Brewing with green malt for an energy and water efficient process: challenges and opportunities

Celina Angela Dugulin

Doctoral dissertation submitted to the Department of Microbiology, Brewing and Biotechnology, School of Biosciences, **University of Nottingham** and to the Faculty of Engineering Technology, **KU Leuven**.

Supervisors:

Prof. David Cook (University of Nottingham)

Dr. Chris Powell (University of Nottingham)

Prof. Luc De Cooman (KU Leuven)

Dr. Ing. Gert De Rouck (KU Leuven)

Examination Committee:

Prof. Jon Hobman (Chair)

Prof. Charles Bamforth

Prof. Imogen Foubert

Dr. Stephen Lawrence

Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in Brewing Science (University of Nottingham) and Doctor in Engineering Technology (KU Leuven)

April, 2021

This project received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 722166.

Acknowledgement

First of all, I would like to thank my supervisors Prof David Cook and Dr. Ing. Gert De Rouck whose insight and knowledge into the subject matter steered me through this PhD journey. Thank you so much for your help and support!

I would like to thank Prof Luc De Cooman for his feedback and his input at various stages of this project. A special thanks to Prof Aleksander Poreda from the University of Agriculture in Krakow who made this '*EJD'* journey possible. I would also like to thank my industrial supervisor, Dr. Irina Bolat for her support within this project, kindly donating us the malt samples for our brewing trials and allowing me to pursue an internship at Boortmalt.

I would also like to thankfully acknowledge Susan Clegg and Emily Fong from the University of Nottingham, as well as Dr. Barbara Jaskula-Goiris, Johanna Schlich and Agata Soszka from KU Leuven for their contributions and support to the data collection and analysis for this thesis. Further, I would like to thank Jasper Buyse for all the fun trips from Ghent to Antwerp picking up green malt, but most importantly for 'sharing' his brewing expertise and helping me to successfully brew with green malt.

In the last three years, I had the chance to meet so many amazing people, who supported me both on a professional and personal level. Huge thanks to my fellow ESR's – Jonas Trummer, Marcus Pagenstecher, Magda Costa, Tuur Mertens, Weronika Filipowska and Maciej Ditrych - for their continuous support, help and friendship; I could not have asked for a better group! I would also like to thank my friends and colleagues of the Bioenergy and Brewing Science Department, University of Nottingham - Marit, Christina, James, Eoin, Imogen, Chris, Saskia, Ola, Steph, Jo, Claire, Neil; and the Fermentation and Brewing Technology team at KU Leuven - Paula, Jeroen, Agata, Jasper, Luisa and Maxime. You really made this experience unique!

Danke, Thank you, Gracias, Dziękuję, Obrigada, Dank je

I would like to dedicate this thesis to my family. Mama, Papa, Maximo, Oma, Tante Vanessa, Lukas, Ruth - thank you for your love, support and understanding. Without you this would not have been possible.

Abstract

Brewing beer using 'green' (germinated, but not dried) malt, thus saving the substantial energy input associated with kilning and conserving the water contained in the green malt, could be a solution to making the malting industry more energy and water efficient. The overall aim of this research project was to evaluate the feasibility of brewing with green malt and propose solutions to some perceived technical and biochemical (flavour) barriers. Early research was dedicated intensively to laboratory scale development to enable this alternative to conventional brewing processes. Of particular concern were the elevated lipoxygenase (LOX) activity and its products, as well as the DMS potential. Furthermore, rootlets were identified to substantially contribute to this problem. Results indicated that LOX levels in green malt can be regulated to a substantial degree by using either a wet 're-steeping' treatment or a 1-hour heat treatment of green malt at 65°C. However, the results indicated further that the brewing process would need to be optimised to deal with the elevated levels of S-methyl methionine (SMM; DMS precursor) and hexanal in green malt worts. On the other hand, results showed that green malt is rich in α- and β-amylase (diastase enzymes), with great capacity to convert starch into fermentable sugars. Subsequent pilot scale brewing trials (Chapter 3) aimed to compare between key quality parameters of worts and beers made from green malt and kilned malts (prepared from the same batches of green malt). 100% green malt was used in these experiments both as a technical challenge and to emphasise key quality factors. Additionally, beers were brewed whereby the green malt was pre-steeped under de-aerated water for 1 hour as this procedure had previously been shown to lower LOX activity in green malt. Here it was demonstrated that beers without significant taints or obvious defects can be brewed directly from green malt without prior removal of rootlets, even though further technological and process optimisations are undoubtedly required. Most remarkably, DMS levels in all green malt beers did not significantly differ from the reference brews, even though SMM levels were 2-3 times higher at the onset of mashing compared to the respective reference brew. Furthermore, a satisfactory beer colour was attained when using 100% green malt; this was considered to originate from natural yellow pigments (polyphenols, water-soluble riboflavin) present in malt. Even though re-

