation and all adjunct beer samples contained lower concentrations of iron and copper compared to the all-malt control. This trend was even more significant with wheat and YeastLife incorporation. This is partly a result of differences in the raw materials, as was observed for iron and copper concentrations in wort, but also partly a result of the FAN supplementation. It could be hypothesised that FAN supplementation has improved yeast growth and caused an increased uptake of iron and copper. However, further analysis of the yeast behaviour during fermentation would be required to determine this.

4.3.2.7. Fermentation volatiles

The quality of beer is influenced by its flavour and aroma profile and an important part of the overall profile is contributed by flavour-active yeast metabolites as discussed in detail in Section 1.5.4. Table 4.13 reports the quantification of major higher alcohols in the adjunct and control beers.

Table 4.13. Higher alcohol composition of adjunct beers

Sample	FAN	Propanol	Isobutanol	Phenethyl Alcohol	Isoamyl Alcohol	Tyrosol	Tryptophol	Total Higher Alcohols
100% Malt	-	8.41	10.76	19.63	48.79	16.39	1.16	105.14
15% B(B)	-	9.09	11.64	19.66	53.45	**12.19****	1.09	107.12
25% B(B)	FB	7.26	9.45	24.2	41.74	15.69	**1.82****	100.16
35% B(B)	FB	6.77	9.14	20.06	42.17	16.3	1.15	95.59
45% B(B)	FB	7.25	9.82	18.2	45.62	16.17	1.51	98.57
25% B(B)	YL	9.49	11.71	20.18	51.57	15.19	1.31	109.45
35% B(B)	YL	8.86	12.26	20.16	54.29	**13.29****	1.24	110.1
45% B(B)	YL	9.38	12.26	20.2	45.62	11.93	1.25	100.64
15% B(A)	None	9.01	10.72	20.47	51.22	14.71	**2.19****	108.32
25% B(A)	YL	9.03	11.53	19.42	55	14.66	**2.03****	111.67
35% B(A)	YL	8.63	12.17	20.34	55.84	**10.54****	1.09	108.61
45% B(A)	YL	**10.14****	12.25	20.28	**54.81***	**13.09****	1.42	111.99
15% W	None	8.83	10.18	19.33	49.62	14.99	1.16	104.11
25% W	YL	8.74	11.99	20.49	55.5	15.2	0.83	112.75
35% W	YL	9.43	12.36	20.64	53.27	**12.01****	1.23	108.94
45% W	YL	**9.93***	**13.17***	19.99	**57.00***	**11.85****	**1.63****	**113.57***
Mixed model analysis of variance of the higher alcohols								
Adjunct type		0.504	0.44	0.995	0.664	0.811	**<0.0001**	0.904
Incorporation rate (%)		0.122	0.079	0.244	0.349	**0.003**	**< 0.0001**	0.517
FAN supplementation method		**<0.0001**	**< 0.0001**	0.182	**<0.0001**	**0.001**	**0.002**	<**0.0001**

One-way ANOVA with Dunnett's post hoc analysis was used to compare the higher alcohol concentration. Asterisks indicate samples that were significantly different in the samples compared to the all malt control (*p<0.01)

The type of FAN supplementation had a significant effect on all higher alcohol concentrations except phenethyl alcohol in the adjunct beers (Table 4.13). The addition of YeastLife tended to slightly increase the higher alcohol concentrations whereas the use of FAN boost resulted in a decrease (Figure 4.10). Previous studies found that the use of Protamex, an exogenous serine protease, decreased the higher alcohol content (Lei *et al.*, 2013b) in agreement with the present data for FAN boost trials. The study showed that worts treated with the serine protease had a higher concentration of valine, phenylalanine and leucine. It might be expected that the increased concentrations of valine and leucine would induce the formation of isobutanol and isoamyl alcohol upon assimilation. However, the addition of the serine protease resulted in a lower assimilation of these amino acids as the serine protease treated worts had lower yeast growth, FAN consumption and fermentability compared to a control or when using other exogenous proteases, and thus a lower concentration of higher alcohols were observed (Lei *et al.*, 2013b; Ferreira *et al.*, 2018).

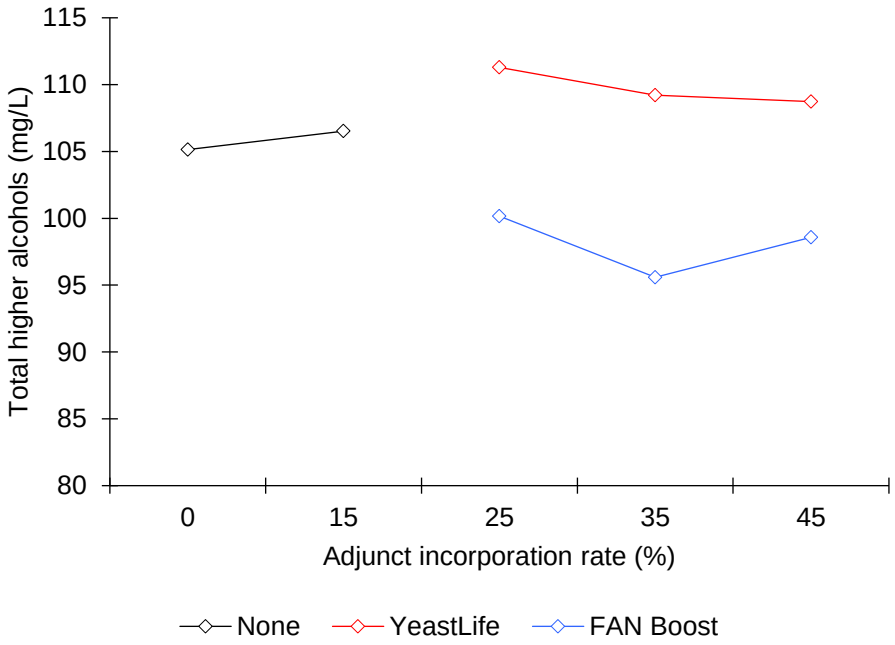


Figure 4.10. The influence of adjunct incorporation rate and FAN supplementation on the total higher alcohol content of beer.

Results are averaged across all adjunct types (barley (A), barley (B) and wheat) at each incorporation rate.

The FAN supplementation method used influenced the concentration of tyrosol in the adjunct beer. Beers produced with FAN boost had a comparable tyrosol concentration to the all-malt control whilst at higher YeastLife treatment rates

tyrosol decreased. Tyrosol is formed during fermentation via the Ehrlich pathway from tyrosine. The use of YeastLife or the incorporation of adjunct material decreased the tyrosine concentration in wort (Table 4.11) correlating to the decrease in tyrosol in the beer. Lei *et al.* (2013b) found that the addition of proteases increase tyrosine in the wort which could explain why the tyrosol concentration of the FAN Boost samples are comparable to the all-malt control. The addition of YeastLife to the adjunct wort significantly increased wort zinc concentrations which could give a reason as to why an increase in higher alcohols was seen with YeastLife addition. In agreement with our findings, Nicola *et al.* (2009) found that increasing zinc concentrations produced more higher alcohols without a significant increase in ethanol and it is believed this could be a result of zinc causing a reversion of acetaldehyde to pyruvate and eventually producing higher alcohols (Verbelen *et al.*, 2008).

The differences in higher alcohol concentration in the adjunct beers are minimal and we would predict that they would not cause a perceptible sensory impact. For most higher alcohols, statistically significant differences only occurred with 45% adjunct incorporation with YeastLife included. This shows that both types of FAN supplementation have successfully balanced flavour 'defects' of low nitrogen fermentation which was determined as a problem with brewing with unmalted adjuncts in Chapter 3.

Table 4.14 shows the ester profile for the adjunct beers with different nitrogen supplementation. The concentrations of isoamyl acetate, ethyl butyrate, ethyl octanoate, ethyl heptanoate and ethyl decanoate were not significantly different across the sample set proving that a similar ester profile can be produced with the use of 45% adjunct material when nitrogen content is balanced.

The type of nitrogen supplementation used had a significant effect on certain acetate ester concentrations (ethyl acetate, isobutyl acetate and phenethyl acetate) whereby FAN boost tended to lower the acetate ester concentrations whilst YeastLife tended to increase acetate ester concentration. This could be a result of the change in wort pH described in Section 4.3.2.2. Piddocke *et al.* (2011) found that fermentations with protease supplementation resulted in slightly lower beer pH and thus slightly lower concentrations of acetate esters. It is possible that lower intracellular pH of yeast cells can affect the activity of esterases or acyltransferases resulting in lower concentrations of acetate esters. However, even with slight changes, the ester profile of the adjunct beers were not strongly significantly different from the all malt control.

The acetaldehyde concentration in the beer samples ranged from 1.54- 6.74 mg/L without a clear trend between adjunct type, incorporation rate or FAN supplementation causing the changes in acetaldehyde concentration. The FAN supplementation techniques used to elevate FAN should have balanced acetaldehyde concentrations in adjunct beers by reducing yeast stress and improving fermentation. Therefore, other yeast nutrition factors may have influenced acetaldehyde concentration. Acetaldehyde concentration can be influenced by fermentation temperatures, pitching rate and fermentation tank filling time. These parameters all influence the level of oxygen dissolved in the fermenting wort and directly modify the unsaturated fatty acids, bilayer and mitochondrial structures of growing yeast cells and their potential to biosynthesise acetaldehyde (Stewart, 2017; Kucharczyk *et al.*, 2020; Petkova *et al.*, 2010). Although fermentation conditions were standardised across the beers as consistently as possible, the level of dissolved oxygen was not measured in this study and may have varied between each brew, influencing acetaldehyde concentration.

Table 4.14. Volatile composition of adjunct beers: esters and acetaldehyde

Sample	Acetaldehyde (mg/L)	Ethyl Acetate (mg/L)	Ethyl Butyrate (mg/L)	Isoamyl Acetate (mg/L)	Isobutyl Acetate (mg/L)	Phenethyl Acetate (mg/L)	Ethyl Hexanoate (mg/L)	Methyl Octanoate (mg/L)	Ethyl Octanoate (mg/L)	Ethyl Heptanoate (mg/L)	Ethyl Decanoate (mg/L)	Ethyl Trans-4-Decenoate (mg/L)
100% Malt	3.82	13.78	0.063	0.93	0.07	0.16	0.09	0.07	0.201	1.44	5.77	3.66
15% B(B)	3.82	13.70	0.065	0.86	0.07	0.13	0.09	0.07	0.213	1.21	5.66	2.93
25% B(B) FB	2.41	12.38	0.06	0.79	**0.05****	0.11	0.09	0.07	0.188	1.51	5.31	3.57
35% B(B) FB	3.49	12.17	0.06	0.86	**0.05****	0.11	0.1	0.09	0.233	1.55	5.65	**4.90****
45% B(B) FB	4.36	11.56	0.06	0.86	0.06	0.11	0.09	0.07	0.217	1.52	5.82	**5.57****
25% B(B) YL	**6.21***	13.66	0.065	0.83	0.07	0.11	0.12	0.07	0.16	1.43	5.32	**1.90****
35% B(B) YL	1.54	13.01	0.06	0.86	**0.05****	0.21	0.1	0.1	0.238	1.53	5.89	**4.91****
45% B(B) YL	3.81	13.02	0.056	0.82	0.06	0.23	0.11	0.08	0.211	1.52	5.69	**5.67****
15% B(A)	2.21	12.64	0.063	0.85	0.06	0.16	0.09	0.07	0.275	1.31	4.51	3.67
25% B(A) YL	**5.98***	13.65	0.058	0.79	0.06	0.18	**0.12****	0.07	0.205	1.5	5.51	**1.67****
35% B(A) YL	2.00	**17.20****	0.066	0.85	0.06	**0.26****	0.11	0.07	0.215	1.44	5.64	**6.11****
45% B(A) YL	**6.07***	13.01	0.058	0.79	0.06	**0.25****	0.09	0.08	0.229	1.6	5.94	4.13
15% W	5.31	11.79	0.055	0.8	0.06	**0.09****	**0.13****	0.06	0.19	1.53	5.55	**0.87****
25% W YL	**6.74***	13.38	0.065	0.98	0.06	0.15	**0.13****	**0.04****	0.213	1.52	5.07	**0.98****
35% W YL	**6.65***	13.81	0.065	0.79	0.05	0.19	0.09	0.09	0.205	1.61	5.49	3.66
45% W YL	2.53	13.50	0.06	0.8	0.06	**0.22****	0.09	0.07	0.24	1.34	5.36	**5.22****
Mixed model analysis												
Adjunct type	0.234	0.921	0.667	0.19	0.100	0.055	0.166	0.06	0.193	0.619	0.292	**<0.0001**
Incorporation rate (%)	**0.014**	**0.012**	0.132	0.065	0.103	**<0.0001**	**<0.0001**	**0.00**	0.106	0.258	0.822	**<0.0001**
FAN supplementation method	0.096	**0.022**	0.508	0.486	**0.001**	**0.028**	0.073	0.159	0.435	0.939	0.432	**<0.0001**

One-way ANOVA with Dunnett's post hoc analysis was used to compare the volatile compound concentration. Asterisks indicate samples that were significantly different in the samples compared to the all malt control (*p<0.01)

Table 4.15 illustrates the percentage difference from the malt control for acetaldehyde, esters and higher alcohols of the barley (B) beers supplemented with FAN Boost and YeastLife in this study with the barley (B) beers produced Chapter 3 (Section 3.3.2.4) where nitrogen content was not controlled.

Table 4.15. Volatile composition variance from 100% malt in studies one and two for barley (B) beers (%)

	Chapter 3- Study 1		Chapter 4- Study 2						
	30% B(B)	60% B(B)	15% B(B)	25% B(B)	35% B(B)	45% B(B)	25% B(B)	35% B(B)	45% B(B)
FAN Supplementation	-	-	-	FB	FB	FB	YL	YL	YL
Acetaldehyde	**-27**	**24**	0	-37	-9	14	63	-60	0
Ethyl Acetate	**-39**	**-41**	-1	-10	-12	-16	-1	-6	-6
Isoamyl Acetate	**-51**	**-33**	-8	-15	-8	-8	-11	-8	-12
Ethyl Hexanoate	**-36**	**-36**	-3	-3	11	-5	20	8	18
Ethyl Octanoate	**-27**	**-19**	6	-6	16	8	-20	18	5
Ethyl Butyrate	**-64**	**-53**	3	-5	-5	-5	3	-5	-11
Propanol	**-22**	**-25**	8	-14	-20	-14	13	5	12
Isobutanol	**-12**	**-1**	8	-12	-15	-9	9	14	14
Isoamyl Alcohol	**-16**	**-12**	10	-14	-14	-6	6	11	-6
Phenethyl Alcohol	**-21**	**-8**	0	23	2	-7	3	3	3
Tyrosol	**-36**	**-32**	-26	-4	-1	-1	-7	-19	-27
Tryptophol	**-39**	**-14**	-6	57	-1	30	13	7	8

The data in Table 4.15 show that both types of nitrogen supplementation method have improved the ethyl and acetate ester profile to being much closer to that of the all malt control. Similarly, at higher adjunct incorporation rates in Study 2, the higher alcohol concentrations showed little variation relative to the all malt control. This data show that the acetaldehyde concentration was not balanced by FAN supplementation and other yeast nutrition factors may have been caused variation in the acetaldehyde concentration.

4.3.2.8. Sensory Evaluation

An informal sensory evaluation by four experienced beer tasters was conducted on a selection of the YeastLife adjunct beer samples (0, 25 and 45% of each adjunct) which highlighted that even with nitrogen supplementation and minimal variation in the analytical profile there was still organoleptic differences when adjuncts are included. Differences were identified in the mouthfeel of the beers

and higher adjuncts beers were described as lacking flavour fullness rather than having a characteristic flavour.

4.3.2.9. Principle Component Analysis

PCA analysis was performed using the analytical results for parameters analysed in wort where a statistically significant difference was identified across the adjunct worts. Figure 4.11 shows a biplot of PC1 and PC2 which accounted for 73.18% of the variance in the data.

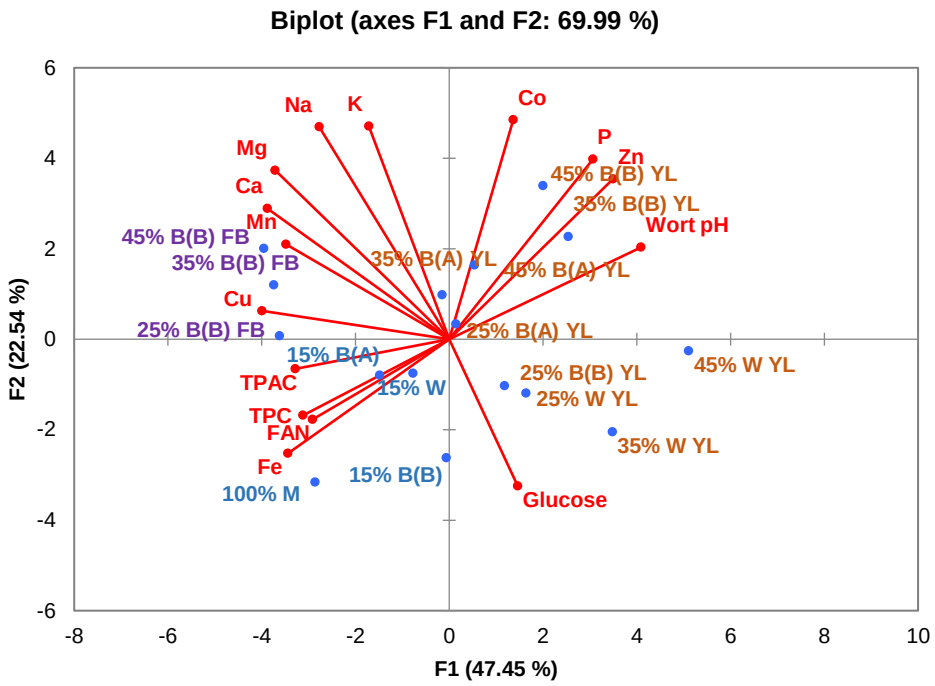


Figure 4.11. PCA Biplot of wort analytical data for the trial beers

Samples supplemented with FAN Boost are in purple, YeastLife samples are in brown, samples with no FAN supplementation are in blue and chemical compounds are in red.

PC1 explains 50.23% of the variation. A positive loading on PC1 corresponds with high amounts of zinc and phosphorous and high wort pH. These components correspond to high additions of YeastLife which can be seen by the 45% adjunct YeastLife grouping in the top right quadrant. Negative loadings on PC1 correspond to higher total polyphenol content, total proanthocyanidin content, FAN, copper and manganese ions. These components correspond with the adjunct worts that were supplemented with FAN boost. Interestingly, the all-malt control wort loads negatively on the PC2, showing similarities with the

adjunct wort produced with FAN boost. PC2 explains 22.95% of the variation. A positive loading on PC2 corresponds to higher amounts of sodium, potassium, cobalt, and therefore negative loadings on PC2 imply lower concentrations of these ions. A cluster of the wheat adjunct beers is observed in the bottom right quadrant. This reflects the fact that these samples typically had the lowest concentrations of the compounds with loading vectors in the upper left quadrant (Na, K, Mg, Ca, Mn, Cu and TPA). When comparing the adjunct wort samples against the 100% malt control wort, it can be seen that the 15% adjunct and the FAN Boost supplemented worts were closer in composition to an all-malt wort than the YeastLife supplemented adjunct worts.

A further PCA analysis was performed on the statistically significant components analysed in the finished beers. Figure 4.12 shows a biplot of PC1 and PC2 which accounted for 51.39% of the variance in the data set.

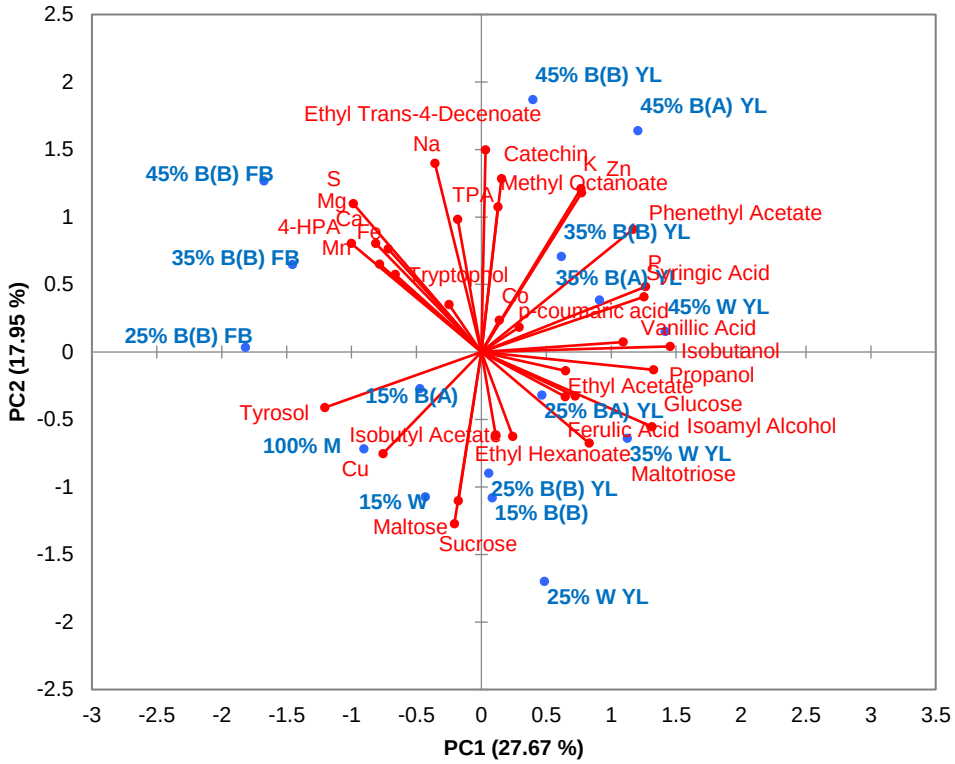


Figure 4.12. PCA biplot of the beer composition for the adjunct beer

Beer samples are in blue and chemical compounds are in red.

The main source of variance in the PCA plot is caused by the FAN supplementation methods. Negative loadings on PC1 corresponded with a cluster of the FAN boost supplemented samples associated with higher concentrations of magnesium, sulphur, manganese and calcium ions in beer. Tyrosol and the fermentable sugars projects the most negatively in the lower

left quadrant of the biplot correlating to the higher concentrations observed in the all-malt beer and lower adjunct concentration beers without FAN supplementation. However, 15% barley (B) does not fall in this cluster. The positive loadings on PC1 correspond to higher concentrations of higher alcohols and ethyl acetate associated with YeastLife supplemented beer samples. The variation across PC1 shows the impacts of including wheat in the grist, as the unmalted wheat-based beers typically had a lower concentration of the magnesium, sulphur, manganese and calcium, similarly lower concentrations of total polyphenols and catechin. PC2 positive loadings correspond to higher concentrations of phenolic compounds such as p-coumaric acid, catechin, vanillic and syringic acid, as well as concentrations of the minerals zinc and phosphorous. Negative loadings on PC2 were mainly associated with samples that have lower concentrations of the phenolic compounds, zinc and phosphorus. Similar sample positions can be observed in both the wort sample PCA and beer sample PCA in Figure 4.11 and Figure 4.12.

4.4. Conclusions

This study showed that the use of nitrogen supplementation created beers with analytical profiles closer to that of an all-malt control beer. The incorporation of wheat especially at 45% of grist showed the greatest variance from the all-malt control, especially with regard to the mineral and polyphenolic composition. Although this may make the beer quality differ from the control, the use of a small portion of wheat may be beneficial when considering quality parameters such as flavour stability. The incorporation of wheat decreased manganese, copper and iron in the wort and beer, these transition metals being associated with oxidative flavour deterioration. It would be interesting to see the impacts of mixing adjuncts such as barley (B) and wheat in different proportions to observe if a balance of their positive attributes would be observed.

The choice of FAN compensation method when brewing with high proportions of unmalted adjuncts is of great importance. Among the two studied options, use of an exogeneous protease led to faster fermentations, comparable wort characteristics and beer quality specifications, including the fermentation volatile profile. Despite the fact that YeastLife could successfully increase assimilable nitrogen, the mineral composition and ammonium chloride nitrogen source in YeastLife led to variability in the wort and beer and unfavourable results such as high pH, zinc concentrations and increased higher alcohol

concentrations. Also, YeastLife did not have a positive impact on the fermentation progression for the adjunct beers. Therefore, the use of a proteolytic enzyme was found to be more favourable for controlling assimilable nitrogen when brewing with adjuncts.

Limited sensory evaluation highlighted that even with nitrogen supplementation and minimal variation in the analytical profile there were still organoleptic differences when adjuncts are included. Further analysis is required to determine the cause of the reported change in mouthfeel. Investigating the metabolomics of the beer and exploring a wider range of non-volatile compounds could provide more information on the cause of this organoleptic variance. One of the most important factors to consider when brewing with adjuncts is consumer acceptability. There may be identifiable differences analytically and sensorially, but it is of interest to investigate if consumers would approve and ultimately purchase adjunct beers.

Chapter 5 Investigating the sensory and analytical drivers of consumer liking of unmalted cereal adjuncts in beer

5.1. Introduction

Large commercial breweries hold a near monopoly on the beer market and have previously brewed, almost exclusively, light lager beers, often with the use of unmalted cereal adjuncts (e.g. barley, wheat, rice and maize) (Jaeger *et al.*, 2020). Brewing with a small proportion of unmalted adjuncts had previously been less of a cost-cutting measure and more a response to consumer demand for beers that are faster to drink, without food and in more social settings (Betancur *et al.*, 2020; Dighe, 2016)

Although the use of unmalted adjuncts is well established in some of the most consumed beers worldwide, there is little comprehensive data exploring consumer acceptability of adjunct beer at different incorporation rates, and furthermore the significant sensory drivers for consumer preference. Traditional descriptive profiling methods such as quantitative descriptive analysis can be analysed alongside consumer preference data via Preference Mapping to provide sensory drivers of consumer liking. However, descriptive analysis can be costly and time consuming and access to a trained panel is not always possible. Rapid sensory profiling methods can be used with consumers (or naïve assessors) to substitute descriptive analysis when investigating sensory drivers of liking. Check-All-That-Apply (CATA) has gained popularity in sensory evaluation due to its simple format, lower level of cognitive effort required and rapid elicitation of sensory characteristics of the examined products from participants (Ares *et al.*, 2015b). Several studies have compared the results obtained with CATA methodologies conducted with naïve consumers with traditional descriptive profiling methods with a trained panel (Ghanbari *et al.*, 2017; Oliver *et al.*, 2018; Bruzzone, 2012; Dooley *et al.*, 2010). Oliver *et al.* (2018) compared the results from CATA method with the descriptive profiling method with a trained panel by means of regression vector (RV) coefficient which is used to assess similarities between two sets of data. The RV is regarded as a correlation coefficient in multidimensional spaces, therefore the closer the RV is to 1, the more similar the sample configurations of the two

spaces are. The study found that CATA produced moderately comparable sample configurations to descriptive analysis (RV coefficient= 0.760). In agreement, Ghanbari *et al.* (2017) found that the sample configurations were most comparable between CATA and descriptive analysis (RV coefficient= 0.938), thus establishing CATA as a time and cost-efficient alternative to descriptive analysis methodologies. In addition, CATA techniques can be used in conjunction with hedonic questions, allowing direct examination of drivers of liking in the sample set (Laureati *et al.*, 2017; Hiscock *et al.*, 2018). An advantage is that this approach does not require both separate sensory and consumer panels, as both techniques can be conducted by the same participants. Open-ended questions asking consumers to communicate the reason for their liking scores can be a useful complement to interpreting sensorial characteristics and preference findings (ten Kleij *et al.*, 2003; Symoneaux *et al.*, 2012). Symoneaux et al (2012) used open-ended questions to ask consumers what they liked and disliked about different batches of apples, as a complementary method for determining preference. This proved useful in identifying consumer's key sensory drivers (Symoneaux *et al.*, 2012). Although open-ended questions create rich textual data on the consumers perception of the products, the analysis of the outputs are more involved than quantitative methods such as CATA. The analysis of textual data is time-consuming and labour-intensive and since the results are in consumers' own language it can often be ambiguous and difficult to interpret. Consumers can also find it difficult to articulate their sensory perception of products or what they like or dislike about a product (Piqueras-Fiszman, 2015).

It is well understood that the chemical composition of beer impacts on the perceived sensory profile, as was discussed in detail in Chapter 1. The results from Chapter 3 showed that changes in volatile composition of the beer when incorporating adjunct material had a direct effect on the perceived sensory profile. Several studies have characterised the volatile and non-volatile composition of beer in order to determine factors which influence consumer preference (Lafontaine *et al.*, 2020; Ramsey *et al.*, 2021; da Costa Jardim *et al.*, 2018). The combination of sensory analysis and instrumental analysis provides a platform for identifying key flavour compounds associated with consumer preference. Understanding the chemical indicators which drive preference when brewing with adjunct materials can assist product development and increase marketability of high adjunct beers.

The objectives of the present research were to determine consumer perception, acceptance and willingness to buy for beers produced with adjunct incorporation rates of up to 45%. The use of CATA methodology and open-ended questions were used to identify the key sensory attributes driving liking and disliking in adjunct based beers. Furthermore, the analytical profile of each adjunct beer was determined to establish chemical indicators which could be associated with consumer preference. Based on our review of existing literature, this is the first study to explore consumer sensory perception and preference of beers brewed with different incorporation rates of unmalted cereal adjuncts.

5.2. Materials and methods

The brewing materials and analytical methods used in this study are described in Chapter 2.

5.2.1. Determining YeastLife and FAN Boost treatment rate required for adjunct wort production

Wort samples were produced using differing incorporation rates of barley (A), barley (B) and wheat (Table 5.1) and the dosing rates of FAN Boost or YeastLife required to increase wort FAN to >200 mg/L identified.

Table 5.1. Grist composition used in FAN Boost and YeastLife dosing trials

	FAN supplementation	Malt (%)	Wheat (%)	Barley (A) (%)	Barley (B) (%)
35% B(B)/W	FAN Boost	65	10	0	25
45% B(B)/W	FAN Boost	55	10	0	35
35% B(A)/W	FAN Boost	65	10	0	25
45% B(A)/W	FAN Boost	55	10	0	35
35% B(B)/W	YeastLife	65	10	0	25

Wort samples were produced using an R8 mash bath (1-Cube, Havlickuy Brod, Czech Republic). All grist materials were milled using a laboratory DFLU disc mill (Buehler Miag, Uzwil, Switzerland). For each wort sample, a liquor to grist ratio of 2.5:1 was attained by mixing 250 mL of reverse osmosis (RO) water at 65 °C with 100 g of mixed grist weighed into a metal mashing beaker. The mash was stirred throughout the mash profile and the same mash profile used in

Chapter 4 was applied (65 °C for 60 min, 72 °C for 10 min and 78 °C for 5 min). The wort samples were then cooled to 20 °C and adjusted with RO water and filtered through pleated filter paper (Whatman, 2555 ½, 320 mm, Sigma-Aldrich, Dorset, UK).

In order to determine the required FAN Boost dosage in the pilot scale brewing trials, FAN boost was added immediately before the mash profile was started at treatment rates of 0.5, 0.65, 0.75, 0.85 and 1 g/kg. Wort samples were produced with a grist composition of 35% B(B)/W (Table 5.1) and YeastLife was added in dosing concentrations of 0.5, 0.65, 0.75, 0.85 and 1 g/L to determine the required YeastLife dosing rate to increase FAN concentration over 200 mg/L. All YeastLife and FAN boost treatments were performed in triplicate. The specific gravity and free amino nitrogen content of each wort sample was determined according to the method described in section 2.5.3.

5.2.2. Pilot scale brewing procedure for adjunct beers and control

The seven adjunct beers and all malt control used for the consumer study were produced using a 40 L pilot scale nanobrewery (Briggs plc, Burton-on-Trent, UK) at the University of Nottingham. Table 5.2 shows the grist composition used for the eight beers brewed. All adjunct beers included a base of 10% unmalted wheat with differing amounts of barley (B) and barley (A). The introduction of a small portion of wheat investigated in this research was to align with industry usage of unmalted adjuncts and to investigate whether a small portion of wheat may be beneficial to quality parameters such as flavour stability which was observed in Chapter 4.

Table 5.2. Grist composition and FAN supplementation additions for adjunct and control beer production

	Malt (kg)	Wheat (kg)	Barley (A) (kg)	Barley (B) (kg)	FAN Boost Treatment (g/kg)	YeastLife Treatment (g/L)
100% Malt	10.0	-	-	-	-	-
25% B(B)/W (1)	7.5	1.0	-	1.5	-	-
25% B(B)/W (2)	7.5	1.0	-	1.5	-	-
35% B(B)/W FB	6.5	1.0	-	2.5	0.9	-
35% B(B)/W YL	6.5	1.0	-	2.5	-	1.0
45% B(B)/W FB	5.5	1.0	-	3.5	1.1	-
35% B(A)/W FB	6.5	1.0	2.5	-	0.9	-
45% B(A)/W FB	5.5	1.0	3.5	-	1.1	-

In this study, four of the adjunct beer samples with higher adjunct incorporation (over 25%) were supplemented with FAN boost at the start of the mash (FAN boost). In Chapter 4, it was determined that the addition of FAN boost produced beers with closer wort characteristics and fermentation volatile profile to an all malt control. Whereas the use of YeastLife led to unfavourable wort and beer characteristics such as high pH, zinc concentrations and increased higher alcohol concentrations. Here, one sample (35% B(B)/W YL) was nitrogen supplemented by adding YeastLife to the cold wort to facilitate a comparison of the consumer sensory perception of the two approaches to FAN supplementation. Two replicates of 25% B(B)/W were brewed to evaluate the reproducibility of the brewing process in determining the sensory and analytical profiles.

The grist compositions shown in Table 5.2 were weighed and mixed with a Roppi-250 roller mill (Robix, Hungary) at a gap setting of 0.4 mm. The grist to liquor ratio used in the mash was 2.5:1 with an addition of 90 mg/L calcium chloride. When FAN boost was included in the recipe, a laboratory predetermined treatment was added at the point of mash-in to the wet mash as described in Section 5.2.1. Mash-in temperature was 65 °C and held for 60 min, followed by a 10 min stand at 72 °C with final mash-off at 78 °C. The mash was separated using a lauter tun vessel and 8 L of sparge water were passed through the grain bed. The resulting wort was boiled with T90 pellets (variety: Zeus) for 60 minutes with a target final bitterness of 20 BU. The wort was adjusted with brewing liquor to 16 °P at the end of boil in the kettle. When YeastLife was included in the sample, the determined amount required from the FAN calculation was added after dilution in the kettle. Approximately 25-30 L of cold wort was produced and split evenly into two 30 L FastFerment plastic conical fermenters. SafLager W34/70 yeast (1 g/L dosing rate) was added to the cool wort and fermented at 15 °C for 10-12 days in line with the recommended fermentation temperature for W34/70 yeast (12-18 °C), followed by maturation at 4 °C for 4 days. Each sample was brewed twice to increase the amount of beer produced due to quantity requirements for this study, producing four fermenters (approximately 50-60 L) of green beer for each sample. The high gravity green beer was blended from the four fermenters and transferred into a 50 L stainless steel keg. The rough high gravity beer was filtered using a Carlson filtration sheet filter with kieselguhr impregnated sheets (Lancashire, UK) to a bright high gravity beer. The bright high gravity beer was manually diluted using de-aerated brewing liquor to a target of 4% ABV. The

beer was manually carbonated using carbon dioxide (CO_2) top pressure into headspace in the keg with manual agitation to a target carbonation level of 2.25 volumes of CO_2. The carbonated beer was bottled into 330 ml brown glass bottles using a Cime Careddu monoblock pressurised bowl filler (6 head rinser, 6 head filler, single head crowner) (Calamandrana, Italy). The bottles were microbiologically stabilised by pasteurisation using an automated batch pasteuriser using a shower of temperature-controlled water programmed to target 15 pasteurisation units (PU). All beers were stored at 4 °C until required for analysis.

5.2.3. Sensory consumer evaluation

5.2.3.1. Consumer recruitment

Before conducting this study, ethics approval was requested and accepted by the Faculty of Medicine & Health Sciences Research Ethics Committee at the University of Nottingham (Ethic Reference No. 94-0920). All participants provided consent to partake and were offered a disturbance allowance for their participation. Consumers (n=104) were recruited from the University of Nottingham based on their self-identified consumption (at least once a month) and their interest and availability to participate. Consumers also provided information regarding the frequency of consumption for several types of beer styles.

5.2.3.2. Beer samples

The beer samples used for this consumer study consisted of an all malt beer and seven adjunct beers as shown in Table 5.2. A commercial beer was provided as a dummy sample for the familiarisation session (Section 5.2.3.3). The commercial beer selected for the familarisation sessions had a similar sensory profile to the test beers. All samples were packaged in 330 ml amber bottles labelled with 3-digit blinding codes and stores at 4 ± 2°C.

5.2.3.3. Procedure

Consumers were invited to take part in two online evaluation sessions; the first session consisted of a 15 min familiarisation session and 45 min test session

and the second session consisted of a 45 min second test session. The objective of the 15 min familiarisation session was to familiarise consumers with the sensory attributes and the evaluation procedure, whilst in the test sessions, consumers reported their liking and perception of key sensory attributes using a Check-All-That-Apply (CATA) question.

All participants collected sample kits for the online evaluation sessions from the Sensory Science Centre at the University of Nottingham. The sample kits included the nine beer samples (8 test samples and 1 dummy sample), palate cleansers and serving cups. Transparent 50 ml plastic cups with 25 ml graduated markers were provided to ensure consistency for drinking containers and crackers (Carr's, Carlisle, UK) and Evian water (Danone, Paris, France) were provided for palate cleansing. Participants were instructed to refrigerate the beer samples for at least 24 hours before the evaluation session to ensure the beer samples were sufficiently refrigerated at the point of evaluation. Participants were also instructed not to open any samples until instructed during the online evaluation sessions. The online evaluations were conducted using the online video conferencing program Microsoft Teams (Version 1.3.00.30866). A maximum of 12 participants were invited to each online session which was hosted and monitored by the researcher. All online sessions were conducted between 1 pm and 7:30 pm. Participants were asked to join the session with their video camera and microphones connected to confirm their participation.

In the first online session, a familiarisation task (15 min) was performed to improve sample discrimination (Jaeger *et al.*, 2017). The task involved a presentation to outline the evaluation process and the pouring protocol for the beer samples to reduce any variation across participants. The attributes and descriptions used in the CATA questions is shown in Table 5.3, which were previously developed by a trained panel in Chapter 3 (Section 3.3.1.1). The procedure for completing the CATA question and the attribute lexicon was explained to ensure participants understood the attribute descriptors. The participants then practiced the full evaluation process using the dummy sample provided and this data was discarded. Data were collected using Compusense Cloud (Compusense, Guelph, ON, Canada).

Table 5.3. The adjunct beer attribute lexicon, description and reference standards developed in Chapter 3 used for CATA analysis

Modality	Attribute	Definition
Aroma	Green Apple/Emulsion Paint	The skin of a green apple and the aroma of emulsion paint
	Banana/Pear Drops	Artificial banana and pear drop sweets
	Sweetcorn/Cooked Vegetable	Tinned sweetcorn and cooked vegetables
	Smoky	Stale acrid smoke such as stale cigarette smoke or faint burnt rubber
	Malty	Sweet processed or cooked malt grains
	Grainy	Dry light barley malt grains
	Plum Fruit	Cooked plum fruit (plums, damsons and prunes)
Flavour	Green Apple/Emulsion Paint	The skin of a green apple and emulsion paint
	Banana/Pear Drops	Artificial banana and pear drop sweets
	Apple	Bruised sweet red apple
	Malty	Sweet processed or cooked malt grains
	Grainy	Dry light barley malt grains
	Plum Fruit	Cooked plum fruit (plums, damsons and prunes)
Taste	Sweet	Intensity of sweetness after swallowing
	Sour	Intensity of sourness after swallowing
	Bitter	Peak intensity of bitterness after swallowing
	Lingering Bitterness	Intensity of bitterness 20 seconds after swallowing
Mouthfeel	Astringent	Drying sensation in the mouth after swallowing

Four test beers were assessed in each of the test sessions, presented monadically using a randomised balanced design according to a Williams Latin Square. Consumers were provided with a 30 second timer before the start of each beer evaluation to open the sample and pour a 25 ml sample immediately before the evaluation to ensure the aliquots were fresh and carbonation and volatiles were not affected. Consumers assessed their overall liking of the adjunct beers using a 9-point hedonic scale ranging from ‘dislike extremely’ to ‘like extremely’. Consumers were asked to freely state separately over two open ended questions what they liked and what they disliked about the sample. There was no limit on word count and each consumer wrote their comments without

guidance, in their own personal style. Purchase intent was recorded on a 5-point scale ranging from ‘definitely would not buy’ to ‘definitely would buy’. A one-minute break for palate cleansing break was enforced, during which they were instructed to pour a fresh 25 ml sample for the CATA question. Consumers were asked to assess aroma first, checking all the attributes that ‘apply’ to that sample before assessing taste, flavour and mouthfeel. Consumers were instructed to familiarise themselves with the attribute list provided and the position of the attributes on the screen before assessment. The attributes were randomised across participants to minimise order bias, although panellists kept the same attribute layout throughout the assessment.

5.2.4. Statistical analysis

5.2.4.1. Instrumental analysis

One-way ANOVA with Dunnett’s test was used to compare adjunct samples to the 100% malt control for the analysis for all chemical analysis. One-way ANOVA with Tukey’s Honest Significant Difference (HSD) was applied to the analysis of mineral composition of the cereal grains used in this study. For the mineral composition of the cereal grains Dunnett’s test was not applied with one-way ANOVA as a direct comparison to a control (malt) was not required. Significance was derived at $p = 0.05$.

5.2.4.2. Overall liking and purchase intent

Mixed model two-factor ANOVA (sample, panellist) with panellist as a random effect was conducted on the overall liking scores for the adjunct beers. Tukey’s Honest Significant Difference (HSD) was applied to identify significant differences between samples for overall liking. Cluster analysis was performed on the overall liking data to determine patterns in liking data across consumers using agglomerative hierarchical clustering with Euclidean distance and Ward’s method in agglomeration was applied. Two-factor ANOVA with Tukey’s HSD was then applied to overall liking samples scores to determine significant differences between samples in each cluster and was also applied to each sample to determine significant differences between each cluster. For purchase intent data, two-way ANOVA was applied to the purchase intent scores and significant differences were identified in the purchase scores for all consumers

and the three separate clusters. To explore relationships between liking and purchase intent, Pearson's correlation coefficient was calculated.

5.2.4.3. Analysis of CATA data

The frequency of times that each participant selected each attribute for each sample was calculated to form a contingency table. The non-parametric Cochran's Q test with Bonferroni comparison test was performed to determine significant differences ($p<0.05$) in citation frequency among the beer samples. Liking and sensory attribute data were subjected to penalty-lift analysis to identify potential drivers for consumer like and dislike.

5.2.4.4. Comment analysis of open-ended questions

The analysis of the open-ended questions explaining drivers for liking and disliking for each sample required pre-processing before statistical analysis. Pre-processing included correcting any spelling mistakes, removing stop words, auxiliary terms and connecting words (e.g., the, I, will, etc.). Terms were then grouped into modalities (aroma, taste, flavour, mouthfeel, abstract and carbonation) whilst simultaneous lemmatisation step which converted every word to its standardised form. Ambiguous comments were not removed, and all terms were retained to avoid over-interpretation or over-grouping of terms by the researcher. In order to simplify the data set, hedonic terms (good, nice, bad) were removed as each response was already connected to liking and disliking. Stemming was applied to group deviates of the same word under one category term (e.g., sour and sourness) and a contingency table created to show how often a category term had been used to describe each of the samples. The initial table consisted of 143 terms, therefore, only category terms which have at least 10% of the consumers responses were retained for further analysis. Fisher's exact test was used to test the independence between samples and category terms of the contingency table. Fisher's was then applied to each cell identifying the source of variation in the data. Correspondence analysis was performed on the contingency table to provide a visual depiction of the relationship between samples and the terms used by the consumers.

5.2.4.5. Relationship between sensory and analytical data

Principle component analysis (PCA) was conducted on the CATA frequency terms and analytical characteristics to provide a visual depiction of the relationship between the sensory and analytical characteristic of the adjunct beers and all malt control.

5.3. Results and discussion

5.3.1. Preliminary laboratory wort experiments

Laboratory mashing trials were conducted to determine the addition rates of YeastLife and FAN boost required to increase wort FAN concentration over 200 mg/L as described in Section 5.2.1. Figure 5.1 shows the increase of FAN concentration when adding FAN boost to the mash. In agreement with the findings from Chapter 4 (Section 4.3.1.2), adjunct incorporation rates influenced the effectiveness of FAN boost. At 35% adjunct usage, wort FAN content increased more steeply with increasing dose rate of FAN boost than for 45% adjunct formulations. Thus a lower FAN boost dosage was required at the 35% adjunct level to attain target wort FAN (>200 mg/L); the linear regression lines shown in Figure 5.1 were used to calculate the required dose rates in each case. Figure 5.2 shows the increase of FAN concentration when adding YeastLife to 35% B(B)/W wort. The results from these laboratory trials enabled accurate additions to ensure a wort FAN level of over 200 mg/L when producing the adjunct beers for this study.

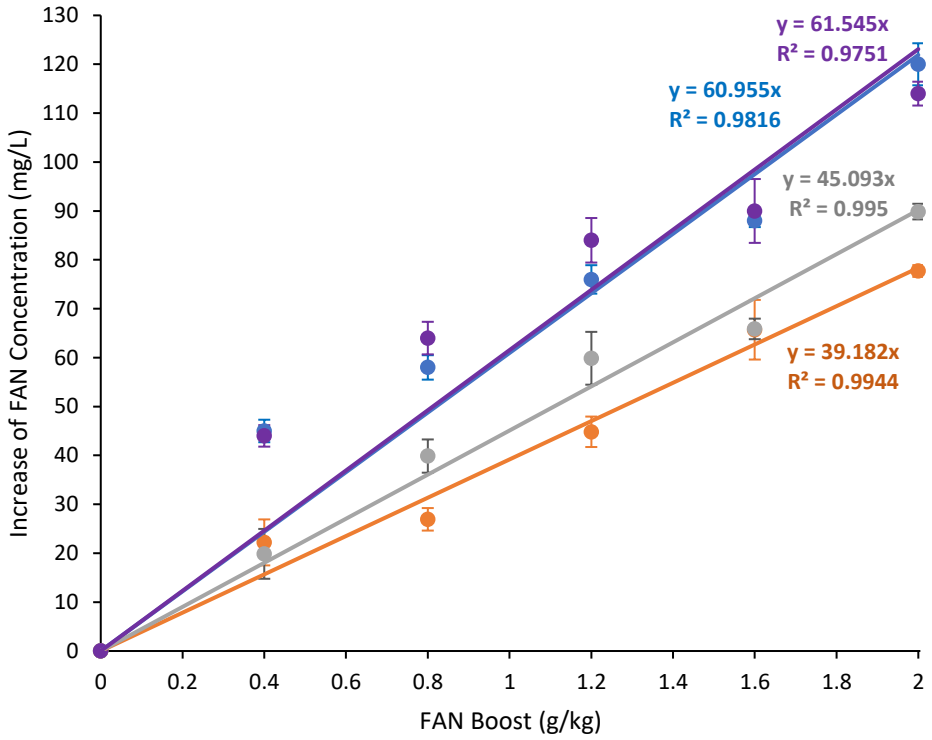


Figure 5.1. The impact of FAN Boost dosing on the increase of wort FAN concentration.

Blue markers indicate 35% B(B)/W samples, orange markers indicate 45% B(B)/W, purple markers indicate 35% B(A)/W and grey markers indicate 45% B(A)/W

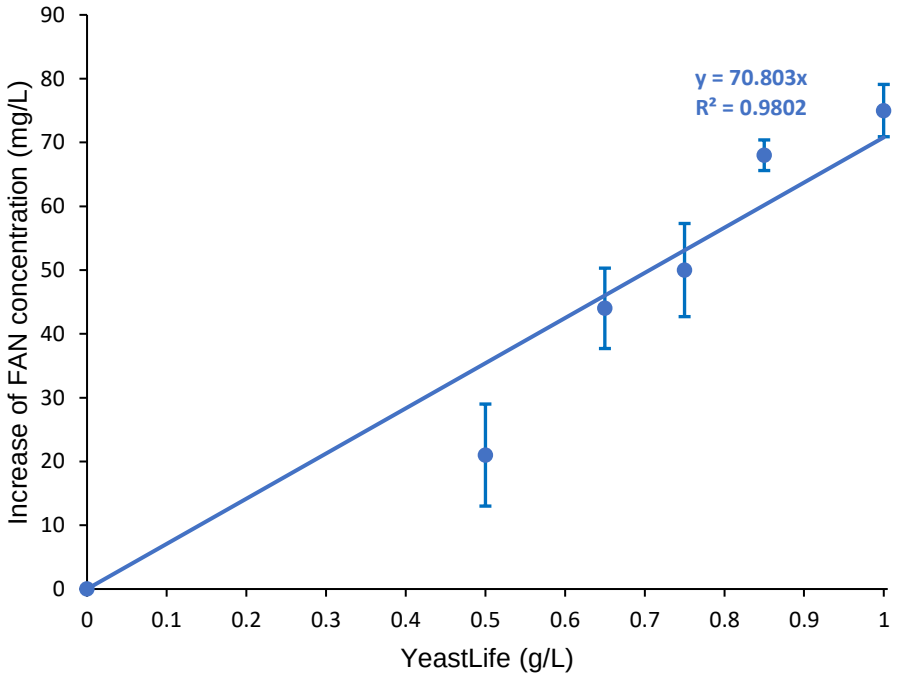


Figure 5.2. The impact of YeastLife dosing on the increase of 35% B(B)/W wort FAN concentration

5.3.2. Physiochemical analysis

5.3.2.1. Extract and carbohydrate composition of adjunct wort and beer

All wort samples were adjusted with brewing liquor after the boil to yield a target final extract of 16 °P. This resulted in wort samples with a measured extract content between 15.8 to 16.7 °P (Table 5.4). The final gravity increased with increasing adjunct material from the lowest of 2.3 °P in the 100% malt up to 4.0 °P with 45% B(B)/W incorporation. A similar decrease in attenuation with the incorporation of unmalted adjuncts was previously observed in the course of this work (Chapter 4) and in prior literature (Goode *et al.*, 2005). The final high gravity ABV for the sample set ranged from 6.6-7.9% with the 100% malt sample having the highest ABV. This was to be expected, as this fermentation had both the highest original gravity and the lowest final gravity, thus resulting in the highest ABV. All beer samples were diluted to an ABV between 3.8-4.1% before packaging.