steeping seemed a promising technique by which to reduce LOX activity in green malt at laboratory scale, it did not have a significant impact on the flavour stability indicators which it was designed to improve. Thus, it can be concluded that the LOX activity was sufficiently controlled in the original green malt brewing process. Therefore, the focus of the subsequent studies (Chapters 4 & 5) was to elucidate the impacts of using this green malt and thus indirectly malt kilning on the grain, wort and beer quality. Fresh beers from kilned malt resulted in similar concentrations of free staling aldehydes compared to green malt beers – despite the high aldehyde concentrations in the kilned malt and particularly at the onset of the brewing process. Nevertheless, these aldehydes might bind to other compounds forming non-volatile adducts, which may dissociate and release the free aldehydes during beer storage. Thus, forced ageing of beers was required to predict the flavour stability of a beer (style). Additionally, results revealed that worts and beers produced from untreated green malt had a significantly better oxidative stability (by Electron Spin Resonance Spectroscopy) than both the re-steeped green malt and the reference beers. Lastly, to identify the beer chemistry changes during staling, beers were subjected to ageing at 30°C for 30, 60 and 90 days. An increase in concentrations of undesirable staling aldehydes and a decrease in concentrations of desirable compounds (acetate esters, bitter acids) were noted in all beers. Interestingly, the results showed that (untreated) green malt beers were less susceptible to beer ageing flavour change than kilned malt beers, due to a lower formation, or release, of staling aldehydes. In principle, fewer aldehydes are available for adduct formation when using green malt, and thus, an improved endogenous ageing potential in green malt beers is hypothesised, provided lipoxygenase activity can be controlled by applying LOX hostile mashing conditions (pH 5.3; >63°C; oxygen free). Brewing with green malt is a disruptive technology and the process needs to be further optimised before it could be implemented in present day breweries. Nevertheless, this research proved that wort and beer without any flavour defects, and with promising flavour stability metrics and decreased aldehyde formation during storage can be produced from 100% green malt.

Samenvatting

Het moutproces kan worden onderverdeeld in drie hoofdstappen: weken, ontkiemen en eesten - de laatste is de meest energie-intensieve stap, verantwoordelijk voor ~78% van de totale energiebehoefte. Dit maakt het eesten het belangrijkste aandachtspunt om de ecologische voetafdruk van mouterijen te verkleinen. Bovendien omvatten mouten en brouwen opeenvolgende stappen van zowel bevochtigen als drogen. Vanuit een energie- en watergebruik-standpunt maakt dit weinig zin. Bierbrouwen met 'groene' (gekiemde, maar niet gedroogde) mout zou een oplossing kunnen zijn om de moutindustrie energie- en waterefficiënter te maken. Een dergelijk proces zou de aanzienlijke energie-input, die gepaard gaat met eesten, verminderen en het water in de groene mout conserveren. Brouwen met ongedroogde mout vertegenwoordigt echter een zeer disruptieve technologie en er moeten meerdere aspecten in overweging worden gebracht om succesvol te kunnen brouwen met dit 'nieuwe' graanmateriaal. Het algemene doel van dit project was het aantonen van de haalbaarheid van het brouwen met groene mout en oplossingen voor te stellen voor een aantal vermeende technische en biochemische (smaak)barrières.

Om dit alternatief op het conventionele brouwproces mogelijk te maken, werd er intensief voorlopend onderzoek gewijd aan diens ontwikkeling op laboratoriumschaal. We onderzochten de belangrijkste kwaliteitsproblemen in verband met groenmout: lipoxygenase(LOX)-activiteit, S-methylmethionineniveaus, oxidatieproducten-ontwikkeling en wortelverwijdering. Deze resultaten vormden de basis voor latere brouwproeven op pilootschaal aan de KU Leuven. Daar was het doel het vergelijken van de belangrijkste kwaliteitsparameters van wort en bieren gemaakt van groenmout en geëeste mout (beiden bereidt uit dezelfde mout-batch). Bij deze experimenten werd 100% groene mout gebruikt, dit omwille van zowel de technische uitdaging, als het benadrukken van de impact op de belangrijkste kwaliteitsfactoren. Er werden monsters genomen tijdens het groenmout-brouwproces. Deze monsters werden vervolgens vergeleken met de wort- en biermonsters geproduceerd uit een geëeste pilsmout (uit dezelfde mout-batch). Hierbij werd aangetoond dat bieren, zonder noemenswaardige taints of duidelijke gebreken, rechtstreeks uit groenmout kunnen worden