Table 5.4. Fermentation parameters of adjunct wort and beer

	Extract (°P)	Final gravity (°P)	High gravity ABV (%)	Beer ABV (%)
100% Malt	16.7 ± 0.2	2.3 ± 0.2	7.9	4.1
25% B(B)/W (1)	16.5 ± 0.2	3.4 ± 0.2	7.2	4.0
25% B(B)/W (2)	16.2 ± 0.5	3.4 ± 0.1	6.9	4.0
35% B(B)/W FB	16.5 ± 0.2	3.9 ± 0.1	6.8	4.0
35% B(B)/W YL	16.4 ± 0.1	3.9 ± 0.1	6.9	3.8
45% B(B)/W FB	16.1 ± 0.1	4.0 ± 0.1	6.9	4.0
35% B(A)/W FB	15.8 ± 0.6	3.8 ± 0.1	6.6	4.1
45% B(A)/W FB	16.6 ± 0.4	3.9 ± 0.1	6.9	3.8

5.3.2.2. Carbohydrate composition of adjunct worts and beers

The fermentable carbohydrate composition (glucose, sucrose, fructose, maltose and maltotriose) of all worts produced in the study were determined (Table 5.5).

Table 5.5. Composition of fermentable sugars in adjunct wort samples

	Wort sugars standardised to 16 °P (g/L)					
	Glucose	Fructose	Sucrose	Maltose	Maltotriose	Total Sugars
100% Malt	12.54 ± 1.07	3.74 ± 0.33	9.31 ± 0.37	64.78 ± 3.33	19.82 ± 1.77	110.19 ± 4.00
25% B(B)/W (1)	**11.12 ± 0.24****	**3.43 ± 0.15****	**8.51 ± 0.59***	61.96 ± 1.26	19.58 ± 0.82	104.60 ± 1.17
25% B(B)/W (2)	**11.35 ± 0.58****	**3.21 ± 0.33****	**8.54 ± 0.83***	63.50 ± 3.75	19.82 ± 1.06	106.42 ± 5.82
35% B(B)/W FB	**10.44 ± 0.31****	**2.63 ± 0.32****	**8.12 ± 0.73***	**55.50 ± 2.11****	17.97 ± 1.32	**94.66 ± 3.59****
35% B(B)/W YL	**10.26 ± 0.23****	**3.10 ± 0.62****	**8.59 ± 0.62****	**56.50 ± 1.59****	18.50 ± 0.75	**96.95 ± 1.54****
45% B(B)/W FB	**10.17 ± 0.43****	**2.87 ± 0.32****	**7.74 ± 0.45****	**57.84 ± 2.25****	19.44 ± 0.87	**98.46 ± 3.32****
35% B(A)/W FB	**10.98 ± 0.92****	**3.21 ± 0.46****	8.68 ± 0.62	**61.08 ± 6.49****	18.81 ± 2.77	**102.76 ± 10.48****
45% B(A)/W FB	**9.91 ± 0.30****	**2.79 ± 0.55****	**7.20 ± 0.70****	**52.82 ± 1.58****	18.50 ± 0.75	**91.22 ± 1.54****

ANOVA with Dunnett's post hoc analysis was used to compare the fermentable sugars in adjunct wort. Asterisks indicate samples that were significantly different compared to the all-malt control (* $p<0.05$ and ** $p<0.01$).

The incorporation of adjunct material at any proportion significantly decreased the concentration of all sugars except maltotriose ($p<0.05$) (Table 5.5). The total fermentable sugars significantly decreased with adjunct incorporation rates above 25%. This result is expected, as most fermentable sugars are produced during the mash by the action of diastatic enzymes on starch (Fangel *et al.*, 2018). Therefore, the reduction in wort fermentable sugars reflects lower enzymatic activity in unmalted grains. At lower incorporation rates the enzymatic activity present in the base malt can convert starch in the mash successfully without the requirement of supplementary enzymes. However, at higher incorporation rates there is a limit to the digestion of starch from the malt enzymes. Therefore, the effect was most prevalent in the 45% B(A)/W FB wort which typically had significantly lower concentrations of most sugars.

Table 5.6 shows the composition of fermentable sugars remaining in the adjunct beer samples.

Table 5.6. Composition of fermentable sugars in adjunct beer samples

	Beer Sugars (g/L)				
	Glucose	Sucrose	Maltose	Maltotriose	Total Sugars
100% Malt	0.087	0.188	1.550	0.904	2.188
25% B(B)/W (1)	0.119	0.150	**1.814****	**0.756****	**2.831****
25% B(B)/W (2)	0.117	0.157	**1.906****	0.824	**2.998****
35% B(B)/W FB	**0.112***	0.163	**0.706****	**0.593****	**1.574****
35% B(B)/W YL	**0.094***	0.150	**0.877****	**0.534****	**1.656****
45% B(B)/W FB	**0.074****	**0.124****	**0.233****	**0.480****	**0.912****
35% B(A)/W FB	**0.105***	0.165	**0.459****	**0.504****	**1.258****
45% B(A)/W FB	**0.086****	**0.144****	**0.323****	**0.398****	**0.996****

ANOVA with Dunnett's post hoc analysis was used to compare the fermentable sugars in adjunct beers. Asterisks indicate samples that were significantly different compared to the all malt control (* $p<0.05$ and ** $p<0.01$).

The data set shows that the wort samples with the lowest fermentable sugars produced beers with the lowest total residual sugars after fermentation. All sugars except maltotriose differed significantly amongst the eight samples ($p<0.05$) with the highest incorporation of adjuncts (45% B(B)/W and 45% B(A)/W) resulting in the lowest amounts of fermentable sugars in finished beer. The 25% B(B)/W (1) and (2) beer samples contained significantly higher amounts of total sugars, especially maltose, compared to the all malt control ($p<0.01$). Beer carbohydrates have a direct effect on beer flavour, as fermentable sugars contribute to the sweetness of beer and carbohydrates with more than four glycosyl units contribute to body and mouthfeel (Li *et al.*, 2020a).

5.3.2.3. Mash, wort and beer pH

In this study, the mash pH ranged from 5.49-5.61 and adjunct material did not impact the mash pH (Table 5.7). The mash pH falls in the optimum pH range of 5.5-5.6 in respect of attenuation, protein breakdown, viscosity and lautering rate (Kunze *et al.*, 2004). The mash and wort pH (Table 5.7) are similar to the wort samples tested previously, both during the course of this work (Chapter 3 and 4) and in current literature (Bamforth, 2001; Kunze *et al.*, 2004). As discussed previously (Section 4.3.2.2), the use of YeastLife increased wort and beer pH. It would be recommended to adjust the wort pH to 5.0-5.2 prior to fermentation to reach reduce the beer to a suitable pH range.

Table 5.7. pH Analysis throughout the brewing process

	Mash pH	Wort pH	Beer pH
100% Malt	5.56 ± 0.02	5.50 ± 0.02	4.57
25% B(B)/W (1)	5.61 ± 0	5.55 ± 0	4.57
25% B(B)/W (2)	5.54 ± 0.01	5.44 ± 0.01	4.57
35% B(B)/W FB	5.54 ± 0.01	5.40 ± 0.01	4.51
35% B(B)/W YL	5.54 ± 0.05	6.02 ± 0.02	4.61
45% B(B)/W FB	5.50 ± 0.05	5.43 ± 0.04	4.51
35% B(A)/W FB	5.58 ± 0.04	5.47 ± 0.04	4.50
45% B(A)/W FB	5.49 ± 0.01	5.40 ± 0.01	4.52

5.3.2.4. Nitrogenous composition of adjunct wort and beers

The findings of Chapter 4 established the benefit of balancing FAN content with the use of an exogenous protease or yeast nutrient to produced adjunct wort and beer with comparable characteristics to that of an all malt beer. In this chapter, all adjunct worts with adjunct incorporation higher than 25% were supplemented with either FAN boost or YeastLife, producing wort with FAN concentration between 238-250 mg/L (Table 5.8).

Table 5.8. Free amino nitrogen and total nitrogen analysis of wort and beer samples

	FAN concentration standardised to 16 °P (mg/L)		Beer FAN (mg/L)	Wort Total Nitrogen (mg/L)	Beer Total Nitrogen (mg/L)
	Before YeastLife Addition	Cold Wort			
100% Malt	-	250 ± 6	51	1222 ± 2	450
25% B(B)/W (1)	-	199 ± 1	39	1054 ± 80	300
25% B(B)/W (2)	-	201 ± 3	36	911 ± 147	292
35% B(B)/W FB	-	238 ± 2	44	1267 ± 82	472
35% B(B)/W YL	182 ± 1	235 ± 4	61	1015 ± 47	328
45% B(B)/W FB	-	225 ± 5	49	1325 ± 58	398
35% B(A)/W FB	-	226 ± 4	48	1257 ± 82	515
45% B(A)/W FB	-	234 ± 2	49	1305 ± 65	414

In agreement with the findings of Chapter 4, the use of FAN boost increased the wort total nitrogen to above that of the 100% malt control. The use of a serine protease causes hydrolysis of the cereal proteins liberating polypeptides and peptides thus increasing total nitrogen content. When using YeastLife as a method of nitrogen supplementation, the total nitrogen content was lower than the concentration of the all malt control in both wort and beer.

5.3.2.5. Beer bitterness

The hop addition during beer production was targeted at a final bitterness of 20 BU. Table 5.9 shows the analysed bitterness units for the beer samples.

Table 5.9. Bitterness units (BU) of adjunct and control beer

	BU
100% Malt	17
25% B(B)/W (1)	19
25% B(B)/W (2)	17
35% B(B)/W FB	19
35% B(B)/W YL	17
45% B(B)/W FB	18
35% B(A)/W FB	18
45% B(A)/W FB	20

There was slight variation as the bitterness ranged from 17 to 20 BU across the sample set. Hahn *et al.* (2018) found that at low levels of BU, the relationship between perceived sensory bitterness intensity and the BU appeared to be linear. However, the variation in BU in this sample set is marginal so it would be unlikely to cause significant sensory differences in the beers.

5.3.2.6. Polyphenolic content of adjunct wort and beers

Polyphenolic compounds and proanthocyanidins are known for imparting bitterness and astringency in beer (Section 1.8.4). The total phenolic content (TPC) and total proanthocyanidin content (TPA) were determined for the adjunct and control wort and beer (Table 5.10).

Table 5.10. Total polyphenol and proanthocyanidin content of wort and beer samples

	Wort TPA (mg/L)	Beer TPA (mg/L)	Wort TPC (mg/L)	Beer TPC (mg/L)
100% Malt	108 ± 1	11	312 ± 9	130
25% B(B)/W (1)	96 ± 2	11	**250 ± 2****	138
25% B(B)/W (2)	94 ± 5	9	**243 ± 8****	133
35% B(B)/W FB	91 ± 2	9	**287 ± 9****	145
35% B(B)/W YL	94 ± 1	8	**282 ± 7****	140
45% B(B)/W FB	**78 ± 5****	9	**279 ± 13****	**151****
35% B(A)/W FB	98 ± 3	**16****	**294 ± 12***	138
45% B(A)/W FB	**88 ± 1****	**18****	**300 ± 2***	**149****

ANOVA with Dunnett's post hoc analysis was used to compare the total polyphenol and proanthocyanidin content in the adjunct wort and beer. Asterisks indicate samples that were significantly different compared to the all malt control (* $p<0.05$ and ** $p<0.01$).

ANOVA analysis with Dunnett's post hoc analysis showed that the wort TPC significantly decreased with the incorporation of any adjunct material whilst the wort TPA also decreased with adjunct material, especially at 45% adjunct incorporation rates. This result is expected as phenolic compounds are released from barley during malting thus increasing the polyphenolic content of malted barley. This is in agreement with prior findings during the course of this work (Chapter 4) and in published literature (Pejin *et al.*, 2009; van Donkelaar *et al.*, 2016). The beer TPC and TPA were comparable to the all malt control up to 45% adjunct incorporation, where higher incorporation rates of barley significantly increased the total polyphenol and proanthocyanidin content. Phenolic substances decrease during fermentation as polyphenols bind with proteins and polysaccharides such as yeast mannoproteins. The mannoprotein production during fermentation depends on yeast strain and their nutritional conditions (Fumi *et al.*, 2011). Increasing the barley (A) and barley (B) rate affects the nutritional conditions of the wort so this could give reason as to why the TPC and TPA decrease with adjunct incorporation in the wort but increase with adjunct incorporation after fermentation.

5.3.2.7. Mineral composition of cereal grain, adjunct wort and beer

Metal ions play an important role in yeast performance and thus influence the beer flavour and stability (Section 1.7.4). The mineral ion composition of the grist material used in this study is shown in Table 5.11.

Table 5.11. Mineral ion composition of grist material used in this study (mg/kg)

	Na	Mg	K	Ca	Mn	Fe	Cu	Zn
Malt	69.0 ± 5.6^{a}	1086.7 ± 30.7^{a}	4099.1 ± 235.7^{b}	521.1 ± 9.6^{a}	9.2 ± 0.6^{b}	48.8 ± 1.6^{a}	3.2 ± 0.2^{b}	29.6 ± 0.9^{a}
Barley (A)	35.0 ± 0.4^{b}	968.3 ± 23.3^{a}	4430.2 ± 139.0^{a}	510.9 ± 33.9^{a}	9.5 ± 0.7^{b}	36.3 ± 6.5^{a}	4.7 ± 0.4^{a}	25.9 ± 0.7^{a}
Barley (B)	32.8 ± 2.5^{b}	918.9 ± 8.5^{a}	4378.8 ± 15.5^{a}	363.6 ± 12.7^{b}	8.3 ± 0.4^{b}	28.7 ± 0.1^{b}	4.3 ± 0.1^{a}	27.3 ± 0.5^{a}
Wheat	6.9 ± 1.0^{c}	600.6 ± 9.5^{b}	3469.0 ± 15.9^{b}	391.0 ± 21.1^{b}	16.8 ± 1.3^{a}	26.5 ± 1.1^{c}	2.3 ± 0.1^{c}	15.2 ± 0.8^{b}

ANOVA with Tukey's HSD post hoc analysis was used to compare the mineral ion composition of cereal grist material. All mineral ions were significantly different across the sample set ($p<0.0001$). Superscript letters indicate post-hoc groupings of cereal grains according to Tukey's HSD test.

The concentrations of mineral ions were significantly different across the four cereal grains analysed ($p<0.001$). For most of the mineral ions analysed, unmalted wheat contained significantly lower concentrations compared to malted barley. This result is in agreement with the findings from mineral composition analysis of grist material in Chapter 4 (Section 4.3.2.6). The manganese concentration was significantly higher in wheat compared to the other cereal grains, in agreement with results already reported in Chapter 4 (Section 4.3.2.6) and prior literature (Poreda *et al.*, 2015). The concentrations of potassium and copper ions were significantly higher in barley (A) and barley (B) compared to malt, which was not previously observed in Section 4.3.2.6. Different sources of grist materials were used across the two studies which can impact the mineral composition of the grain. Several studies have found that mineral ion content can vary widely between different barley samples due to environmental conditions such as climatic conditions, soil fertility and genetic makeup (Yan *et al.*, 2016; Hussain *et al.*, 2021; Rodehutscord *et al.*, 2016).

Fermenting yeast requires adequate mineral levels from the wort to support optimum growth, healthy fermentation and to produce a balanced flavour profile in the beer (Section 1.7.3). The mineral composition of the adjunct and all malt control wort produced in this study are shown in Table 5.12.

Table 5.12. Mineral ion composition of adjunct wort samples

Sample	Na (mg/L)	Mg (mg/L)	K (mg/L)	Ca (mg/L)	Mn (µg/L)	Fe (µg/L)	Cu (µg/L)	Zn (µg/L)
100% Malt	44.96 ± 2.38	119.60 ± 8.23	819.09 ± 45.09	35.30 ± 2.64	189.86 ± 14.73	129.75 ± 9.64	164.39 ± 12.83	174.25 ± 17.06
25% B(B)/W (1)	**38.81 ± 1.69****	116.57 ± 6.23	895.58 ± 47.02	32.38 ± 1.79	209.64 ± 11.09	**96.56 ± 14.17****	153.09 ± 9.39	161.40 ± 7.67
25% B(B)/W (2)	**34.22 ± 2.02****	105.53 ± 7.42	834.43 ± 47.87	**26.60 ± 2.29****	188.39 ± 14.09	**101.85 ± 12.23****	162.81 ± 16.04	158.23 ± 10.73
35% B(B)/W FB	**32.70 ± 1.69****	109.64 ± 8.40	905.16 ± 35.18	**22.78 ± 1.78****	192.32 ± 14.64	**52.17 ± 7.38****	**137.99 ± 19.10***	158.82 ± 35.58
35% B(B)/W YL	**35.75 ± 0.80****	103.38 ± 2.42	**963.58 ± 33.35****	**28.22 ± 2.78****	176.04 ± 12.05	**71.56 ± 27.70****	**129.55 ± 5.34****	**1252.60 ± 59.74****
45% B(B)/W FB	**34.05 ± 1.03****	**122.17 ± 7.64***	**950.67 ± 28.03****	**25.50 ± 3.38****	194.31 ± 12.08	**64.04 ± 2.00****	139.48 ± 6.77	144.65 ± 16.16
35% B(A)/W FB	**36.08 ± 2.51****	108.84 ± 8.55	**954.46 ± 66.52****	**26.10 ± 1.86****	200.29 ± 24.09	**68.08 ± 10.86****	140.35 ± 12.77	156.20 ± 27.14
45% B(A)/W FB	**32.39 ± 2.89****	**100.53 ± 7.25***	**1004.67 ± 68.68****	**22.96 ± 3.43****	193.09 ± 26.55	**60.11 ± 3.77****	**111.80 ± 12.95****	149.21 ± 33.01

ANOVA with Dunnett's post hoc analysis was used to compare the mineral ion composition of adjunct wort. Asterisks indicate samples that were significantly different compared to the all malt control (* $p<0.05$ and ** $p<0.01$).

The concentrations of sodium and iron significantly decreased compared to the all malt control with all incorporation of adjunct materials. This result was expected, based on lower concentrations observed in barley (A), barley (B) and wheat in the grain analysis (Table 5.11). The wort manganese concentration did not significantly change with adjunct incorporation ($p > 0.05$). This result could be explained by the 10% proportion of unmalted wheat included in the grist bill when brewing the adjunct beers. The high concentration of manganese in the wheat grain could have balanced the decrease in manganese caused by incorporating barley (A) and barley (B). In agreement with the results found in Chapter 4 (Section 4.3.2.6), the inclusion of YeastLife increased wort zinc concentrations significantly ($p<0.01$). Zinc is essential in the operation of several enzymes which regulate the production of several flavour active fermentation compounds (Section 1.7.3). Wort zinc concentrations lower than 0.1 mg/L are generally considered too low and may lead to sluggish fermentation (De Nicola *et al.*, 2011). In this case, all wort samples had a zinc concentration over 0.1 mg/L, however the use of YeastLife increased wort zinc concentration 10-fold to 1 mg/L. Although YeastLife increased the wort zinc concentration significantly, previous studies have found that it requires over 10 mg/L zinc in the wort to impact the fermentation flavour profile of beer (Quilter *et al.*, 2003; Nicola *et al.*, 2009).

The mineral ion composition of the adjunct beers and all malt control was determined using ICP-MS. Table 5.13 shows the concentration of key mineral ions in the beer samples.

Table 5.13. Mineral ion composition of adjunct beer and control samples

Sample	Na (mg/L)	Mg (mg/L)	K (mg/L)	Ca (mg/L)	Mn (µg/L)	Fe (µg/L)	Cu (µg/L)	Zn (µg/L)
100% Malt	26.32	50.22	331.15	31.63	110.31	75.78	109.52	44.99
25% B(B)/W (1)	23.64	57.26	**409.51****	29.19	113.57	77.97	106.59	44.99
25% B(B)/W (2)	25.41	55.91	**397.07****	33.13	118.74	71.04	107.05	40.70
35% B(B)/W FB	23.86	55.16	**417.79****	27.98	**154.91****	**53.91****	**98.57***	39.20
35% B(B)/W YL	**31.85****	54.88	**391.92****	**66.22****	**169.46****	76.48	108.60	47.89
45% B(B)/W FB	23.61	53.63	**450.40****	41.42	**150.56****	71.70	104.46	49.15
35% B(A)/W FB	24.41	59.13	**474.52****	21.97	**144.55****	**69.88****	**99.81***	42.45
45% B(A)/W FB	**29.35****	52.34	**416.01****	**73.86****	**172.67****	**61.57****	107.07	47.46

ANOVA with Dunnett's post hoc analysis was used to compare the mineral ion composition of adjunct beer. Asterisks indicate samples that were significantly different compared to the all malt control (* $p<0.05$ and ** $p<0.01$).

ANOVA with Dunnett's post hoc analysis determined that the concentration of potassium and manganese in the beer samples significantly increased with any type of adjunct incorporation ($p<0.01$). This correlates to the higher concentrations of these mineral ions observed in the wort which has carried through fermentation into the finished beer. Although there was a significantly higher concentration of zinc in the 35% B(B)/W YL wort ($p<0.01$), the zinc concentration in the beer was comparable to that of the sample set, indicating that the zinc had been fully utilised during the fermentation. The transition metals iron and copper are important for flavour stability (Section 1.7.4). In this present study, the concentration of iron and copper in beer decreased with adjunct incorporation rates. However, not to the same extent as observed in Section 4.3.2.6 (Table 4.12). A decrease in iron and copper could be a result of differences in the raw materials as significant decreases were also observed in wort produced with adjuncts.

5.3.2.8. Analysis of flavour-active fermentation volatiles

The volatile constituents of beer are important for its quality and palatability (Section 1.5.4). Table 5.14 reports the analysed concentrations of fermentation-derived volatiles in the adjunct beers and the all malt control.

The concentration of higher alcohols (propanol, isobutanol and phenyl ethyl alcohol) did not significantly change with adjunct incorporation ($p >0.05$). Higher alcohol production is closely related to wort nitrogen composition (He *et al.*, 2014). In this case, the use of FAN supplementation has balanced the wort nitrogen composition and successfully balanced the higher alcohol profile closer to that of the all-malt control.

The data in Table 5.14 indicate that most of the identified acetate and ethyl esters reduced in concentration with the addition of any unmalted adjunct. This result is most significantly observed with the 45% B(A)/W sample where eight of the key esters were significantly lower in concentration compared to the all-malt control ($p<0.01$). The lower wort sucrose level observed in the 45% B(A)/W sample could have attributed to the lower concentration of esters. Previous studies have reported that decreased wort sucrose levels reduce the production of esters during fermentation (Younis *et al.*, 1998; He *et al.*, 2014) which is discussed in more detail in Section 1.3.4.

Table 5.14. Fermentation volatile composition of adjunct beers versus an all malt control

	100% Malt	25% B(B)/W (1)	25% B(B)/W (2)	35% B(B)/W FB	35% B(B)/W YL	45% B(B)/W FB	35% B(A)/W FB	45% B(A)/W FB
Acetaldehyde (mg/L)	1.23	**1.77***	0.91	1.17	**2.17****	1.25	**1.92****	1.35
Propanol (mg/L)	5.28	5.83	5.38	5.60	5.38	5.61	5.70	5.23
Isobutanol (mg/L)	9.26	9.15	9.24	9.11	8.87	9.09	9.57	9.32
Isoamyl Alcohol (mg/L)	34.67	**38.47****	**40.98****	**42.94****	36.35	**44.41****	**44.94****	38.50
Phenethyl Alcohol (mg/L)	10.33	10.26	10.67	9.77	9.01	11.05	9.95	9.84
Ethyl Acetate (mg/L)	4.02	3.87	3.22	3.82	3.84	3.25	3.84	3.37
Isoamyl Acetate (mg/L)	0.59	0.57	0.55	0.53	**0.47****	0.54	0.55	**0.46****
Isobutyl Acetate (mg/L)	0.06	0.05	0.06	**0.07****	0.06	0.06	0.06	**0.04****
Phenethyl Acetate (mg/L)	0.18	0.19	0.17	**0.12****	**0.10****	**0.13****	**0.13****	**0.07****
Ethyl Hexanoate (mg/L)	0.07	0.07	0.07	0.06	0.06	0.06	0.07	**0.05****
Ethyl Octanoate (mg/L)	0.18	0.21	0.19	0.15	0.19	**0.12****	**0.12****	**0.11****
Ethyl Butyrate (mg/L)	0.03	0.02	0.02	0.02	0.02	0.03	0.02	0.02
Ethyl Decanoate (mg/L)	4.83	4.47	4.51	4.83	**2.80****	**2.67****	**2.40****	**2.32****
Methyl Octanoate (mg/L)	0.04	**0.02****	**0.02****	**0.02****	**0.01****	**0.00****	**0.02****	0.03
Ethyl Isobutyrate (µg/L)	5.68	**4.77****	5.03	5.53	5.42	**4.50****	5.12	**4.47****
Ethyl Pentanoate(µg/L)	0.04	0.04	0.04	0.04	**0.05***	0.04	0.03	**0.05***
Ethyl Heptanoate (µg/L)	1.98	**1.47****	**1.76****	1.93	2.27	**2.49****	2.02	**1.76****
Ethyl Trans-4-Decenoate (µg/L)	3.99	**5.78***	3.43	5.16	**2.21***	**5.81***	4.56	3.71

ANOVA with Dunnett's post hoc analysis was used to compare the fermentation volatile concentrations. Asterisks indicate samples that were significantly different compared to the all malt control (* $p<0.05$ and ** $p<0.01$).

The standard beer quality and chromatographic flavour analysis used in this study on the adjunct beers has confirmed that use of FAN supplementation can improve nitrogen content when using FAN deficient unmalted adjuncts. Although the improvement of nitrogen in the wort has enabled a more balanced fermentation profile when considering the formation of higher alcohols, a significant change in ester and carbohydrate profile was observed at 45% adjunct incorporation rates. Consumer analysis was used to determine if there are any noted changes in sensory attributes caused by the differences in chemical composition and ultimately if this had an impact on consumer preference and acceptance.

5.3.3. Consumer analysis

5.3.3.1. Overall liking and purchase intent

Two-factor ANOVA showed significant differences amongst the 8 beers ($p<0.0001$) for overall liking. The mean overall liking scores (Table 5.15) show that 35% B(B)/W YL had a significantly lower overall liking (4.81) compared to the rest of the sample set, with the highest mean score for the sample 25% B(B)/W (1) (6.13) corresponding to 'like slightly' on the 9-point hedonic scale. This indicates that consumers preferred adjunct beers supplemented with FAN boost samples over the one with YeastLife. Interestingly, sample 25% B(B)/W (1) obtained a slightly higher mean overall liking than the all-malt beer, suggesting acceptability of small incorporation of adjuncts. The adjunct incorporation rate of this sample is aligned with typical industry usage for adjunct beers, thus potentially being familiar to consumers. Overall, the type of adjunct and amount of adjunct included did not appear to have a significant impact on overall consumer acceptance indicating that the majority of consumers accepted all adjunct types at all incorporation rates. However, on closer inspection of the data there appeared to be differences between consumers regarding their liking.

Table 5.15. Mean overall liking scores (standard deviations) for the beer samples for all consumers and for the three consumer clusters identified in cluster analysis

Sample	All Consumers (n=104)	Cluster 1 (n=43)	Cluster 2 (n=36)	Cluster 3 (n=25)
100% Malt	6.06^{a} (1.72)	6.93^{abA} (1.26)	5.44^{aB} (1.84)	5.44^{bcB} (1.53)
25% B(B)/W (1)	6.13^{a} (1.69)	6.09^{bcA} (1.10)	5.42^{aB} (1.73)	6.72^{abA} (1.37)
25% B(B)/W (2)	6.03^{a} (1.75)	7.02^{abA} (1.74)	5.39^{aB} (1.83)	5.60^{abcB} (1.70)
35% B(B)/W FB	5.97^{a} (1.80)	7.12^{aA} (1.13)	4.75^{abB} (1.90)	5.68^{abcB} (1.84)
35% B(B)/W YL	4.82^{b} (1.93)	5.86^{cA} (1.90)	4.00^{bB} (2.04)	4.72^{cAB} (1.62)
35% B(A)/W FB	5.85^{a} (1.89)	6.88^{abA} (1.40)	5.22^{abB} (1.90)	4.84^{cB} (1.92)
45% B(B)/W FB	5.98^{a} (1.90)	6.98^{abA} (1.28)	4.11^{bB} (1.63)	6.88^{aA} (0.92)
45% B(A)/W FB	5.83^{a} (1.95)	6.26^{bcA} (1.78)	4.47^{bB} (1.85)	6.92^{aA} (0.81)

Different letters within a beer sample^{ab} or cluster^{AB} represent post-hoc groupings using Tukey's HSD ($p < 0.05$)

Therefore, agglomerative hierarchical clustering analysis was applied to overall liking scores to determine if there were differences between consumers in their liking of the beers not evident from the mean liking scores. Three distinct groups of consumers were distinguished (Figure 5.3) and ANOVA revealed significant

differences between clusters, indicating the consumers interpreted the beers differently across the clusters (Table 5.15).

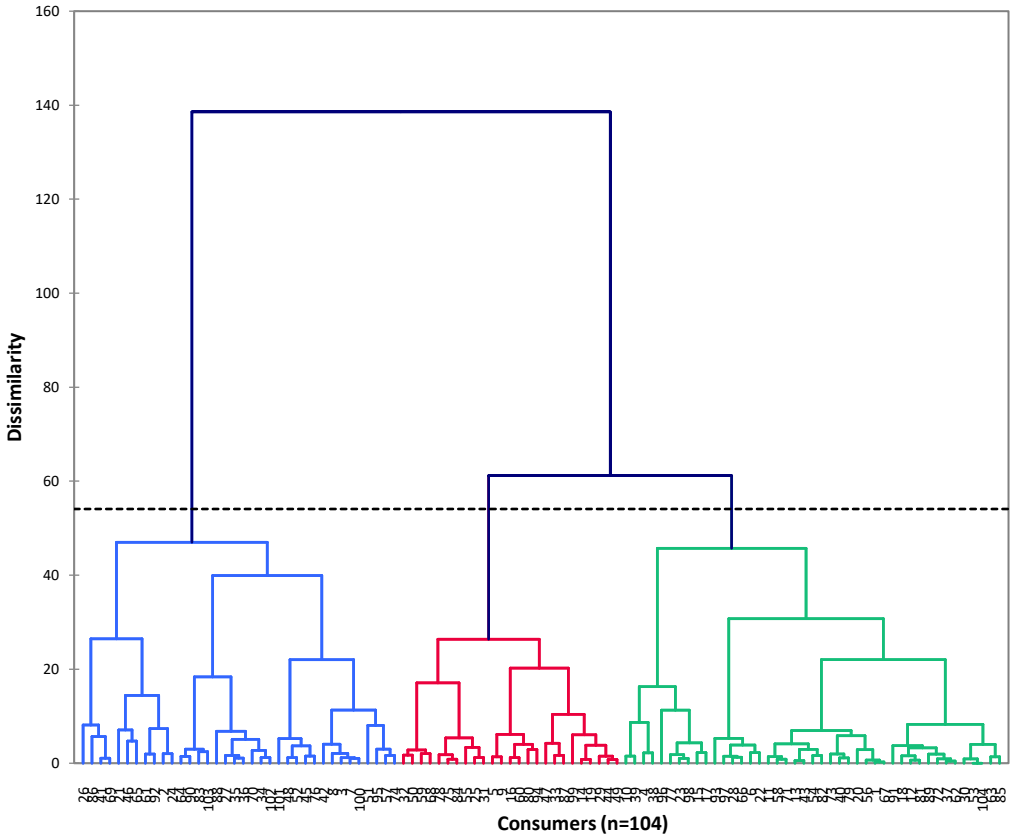


Figure 5.3. Dendrogram of the three consumer clusters identified based on overall liking of adjunct beers

Cluster 1 (C1) was the largest group consisting of 43 consumers (41%). C1 showed higher liking for all samples compared to C2 and C3, specifically C2 which showed significant differences across the clusters for all samples ($p<0.0001$). The consumers in C1 could be described as 'all beer likers' as most samples were rated as 'like slightly' or 'like moderately', with highest liking for the 35% B(B)/W FB sample. In contrast, cluster 2 (C2) comprised of 36 consumers (35%) who had statistically lower overall liking scores compared to C1. C2 showed lower liking, rating all samples as 'dislike slightly' to 'neither like nor dislike'. However, further inspection of this cluster showed higher liking for 100% malt and lower adjunct incorporation rates, as 25% B(B)/W (1) and (2) were significantly more liked than the 45% adjunct beers. Therefore, this cluster could be described as 'adjunct dislikers'. Cluster 3 (C3) comprised of 25 (24%) consumers and had the most varied mean scores out of the three clusters with a marked preference for 45% B(B)/W and 45% B(A)/W. The consumers in C3 showed the opposite preference to those of C2 and could be described as

‘adjunct likers’ as they preferred higher adjunct beers to those containing lower or no adjunct material. These results support the findings of previous studies that have determined that beer consumers are not uniform in their consumption preferences and form different consumer segments (Calvo-Porral *et al.*, 2018). Determining the key liking and disliking drivers for the results obtained for each consumer segment could help brewers develop adjunct based products better targeted at each cluster.

Participants were asked to rate their purchase intent on a 5-point scale for each beer sample immediately after determining the overall liking (Table 5.16).

Table 5.16. Mean purchase intent scores (standard deviations) for the beer samples for all consumers and for the three consumer clusters

Sample	All consumers (n=104)	Cluster 1 (n=43)	Cluster 2 (n=36)	Cluster 3 (n=25)
100% Malt	2.92^{a} (1.02)	3.42^{ab} (0.88)	2.86^{a} (1.05)	2.52^{bc} (1.19)
25% B(B)/W (1)	3.10^{a} (1.13)	3.09^{ab} (0.85)	2.81^{a} (1.11)	3.20^{ab} (1.28)
25% B(B)/W (2)	2.96^{a} (1.16)	3.60^{a} (1.16)	2.64^{a} (1.24)	2.64^{abc} (1.04)
35% B(B)/W FB	3.00^{a} (1.23)	3.72^{a} (1.22)	2.39^{a} (1.12)	2.64^{abc} (1.26)
35% B(B)/W YL	2.48^{b} (1.28)	2.75^{b} (1.05)	2.11^{b} (1.15)	2.16^{c} (1.25)
35% B(A)/W FB	3.00^{a} (1.23)	3.50^{a} (1.05)	2.58^{a} (1.22)	2.48^{bc} (0.98)
45% B(B)/W FB	3.05^{a} (1.11)	3.55^{a} (0.95)	2.14^{a} (0.36)	3.52^{a} (0.82)
45% B(A)/W FB	2.85^{a} (1.26)	3.11^{ab} (1.25)	2.14^{a} (0.00)	3.40^{ab} (0.95)
p-value	<0.0001	<0.0001	<0.0001	<0.0001
Pearson’s R	0.821	0.841	0.816	0.789

P value shows significant differences for purchase intent (alpha 0.05) within a column. Different letters within a beer sample^{ab} represent post-hoc groupings using Tukey’s HSD ($p < 0.05$). Pearson’s R measures the relationship between overall liking scores and purchase intent scores.

In agreement with the results for overall liking, 35% B(B)/W YL obtained a significantly lower purchase intent score compared to the rest of the sample set. Pearson’s correlation coefficient was performed to measure the strength of association between the overall liking and purchase intent score. There was a positive correlation (R 0.821) between overall liking and purchase intent indicating a strong relationship between both measures. However, it is important to note that mean scores for purchase intent were between ‘would not buy’ and ‘might or might not buy’ indicating that the liking scores may not be high enough for purchase to be considered. It seems likely that consumers assessing the beers in uninformed conditions may have negatively impacted the purchase intent from the consumer as a decision to purchase a beer sample is influenced by intrinsic and extrinsic cues of the product’s properties (Zanetta *et al.*, 2020; Chaya *et al.*, 2015). This study focused on intrinsic cues relating to the physicochemical characteristics such as taste, aroma and flavour. Extrinsic cues (brand, packaging, advertising and price) were not provided to the

consumer when making their purchase intent decision which could have influenced the consumer's purchase intent score. In some studies, extrinsic cues have been suggested as a primary influence and can impact willingness to pay more than intrinsic cues (Aquilani *et al.*, 2015; Lee *et al.*, 2006; Galizzi *et al.*, 2012).

However, purchase intent scores did follow liking trends for clustered data. C1 (all beer likers) showed a higher willingness to buy for most samples compared to the C2 and C3. 60% of C1 indicated that they would purchase 35% B(B)/W FB which was the sample that obtained the highest mean overall liking score for C1 (Table 5.15). Consumers in C2 (adjunct beer dislikers) showed their disliking for high adjunct beers with over 95% of the consumers in this cluster indicating they would not buy (rating ≤ 2) beers produced with 45% incorporation rates of adjuncts. In contrast, over 60% of consumers in C3 indicated they would buy (rating ≥ 4) the sample 45% B(B)/W FB.

5.3.3.2. Check-all-that-apply (CATA) sensory attribute data

The frequency table of terms checked by all consumers (n=104) to describe the eight adjunct beers is reported in Table 5.17.

The most frequently used terms were 'bitter', 'green apple aroma' and 'malty flavour'. The least used terms were 'smoky aroma', 'plum fruit aroma' and 'plum fruit flavour'. Cochran's Q test was used to evaluate the discrimination across the samples for each attribute. Significant differences ($p<0.05$) were identified in 9 out of the 18 terms used to describe the aroma and flavour of the adjunct beers. Significant differences were found for five aroma attributes (green apple, banana, grainy, sweetcorn and smoky), two flavour (banana and grainy) and two taste (bitter and lingering bitterness) attributes. This suggests that this type of question was able to detect differences in consumers' perception of the sensory characteristics of the evaluated beers, despite the small variation in consumers liking scores. No significant differences ($p>0.05$) were observed for the remaining attributes indicating that the consumers could not significantly discriminate between the samples for these sensory terms.

Table 5.17. Frequencies (percentage) of selection of the CATA terms used by consumers to describe adjunct beers, and results from Cochran's Q test for comparison between samples

Attributes	p value	Sample							
		100% Malt	25% B(B)/W (1)	25% B(B)/W (2)	35% B(B)/W FB	35% B(B)/W YL	35% B(A)/W FB	45% B(B)/W FB	45% B/(A)W FB
Aroma									
Green Apple/Emulsion Paint**	0.005	37 (35.6)	32 (30.8)	43 (41.3)	50 (41.3)	33 (31.7)	35 (33.7)	48 (46.2)	29 (27.9)
Banana/Pear Drops**	0.000	27 (26.0)	34 (32.7)	34 (32.7)	32 (30.8)	31 (29.8)	40 (38.5)	29 (27.9)	11 (10.6)
Malty	0.566	31 (29.8)	35 (33.7)	23 (22.1)	26 (25.0)	25 (24.0)	30 (28.8)	28 (26.9)	28 (26.9)
Grainy**	0.000	22 (21.2)	24 (23.1)	26 (25.0)	30 (28.8)	17 (16.3)	13 (12.5)	26 (25.0)	36 (35)
Plum Fruit	0.189	23 (22.1)	20 (11.5)	12 (11.5)	15 (14.4)	19 (18.3)	25 (24.0)	16 (15.4)	21 (20.2)
Sweetcorn/Cooked Veg.**	0.002	27 (26.0)	24 (23.1)	17 (16.3)	19 (18.3)	35 (33.7)	16 (15.4)	18 (17.3)	31 (29.8)
Smoky*	0.035	9 (8.7)	5 (4.8)	11 (10.6)	12 (11.5)	13 (12.5)	10 (9.6)	7 (6.7)	19 (18.3)
Taste, Flavour and Mouthfeel									
Green Apple/Emulsion Paint	0.142	17 (16.3)	15 (14.4)	28 (26.9)	27 (26.0)	20 (19.2)	26 (25.0)	23 (22.1)	19 (18.3)
Banana/Pear Drops**	0.001	18 (17.3)	23 (22.1)	24 (23.1)	24 (23.1)	29 (27.9)	38 (36.5)	25 (24.0)	12 (11.5)
Malty	0.472	42 (40.4)	37 (35.6)	33 (31.7)	33 (31.7)	33 (31.7)	36 (34.6)	27 (26.0)	35 (33.7)
Grainy**	0.006	31 (29.8)	34 (32.7)	38 (36.5)	23 (22.1)	28 (26.9)	25 (24.0)	24 (23.1)	42 (40.4)
Plum Fruit	0.084	15 (14.4)	16 (15.4)	8 (7.7)	9 (8.7)	19 (18.3)	14 (13.5)	11 (10.6)	7 (6.7)
Apple	0.690	26 (25.0)	28 (26.9)	24 (23.1)	23 (22.1)	20 (19.2)	27 (26.0)	28 (26.9)	20 (19.2)
Sweet	0.209	36 (34.6)	30 (28.8)	29 (27.9)	33 (31.7)	38 (36.5)	35 (33.7)	32 (30.8)	22 (21.2)
Sour	0.599	25 (24.0)	28 (26.9)	22 (21.2)	26 (25.0)	28 (26.9)	24 (23.1)	28 (26.9)	34 (32.7)
Bitter**	0.008	39 (37.5)	48 (46.2)	55 (52.9)	39 (37.5)	38 (36.5)	40 (38.5)	44 (42.3)	56 (53.8)
Lingering Bitterness**	0.000	24 (23.1)	25 (24.0)	17 (16.3)	34 (32.7)	24 (23.1)	25 (24.0)	30 (28.8)	43 (41.3)
Astringent	0.366	28 (26.9)	28 (26.9)	25 (24.0)	36 (34.6)	29 (27.9)	22 (21.2)	28 (26.9)	32 (30.8)

Asterisks indicates significant differences between samples according to Cochran's Q test (* $p<0.05$, ** $p<0.001$)

The incorporation of 45% B(A)/W incurred significantly higher frequencies compared to the sample set for several attributes (smoky aroma ($p=0.035$), grainy aroma ($p=0.000$) and grainy flavour ($p=0.006$) bitter ($p=0.008$) and lingering bitterness ($p=0.000$)). The higher frequencies observed for bitter and lingering bitterness attributes could be related to beer TPC as polyphenols are known for imparting bitterness in beer (Section 1.8.4). However, higher concentrations of TPC in beer were also observed in 35% B(A)/W which did not have such high selection frequencies of bitter and lingering bitterness attributes. Similarly, proanthocyanidins can influence the bitterness in beer (Section 1.8.4). The beer TPA was highest in 45% B(A)/W and 45% B(B)/W, however the sample produced with barley (B) did not have high frequencies of bitter and lingering bitterness in the CATA results. This indicates that there is a different source for the bitterness perceived in 45% B(A)/W. The increased bitterness observed in the 45% B(A)/W supports previous findings in Chapter 3 which identified beer brewed with 60% barley (A) as having significantly higher bitterness and lingering bitterness compared to 60% B(B) ($p<0.001$). The 45% B(A)/W sample also received the lowest CATA frequencies for several attributes (green apple aroma ($p=0.005$), banana aroma ($p=0.000$) and flavour ($p=0.001$)) making the sample the most distinct from the sample set. The low banana aroma and flavour frequencies determined with 45% B(A)/W could be a result of the low ester concentrations identified in this beer (Table 5.14), especially significantly lower concentrations of isoamyl acetate which is known for imparting banana aroma and flavour. Green apple aroma is normally associated with acetaldehyde concentration, in this case, 45% B(A)/W did not have the lowest acetaldehyde concentration. However, this sample had significantly lower concentrations of ethyl hexanoate and ethyl octanoate compared to the all-malt control, which are esters known for also imparting apple aroma.

The most liked 25% B(B)/W (2) sample showed lower frequencies of lingering bitterness compared to 45% B(B)/W. The two 25% B(B)/W replicate samples were perceived slightly differently by consumers, with 25% B(B)/W (1) obtaining higher attribute frequencies for malty aroma and flavour, plum fruit flavour and aroma compared to 25% B(B)/W (2). The sample 35% B(B)/W YL which obtained the lowest overall liking score for all consumers and clusters (Table 5.15) was described by significantly higher sweetcorn aroma ($p=0.002$) and lower bitterness ($p=0.000$) frequencies compared to the sample set. However, the bitter frequency score obtained for 35% B(B)/W YL was comparable to that of 100% malt and 35% B(B)/W FB.

5.3.3.3. Penalty-lift analysis

In penalty-lift analysis, liking scores and CATA attribute data are combined to explore the impact on liking when an attribute is not selected. Liking ratings are averaged across all observations (consumers and products) for which the attribute in consideration was used, and across all observations for when the attribute was not used. The difference between the two means provides an estimate of how much liking changes when an attribute is applied (Ares *et al.*, 2014). This analysis can be used to explore the main drivers for liking and disliking for consumer preference and was applied here to each cluster. Figure 5.4 shows a visualisation of the results of the penalty-lift analysis for all three clusters that compares the percentage of entries of an attribute (y-axis) with the impact on the mean score (x axis).

For C1 (overall beer likers, red dots), 14 of the 18 terms positively impacted the mean score. Malty aroma, malty flavour, sweet and apple flavour having a statistically significant ($p<0.05$) impact on overall liking, indicating that these attributes are significant drivers for the liking observed in C1. For C2 (adjunct beers dislikers, purple squares), the only significant driver ($p<0.0001$) was lingering bitterness which had the strongest driver (-1.26) of all attributes across all three clusters. C3 (adjunct likers, green triangles) did not have any statistically significant drivers for liking and disliking. However, green apple aroma and apple flavour were the highest positive drivers of liking for C3. The results determined from penalty-lift analysis can been used to identify recommendations for product improvement, specifically for consumers in C2. The main improvement for C2 would be to minimise lingering bitterness in beer samples to increase the overall liking. However, there was not a clear source for lingering bitterness that could be identified in the standard analytical tests performed in this study, therefore further research would be needed to determine the causation of lingering bitterness to increase overall liking. By means of evaluation of penalty-lift analysis there is still no defined clear driver for the liking of the 35% B(B)/W YL sample.

Understanding the cause and changing physiochemical characteristics of the beer in order to reduce attributes associated with a negative mean impact might not lead to a large increase in overall liking scores, since the mean impact for most attributes was low. However, this type of analysis has enabled identification of specific and actionable recommendations for improving liking for specific consumer clusters for each product based on overall liking. A

limitation to this method is the selection of CATA terms; the 18 used may have missed key drivers for liking or disliking.

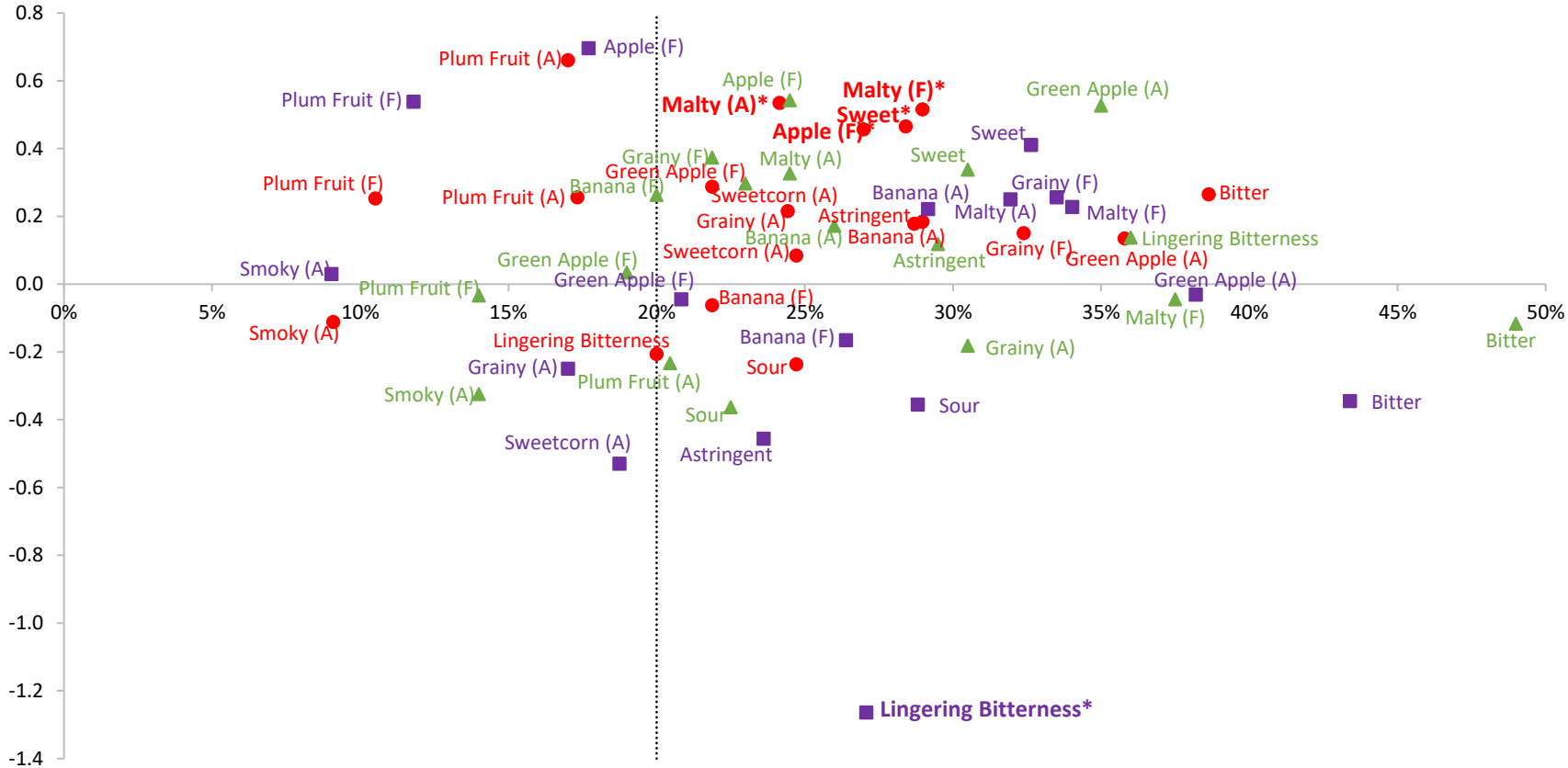


Figure 5.4. A visualisation of the results of the penalty-lift analysis for all three clusters that compares the percentage of consumers which selected an attribute (y-axis) with the impact on the mean overall liking score (x axis). Attributes highlighted with an asterisk and bold font correspond to those with a significant decrease or increase corresponding with more than 20% of the consumers (*p<0.05). Cluster 1 attributes are red circles markers and font, cluster 2 attributes are green triangles in green font and cluster 3 are purple squares in purple font

5.3.3.4. Comment analysis on liking and disliking

Consumers were asked to comment after giving their liking score, by expressing what they liked and disliked about each product in open questions. By separating liking and disliking questions, key drivers were obtained without the need for further interpretation. Table 5.18 shows the most used category terms identified by comment analysis consisting of 17 liking terms and 14 disliking terms.