geproduceerd—en zonder voorafgaande verwijdering van de kiemwortels. Verdere technologische en proces-optimalisaties zijn ongetwijfeld vereist. Bovendien toonden de resultaten, van de brouwsels met onbehandelde groenmout, veelbelovende indicatoren aan in vermand met de smaakstabiliteit. Hieromwille lag de nadruk van de daaropvolgende studie op het ophelderen van de smaakstabiliteitseffecten van het brouwen met groene mout. Dit was belangrijk om te controleren, omdat een verminderde warmtebelasting de smaakstabiliteit zowel kan verbeteren (verminderde hoeveelheid aan ouderdomsgerelateerde aldehyden) als kan verslechteren (omdat, bijvoorbeeld, lipoxygenase-activiteit wordt gereguleerd door warmtebehandeling tijdens het eesten). De resultaten toonden aan dat verse bieren, gebrouwen met gedroogde (pilsener-stijl) mout, vergelijkbare concentraties hadden aan vrije ouderdomsgerelateerde aldehyden als de groenmoutbieren. Dit was verrassend, aangezien de ouderdomsgerelateerde aldehyde-concentraties in de geëxtraheerde mout—vooral aan het begin van het brouwproces—significant hoger waren. Dat gezegd zijnde kunnen deze aldehyden zich aan andere componenten binden, waardoor niet-vluchtige adducten worden gevormd. De huidige theorie is dat, tijdens de opslag van bier, deze adducten—onder specifieke omstandigheden (temperatuur, pH-waarde, redoxpotentiaal, bindingssterkte, thermodynamische stabiliteit)—kunnen dissociëren en de aldehyden in hun ongebonden vorm kunnen worden vrijgeven. Om de smaakstabiliteit van een bier(stijl) te voorspellen was geforceerde veroudering van de bieren vereist. Bovendien werd de oxidatieve stabiliteit van de wort- en biermonsters bepaald met behulp van EPR-spectroscopie aan de Universiteit van Kopenhagen. De resultaten toonden aan dat wort en bier, geproduceerd uit onbehandelde groenmout, een significant betere oxidatieve stabiliteit hadden in vergelijking met de referentie-droogmoutbieren. Tot slot werden de bieren 30, 60 en 90 dagen verouderd bij 30°C om de chemische veranderingen van het bier tijdens het verouderen te identificeren. Bij alle bieren werd een concentratietoename aan ongewenste ouderdomsgerelateerde aldehyden, en een concentratieafname aan gewenste verbindingen, opgemerkt. Opvallend genoeg toonden de resultaten aan dat groenmoutbieren minder vatbaar waren voor smaakverandering bij bierveroudering dan de geëeste moutbieren, vanwege een lagere vorming aan (of afgifte van) ouderdomsgerelateerde aldehyden. Over het algemeen toonden de resultaten aan dat groenmoutwort en -bieren een potentieel

voordeel kunnen hebben met betrekking tot biersmaakstabiliteit, op voorwaarde dat de lipoxygenase-activiteit onder controle kan worden gehouden door het aanwenden van lipoxygenase-vijandige maischomstandigheden (pH 5,2; > 63°C; zuurstofvrij).

Brouwen met groene mout is een disruptieve technologie en het proces moet verder worden geoptimaliseerd voordat het in de huidige brouwerijen kan worden geïmplementeerd. Desalniettemin heeft dit onderzoek aangetoond dat er wort en bier uit 100% groenmout (met intacte wortels) kan worden geproduceerd, zonder enige smaakgebreken en met een veelbelovende smaakstabiliteit.

Table of Contents

List of abbreviations

ANOVA	analysis of variance
CO_2	carbon dioxide
d.b.	dry base
DMS	dimethyl sulphide
DMS-P	dimethyl sulphide precursor
e.g.	for example
FAN	free amino nitrogen
g	gram
GC-MS	gas chromatography – mass spectrometry
GM	green malt
h	hour
HPLC	high performance liquid chromatography
KM	kilned malt
L	litre
LC-MS	liquid chromatography – mass spectrometry
LOX	lipoxygenase
mg	milligram
mQ water	milli-q water (purified and deionized)
µg	microgram
mL	millilitre
PCA	principal component analysis
RGM	re-steeped green malt
RKM	kilned malt (control for re-steeping trials)
RO	reversed osmosis
rpm	revolutions per minute
SD	standard deviation
sec	second
SMM	S-methyl methionine
THFA	trihydroxy fatty acids
UPLC	ultra performance liquid chromatography
%	percent
ºC	degree centigrade

Aldehydes

2-MP	2-methylpropanal
2-MB	2-methylbutanal
3-MB	3-methylbutanal
FUR	furfural
HEX	hexanal
MET	methional
PHE	phenylacetaldehyde
T2N	*trans*-2-nonenal

Amino acids

Ala	alanine
Arg	arginine
Asn	asparagine
Cys	cysteine
His	histidine
Gln	glutamine
Gly	glycine
Ile	isoleucine
Leu	leucine
Lys	lysine
Met	methionine
Phe	phenylalanine
Pro	proline
Ser	serine
Thr	threonine
Trp	tryptophan
Val	valine

Esters and higher alcohols

1Prop	1-propanol
3M1B	3-methyl-1-butanol
EAc	ethyl acetate
Ebut	ethyl butyrate
Ehex	ethyl hexanoate
Eoct	ethyl octanoate
IsoAA	isoamyl acetate
IsoBA	isobutyl acetate