The amount of total liking and disliking comments related to the overall liking score of each sample. For example, beer 25% B(B)/W (1) had the highest mean overall liking score (6.13) corresponding to the highest total liking responses (169) and the lowest total disliking responses (84). The most recurrent liking terms were ‘sweet’, ‘light’ and ‘refreshing’. This is agreement with a previous finding which determined ‘refreshing’ ‘light’ and ‘sweet’ as the most desirable sensory attributes in lager beer (Bettenhausen *et al.*, 2020). Contrastingly, the most mentioned dislikes were ‘weak flavour’, ‘carbonation’ and ‘bitter’. It is also to be noted that the low intensity or absence of a characteristic such as ‘weak flavour’ and ‘weak body’ was only associated with disliking whilst ‘strong flavour’ was associated with liking. The three terms ‘bitter’, ‘sweet’ and ‘carbonation’ were associated with both disliking and liking categories indicating the consumer perception of these terms were more complex than the other attributes. The most used term throughout the comment analysis was the disliking term for ‘weak flavour’ with over 106 uses of the term. Interestingly, no samples were significantly higher or lower than expected for this term indicating that all samples could be described by having weak flavour. Investigating the causation of this term and determining a remedy could increase the overall liking and subsequent purchase intent for all of the samples.

Table 5.18. Frequency of terms occurring for each category term to describe adjunct beers

Category Terms	Synonyms	100% Malt	25% B(B)/W (1)	25% B(B)/W (2)	35% B(B)/W YL	35% B(B)/W FB	35% B(A)/W FB	45% B(B)/W FB	45% B(A)/W FB
Liking Categories									
Bitter	bitterness, moderate bitterness, faint bitterness	10	9	17	2(-)*	11	11	13	8
Sweet	just right sweetness, good sweetness, slightly sweet	12	2	11	11	8	5	7	11
fruity	faint fruity	7	8	6	6	7	7	5	5
grainy	nice grainy, slightly grainy	4	2	4	3	3	1	1	6
hoppy	low hoppy, nice hoppy, fragrant hops, balanced hops	3	1	0(-)*	0(-)*	3	5	3	0(-)*
malty	nice malty, roasted malty, slightly malty	10	11	8	8	7	5	7	7
strong flavour	deep flavour, lots of flavour, intense flavour, full flavour, flavourful	4	4	7	5	8	5	7	4
balanced	balanced flavour, rounded	1(-)*	6	6	2	1(-)*	5	9	4
crisp		5	2	2	2	1	3	6	3
drinkable	easy drinking, enjoyable, sessionable	4	8	8	2	7	3	5	2
fresh		8	2(-)*	4	3	1(-)*	5	7	7
light		12	20	10	5	17	9	13	10
refreshing		13	13	13	11	11	15	12	5(-)*
smooth	soft	1	2	5	5	3	2	0(-)*	4
mild	mellow, neutral, subtle	0(-)*	3	6(+)*	2	3	1	1	0(-)*
carbonation	nice carbonation, nice bubbles, light fizz, bubbly	6	8	5	8	8	11	8	10

Table 5.18 continued.

Category Terms	Synonyms	100% Malt	25% B(B)/W (1)	25% B(B)/W (2)	35% B(B)/W YL	35% B(B)/W FB	35% B(A)/W FB	45% B(B)/ W FB	45% B(A)/W FB
Disliking Categories									
bitter	bitterness, moderate bitterness, quite bitter, harsh bitterness	6	12	13	3(-)*	7	17(+)*	8	19(+)*
sour		2	1	7	5	6	7	5	0
sweet		6(+)*	2	2	1	4	2	1	0
artificial		1	1	0	5	3	0	1	2
weak flavour	not strong flavour, lacking flavour, low overall flavour	15	19	10	9	13	8	16	16
astringent	good astringency, harsh astringency	0(-)*	3	1	5	4	2	2	3
dry	slight dry, bit dry	5	5	5	0(-)*	3	6	5	1(-)*
weak body	no body, lacking body	4	4	2	0(-)*	5	0(-)*	4	0(-)*
thin		2	3	2	2	3	0(-)*	2	2
watery		1(-)*	6	6	2	5	5	3	5
bland	boring, no character, flat	5	4	3	4	0	1	4	2
carbonation	very carbonated, very fizzy, too fizzy, tongue-biting carbonation	5	3	4	6	3	3	4	0(-)*
lingering bitterness	bitter aftertaste	3	5	4	6	4	0(-)*	5	13(+)*
	Total Like Comments	153	169	164	124	157	133	175	127
	Total Dislike Comments	97	101	84	109	101	101	97	101

(+) or (-) indicates that the observed value is higher or lower than the expected theoretical value.
*p < 0.05; effect of the Fisher's test per cell..

Fisher's test was applied to the contingency table (Table 5.18) to provide a statistical evaluation of the comment analysis data and showed a significant result ($p = 0.007$) revealing that the consumers used different terms for describing their likes and dislikes depending on specific beer samples. Fisher's test significance per cell was used to identify if the observed value for a beer sample was significantly higher or lower than the expected theoretical value (Table 5.18). The 45% B(A)/W sample was characterised by the most significantly different terms. The sample was characterised by higher disliking for 'bitterness' and 'lingering bitterness' and lower frequency for liking terms 'hoppy', 'mild' and 'refreshing' compared to the expected value. The 35% B(A)/W sample had a significantly higher use of the 'bitterness' disliking term. Indicating the consumers found a disliking to barley based beers which was not observed in the remaining sample set. The 45% B(B)/W sample was characterised by the least amount of significant terms and was only characterised by significantly lower frequency of the term 'smooth'. All four beer samples that included barley (B) did not present any disliking terms which were significantly different to the expected value. Although 35% B(B)/W YL was the least liked sample determined by the consumers, there were no clear drivers determined from the comment analysis. The sample had significantly lower frequency of the liking term for 'bitterness' whilst also having the lowest frequency for disliking 'bitterness'. Therefore, indicating that the quality of bitterness in the 35% B(B)/W YL could have reduced overall liking for this sample.

In order to visualise the beer samples based on the comments cited by the consumers, a correspondence analysis (CA) was performed presented in Figure 5.5.

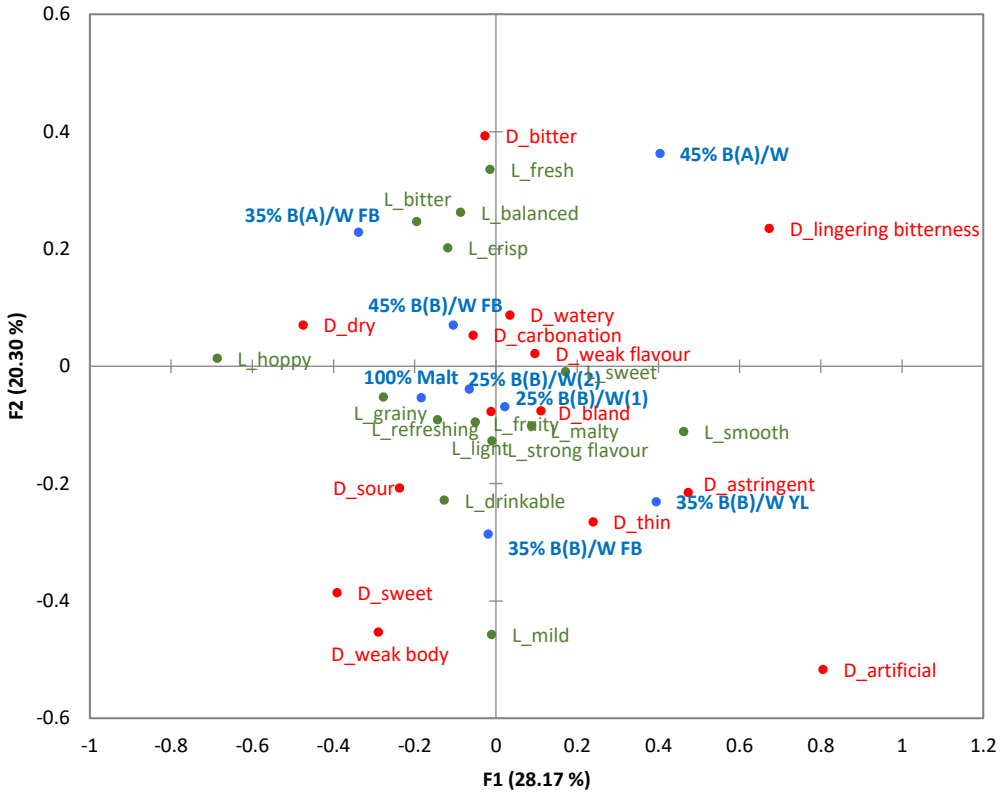


Figure 5.5. Correspondence analysis of the contingency table (Table 5.18) showing the beer samples with the main liking and disliking terms cited by consumers.

Samples are in blue, liking terms are in green and disliking terms in red. (Axes F1 and F2: 48.47%)

The first two dimensions of the CA represented 48.47% of the total variation. The most liked samples (25% B(B)/W (1) and (2) and 100% malt) were central to the CA and associated with 'sweet', 'fruity' and 'malty' liking terms whilst also associated with 'bland', 'carbonation' and 'weak flavour' disliking terms. The 35% B(B)/W YL, the least liked sample, was associated with 'astringent' and 'artificial' disliking terms. However, the Fisher's test did not show these terms as significant for this sample indicating that they might not be the key drivers for the disliking. Comment analysis has therefore not determined an obvious driver as to why the consumers had a lower preference for the 35% B(B)/W YL sample. The close similarities in the composition of recipe for each sample may

have made it harder for consumers to discriminate what they liked and disliked about each beer.

5.3.3.5. CATA and comment analysis

Further analysis of the consumer responses to open liking and disliking questions with relation to the CATA results provides insight and interpretation to understand consumers perception of adjunct based beers. The results obtained in the penalty-lift analysis correspond with the drivers of liking and disliking obtained in the open-ended questions as 'grainy', 'malty' and 'fruity' were all positive terms in the comment analysis. 'Sweet' was a term classified by liking and disliking terms but more frequently used to describe liking in the comment analysis. 'Sour' and 'lingering bitterness' were only described as disliking which corresponds to their negative impacts in the penalty analysis.

The most liked sample 25% B(B)/W (2) and a least preferred sample 45% B(A)/W both obtained the highest frequencies for the bitter term in the CATA analysis (Table 5.17). The use of open-ended questions has enabled further understanding of the differences in the bitterness. The 25% B(B)/W (2) sample had the highest frequency of 'bitter' as a liking term whilst 45% B(A)/W had the highest frequency of 'bitter' as a disliking term (Table 5.18), indicating that although higher bitterness is prevalent in both samples, consumers perceive the bitterness differently across the two samples. Terms such as 'harsh' and 'weird' bitterness were used when describing disliking for 45% B(A)/W whilst 'nice' and 'just right' bitterness were used to describe liking for 25% B(B)/W (2). Bitterness is an important flavour attribute that consumers expect and enjoy to a varying degree during consumption and bitterness quality in beer can be said to be a combination of intensity, temporal and spatial characteristics (Oladokun *et al.*, 2017). Therefore, higher incorporation of barley may change the quality and perception of the type of bitterness in the beer to an unfavourable bitterness, which could correspond to the lingering bitterness. The quality and intensity of bitterness was also an important feature with the 35% B(B)/W YL sample. This sample obtained the lowest frequency of bitterness in the CATA term frequencies which also corresponded to significantly lower than expected liking and disliking bitterness in the open-ended questions, suggesting that bitterness could be a factor feeding into the disliking observed by the consumers for this sample. This underlines the importance and complexity of bitterness quality in beer and the influence of adjunct use with bitterness

perception. Physicochemical constituents such as phenolic compounds and α-acids in beers have an impact on the quality of bitterness but also sensory perception of bitterness can vary greatly between individuals (Higgins *et al.*, 2020). One of the most important factors influencing individual difference in oral sensations is genetic variation. PROP taster status (PTS) is a well-known taste phenotype and classifies individuals as non-tasters, medium tasters or supertasters dependent on the intensity of bitterness they perceive whilst holding 5-n-propylthioracial (PROP) in their mouth. Studies have reported that PROP tasters have a heightened sensitivity to bitter compounds as well as other tastes (sweet, salty and sour) (Yang *et al.*, 2018). Those who perceive bitterness from PROP also find some type of beers more bitter (Intranuovo *et al.*, 1998; Duffy *et al.*, 2004). The role of personality traits can also impact perception and acceptance trends where it is found that sensation seeking individuals report increased liking of higher bitterness beer (Higgins *et al.*, 2020). Although lack of liking for bitterness may be a contributing factor for the consumer disliking of the 35% B(B)/W YL sample, the CATA and open-ended questions have not highlighted any unique sensory characteristics that relate to disliking in this beer. As the consumers were not trained beer panellists, they may not have been able to articulate the drivers for their disliking. Therefore, it would be useful to present this sample to a trained beer panel to discriminate the differences between the sample set that may have caused this disliking driver.

Higher frequencies of ‘malty’ aroma and flavour were obtained for low adjunct beers such as 100% malt and 25% B(B)/W (1) with decreasing frequency with increasing adjunct material. Similarly, lower adjunct beers had higher frequencies for ‘malty’ terms in the open questions. Malty was only associated as a liking driver in the open questions, indicating that the consumers perceived maltiness as a positive attribute to adjunct lager beer which was mainly observed in lower adjunct beers.

Notably, three of the most used positive and negative terms (‘light’, ‘refreshing’ and ‘weak flavour’) were not used in the adjunct beer attribute lexicon used in the CATA analysis, signifying that the comment analysis has highlighted drivers for preference that were not previously considered and therefore missed by the use of CATA. This highlights the importance of developing consumer defined lexicons for CATA evaluations with consumers.

5.3.4. Correlation between beer physicochemical and CATA analysis

Correlation analysis was performed to visualise the relationships between the samples, CATA terms and chemical composition. Figure 5.6 shows a biplot of F1 and F2 which accounted for 49.14% of the variance in the data set. As expected, the fruity attributes such as banana aroma and flavour, plum fruit aroma and flavour and apple flavour were projected similarly to the ethyl and acetate esters in the negative F1 plot. Banana aroma and flavour attributes were projected with isoamyl acetate, isobutyl acetate and isoamyl alcohol which are all flavour compounds that are known to impart banana aroma (Holt *et al.*, 2019). The esters ethyl hexanoate and ethyl-trans-4-decenoate which are known to impart sweet, apple aromas to beer were projected with the apple flavour and sweet sensory attributes. Ethyl hexanoate and ethyl octanoate have been previously shown as positively improving consumer overall liking, which correlates to the position of the most liked beers being associated with these compounds (Gonzalez Viejo *et al.*, 2019). The negative F2 loading corresponds to higher concentrations of green apple aroma and flavour sensory attributes which were projected with acetaldehyde which imparts green apple aroma. The fermented sugars were projected with the malty aroma and flavour sensory attributes, this agrees with previous findings that beers with higher residual sugars are often described as more malty, honey and sometimes correlated with palate fullness (Bellut *et al.*, 2019; Ramsey *et al.*, 2021). A positive loading on F1 corresponds to high concentrations of bitter, astringent and lingering bitterness sensory attributes exemplified by the 45% B(A)/W beer and associated with slightly higher concentrations of proanthocyanidin content. The most overall liked beer samples (100% malt, 25% B(B)/W (1) and 25% B(B)/W (2) are associated with the malty, apple flavour and sweet attributes which have been determined as the key drivers for liking in adjunct beer. The physiochemical analysis indicates that the fermentable sugars and ethyl esters can contribute to these favourable characteristics. There were no clear physiochemical and sensorial aspects that were closely associated with the lowest overall liked sample 35% B(B)/W YL.

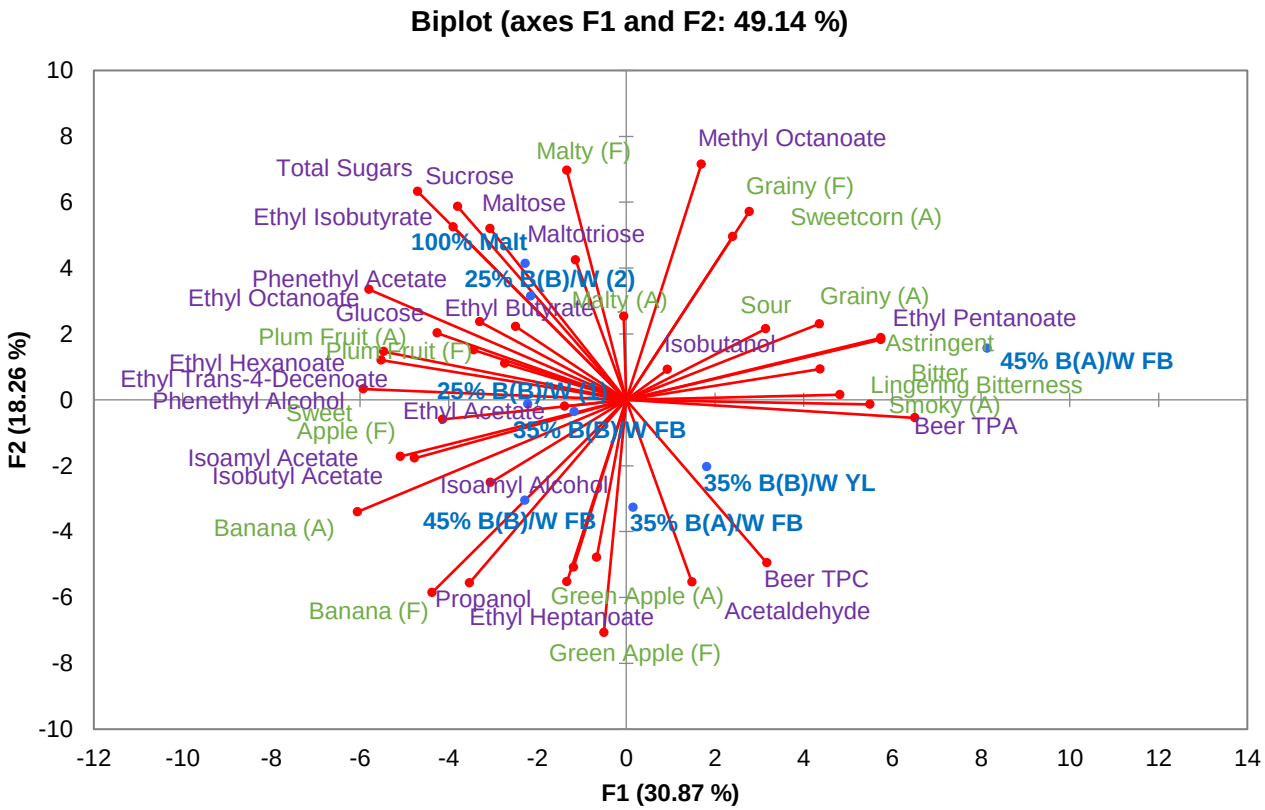


Figure 5.6. Principal Component Analysis (PCA) of normalised sensory and analytical profiles on principal components 1 and 2.

Samples are in blue, sensory attributes in green and chemical compounds in purple. Sample Codes: (A) aroma; (F) flavour.

5.4. Conclusions

Results from this study found that FAN supplementation methods when producing wort up to 45% incorporation rates increased the total and soluble nitrogen to concentrations similar to that of the all-malt control. However, the use of adjuncts up to 45% incorporation changed the wort composition and properties and consequently the analytical profile of the resulting beer. Increasing usage of unmalted adjuncts significantly decreased the wort and beer fermentable sugar concentrations and decreased the concentration of key ethyl and acetate esters in adjunct beers. The use of unmalted adjuncts also affected the mineral composition, as a decrease in sodium, iron and copper concentration was observed in the wort and beer when incorporating adjunct material. This study identified that the use of 45% barley(A)/wheat of grist produced an analytical profile most dissimilar to the all-malt control sample.

The check-all-that-apply technique used in the consumer testing determined that consumers detected differences in 9 out of the 18 attributes (‘smoky’, ‘sweetcorn’ and ‘green apple’ aroma, ‘banana’ and ‘grainy’ aroma and flavour, ‘bitter’ and ‘lingering bitterness’) across the sample set of beers. The beer produced with 45% B(A)/W was the most distinct sample which was characterised by higher frequencies of ‘smoky aroma’, ‘grainy aroma and flavour’, ‘bitter’ and ‘lingering bitterness’ with lowest frequencies for ‘green apple aroma’, ‘banana aroma and flavour’. The significantly lower concentration of ethyl and acetate esters may have contributed to the lower apple and banana attributes. This result indicates that 45% may be the maximum incorporation of barley (A) before the sensory and analytical profile are significantly altered compared to the all-malt control.

Although, analytical and sensory properties did differ across the samples, this did not appear to impact consumer preference as the overall liking and purchase intent of adjunct beers were not significantly different compared to the all-malt control. The beer sample produced with YeastLife as a FAN supplementation method, showed significantly lower overall liking and purchase intent scores, indicating consumers preferred beers produced with FAN boost to beers produced with YeastLife. This highlights the importance of the FAN supplementation technique used when producing adjunct beers.

This study has also confirmed that beer consumers are not consistent in their preference when considering adjunct beers, forming three different clusters with

different liking patterns. Interestingly, one cluster (24% of consumers in the present study) showed a marked preference for beers produced with 45% incorporation of adjunct material. It would be interesting to further investigate the drivers of liking of consumers in this cluster with the aim of developing beers with high adjunct incorporation as a selling point.

The combination of overall liking, CATA and open-ended questions has provided key sensorial characteristics that could be used to improve beers brewed with higher adjunct rates and improve their desirability in the beer market. 'Malty aroma and flavour', 'apple flavour' and 'sweet' attributes were all significant positive drivers for liking. The attribute 'lingering bitterness' was the only significant negative driver for liking. PCA correlations between the sensory attributes of adjunct beers and their underlying chemical composition identified compounds which could be associated with the sensory attribute drivers. Ethyl esters were projected with sweet and apple attributes, fermentable sugars were projected with malty attributes. The unfavourable attribute, lingering bitterness, was projected with higher concentrations of total proanthocyanidin content. This suggests specific chemical components to focus on controlling when brewing with unmalted adjuncts to improve consumer acceptance and desirability.

This is the first study to investigate consumer perception and acceptance of adjuncts in beer using a consumer panel and instrumental techniques, evaluating the differences in the adjunct beers in terms of physiochemical properties and consumer perception of their sensory attributes and their influence on consumer liking. This research is important for the brewing industry as it builds on the growing knowledge on the impact of using adjuncts on the sensory and analytical profile of beer. Improved understanding of consumer acceptance of unmalted cereal adjuncts in beer can provide breweries confidence to increase adjunct usage in lager beers, facilitating the benefits that using adjunct material can provide.

Chapter 6 Untargeted metabolomic profiling of the differences between 100% malt beers and those made with unmalted barley adjunct

This work was conducted in collaboration with Dr Tristan Dew. The author gives thanks to Dr Tristan Dew for conducting the UHPLC-QTOF-MS analysis, completing the processing and statistical analysis of the data.

6.1. Introduction

Metabolomics is the study of small molecular weight compounds (metabolites that are less than 1000 Da) that are reactants or products of metabolism. Beer metabolites originate from a variety of sources including malted barley, adjuncts, hops and yeast (Heuberger *et al.*, 2012). Metabolomic analyses can be classified into targeted and untargeted modes. Targeted analyses focus on a selected pre-defined group of metabolites with the aim of identifying and quantifying the selected compounds of interested. The flavour-active volatile metabolites in beer including aldehydes, higher alcohols and esters have been previously characterised by targeted gas chromatography methods (Riu-Aumatell *et al.*, 2014; Rossi *et al.*, 2014). The non-volatile metabolites of beer such as carbohydrates, phenolic compounds and hop acids have been determined by targeted liquid chromatography methods (Oladokun *et al.*, 2016a; Das *et al.*, 2014; Cheiran *et al.*, 2019). In contrast, untargeted metabolomics focuses on the detection of many groups of metabolites to obtain a metabolite fingerprint of the sample without necessarily identifying or quantifying all the features detected. Statistical analysis can then be applied to the extracted molecular features to identify key differentially expressed molecular features between beer samples.

Untargeted metabolomic profiling by means of mass spectrometry has recently been used to determine differential metabolites between alcoholic and non-alcoholic beer (Andrés-Iglesias *et al.*, 2014), beers stored at different storage temperatures (Heuberger *et al.*, 2012) and beers produced with different brewing procedures (Gallart-Ayala *et al.*, 2016). Hughey *et al.* (2016) showed electrospray ionisation quadrupole time of flight mass spectrometry (ESI q-TOF MS) is a useful tool for identifying key differential compounds relating to flavour

when investigating the use of different hop varieties in India pale ales. Therefore, metabolomic profiling could prove useful for identifying key molecular features that influence the sensory properties of beers produced with unmalted cereal adjuncts.

The findings throughout the course of this work (Chapter 3-5) have determined that the incorporation of unmalted barley can impact the sensory profile of the beer. Beers produced with barley (A) up to 60% incorporation rates were characterised by higher bitterness, lingering bitterness and astringency compared to an all-malt control. Terms such as 'harsh' and 'weird' bitterness were used by consumers to describe beers produced with barley (A). Therefore, incorporation of barley (A) has been found to unfavourably change the quality and perception of bitterness. Bitterness congeners, hop acids and phenolic compounds, are normally associated with the quality of bitterness of beer (Oladokun *et al.*, 2016b). However, throughout the course of this work, HPLC targeted analyses identified minimal variance in phenolic compounds between all malt beer and beer produced with unmalted adjuncts. Therefore, it is unlikely that the simple phenolic acids and allied compounds that were analysed previously by HPLC are the main cause of the reported change in bitterness and astringency when brewing with barley (A). Untargeted metabolomic analysis was proposed to try and identify previously unexplored compounds associated with incorporation of unmalted barley which could contribute to the sensory differences obtained with adjunct beers.

The objective of the present research was thus to use a non-targeted metabolomics approach to determine differential non-volatile metabolites which could contribute to flavour differences caused by the incorporation of unmalted barleys. Three beer samples (100% malt, 15% barley (A) and 15% barley (B)) were analysed using ultra-high performance liquid chromatography (UHPLC) coupled to a quadrupole time-of-flight mass spectrometer with electrospray ionisation source (ESI). The metabolomic profile of the three beers were compared using principal component analysis (PCA) to determine significantly differential molecular features across the sample set. Additionally, the data obtained from the tandem quadrupole-time of flight mass spectrometer was analysed to putatively identify the significantly differential compounds in the beer. The properties of the key differential molecular features were then

investigated to determine if these compounds could contribute to the sensory differences observed with unmalted barley.

6.2. Materials and methods

6.2.1. Materials

LCMS grade formic acid and acetonitrile were obtained from Fisher Scientific (Loughborough, UK). Type 1 high purity water was obtained using a Lab Pro PURA Q+20 system (SLS, Nottingham, UK).

6.2.2. Beer samples and sample preparation

The three beer samples used for this metabolomics study included an all-malt control (100% malt), a beer produced with 15% barley (A) (15% B(A)) and 15% barley (B) (15% B(B)). These samples were previously produced for the work of Chapter 4 (Section 4.2.4). The beer samples selected for this analysis were not designed specifically for this metabolomic study. The sample produced with 15% incorporation rates were chosen for this investigation as these beers were produced without balancing the C:N ratio of wort or micronutrient levels. Thus, ensuring that any substantial metabolomic differences observed are most likely due to changes in adjunct incorporation rates and not caused by FAN supplementation techniques.

Sample preparation and high-resolution MS data acquisition were based on the approach of Gallart-Ayala *et al.* (2016) with some modifications. The test beers were degassed via sonication (20 min), diluted 1:1 with distilled water in replicates of 5 and filtered using 0.2 µm Whatman puradisc syringe filters (Sigma-Aldrich, Dorset, UK). The filtered samples were then aliquoted to microcentrifuge tubes and frozen. On the day of analysis, samples were thawed at 5 °C, vortexed for 1 minute and placed into 2 mL amber HPLC vials and capped. A single pooled QC sample was produced by mixing together small aliquots of the 23 beers produced for the work of Chapter 4 (Section 4.2.4) by combining equal volumes to create a combined sample. The pooled QC represents both the sample matrix and metabolite composition of the samples evaluated. This present research focuses on the metabolic profile of three samples (100% malt, 15% barley (A), 15% barley (B)). However, the metabolic

profiles were analysed for the 23 beer samples giving reason as to why the QC sample includes all beer samples.

6.2.3. Metabolomic analysis by UHPLC-qTOF-MS

LC-MS analysis was performed using an Agilent 1260 Infinity II UHPLC, coupled to an Agilent 6546 tandem Quadrupole – Time of Flight mass spectrometer (Agilent Technologies, Cheadle, UK). The chromatographic separation was performed using a Kinetex C18 column (2.6µ, 150 x 2.1mm; Phenomenex, Macclesfield, UK) held at 40 °C. Solvents A and B comprised 5% or 95% respectively of acetonitrile in water (v/v) plus 0.1% formic acid. The gradient flowing at 0.3 ml/min, started from 0% B, to 20% at 10 minutes, rose to 100% at 13 minutes, was held until 15.5 minutes, then returned to starting conditions by 15.6 minutes, with a further 3.4 minutes of reconditioning.

The ESI source used gas and sheath gas temperatures of 320 ˚C, and 350 ˚C respectively, drying gas and sheath gas flows of 8 and 11 L/min respectively, with the nebuliser set to 35 psi. VCAP and nozzle voltages were set to 3500 V and 1000 V respectively, with fragmenter, skimmer and octopole RF voltages of 110, 65 and 750 V respectively. MS1 data were acquired in both (+) and (-) ESI, between 100 m/z-3200 m/z. MS2 data were acquired in Auto MS-MS mode with collision energies fixed at 10, 20 and 40 V, using separate preferred ion lists to avoid co-elution of targets within 0.2 min.

For MS1 acquisition, the column was conditioned with 8 x 5 uL injections of QC samples. Thereafter, samples were injected in a randomised order, with a QC sample injected every 5 analytical runs.

For data analysis, the TICs of repeat QC injections were visually assessed to check comparability of runs throughout the data set. Global MS1 features (peak height >4000) were first extracted using Mass Profiler software (MP; v10, Agilent Technologies), and exported to a common .CEF file for each polarity. Thereafter, files for each replicate group were time aligned to the central QC sample in Profinder software (v10, Agient Technologies), then features extracted (peak height >3000) in ‘batch targeted’ mode using the MP .CEF file as a template. Extracted files were then exported to Mass Profiler Professional (MPP; v15, Agilent Technologies) for statistical analysis, and initial PCA plots generated to assess data quality. To isolate features of interest between adjunct incorporation rates, features were initially filtered on the basis of significant

difference from control ($p<0.01$; one way ANOVA) and then fold change (typically >50) between treatments under consideration. The shortlisted features were further limited to peak heights of >10,000 and exported as preferred inclusion lists for MS2 analysis. Following this second round of data acquisition, compounds were extracted using Mass Hunter Qualitative Analysis software (v10, Agilent Technologies), and the resulting spectra exported to Sirius GUI software (v4; Dührkop *et al.* (2019) for putative identification. The Sirius, CSI:Finger ID (Dührkop *et al.*, 2015) and Canopus (Feunang *et al.*, 2016; Dührkop *et al.*, 2021) modules were employed to provide *de novo* chemical formulae prediction, identity match scores and to predict chemical class, respectively. All searchable databases (as of Spring 2021) were employed for CSI:Finger ID analysis.

6.3. Results and discussion

6.3.1. Principal component analysis

Principal component analysis was conducted on the initial extracted features (>3000 peak height) to provide a visual depiction of the differences in metabolite profile in negative ionisation mode (Fig 6.1A) and positive ionisation mode (Fig 6.1B) of the 15% barley (A), 15% barley (B), all-malt control and QC sample.

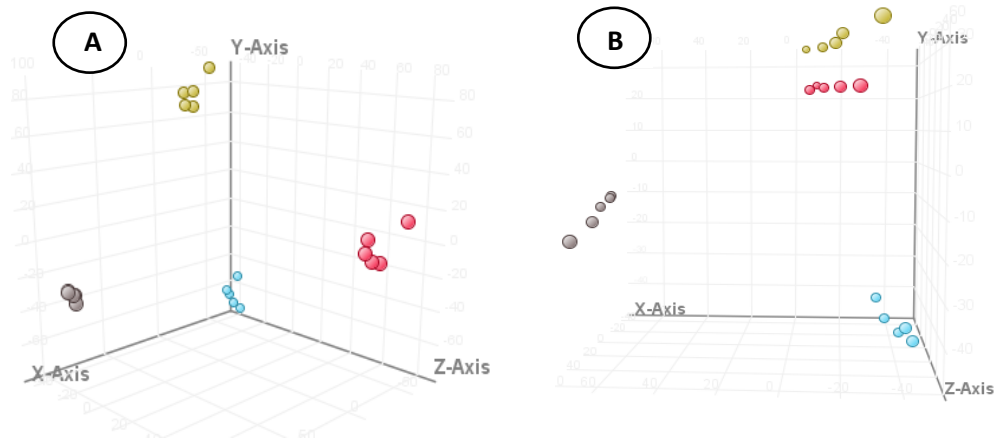


Figure 6.1. 3D Principal component analysis (PCA) plots for the UPLC-MS data of the extracted feature (>3000 peak height). A) ESI+ and B) ESI-. QC samples are the grey circles, 15% barley (A) are the yellow circles, 15%

barley (B) are the red circles and 100% malt are the blue circles. All samples were analysed in 5 replicates as shown on the PCA plot.

The 3D PCA plot obtained from the ESI(+) data (Figure 6.1A) showed a clear separation in the metabolomic features extracted from the three beer samples along the Y- and Z- axis. Less variation was observed along the X-axis explained by minimal variance between sample replicates. In Figure 6.1A, the 15% barley (A) samples showed most variation relative to the all malt control samples along the Y-axis. In contrast, the 15% barley (B) showed most variation from the all malt in the Z-axis. Most of the differential metabolites were detected as protonated ions supporting the increased variation observed in the ESI(+) 3D PCA plot. The variation observed in the PCA plot shows that not only are the metabolomic features of the adjunct beers different from the all malt control, but there is also variance between the two adjunct beers.

In the 3D PCA plot obtained from the ESI(-) data (Figure 6.1B) most of the variation between the samples exists in the Y-axis, where the most variation is observed between the all malt beers and 15% barley beers. There is also variation shorter distance between groupings for the two barley adjunct beers in the 3D PCA plot from ESI(-) data. Fewer features were detected as deprotonated ions $[M-H]^-$ which could be one factor explaining the smaller separation of the beers in the PCA for the negative mode. In both 3D PCA plots the QC samples have high variation from the beer samples along the X-axis, this is because the QC sample consisted of an equal mix of the 23 beers produced in Chapter 4 (4.2.4) creating variation from the three samples that are the focus of this study.

6.3.2. Differential analysis of molecular features with increased levels in 15% barley (A) and 15% barley (B)

In order to shortlist differential metabolite features that showed significant change with adjunct incorporation, analysis was applied to filter metabolomic features. Three criteria were applied: (i) the feature is significantly different ($p<0.01$) to another sample in the sample set; (i) the peaks differed with a >50 fold change, (iii) and the feature had a peak height of >10,000. The most abundant features were focused on in order to facilitate downstream

identification of the differentially expressed features. This does not mean that low-abundance molecular features would not be important for organoleptic properties. However, high abundance molecular features have an increased likelihood of positive identification. From this criteria, 88 features (68 ESI+ and 19 ESI-) were significantly higher in 15% barley (B) compared to 15% barley (A) and/or 100% malt. It was also determined that 92 features (79 and 13, ESI+ and ESI- respectively) were significantly higher in 15% barley (A) compared to 15% barley (B) and/or 100% malt.

In an effort to identify the shortlisted molecular features, the MS spectra were searched against Sirius GUI software and CSI:Finger ID scores were employed to provide identity match scores for the predicted chemical formulae and chemical class compared to a searchable database. For the whole data set, the SIRIUS score ranged from 0-100% across all features, with 100% being the best match. The CSI:Finger ID score ranged from -23 to -580 across all features, with a score closest to 0 being the best match. With the aim of consolidating to the key differential metabolites, further investigation was only applied to metabolites with a SIRIUS score over 50% or a CSI:Finger ID score higher than -150. The differential metabolites were concentrated and 34 features (31 and 3, ESI+ and ESI- respectively) were significantly higher in 15% barley (B) compared to 15% barley (A) and/or 100% malt. Additionally, 36 features (34 and 2, ESI+ and ESI- respectively) were significantly higher in 15% barley (A) compared to 15% barley (B) and/or 100% malt (see Appendix Table A.1 and A.2). Differential metabolites between the all malt control, 15% barley (A) and 15% barley (B) were representative of a range of chemical classes such as peptides, purines, secondary alcohols, oligosaccharides, and benzoic acid derivatives. In this study, these components are understood as being ‘tentatively identified’ because it is acknowledged that additional analysis with independent standards is necessary to provide full identification.

6.3.2.1. Peptides

The most recurrent chemical class identified in the differential compounds were peptides (Appendix Table A.1 and A.2). A range of di-, tri- and tetrapeptides were identified as differential compounds in the ESI(+) mode. Table 6.1 shows the 11 peptide features that were identified as being significantly higher in the 15% barley (A) beer compared to 100% malt and 15% barley (B). Table 6.2

shows the 6 peptides that were identified as being significantly higher in 15% barley (B) compared to 100% malt and 15% barley (A).

Table 6.1. Differential peptides putatively identified from positive ionization analysis of the three beer samples. Differential metabolites shown are significantly higher in 15% barley (A) compared to 100% malt (100M) and 15% barley (B) (15B(B)). Beer d.c. indicates the beers for which the compound was found to be significantly differential compared to 15% barley (A).

Compound No.	**Compound name (Amino acid residues)**	**Beer d.c.**
7	Valylalanylglycylglutamine (val-ala-gly-glu)	100M 15B(B)
6	Alanylserylprolylproline (ala-ser-pro-pro)	100M 15B(B)
5	α-Glutamylserylleucine (glu-ser-leu)	100M 15B(B)
20	Threonylthreonylleucylthreonine (thr-thr-leu-thr)	100M
21	Alanylvalylvalylalanine (ala-val-val-ala)	100M
22	Isoleucylprolylthreonine (ile-pro-thr)	100M
23	L-Valyl-L-α-glutamyl-L-tryptophan (val-glu-trp)	100M
33	α-Glutamylglutaminylvaline (glu-gln-val)	15B(B)
34	Leucylglutaminylglycylglutamic acid (leu-gln-gly-glu)	15B(B)
35	Leucylglycylthreonylglutamine (leu-gly-thr-gln)	15B(B)
36	α-Glutamylalanylleucine (glu-ala-leu)	15B(B)

Table 6.2. Differential peptides putatively identified from positive ionization analysis of the three beer samples. Differential metabolites shown are significantly higher in 15% barley (B) (15B(B)) compared to 100% malt (100M) and 15% barley (A) (15B(A)). Beer d.c. indicates the beers for which the compound was found to be significantly differential compared to 15% barley.

Compound No.	**Compound name (Amino acid residues)**	**Beer d.c.**
40	Prolylprolylglycylserine (pro-pro-gly-ser)	100M
42	Isoleucylprolylthreonine (ile-pro-thr)	100M
54	Prolylserylglycylproline (pro-ser-gly-pro)	15B(A)
56	Prolyl-α-aspartylthreonylproline (pro-asp-thr-pro)	15B(A)
57	Seryl-α-glutamylleucine (ser-glu-leu)	15B(A)
58	α-Glutamylglycylleucine (glu-gly-leu)	15B(A)

The peptide fraction of beer arises from the degradation by-products of proteolytic activity of barley derived proteins creating a broad range of molecular size peptides. Amongst the 17 peptides identified, 11 peptides contained glutamine or proline residues. Prolamins, also known as hordeins in barley, are monomeric polypeptide chains that are rich in proline and glutamine (20-55%) (Mickowska *et al.*, 2012). The S-poor prolamin (C-hordein in barley) accounts for approximately 10-20% of the total prolamins with an amino acid composition of 40-50% glutamine, 20-30% proline and 7-9% phenylalanine (Strouhalova *et al.*, 2012). During malting, hordeins are in part degraded to amino acids and small peptides by a range of proteolytic enzymes. The amount of hordeins decrease during the malting process, in the case of C-hordein, the protein decrease is approximately 65% (Tatham *et al.*, 2012; Flodrova *et al.*, 2012). It has been shown that well-modified malt contains less than half the

amount of hordeins present in the original barley (Celus *et al.*, 2006). The D-hordeins (sulphur rich proteins) are degraded more rapidly than B and C-hordeins during malting (Picariello *et al.*, 2011) . Colgrave *et al.* (2012) identified C-hordein fragments in wort and trace levels of C-hordein peptides in the beer. Many of the peptide fragments contained runs of glutamine and proline. When brewing with unmalted barley, the hordeins will not be degraded during malting leading to increased intact proteins in the mash. Proteolytic enzymes from the proportion of malt in the mash will start to break down the intact proteins in the unmalted barley but not to the same extent as during the malting process, explaining the higher concentration of peptides in beer produced with barley (A) and barley (B) compared to the all malt control.

It was found that 11 of the 17 peptides were higher in 15% barley (A) compared to 15% barley (B). Holopainen *et al.* (2012) found that barley (B) by removing 16-17% of the outside significantly decreased C-hordeins by 21%. Therefore, the use of barley (B) would potentially change and lower the protein composition of the mash and changing the peptides found in the beer. This could be one reason as to why more differential peptides are identified in 15% barley (A) compared to 15% barley (B).

It is known that cysteine proteinases play the biggest role in solubilising proteins during mashing (Jones *et al.*, 2005). Davy *et al.* (1998) investigated two cysteine endoproteases, EP-A and EP-B, which are known to play a central role in the breakdown of barley endosperm and storage proteins which are secreted into the starchy endosperm during germination. It was found that the cysteine endoproteases specified to hydrolytic sites with amino acids in order Leu > Phe > Val > Pro > Ser. This could help explain the peptide sequences that were identified as differential peptides, as 6 of the peptides have terminal leucine residues (Table 6.1 and 6.2). This suggests that cysteine proteinases in the mash breakdown the intact hordeins in the mash forming different sequences of peptides.

Peptides can induce taste and mouthfeel sensations in food and beverage products and the taste of peptides depends on the amino acid composition and sequence. The presence of glycine, alanine, valine, leucine, tyrosine and phenylalanine in peptides can impart bitterness (Zhao *et al.*, 2016). Four of the tripeptides identified, compounds 5, 36, 57 and 58 (Table 6.1 and 6.2) have a leucine residue located at the C-terminus of the tripeptide. Ishibashi *et al.* (1987)

found that peptides with a leucine residue at the C-terminus have noticeably higher bitterness compared to peptides which have leucine residues located at the N-terminus or in the middle. It was proposed that the hydrophobic residue of the peptide acts a binding site between bitter peptides and gustation cells. Similarly, the tripeptide val-glu-trp has a bulky hydrophobic tryptophan residue at the C-terminus of the tripeptide. This is important for providing bitterness to the tripeptide as the hydrophobicity of the C-terminus amino acid influences the bitterness of the tripeptide. (Kim *et al.*, 2006; Xu *et al.*, 2019). The hydrophobic valine residue at the N-terminal would also increase bitterness because the combination of hydrophobic amino acids at the C- and N-terminus can also increase bitterness in tripeptides (Kim *et al.*, 2006).

For tetrapeptides bulky hydrophobic amino acids at the C-terminus and bulky basic or hydrophobic amino acids at the N-terminal relate to bitterness in peptides. The tetrapeptide ala-ser-pro-pro (compound 6, Table 6.1) consists of bulky hydrophobic proline residues and hydrophobic alanine. Proline is considered a major contributor to bitter taste in peptides (Ishibashi *et al.*, 1988). Similarly, compound 40, 54 and 56 (Table 6.2) contain two proline residues which would increase the bittering effect of these four tetrapeptides. It has been proposed that bitter tetrapeptides produce bitterness due to a bulky hydrophobic group of the peptide functioning as a binding unit for the bitter taste receptor whilst the adjacent bulky basic or bulky hydrophobic group can plays a role as a stimulating unit, increasing bitter taste (Maehashi *et al.*, 2009).

A range of bittering peptides have been identified to be differential features in the 15% barley (A) and 15% barley (B) which could contribute to the change in quality of bitterness in beer produced with unmalted barley. The findings of Chapter 4 and 5 focused on the importance of proteolytic enzymes when brewing with unmalted barley for the requirement of free amino nitrogen in the formation of flavour-active volatile compounds. However, these results show the importance of proteolytic enzymes and the hydrolysis of proteins in the formation of taste inducing peptides in the beer. The differential features identified show that peptides could be important for beer flavour quality.

6.3.2.2. γ-glutamyl peptides

A peak was elicited in the ESI(+) mode centred at 2.73 min which was ascribed to γ-glutamyl tyrosine (Compound 37, Table 6.1), with a strong SIRIUS match score of 99% and CSIfinger score of -23.10. The compound was statistically significantly higher in the 15% barley (A) compared to the 15% barley (B) beer ($p<0.01$). γ-glutamyl peptides are small molecular peptides that contain a γ-carboxyl group of glutamic acid at the N terminus of the peptide. Many γ-glutamyl di- and tri-peptides including, γ-glutamyl tyrosine have been detected in many fermented foods such as cheese, soy sauce and fermented fish (Zhao *et al.*, 2016). γ-glutamyl peptides have also been previously identified in beers, Miyamura *et al.* (2015) found kokumi peptide γ-glu-val-gly in a range of beers at a concentration from 0.08-0.18 mg/L.

γ-glutamyl peptides are recognised because of their ability to impart kokumi-active taste in food products (Li *et al.*, 2020b). These compounds impart minimal flavour in water, however when introduced into food products they can substantially enhance continuity and mouthfulness of the taste present in the food in which they have been added. These compounds are typically referred to as kokumi-active substances (Miyaki *et al.*, 2015). The functional taste properties of γ-glutamyl peptides involve their ability to activate the calcium-sensing receptor (CaSR) channels on the tongue leading to a release of intracellular Ca^{2+} in the surrounding taste cells (Ohsu *et al.*, 2010). Although kokumi substances tend not to have a specific taste themselves at food product concentrations, some γ-glutamyl peptides can exhibit bitterness and/or astringency in aqueous solutions depending on the C-terminus amino acid residue in the peptide (Yang *et al.*, 2019; Liu *et al.*, 2015). γ-glutamyl tyrosine can impart astringency and bitterness in aqueous solutions. Toelstede *et al.* (2009) determined the intrinsic orosensory threshold concentration for γ-glutamyl tyrosine and found a slight astringent mouth coating characteristic at 2.5 mmol/L and bitterness at 5 mmol/L. This is in agreement with Liu *et al.* (2015) who determined that γ-glu-tyr imparts bitterness at 5 mmol/L in water. Thus, the sensory properties that γ-glu-tyr induce in food products could be a reason why the incorporation of barley (A) has a different sensorial taste profile to beers produced with barley (B).

γ-glutamyl peptides can be enzymatically generated by γ-glutamyl transpeptidase or γ-glutamyl transferase (GGT) which has been previously identified in S*accharomyces cerevisiae* (Sofyanovich *et al.*, 2019). γ-glutamyl peptides can be formed by the enzyme binding to glutamate to form a γ-

glutamyl-enzyme conjugate, which can react with other free amino acids in fermenting material producing the corresponding γ-glutamyl dipeptides (Sofyanovich *et al.*, 2019; Toelstede *et al.*, 2009). The amino acid analysis performed on the adjunct wort in Chapter 4 (Section 4.3.2.3) determined that wort produced with 35% barley (A) had a higher tyrosine concentration compared to wort produced with 35% barley (B) (107.2 mg/L and 94.3 mg/L respectively). Although the difference in tyrosine concentration was not substantial between the two adjunct worts, the higher tyrosine concentration in wort produced with unmalted barley may have increased the production of γ-glutamyl tyrosine during fermentation.

6.3.2.3. Purines

Three differential features were identified as purine based chemical classes. Adenine (purine) and 6,7-dihydropurin-6-ylium (purine derivative) were tentatively identified as significantly higher in 15% barley compared to both 100% malt and 15% barley (B). Compound 50 was determined as a purine nucleoside and was identified as significantly higher in 15% barley (B) compared to 15% barley (A) (Table A.2).