1 Introduction

Beer is one of the oldest alcoholic beverages (Michel et al., 1993), it has accompanied the development of whole civilisations and has been continuously evolving within time. Nowadays, beer is one of the most consumed alcoholic beverages in the world, with an annual global production of 1.94 billion hL measured in 2018 (Conway, 2019). With the rising demand in beer, simultaneously the demand of malted cereals, such as malted barley grows. Malt is a key component for beer production - it is the key starch source and imparts the typical flavour and colour to the beer. The contribution of malt to beer is remarkable as changing the grade and type of barley, processing under different conditions (kilning or roasting), numerous different types of malt, at different colour and flavour profiles can be prepared (Briggs, 1998a, Huang et al., 2016, Müller et al., 2013, Yahya et al., 2014). Globally about 23 million tonnes of malt are produced annually, of which Europe contributes almost 9.7 million tonnes (Euromalt, 2017). In the UK alone, emissions of more than 300,000 tonnes CO_2 per year are produced through the manufacturing of more than 1.6 million tonnes of malt (CarbonTrust, 2011, Euromalt, 2017) –this corresponds to annual CO_2 emissions of about 14,851 British households (Buchs and Sylke, 2013). Thus, the malting industry is always interested in improving its energy efficiency. Furthermore, as climate change targets are tightened, several industries have been under a lot of legal, social and economic pressure to take actions to reduce their impact on the environment. Trying to support this carbon-cut policy, governments of several countries have implemented taxes on carbon emissions and energy consumption based on the carbon footprint.

The malting process can be separated into three key steps: steeping, germination and kilning – the latter being the most energy intensive step (CarbonTrust, 2011, Davies, 2010, Doug, 2010, Manger, 2017) accounting for ~78% of the total energy demand. Naturally, the kilning process becomes the main point of interest to reduce the maltings energy footprint. Furthermore, malting and brewing involve sequential wetting and drying steps. Viewed solely from an energy and water use perspective these processes make little sense. If omitting the kilning process entirely, the brewer must brew with

freshly germinated (green) malt, which introduces new technical and biochemical (flavour) challenges but offers the reward of significantly lower energy and water usage.

Brewing with green malt represents very disruptive technology and multiple aspects need to be considered to successfully brew with this 'novel' grist material. Thus, the following literature review is separated into two main parts. The first part provides a brief overview of barley malt, one of the four major ingredients in beer, as well as an introduction to the conventional malting and brewing process. This is followed by a discussion on beer flavour stability and the most common biochemical pathways causing beer staling. The second part of this review focuses on the technical feasibility of brewing using freshly germinated (green) malt, with omission of the kilning step. Similarly, the main focus is on the influences on flavour and flavour stability, storability and extractability due to different biochemical compositions of germinated malt compared to kilned malt. The research objective and thesis structure are presented at the end of this literature review.

The second part of this literature review will be published and corresponds to:
Dugulin CA, De Rouck G, Cook DJ, 2021. Brewing with green malt for an energy and water efficient future – a review. Journal of the American Society of Brewing Chemists. https://doi.org/10.1080/03610470.2021.1902710

1.1 Barley

Even though many different grains are suitable for the production of beer, barley is still the cereal of choice, particularly because of its high starch (63% of barley dry matter), but moderate protein (10-11%) and lipid (1.5-2%) contents, as well as its low husk to endosperm (starch) ratio (Hertrich, 2013b, Kunze, 2014). The barleys used in the brewing industry are husked types, which means that the husk remains on the kernel after threshing. This is important, as the husk fraction is needed in brewing as filtration material during lautering, but most importantly the husk regulates water uptake during malting, and protects the kernel mechanically (Hertrich, 2013b). Barley plants are annual grasses, which can be planted in autumn (winter type) or spring (spring type) and can be subdivided as two-rowed or six-rowed grains, depending on the arrangement of the corns on the ear axis. The grains vary in shape, size and chemical composition. Figure 1.1 illustrates the internal and external structure of a barley kernel.