Nucleic acids make up 0.2-0.3% of barley and few of the nucleic acids present in barley survive malting and mashing but their degradation products, nucleotides, nucleosides and purine and pyrimidine bases are present in beer (Briggs *et al.*, 2004). During malting and mashing, nucleotidases catalyse the degradation of nucleic acids to nucleosides. Then the glycosidic linkages between the purine and the sugar moieties are cleaved by nucleosidases. Therefore, the incorporation of unmalted barley will increase nucleic acid content in the mash as there has not been sufficient break down from the malting process. Harris *et al.* (1958) found that nucleotidases present in the mash function almost optimally at normal infusion mash temperatures (e.g. 65 °C). This means that nucleotidases from the malted barley proportion of the grist bill will enable the breakdown of nucleic acids in the unmalted barley creating a different mixture of nucleic acid breakdown products compared to an all-malt wort.

Some nucleic acid breakdown products are known to have flavour-enhancing properties (Lee *et al.*, 1986). The purine and purine nucleotide class has been

recognised for influencing flavour. Bettenhausen *et al.* (2018) found that purine compounds in malt and beer were associated with corn chip flavour in beer. The purine nucleosides identified in their study were negatively correlated with fruity characteristics (e.g. green apple and watermelon rind) but positively correlated with typical staling characteristics such as cardboard, bitter and astringent. Therefore, the purine based differential features putatively identified in this present study may influence the sensory perception of beers produced with unmalted barley.

6.3.2.4. Hordatines

Hordatines are one of the most abundant but least studied group of phenolic substances in beer. Hordatines consist of polyamides conjugated with hydroxycinnamic acids to form phenolamides (Pihlava, 2014). Several studies have investigated hordatines present in barley in order to understand astringent aftertaste in beer (Kageyama *et al.*, 2011; Kageyama *et al.*, 2013; Kohyama *et al.*, 2013). Kageyama *et al.* (2011) fractionated malt acrospires by preparative HPLC and determined that a fraction exhibited astringency in sensory evaluation by trained panel members. The fraction consisted of three compounds 4'-*O*-β-D-glucopyranosyl hordatine A, 4'-*O*-β-D glucopyranosyl hordatine B and 4'-*O*-β-D-maltosyl hordatine A, which all exhibit astringent properties. Physical removal of acrospires from the malt by polishing led to reduced astringency and improved beer flavour. Kohyama *et al.* (2013) isolated hordatine A-β-D glucopyranoside from ungerminated barley grains and found that the compound is localised in the aleurone layer.

Hordatines are typically synthesised from cinnamoyl-CoA and agmatine by agmatine coumaroyl transferase and subsequent peroxidase-catalysed dimerisation (Wannenmacher *et al.*, 2018). A peptide containing an agmatine residue was identified as significantly higher in 15% barley (A) beer compared to 15% barley (B) and the all-malt control beer. However, the SIRIUS score and CSI:Finger score (0.2% and -154.65 respectively) did not satisfy the match criteria for further investigation. It could be hypothesised that peptides with agmatine residues could be an interesting line of enquiry for astringency caused by incorporation of barley (A). The agmatine residue containing peptides have analogous chemical features to hordatine and could therefore have astringency

inducing properties. It would be of interest to conduct further metabolomic analysis of beers produced with higher proportions of barley (A) and barley (B) to determine if hordatine structures in the beer significantly change with adjunct incorporation.

6.4. Conclusions

The application of non-targeted metabolomic profiling with principal component analysis was a successful method in putatively determining differential metabolites present when comparing all malt beer to 15% barley (A) and barley (B) beers. The large number of features were reduced based on statistical criteria and further shortlisted by assignments of the features from search databases. However, many of the features could not be assigned to identifiable molecules. Further work is required to confirm the actual chemical structures and properties of the differential compounds discussed, in order to confirm if these compounds are important when considering the sensory profile of adjunct-based beers. Despite this, the non-targeted profiling method has provided new insights into non-volatile molecular features that have been previously overlooked with standardised targeted analysis.

The results show that the main differential molecular features of interest were nitrogenous compound groups such as peptides and purine derivatives. The differential peptides identified have bitter inducing properties, including γ-glutamyl tyrosine which can influence bitterness, astringency and induce kokumi taste in beer. Among the 17 peptides identified, 9 were significantly higher in either 15% barley (A) or 15% barley (B) compared to the all-malt control. This shows that replacing even a small portion of the malt grist with barley (A) or barley (B) can impact the nitrogenous composition of the final beer. A reduction in the endogenous enzymes present in the mash that are synthesised and activated during the malting process impacts the metabolomic profile and subsequently the sensory profile of the beer. This result emphasises that even a small proportion of unmalted adjunct usage can impact the metabolomic profile of the beer. Similarly, 11 of the 17 taste inducing peptides were significantly higher in 15% barley (A) compared to 15% barley (B). This shows that barley (B) before use changes the protein and peptide composition in the beer. This could help to explain increased bitterness and astringency sensory characteristics that are observed when brewing with barley (A) which are less pronounced in beers produced with barley (B). Overall, the results

presented have identified key metabolites which have not been considered previously when investigating the cause for the unfavourable bitterness and astringency characteristic of unmalted barley. Processing barley before use or supplementing exogenous proteases to replicate the lost proteolytic activity in the mash can help reduce the bitter peptides identified and improve the sensorial profile of beer produced with unmalted barley.

Chapter 7 Overall conclusions and future work

7.1. Overall conclusions

The aim of this research project was to better understand the sensory and analytical impacts of brewing with unmalted cereal adjuncts on beer quality. In addition, to determine techniques to improve flavour quality when using unmalted adjuncts and understand the consumer preference and acceptance of adjunct beers.

The aim of the work presented in Chapter 3 was to address the lack of detailed sensory descriptive evaluation of beers produced when brewing with unmalted barley, rice, maize and wheat adjuncts. Furthermore, to examine the chemical composition of the adjunct beers to develop understanding of the underlying basis for the sensory properties observed. A trained panel developed and defined a lexicon of 18 attributes which were used to characterise the sensory properties of the beers produced with unmalted adjuncts and an all malt control. In both the analytical and sensory data it was found that beers produced with adjuncts, even at incorporation rates of 60%, had more subtle changes in sensory and analytical characteristics than had been expected. Beers produced with 30% unmalted adjunct incorporation showed insignificant variation for 17 of the 18 attributes in the sensory beer profile ($p>0.05$), however, these beers were characterised by a lack of malty or sweet, fruity character. The use of barley (A) at 60% incorporation rate significantly increased the perception of lingering bitterness ($p<0.05$) and increased bitter and astringent characteristics ($p>0.05$). These sensory characteristics were not observed in the beers produced with 60% barley (B), demonstrating that the processing the barley before incorporation reduces bitter characteristics when brewing with unmalted barley. Physicochemical analysis was used to investigate whether phenolic compounds could be a cause of the bitterness observed with barley (A). However, the beer TPC of 60% barley (A) was 10 mg/L lower than the all malt control (160 and 170 mg/L respectively). This finding is in agreement with published literature which found that increasing the proportion of unmalted barley decreased beer TPC (van Donkelaar *et al.*, 2016). This suggests that there is another class of compounds responsible for the lingering bitterness and astringency associated with high incorporations of unmalted barley. Wort FAN was reduced by 75% with the incorporation of 60% rice and 60% maize which

impacted the beer flavour profile. These beer samples were characterised by high levels of higher alcohols which are flavour defects typical of low nitrogen fermentation. For example, the concentration of tyrosol in the beer was 160% higher in the 60% maize beer compared to the all malt control (31.64 and 12.68 mg/L respectively). Interestingly, the incorporation of 60% unmalted wheat produced beer with an analytical profile closest to that of the all malt control beer.

This was the first study (Chapter 3) to develop a specific adjunct beer sensory lexicon with corresponding physicochemical analyses. These findings represent a significant contribution to our current understanding of the sensory impacts of brewing with higher incorporations of unmalted adjunct material. The results suggest that unmalted adjunct incorporation rates could be pushed higher than the typical current industry usage without negatively impacting the beer quality. Torrefied maize and rice were used in this study to facilitate processing on a pilot scale without the requirement of the cereal cooking step. It is recognised that torrefied adjuncts are relatively expensive materials compared to unprocessed grains. Therefore, the use of torrefied rice and maize up to 60% in a full scale lager brewery may not be cost-effective and the use of expensive torrefied grains could offset the economic benefits of utilising unmalted adjuncts. The utilisation of unprocessed rice and maize would require adjustments to the brewing protocols developed in this study. In order to incorporate unprocessed rice and maize at a high concentration, the grain would have to be cooked in a cereal cooker before addition into an infusion mash which would involve extra costs for installation of a cereal cooker. Otherwise, a decoction vessel could be used where cooked adjuncts constitute the first decoction of a decoction mashing program. The prospect of using high incorporation rates of maize and rice in large scale brewing is dependent on the design of the brewery which influences the processability of unmalted rice and maize. Remodelling a full scale brewhouse to utilise unprocessed grains could be costly and increase overall energy use, outweighing the benefits of unmalted adjunct use.

Dependent on the beer style being produced and the target market, the results of this study show that the incorporation of 30% unmalted adjunct usage can be used to adjust sensory properties by reducing malty, sweet or fruity character in the beer, if desired. The substitution of a portion of the malt in the grist bill

with wheat could facilitate the economic and environmental benefits of adjuncts whilst retaining the beer flavour characteristics of an all-malt beer.

The aim of the study presented in Chapter 4 was to investigate methods of balancing nitrogenous and micronutrient levels in wort to eliminate flavour 'defects' caused by low wort FAN levels that were observed in beers produced with 60% unmalted cereal adjuncts. Beers were produced with the addition of an exogenous thermostable mash protease (FAN Boost) in the mash or yeast nutrient (YeastLife) to the cold wort for the purpose of increasing FAN and micronutrient levels. Results revealed that the use of FAN compensation methods increased FAN sufficiently to produce similar concentrations to the all-malt control. By thus balancing the wort FAN in the adjunct beers, variations in esters and higher alcohol concentrations were decreased to less than 20% for all adjunct beers compared to the all-malt control. Thus, increasing FAN when brewing with adjuncts was shown to minimise flavour defects caused by FAN deficiency and create adjunct beers with analytical profiles comparable to all-malt beers.

The two FAN compensation methods investigated altered the wort and beer quality in different ways. Although YeastLife did increase assimilable nitrogen, unfavourable results were determined in the physicochemical analysis of the wort and beer. The addition of YeastLife increased wort and beer pH. This was shown by the addition of 1 g/L of YeastLife in 45% B(B) YL increased wort pH to 6.17 in comparison to 45% B(B) FB wort pH of 5.32. The addition of YeastLife also significantly increased zinc wort concentration to 0.79 mg/L in 45% B(B) YL compared to 0.18 mg/L in 45% B(B) FB. The increase of wort pH and wort zinc could give reason to an increase in acetate esters and total higher alcohols observed in YeastLife supplemented beers compared to the all-malt control. This is consistent with findings from previous studies conducted by Nicola *et al.* (2009) which found that increasing wort zinc concentration increased higher alcohols and Piddocke *et al.* (2011) which found that fermentations with lower wort pH lead to beer with lower concentrations of acetate esters. The results show that YeastLife supplementation can increase the production of flavour volatiles which could impact flavour consistency and producing a uniform product when brewing on a large scale. For YeastLife to increase FAN concentration to the target wort FAN, the dosing rate required was over 10 times the recommended dosing rate provided by the supplier. This could cause a constraint in scale up use for large breweries as the requirement to use a large

dose of the product could be costly. Therefore, the potential impact on flavour and overall costs by using YeastLife as a FAN supplementation could outweigh the benefit of increased FAN. The use of FAN boost led to faster fermentations and the FAN boost wort samples had comparable wort and beer pH to the all malt control. For example, the wort pH of 45% B(B) FB was 5.32 in comparison to the 100% malt wort pH of 5.39. The use of FAN boost did not significantly impact the concentration of flavour volatile compound and caused a decrease in the content of unfavourable total higher alcohols ($p>0.05$). The 45% B(B) FB sample had lower total higher alcohol content compared to the all-malt control (98.57 and 105.14 mg/L respectively). The results of this chapter showed that the use of the exogenous thermostable protease, FAN boost provided better quality wort and beer compared to the addition of YeastLife the yeast nutrient and was therefore favoured when increasing FAN to improve beer quality.

Analysis of the transition metal ions present in adjunct wort and beer showed that the incorporation of unmalted adjuncts, especially wheat, decreased iron, copper and manganese in the wort and beer. This identifies a potential benefit of utilising adjuncts when considering flavour stability of beer. In agreement with our finding, (Krstanović *et al.*, 2020) found that replacing a proportion of malt with maize and wheat halved the concentration of copper in the beer. Unmalted wheat in conjunction with other unmalted cereals could reduce the formation of specific ageing compounds attributed to oxidative processes catalysed by transition metal ions and thus improve adjunct beer flavour stability.

The findings of Chapter 4 provide a comprehensive analytical evaluation of successful modifications to wort that can improve the quality of beer enabling higher incorporation of adjunct material. The understanding of the effects of FAN supplementation techniques determined in this chapter would be a useful resource to brewers aiming to increase adjunct material whilst retaining effective processability and flavour quality.

The study presented in Chapter 5 investigated the consumer sensory perception and preference of adjunct beers that were produced with FAN supplementation methods. The instrumental analyses performed on wort and beer samples in Chapter 5 found that the incorporation of unmalted adjuncts up to 45% (wheat, barley (A) and barley (B)) decreased the wort and beer fermentable sugar concentrations and concentrations of key esters. The incorporation of 45% B(A)/W showed the highest dissimilarity in ester profile to

100% malt as the 45% B(A)/W beer had a total esters concentration 36% lower than the all-malt control.

The consumer sensory evaluation determined significant differences in citation frequency for 9 out of the 18 sensory attributes in the CATA lexicon ($p<0.05$), indicating that consumers could discriminate sensory properties between adjunct beers and an all-malt control. Although significant sensory and analytical differences were determined across the sample set ($p<0.05$), this did not strongly impact consumer preference. The overall liking and purchase intent of consumers for adjunct beers produced with FAN boost were not significantly different to those for the all-malt control ($p>0.05$). Thus, beers produced with 45% adjunct incorporation and FAN Boost were equally accepted by consumers as for the all-malt control. The utilisation of YeastLife negatively impacted the consumer perception of adjunct beers as the beer produced with YeastLife was significantly lower in overall liking and purchase intent compared to the rest of the samples ($p<0.05$). This result supports the finding of Chapter 4, that FAN boost was the better FAN supplementation method when aiming to match the sensory and analytical characteristics of all-malt beer.

The results in Chapter 5 found that beer produced with 45% unmalted barley/wheat was the most sensorially and analytically distinctive sample. The beer was characterised by significantly lower selection frequencies of green apple aroma, banana aroma and flavour ($p<0.05$) which correlated to significantly lower concentrations of ethyl and acetate esters identified in the chromatographic analysis ($p<0.05$). This beer sample was also characterised by significantly higher selection frequencies of bitter and lingering bitterness attributes ($p<0.05$). It can be concluded from this study and the results from Chapter 3 that 45% is close to the upper limit of incorporation of unmalted barley that can be used before the flavour profile is compromised.

The correlation of consumer sensory evaluation and physicochemical analysis led to the identification of significant sensory and analytical drivers for liking and disliking of adjunct beers ($p<0.05$). ‘Apple flavour’ and ‘sweet’ attributes associated with higher concentrations of ethyl esters and ‘malty aroma and flavour’ associated with higher concentrations of fermentable sugars were significant positive drivers for liking ($p<0.05$). The only attribute that was a significant negative driver for liking was ‘lingering bitterness’, which was associated with higher concentrations of total proanthocyanidin content

($p<0.05$). These results help identify specific chemical components and sensory characteristics that should be controlled when brewing adjunct lager beers, to improve their consumer acceptance and desirability.

Beer consumers were found to have different liking patterns when considering adjunct beers and three consumer clusters were identified. Cluster 1 (41% of consumers) had a higher liking for all samples compared to clusters 2 and 3. In contrast, cluster 2 (35% of consumers) showed lower overall liking for all samples. Cluster 3 (24% of consumers) showed a marked preference for 45% adjunct incorporation beers. This result shows that consumers are not uniform in their preferences for adjunct beers. The identification of a cluster of consumers that prefer higher adjunct beers is significant for product development of adjunct beers. This result shows there is potential in creating higher adjunct beers whereby the properties imparted by adjuncts are seen as a selling point for a certain sector of consumers.

This was the first study to determine consumer perception and preference of beers produced with unmalted cereal adjuncts. Our results give brewers new insights with which to develop recipes for high-adjunct lager beers, based on the knowledge that this need not significantly impact on their consumer acceptability; indeed, for some consumers it may increase the appeal of products.

The metabolomic study in Chapter 6 presented key non-volatile metabolites which could contribute to flavour differences caused by the incorporation of unmalted barleys. Results found that the main differential molecular features were peptides and purine derivatives. The differential peptides identified were mostly significantly higher ($p<0.05$) in the beers produced with 15% barley (A) and barley (B) compared to the all-malt control. Among the 17 peptides identified, 9 were significantly higher in either 15% barley (A) or 15% barley (B) compared to the all-malt control. This shows that replacing even a small portion of the malt grist with unmalted barleys can impact the nitrogenous composition of the final beer. This result can be explained by the removal of the malting process when utilising unmalted barleys. As 88-90% of wort assimilable nitrogen is formed as a result of germination during malting and only a minority is produced by proteolytic activity during mashing (Lekkas *et al.*, 2014). This shows that malting is the most important process for proteolytic activity and the breakdown of proteins and peptides. Therefore, removing the malting process

will introduce more peptides and proteins that are broken down by a specific group of mash peptidases. Removing the proteolytic enzyme activity that occurs during the malting process impacts the sensory profile of the beer. This result emphasises that even a small proportion of unmalted adjunct usage can impact the metabolomic profile of the beer.

Similarly, other peptides were determined to differ significantly when comparing equivalent beers made with either 15% barley (A) or barley (B) ($p<0.05$), highlighting that the processing applied to the barley grain can have an impact on the chemical composition of the beer. Several of the amino acids residues present in the differential peptides identified are known for causing bitter and kokumi taste in beer. The results from this study provided key metabolites which have not previously been considered when investigating the cause of unfavourable bitterness and astringency produced from unmalted barley. These results highlight the importance of the proteolytic enzyme activity breaking down proteins and peptides during malting and provide further significance to the use of exogenous proteases at high incorporation rates of unmalted adjuncts. These results confirm that processing unmalted barley before use can alter the metabolite profile of the beer and potentially improve sensory properties.

7.2. Future work

The following points are raised for consideration to strengthen the findings of the present research or to propose follow-on studies in this field:

- The beer brewed throughout this research has been produced on a 25-35 L pilot scale using either a GrainFather system or Briggs brewline system. Larger scale brewing trials involving unmalted cereal adjuncts and FAN supplementation should be conducted to further investigate the feasibility and processability of using high proportions of unmalted adjuncts in large brewhouses.

- The metabolomic profiles of beer presented in Chapter 6 could be further improved by evaluating beers produced with higher amounts of barley (A) and barley (B). Identification of these differential metabolites could improve understanding of the bitterness and astringency characteristics observed when brewing with unmalted barley. Further

identification and confirmation with standards of the differential taste inducing peptides could improve understanding of the sensory properties these compounds elicit. It would also be useful to analyse the amino acids present in the adjunct beers as amino acids are known for imparting bitter, sour, sweet and umami tastes in beer.

- In Chapter 3 and Chapter 5, the sensory attribute ‘sweetcorn/cooked vegetable’ was found to be significantly different across a sample set of adjunct beers and an all-malt control. Analysis of sulphur compounds by SPME-GC-PFPD conducted in Chapter 3 was inconclusive with regards to determining the sulphur compounds which contribute to this sensory characteristic identified in the 30% rice beer. Further work is thus required to determine the sulphur compounds that could cause the ‘sweetcorn/cooked vegetable’ aroma in the adjunct based beers.

- The results of Chapter 5 found that ‘sweet’ and ‘apple’ sensory attributes associated with ethyl esters and ‘malty’ attributes associated with fermentable sugars were significantly positive drivers for consumer liking. These sensory and analytical drivers correlated to low adjunct beers. It would be of interest to increase esters and fermentable sugars in high adjunct beers to determine if the change in chemical composition could improve consumer liking.

- The rapid profiling techniques used in the sensory consumer evaluation in Chapter 5 were based on the attribute lexicon developed by trained panellists for adjunct beers. However, open-comment analysis showed that consumers used attributes to describe their preference of adjunct beers that were not previously considered by the trained panel. Development of consumer defined lexicons for CATA evaluations could provide better insight into the consumers perception of adjunct beers.

- The results presented in Chapter 5 showed that consumers formed three different clusters with different liking patterns. Further investigation should be performed to establish if these clusters are robust and reproducible amongst a wider section of consumers. The drivers of the

preference differences in the consumer clusters such as genetic variation (e.g., PROP tasters), socioeconomic factors and beer style preferences should be explored to understand if these factors are the cause of the different preference clusters observed. Specifically, understanding the consumer cluster with a marked preference for higher incorporation of adjunct material as this could help develop beers with high adjunct incorporation as a selling point.

7.3. Final conclusions

This research has considerably improved our understanding of the sensory and analytical profile of beers produced with unmalted cereal adjuncts. It has shown that current industry adjunct usage has potential to be pushed further without adversely changing the chemical and sensory profile of the beer. With the help of FAN supplementation techniques, the quality of wort and beer produced with adjuncts can be more closely matched with their all-malt counterparts. Consumer research identified that beers produced with up to 45% unmalted adjunct incorporation had similar acceptance to all-malt beers, and in some cases, were actually preferred. With further optimisation, high adjunct incorporation can be fully utilised in brewing and become an attractive part of the wider portfolio of lager beers.

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Table A.1. Nitrogen content of the grist material used in each study (%)

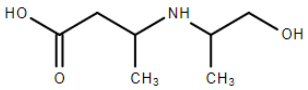
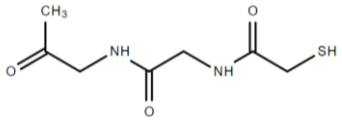
	Chapter 3	Chapter 4	Chapter 5
Malt	1.48	1.82	1.51
Barley (B)	1.74	1.82	1.75
Barley (A)	1.66	1.68	1.70
Wheat	1.96	1.86	2.25
Rice	1.16	-	-
Maize	1.22	-	-

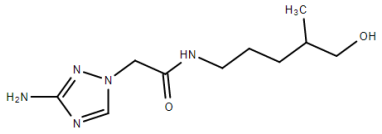
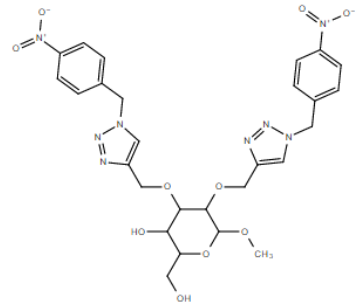
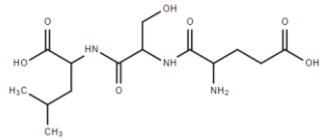
Table A.2. Fermentable carbohydrate content of trial wort samples (g/L)

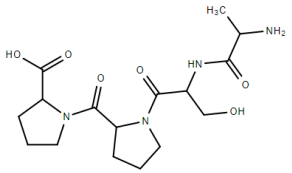
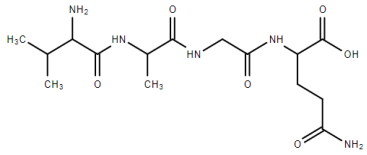
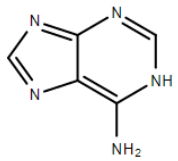
	FAN Supplementation	Glucose	Sucrose	Maltose	Maltotriose
100% Malt	-	10.99	6.25	64.43	18.52
15% Barley (B)	-	11.04	6.10	65.53	18.57
15% Barley (A)	-	11.30	6.14	66.12	18.69
15% Wheat	-	11.13	5.62	67.73	19.11
25% Barley (B)	Fan Boost	8.45	5.83	65.51	18.71
35% Barley (B)	Fan Boost	**8.26****	5.87	61.04	17.81
45% Barley (B)	Fan Boost	8.11	5.87	63.13	19.69
25% Barley (B)	YeastLife	12.34	5.76	72.09	19.49
35% Barley (B)	YeastLife	10.66	6.09	63.02	18.61
45% Barley (B)	YeastLife	9.66	5.71	60.10	19.77
25% Barley (A)	YeastLife	10.40	6.24	63.45	17.85
35% Barley (A)	YeastLife	11.30	6.67	67.73	20.42
45% Barley (A)	YeastLife	9.94	5.72	60.97	18.20
25% Wheat	YeastLife	11.03	5.99	74.93	20.02
35% Wheat	YeastLife	10.72	5.88	65.72	18.24
45% Wheat	YeastLife	8.87	5.85	62.86	17.75
Incorporation rate (%)		**0.036**	0.734	0.549	0.673
Adjunct		**0.001**	0.079	0.060	0.371
Supplementation		**<0.0001**	0.384	0.603	0.512

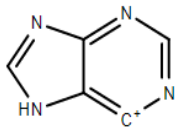
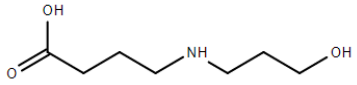
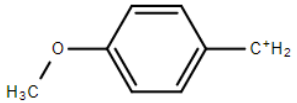
One-way ANOVA with Dunnett's post hoc analysis was used to compare the fermentable carbohydrates. Asterisks indicate samples that were significantly different in the samples compared to the all-malt control (**p<0.01).