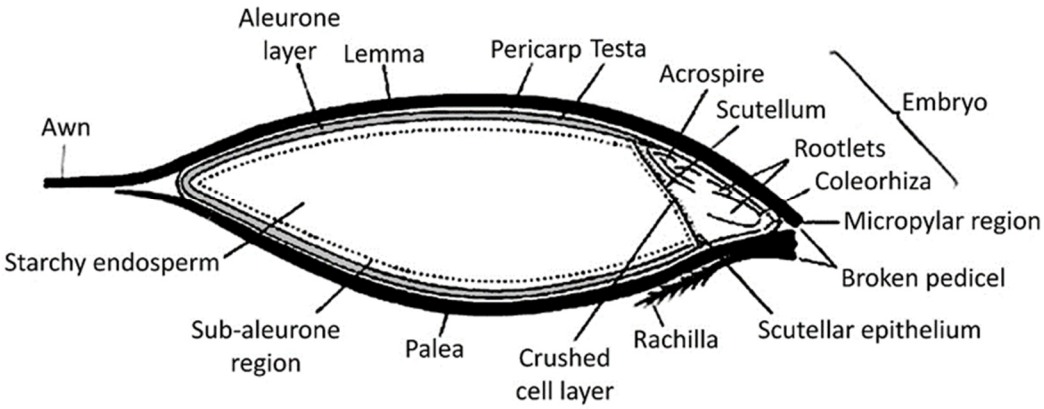


Figure 1.1: Structure of barley the barley kernel according to Briggs D. E. (Briggs, 1998c).

The external structure – the husk (10-13%) - is called lemma on the dorsal, and palea on the ventral side of the kernel and completely encloses the grain (Briggs, 1978). The husk and pericarp consist mostly of cellulose, and small amounts of polyphenols and testinic acid (mixture of polyphenols and proteins). The husk is the only tissue in barley that is lignified, and its outermost layer (external epidermis) is also silicified (Briggs, 1998a). The following two layers are the semipermeable testa, which limits the diffusion of solutes (e.g. ionised salts) that permeate the husk and pericarp, followed by the

aleurone layer (Briggs, 1998b). The internal structure consists of the germ region and the endosperm, separated through the scutellum, displayed in Figure 1.1. The inner face of the scutellum is pressed against the starchy endosperm and is surfaced with a single layer of columnar epithelial cells (scutellar epithelium). The embryo region comprises the acrospire (coleoptile) pointing at the apex of the grain, and the coleorhiza which encloses the embryonic roots that appear at the end of the grain during germination ('chitting'). The endosperm region consists of the starchy endosperm occupying the centre of the grain (76-82% (Briggs, 1998c)) and the surrounding aleurone layer. The large and small starch granules are embedded in a protein matrix, surrounded by cell walls consisting of β-glucan (75%) and arabinoxylan (20%) (Evers and Millar, 2002). Aleurone cells are surrounding the starchy endosperm; a thinner layer of the aleurone extends partly over the surface of the embryo. The aleurone layer does not contain any starch reserves, but consists of protein, lipids, polyphenols and colouring materials (Hertrich, 2013b). The activation or synthesis of enzymes secreted into the starchy endosperm, occurs in the aleurone and scutellar cells via embryonic gibberellin-activated signal transduction pathways (Cohen and Paleg, 1967, Palmer, 1982, Palmer, 1998).

1.2 Introduction to the malting process

Considering the production of pale malted barley, the malting process comprises three main steps: steeping, germination and kilning. Malt quality and functionality is not solely defined by these classical stages. By changing the grade and type of barley, steeping and germinating under different conditions and by kilning or roasting at differing moisture contents and to different temperatures, a range of malt types are prepared (Briggs, 1998a, Huang et al., 2016, Müller et al., 2013, Yahya et al., 2014). Malting is a process that modifies barley until it is suitable to produce beer (both due to physical modification of the grain and the development of key enzyme activities). The proteolytic, amylolytic and cytolytic modification influence the malt quality (Briggs, 1998a). Both, under-modified and over-modified malt lead to poor malt quality. The main factors influencing modification are: steeping degree, germination time and temperature (Brookes et al., 1976, Hertrich, 2013a).

1.2.1 Pre-drying and storage of barley

After the harvest, barley can go through a phase of dormancy, a period of 6-8 weeks (depending on variety and the weather during harvest) where the seedling cannot germinate (Briggs and Woods, 1993, Woonton et al., 2005). Thus, after cleaning and grading, the barley is usually not malted directly but stored until the actual malting process can start. If the moisture content in barley exceeds 12%, the barley has to be dried to prevent the seedling from intercellular respiration and avoid microbial growth (e.g. fungi). Subsequently, the grain can be stored e.g. in silos, while ventilation is necessary to maintain the viability. This guarantees removal of CO_2, water and heat, while simultaneously supplying the grain with oxygen. Storing barley dry and warm can accelerate post-harvest maturation, but can also cause losses in viability (Briggs and Woods, 1993).

1.2.2 Steeping

During steeping, the barley is hydrated (steeped), to increase the moisture content, aiming to trigger germination (> 32%), but also to clean the grain from dust, impurities and germination inhibitors (EUREKA SWAN Project, 2006, Guiga et al., 2008). The steeping process consists of one or more wet and dry cycles. During the wet phase, the grain is submerged in water. The moisture uptake can change depending on barley variety, crop year, kernel size, nitrogen content, dormancy, water sensitivity, or applied steeping parameters (time, temperature, aeration) (Briggs, 1986, Brookes et al., 1976, Turner et al., 2019). After a few hours of steeping, the water is drained off and the dry phase (air rest) commences. During this phase, the internal part of the grain takes up the adhering water faster. During steeping, the grain must be aerated, while the produced CO_2 is removed, failure to do that could result in a so-called “dead steep”. Steeping usually takes around 24 – 48 h until the grain reaches a moisture content of approx. 42 – 46% and rootlets appear at the base.