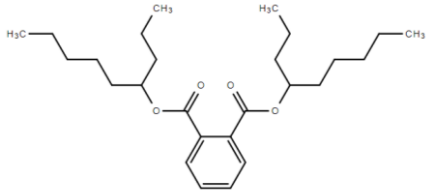
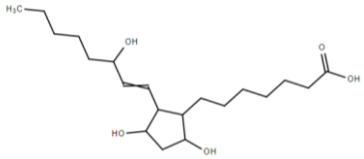
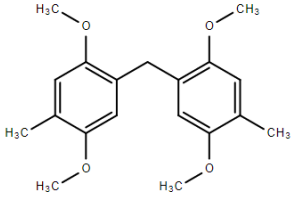
Table A.3. Differential metabolites putatively identified by SIRIUS GUI software from positive $[M+H]^+$ and negative $[M-H]^-$ ionization analysis of the three beer samples. Differential metabolites shown are significantly higher in 15% barley (A) compared to 100% malt (100M) and 15% barley (B) (15B(B)). Elemental composition (E.C.) corresponds to the $[M+H]^+$ and negative $[M-H]^-$ ion. RT is the elution time in min. Beer d.c. indicates the beer for which the compound was found to be significantly differential compared to 15% barley.

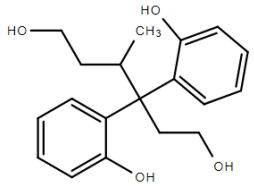
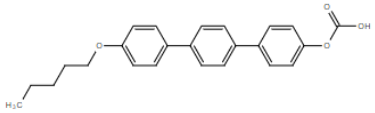
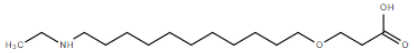
No	Mode	Compound Name	Predicted Class	E.C.	Predicted Structure	m/z	SIRIUS Score (%)	CSI Finger score	Beer d.c.
1	$[M + H]^+$	Compound 1	Amino acid	C7H15NO3		203.14	33.33	-100.49	100M 15B(B)
2	$[M + H]^+$	Compound 2	Dipeptide	C7H12N2O3S		205.06	100	-107.06	100M 15B(B)

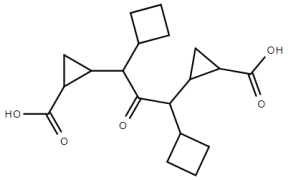
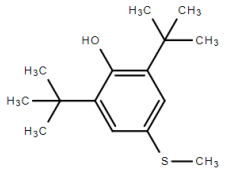
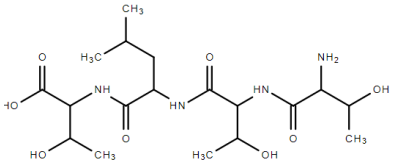
No	Mode	Compound Name	Predicted Class	E.C.	Predicted Structure	m/z	SIRIUS Score (%)	CSI Finger score	Beer d.c.
3	[M + H]$^+$	2-(3-Amino-1H-1,2,4-triazol-1-yl)-N-(5-hydroxy-4-methylpentyl)acetamide	Monoamine	C10H19N5O2		242.16	100	-135.86	100M 15B(B)
4	[M + H]$^+$	2-(hydroxymethyl)-6-methoxy-4,5-bis[[1-[(4-nitrophenyl)methyl]triazol-4-yl]methoxy]tetrahydropyran-3-ol	Peptide	C27H30N8O10		625.2	80.2	-371.66	100M 15B(B)
5	[M + H]$^+$	α-Glutamylserylleucine (glu-ser-leu)	Oligopeptide	C14H25N3O7		389.2	33	-77.61	100M 15B(B)

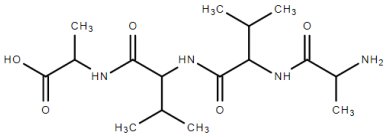
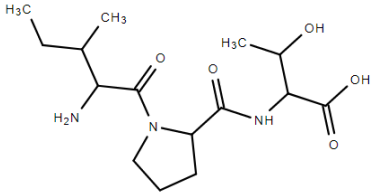
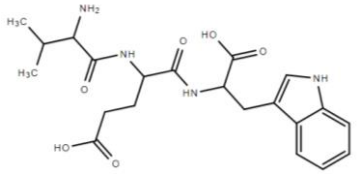
No	Mode	Compound Name	Predicted Class	E.C.	Predicted Structure	m/z	SIRIUS Score (%)	CSI Finger score	Beer d.c.
6	$[M + H]^+$	Alanylserylprolylproline (ala-ser-pro-pro)	Oligopeptide	C16H26N4O6		389.2	94	-123.16	100M 15B(B)
7	$[M + H]^+$	Valylalanylglycylglutamine (val-ala-gly-glu)	Oligopeptide	C15H27N5O6		374.2	24.9	-74.89	100M 15B(B)
8	$[M + H]^+$	Adenine	6-aminopurine	C5H55N5		136.06	97	-37.74	100M 15B(B)

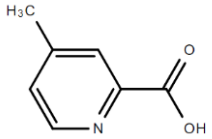
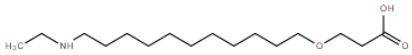
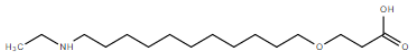
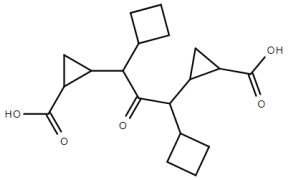
No	Mode	Compound Name	Predicted Class	E.C.	Predicted Structure	m/z	SIRIUS Score (%)	CSI Finger score	Beer d.c.
9	$[M + H]^+$	6,7-dihydropurin-6-ylium	Purines and derivative	C5H3N4		137.05	99	-29.34	100M 15B(B)
10	$[M + H]^+$	4-(3-hydroxypropylamino)butanoate	Fatty acids and conjugate	C7H15NO3		203.14	100	-95.74	100M 15B(B)
11	$[M + H]^+$	(4-methoxyphenyl)-methylium	Benzene substituted derivative	C8H9O		203.12	100	-58.07	100M 15B(B)

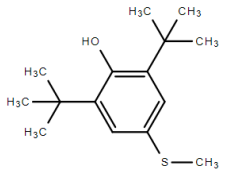
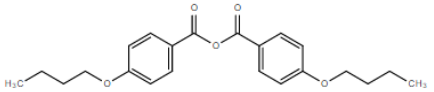
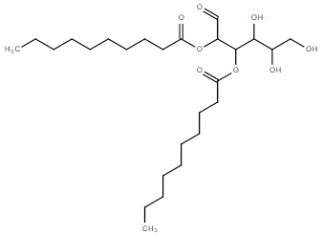
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12	$[M + H]^+$	Compound 12	Benzoic acid derivatives	C26H42O4		419.32	99	-56.32	100M 15B(B)
13	$[M + H]^+$	PGF1beta Prostaglandin F1β	Eicosanoid	C20H36O5		339.25	100	-66.52	100M 15B(B)
14	$[M + H]^+$	Oprea1_437193 bis(2,5-dimethoxy-4-methylphenyl)methane	Prenol lipid	C19H24O4		335.19	50	-167.34	100M 15B(B)

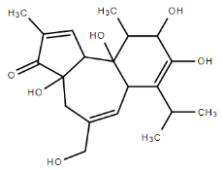
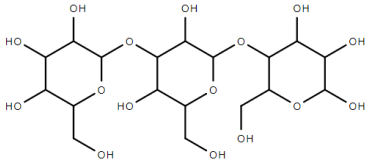
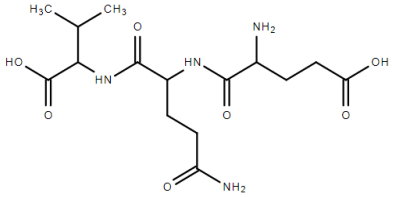
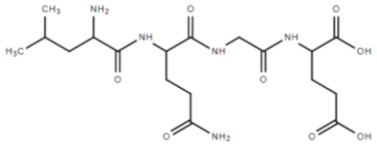
No	Mode	Compound Name	Predicted Class	E.C.	Predicted Structure	m/z	SIRIUS Score (%)	CSI Finger score	Beer d.c.
15	$[M + H]^+$	Compound 15	Cyclohexanone	C19H26O5		335.19	50	-172.44	100M 15B(B)
16	$[M + H]^+$	Compound 16	Flavone	C24H24O4		399.16	98.2	-135.42	100M 15B(B)
17	$[M + H]^+$	**Compound 17**	Secondary Alcohol	C18H36N2O3		310.24	50	-106.09	100M

No	Mode	Compound Name	Predicted Class	E.C.	Predicted Structure	m/z	SIRIUS Score (%)	CSI Finger score	Beer d.c.
18	$[M - H]^-$	Compound 18	Dicarboxylic acid	C19H26O5		393.19	33.33	-194.02	100M
19	$[M - H]^-$	2,6-bis(1,1-dimethylethyl)-4-(methylthio)-phenol	Phenyl propanes	C15H24OS		297.15	99.4	-88.05	100M
20	$[M + H]^+$	Threonylthreonylleucylthreonine (thr-thr-leu-thr)	Oligopeptide	C18H34N4O8		435.25	33	-61.14	100M

No	Mode	Compound Name	Predicted Class	E.C.	Predicted Structure	m/z	SIRIUS Score (%)	CSI Finger score	Beer d.c.
21	$[M + H]^+$	**Alanylvalylvalylalanine** (ala-val-val-ala)	Oligopeptide	C16H30N4O5		359.23	33	-64.05	100M
22	$[M + H]^+$	**Isoleucylprolylthreonine (leu-pro-thr)**	Oligopeptide	C15H27N3O5		330.2	99	-84.87	100M
23	$[M + H]^+$	L-Valyl-L-α-glutamyl-L-tryptophan	Oligopeptide	C21H28N4O6		433.21	88	-128.56	100M

No	Mode	Compound Name	Predicted Class	E.C.	Predicted Structure	m/z	SIRIUS Score (%)	CSI Finger score	Beer d.c.
24	$[M + H]^+$	4-Methylpicolinic acid	Substituted benzene	C7H7NO2		198.11	33	-69.33	100M
25	$[M + H]^+$	Compound 25	Secondary Alcohol	C18H36N2O3		310.24	50	-106.09	100M
26	$[M + H]^+$	**Compound 26**	Secondary Alcohol	C18H36N2O3		310.24	50	-106.09	100M
27	$[M - H]^-$	Compound 27	Dicarboxylic acid	C19H26O5		393.19	33.33	-194.02	100M

No	Mode	Compound Name	Predicted Class	E.C.	Predicted Structure	m/z	SIRIUS Score (%)	CSI Finger score	Beer d.c.
28	$[M - H]^-$	2,6-bis(1,1-dimethylethyl)-4-(methylthio)-phenol	Phenyl propane	C15H24OS		297.15	99.4	-88.05	100M
29	$[M + H]^+$	Compound 29	Coumarins and derivative	C22H26O5		403.21	25	-141.09	15B(B)
30	$[M + H]^+$	Compound 30	Alkyl glycoside	C26H48O8		511.33	78	-131.90	15B(B)

No	Mode	Compound Name	Predicted Class	E.C.	Predicted Structure	m/z	SIRIUS Score (%)	CSI Finger score	Beer d.c.
31	$[M + H]^+$	Compound 31	Secondary alcohol	C18H30O6		347.19	33	-132.58	15B(B)
32	$[M - H]^-$	Hexopyranosyl-(1->3)hexopyranosyl-(1->4)hexopyranose	Oligosaccharide	C18H32O16		503.16	99.7	-56.96	15B(B)
33	$[M + H]^+$	α-Glutamylglutaminylvaline (glu-gln-val)	Oligopeptide	C15H26N4O7		375.19	99	-126.44	15B(B)
34	$[M + H]^+$	Leucylglutaminylglycylglutamic acid (leu-gln-gly-glu)	Oligopeptide	C18H31N5O8		446.23	56	-87.95	15B(B)

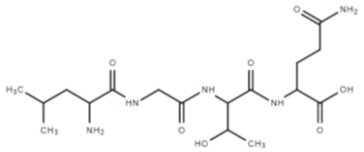
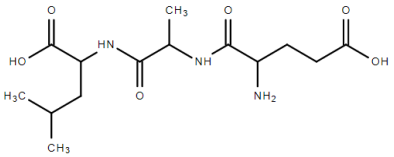
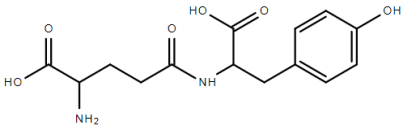
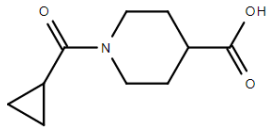
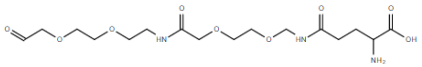
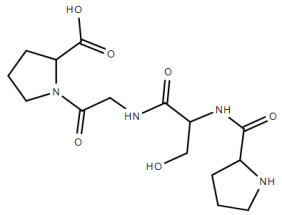
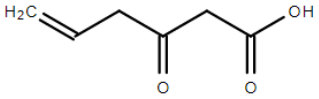
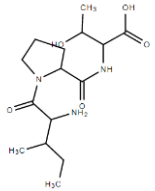
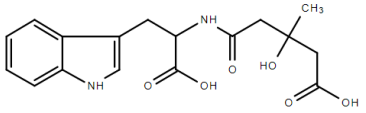
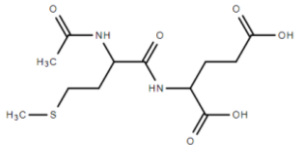
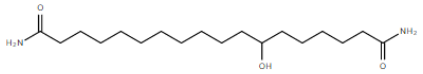
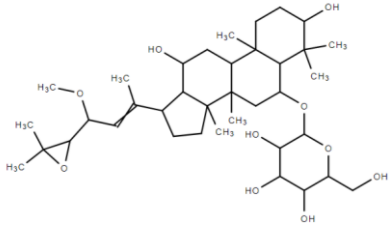
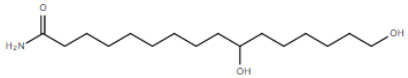
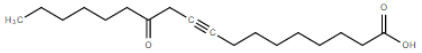
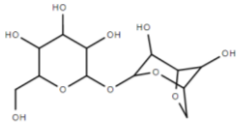
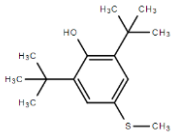
No	Mode	Compound Name	Predicted Class	E.C.	Predicted Structure	m/z	SIRIUS Score (%)	CSI Finger score	Beer d.c.
35	$[M + H]^+$	Leucylglycylthreonylglutamine (leu-gly-thr-gln)	Oligopeptide	C17H31N5O7		418.23	33	-83.26	15B(B)
36	$[M + H]^+$	α-Glutamylalanylleucine (glu-ala-leu)	Oligopeptide	C14H25N3O6		332.18	99	-57.78	15B(B)
37	$[M + H]^+$	γ-Glutamyltyrosine	Gama glutyl amino acids	C14H18N2O6		311.13	99	-23.10	15B(B)

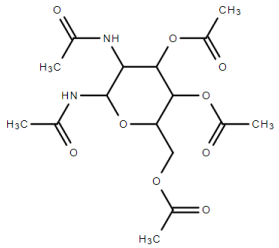
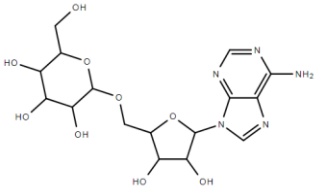
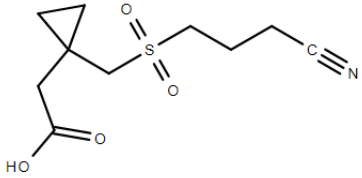
Table A.4. Differential metabolites putatively identified by SIRIUS GUI software from positive [M+H]+ and negative [M-H]- ionization analysis. Differential metabolites shown are of which significantly varied higher in 15% barley (B) compared to 100% malt (100M) and 15% barley (A) (15B(A)). Elemental composition (E.C.) corresponds to the $[M+H]^+$ and negative $[M-H]^-$ ion. RT is the elution time in min. Beer d.c. indicates the beer for which the compound was found to be a differential one compared to 15% barley (B).

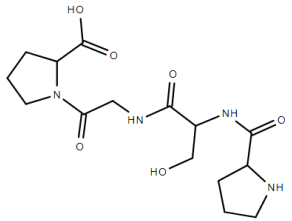
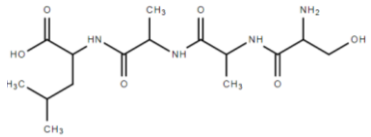
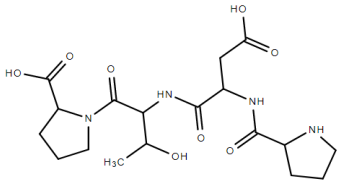
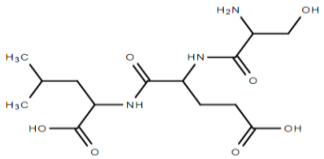
No	Mode	Compound Name	Predicted Class	E.C.	Predicted Structure	m/z	SIRIUS Score (%)	CSI Finger score	Beer d.c.
38	$[M + H]^+$	1-(cyclopropanecarbonyl)piperidine-4-carboxylate	Amino acid	C10H15NO3		198.11	100	-148.07	100M
39	$[M + H]^+$	Compound 39	Dipeptide	C16H29N3O9		446.15	58	-144.03	100M
40	$[M + H]^+$	Prolylprolylglycylserine (pro-pro-gly-ser)	Oligopeptide	C15H24N4O6		389.2	99.98	-150.31	100M

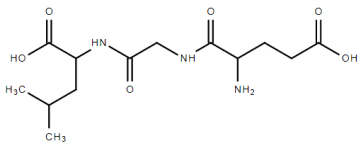
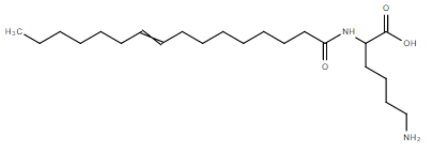
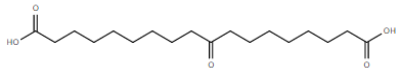
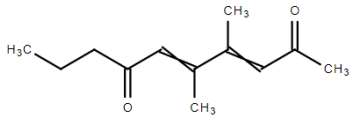
No	Mode	Compound Name	Predicted Class	E.C.	Predicted Structure	m/z	SIRIUS Score (%)	CSI Finger score	Beer d.c.
41	$[M + H]^+$	3-oxohex-5-enoate	Carboxylic acid ester	C6H8O3		170.08	100	-76.52	100M
42	$[M + H]^+$	Isoleucylprolylthreonine (Ile-pro-thr)	Oligopeptide	C15H27N3O5		330.2	99	-89.14	100M
43	$[M + H]^+$	Achillamide N-(4-Carboxy-3-hydroxy-3-methylbutanoyl)tryptophan	N-acyl-alpha amino acids	C17H20N2O6		349.14	49.9	-134.87	100M

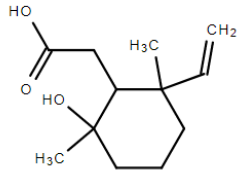
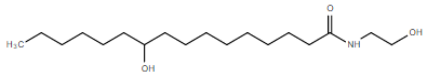
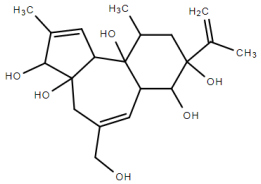
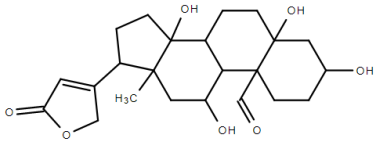
No	Mode	Compound Name	Predicted Class	E.C.	Predicted Structure	m/z	SIRIUS Score (%)	CSI Finger score	Beer d.c.
44	$[M + H]^+$	N-Acetylmethionylglutamic acid	Dipeptide	C12H20N2O6S		321.11	99	-129.5	100M
45	$[M + H]^+$	Compound 45	n-acyl amides	C18H36N2O3		329.28	100	-125.67	100M
46	$[M + H]^+$	Compound 46	Triterpene Saponins	C37H62O10		689.42	44	-117.90	100M
47	$[M + H]^+$	Compound 47	Fatty acid amide	C16H33NO3		310.24	50	-105.40	100M

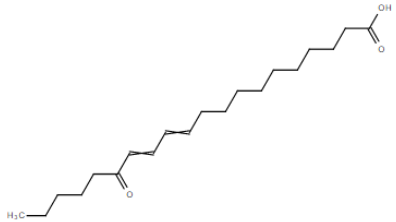
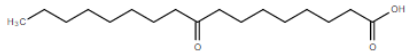
No	Mode	Compound Name	Predicted Class	E.C.	Predicted Structure	m/z	SIRIUS Score (%)	CSI Finger score	Beer d.c.
48	$[M + H]^+$	12-oxo-stearic acid	Cyclic alcohols and derivative	C18H30O3		317.21	99	-102.87	100M
49	$[M - H]^-$	Compound 49	Disaccharide	C12H20O10		323.1	88.5	-106.12	100M
50	$[M - H]^-$	2,6-bis(1,1-dimethylethyl)-4-(methylthio)-phenol	Phenyl propane	C15H24OS		297.5	98.3	-83.47	100M

No	Mode	Compound Name	Predicted Class	E.C.	Predicted Structure	m/z	SIRIUS Score (%)	CSI Finger score	Beer d.c.
51	$[M + H]^+$	2-Acetamido-N-acetyl-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosylamine	Amino acid	C16H24N2O9		421.18	94.5	-228.25	15B(A)
52	$[M + H]^+$	(2S,3S,4S,5S,6R)-2-[[(2R,3S,4R,5R)-5-(6-aminopurin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy]-6-(hydroxymethyl)oxane-3,4,5-triol	Purine nucleoside	C16H23N5O9		430.16	63.4	-108.04	15B(A)
53	$[M + H]^+$	(1-{[(3-Cyanopropyl)sulfonyl]methyl}cyclopropyl)acetic acid	α-amino acid	C10H15NO4S		246.08	100	-161.85	15B(A)

No	Mode	Compound Name	Predicted Class	E.C.	Predicted Structure	m/z	SIRIUS Score (%)	CSI Finger score	Beer d.c.
54	$[M + H]^+$	Prolylserylglycylproline (pro-ser-gly-pro)	Oligopeptide	C15H24N4O6		389.2	99.8	-127.65	15B(A)
55	$[M + H]^+$	Serylalanylalanylleucine (ser-ala-ala-leu)	Oligopeptide	C15H28N4O6		361.21	24.9	-96.63	15B(A)
56	$[M + H]^+$	Prolyl-α-aspartylthreonylproline (pro-asp-thr-pro)	Oligopeptide	C18H28N4O8		447.21	96	-144.37	15B(A)
57	$[M + H]^+$	Seryl-α-glutamylleucine (ser-glu-leu)	Oligopeptide	C14H25N3O7		348.18	49	-61.92	15B(A)

No	Mode	Compound Name	Predicted Class	E.C.	Predicted Structure	m/z	SIRIUS Score (%)	CSI Finger score	Beer d.c.
58	$[M + H]^+$	α-Glutamylglycylleucine (glu-gly-leu)	Oligopeptide	C13H23N3O6		318.17	99	-81.60	15B(A)
59	$[M + H]^+$	**Compound 59**	n-acyl amide	C22H42N2O3		383.33	99	-129.51	15B(A)
60	$[M + H]^+$	9-Oxooctadecanedioic acid	Long chained fatty acid	C18H32O5		311.22	33	-59.71	15B(A)
61	$[M + H]^+$	Compound 61	Sequiterpenoid	C12H18O2		195.14	50	-110.89	15B(A)

No	Mode	Compound Name	Predicted Class	E.C.	Predicted Structure	m/z	SIRIUS Score (%)	CSI Finger score	Beer d.c.
62	$[M + H]^+$	**[(1S,2S,6R)-2-Hydroxy-2,6-dimethyl-6-vinylcyclohexyl]acetic acid**	Prenol lipid	C12H20O3		213.15	50	-90.58	15B(A)
63	$[M + H]^+$	**Compound 63**	n-acyl amide	C18H37NO3		316.29	50	-89.49	15B(A)
64	$[M + H]^+$	**Compound 64**	Cyclic alcohol and derviative	C20H30O6		389.19	79.86	-124.20	15B(A)
65	$[M + H]^+$	Canescegenin (3,5,11,14-Tetrahydroxy-19-oxocard-20(22)-enolide)	Cardenolide	C23H32O7		403.21	24.9	-138.84	15B(A)

No	Mode	Compound Name	Predicted Class	E.C.	Predicted Structure	m/z	SIRIUS Score (%)	CSI Finger score	Beer d.c.
66	$[M + H]^+$	15-Oxo-11,13-eicosadienoic acid	Linoleic acid and derivative	C20H34O3		341.27	25.0	-40.84	15B(A)
67	$[M-H]^-$	9-Oxoheptadecanoic acid	Linoleic acid and derivative	C17H32O3		329.23	50	-73.09	15B(A)