1.2.3 Germination

The initiation of germination triggers a hormonal and enzymatic cascade which affects the breakdown of endosperm components: cell wall materials, protein matrix and some limited breakdown of starch (Briggs, 1998a). Additionally, the grain develops the

acrospire and rootlets, the latter becoming visible towards the end of the steeping phase (Figure 1.2B).

Figure 1.2: Growth of the barley kernel (A) barley after the first steep, (B) appearance of the first rootlet (chit malt) and onset of germination, (C) well-germinated green malt with rootlets.

Malt rootlets are highly hygroscopic, due to their fibre content of up to 15% (Briggs, 1998a, Salama et al., 1997), have emulsification capacities (Kunze, 2014) and are considered to impair the flavour of beer. Therefore, maltsters try to avoid excessive rootlet growth during germination and remove the rootlets, by deculming them after kilning. Long and warm germination conditions lead to increased rootlet growth, so maltsters usually perform germination at the lowest possible temperature and time (Kunze, 2014). In the early growing stages, the embryo releases gibberellin hormones, generated from a precursor in the scutellum (Palmer, 1982, Palmer, 1995, Palmer, 1998), which pass from the embryo to the aleurone layer and the endosperm. This develops or releases hydrolysing enzymes, such as amylolytic, proteolytic and cellulolytic enzymes. Their main function is to break down starch, cellular material and the endosperm cell wall (Palmer, 1982, Palmer, 1995, Palmer, 1998).

Proteolytic enzymes, such as carboxypeptidase and endopeptidases break down high molecular weight proteins (38 – 42% (Kunze, 2014)) for the formation of the new cell tissues.

Cytolysis, the breakdown of the cell walls of the endosperm, facilitates the diffusion of enzymes into the starchy endosperm. The main components of the walls are (1,3)(1,4)-β-D-glucan (75%), arabinoxylan (20%) and protein (5%), with phenolic acid residues

(notably ferulic acid linked to arabinose) (Fincher, 1975). During the growth and modification process, the middle lamella of protein is degraded, followed by the pentosan structure and organic acids by xylanases. Subsequently, the glucans are released from the binding protein by the β-glucan-solubilase and mainly degraded by endo-β-1,4-glucanase, endo-β-1,3-glucanase and exo-β-glucanase. The endosperm of unmalted barley is very hard and softens during the modification processes. Most of the necessary cytolytic degradation of barley (1,3)(1,4)-β-D-glucan via β-glucanases occurs during malting. Thus finished malts should contain low levels of β-glucan for a satisfactory brewing performance and to avoid the process problems associated with the elevated content of β-glucan (poor lautering performance (Bamforth and Martin, 1981, Jin et al., 2004) and colloidal (in) stability of the finished beer (Bamforth, 1999b, Speers et al., 2003).

Amylolytic enzymes help to degrade the starch to sugars during mashing and are thus the most important enzymes for the brewer. β-amylase is already present in barley in its bound inactive form, whereas α-amylase is formed in the aleurone layer during germination (Sopanen and Laurière, 1989). The formation of α-amylase is highly dependent on the presence of oxygen. The seedling uses starch as a nutrient, therefore it is important to prevent significant starch loss by avoiding long germination periods with high temperatures and an excess of air.

The germinating undried grain is called green malt, which is displayed in Figure 1.2C. Green malt contains a high amount of desired enzymes (e.g. α-,β- amylase (Evans et al., 1997, Hämäläinen and Reinikainen, 2007, Sissons et al., 1995)), but also develops lipid degrading enzymes such as lipases, and the two lipoxygenase isoenzymes (LOX-1 and LOX-2) (Franke and Frehse, 1953, Yabuuchi, 1976, Yang and Schwarz, 1995, Yang et al., 1993). The products of lipoxygenase are responsible for the cucumber-like aroma of green malt, and their oxidation products can impair the beer flavour (Section 1.4.1.1). Furthermore, S-methyl methionine (SMM), the precursor of the volatile sulphur compound dimethyl sulphide (DMS), which can give beer a canned corn flavour, is produced and increases in concentration throughout germination (depending on steeping degree and germination temperature; Section 1.8.5) (Pimenta et al., 1998, White and Wainwright, 1976b).

The germination process is allowed to proceed until the desired degree of modification has been achieved (usually after 3.5 - 5 days of germination; acrospire reaches approximately ¾ of the way towards the distal end of the grain) when seed germination is arrested by a heating step – kilning.

1.2.4 Kilning

The main goal of kilning is to lower the moisture content of green malt down to 4%, aiming to start chemical processes, which support the formation of colour and flavour compounds and to get a stable product, which can be stored easily (Johnston, 1954, Palmer and Bathgate, 1976, Whitehurst and Oort, 2010). During conventional kilning, the initial moisture of > 40% is reduced to approx. 12% by forcing dry air through a bed of grain with a stepwise increase in air-on temperature, starting at around 50°C and ramping gently to 70°C. In this stage, free water is removed. In the next phase, the bound water is removed, lowering the moisture to 4-5% by circulating air at temperatures above 80°C (curing stage). After cooling is completed, the rootlets can be removed by abrading them after kilning (deculming), with an associated malting loss of around 4%. The rootlets are then usually sold as animal feed or organic fertiliser. Freshly kilned malt is associated with a poor brewhouse performance (Bamforth et al., 2009, Kunze, 2014, Mallett, 2014, Rennie and Ball, 1979), particularly wort separation– therefore it should be stored at least for 3-4 weeks before further processing.

Kilning is an important process as it reduces the activity of undesired enzymes, such as, lipoxygenase (De Buck et al., 1997, Doderer et al., 1992, Yang and Schwarz, 1995, Yang et al., 1993) and regulates dimethyl sulphide (DMS) precursor levels (White and Wainwright, 1976a, White and Wainwright, 1976b, White and Wainwright, 1977), which will be discussed in more detail in Sections 1.8.4 and 1.8.5, respectively. At temperatures above 70°C, the DMS precursor S-methyl methionine, is decomposed to free volatile DMS and L-homoserine (Anness and Bamforth, 1982, Yang et al., 1998). On the other hand, during kilning another DMS precursor can be formed, which can be reduced to DMS by yeast during fermentation: DMSO (Anness, 1980, Baldus et al., 2013, Yang et al., 1998). Furthermore, kilning reduces the activity of desired enzymes, such as starch degrading β-amylase (Evans et al., 1997) or cytolytic enzymes (β-glucanase), which will be further discussed in detail in Section 1.8.

1.3 Introduction to the conventional brewing process

The manufacturing of beer from malt, hops, water and yeast can be divided into two main sections: i) production of a sweet (sugary) liquid – wort- and ii) fermentation of wort by yeast to convert fermentable sugars to alcohol. Within the first section, the insoluble components of malt are converted into soluble products with the aid of the enzymes generated during malting. Subsequently, the sugars in the wort can then be converted by the yeast to alcohol and carbon dioxide. A basic outline of a standard brewing process is displayed in Figure 1.3, however, the design of the brewhouse can differ in terms of equipment (e.g. choice of milling and mash separation systems).

Commercially kilned malts are friable, due to the low moisture content, and can be broken by a mill (Figure 1.3A) into small fragments. To obtain the optimal grist particle size distribution for the filtration, coarse or fine milling must be chosen. In a lauter tun, the husks are used as filter material, thus the malt needs to be milled coarsely to leave the husks mostly intact. This is done using e.g. a roller mill. On the other hand, if a mash filter is available, the grist should be very finely milled, by using a hammermill or a wet milling system. Wet milling systems in combination with a mash filter are able to improve the brewhouse yield but also grind malts that have a high moisture content (e.g. green malt; https://www.meura.com/products/hydromill.html) (Andrews, 2004, De Rouck et al., 2013a, De Rouck et al., 2013b, Leclercq, 2020, Menger, 2006). Additionally, by injecting CO_2 into the malt inlet and malt bin, this technology increases the protection against oxidation. Mashing is a process where the grist is mixed with the brewing water into a mash kettle (Figure 1.3B). It is a controlled time-temperature process - each mash 'stand' is at a temperature designed to support the targeted activation or deactivation of certain enzymes. During mashing the brewer generates the 'sweet wort' which is rich in sugars, amino acids and other soluble compounds which make their way into the final beer.

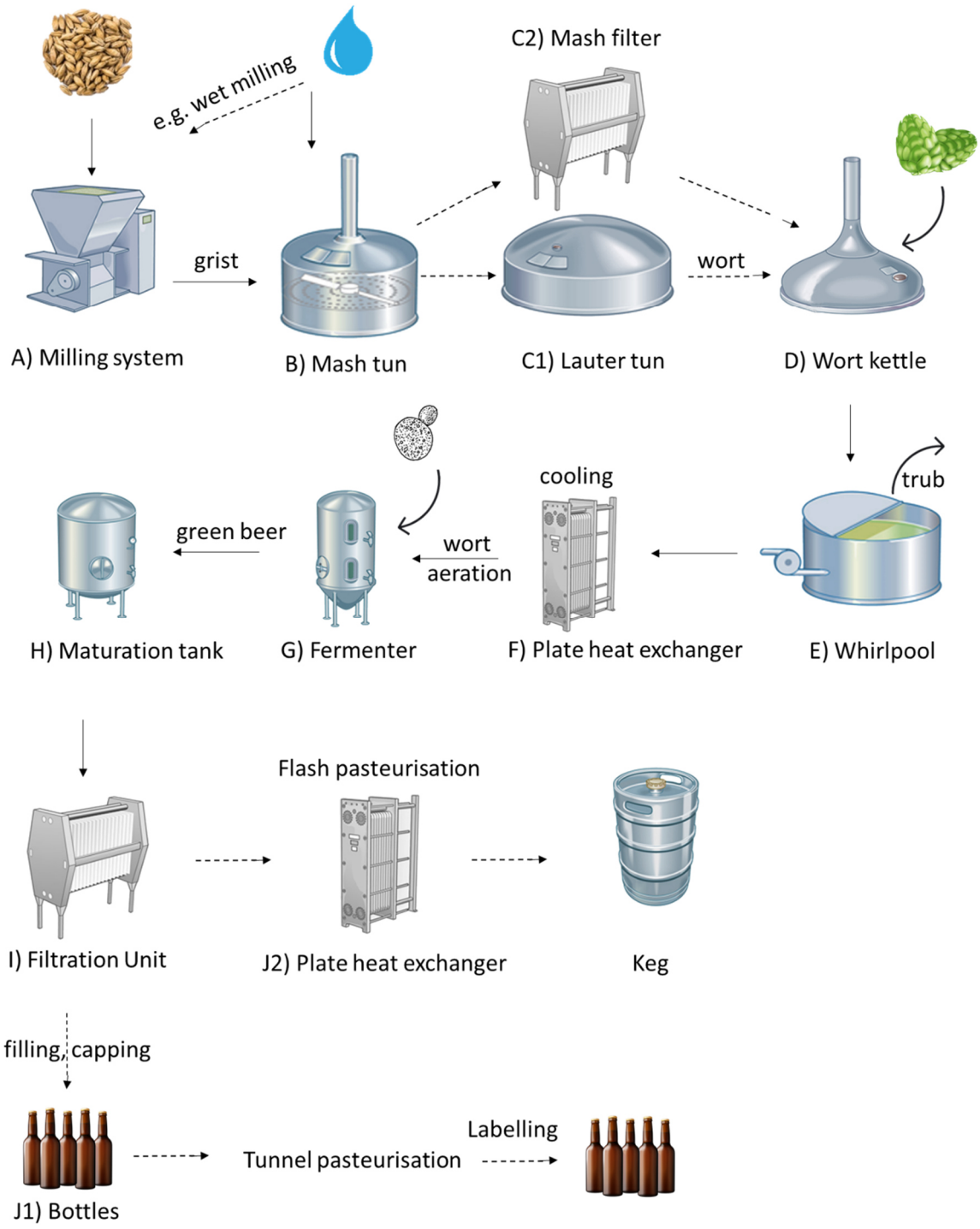


Figure 1.3: An outline of the brewing process. Figure adapted from Encyclopædia Britannica (https://www.britannica.com/topic/beer/Types-of-beer).

When mashing, the milled grist is combined with the brewing liquor (strike water) via a pre-masher. Naturally, when using a wet milling process, the brewing liquor and malt

are already combined during the milling process. The enzymatic breakdown of starch from malt into simpler sugars by the diastatic enzymes generated during malting is a very crucial step throughout the wort production process (Bamforth, 2009, Evans et al., 2008, Henson et al., 2014). These include (i) α-amylase, which hydrolyses α-(1-4) linkages in the long glucose chains of starch to yield smaller fragments, (ii) β-amylase for the splitting of maltose from the non-reducing chain end and (iii) limit dextrinase to hydrolyse α-(1-6) linkages which form the branch points that are most prevalent in amylopectin molecules. The optimum temperature ranges for limit dextrinase, α- and β-Amylase are 50-55°C, 68-72°C and 63-65°C, respectively (O'Rourke, 2015). Many breweries nowadays opt for infusion mashing which starts at temperatures of 62-63°C. However, this is only recommended if a well-modified malt (low β-Glucan, sufficient free amino nitrogen) is used. The acidification of the mash to 5.2-5.4 (Briggs et al., 1981a) or 5.3-5.8 (Bamforth and Simpson, 1995) is practiced in order to increase the extract yield. This is advantageous because β-amylase and α-amylase have their highest activity at a pH of 5.2 (Narziss and Rusitka, 1977) and 5.5 (Greenwood and MacGregor, 1965), respectively, whereas lipase (pH optima 6.8, (Baxter, 1984)) and LOX enzyme activity (pH optima 6.5, (Baert et al., 2012)) can be reduced. Even though lipoxygenase enzymes (LOX-1, LOX-2) are mostly destroyed during kilning, even low residual lipoxygenase activities in pale kilned malt can cause serious flavour deteriorations in the final beer (Hirota et al., 2005, Skadhauge et al., 2005). Thus, to avoid the disadvantageous effects of lipoxygenase, while achieving high extract yields, mashing in at > 63 °C, at a pH in the region of 5.3 under oxygen-limited conditions (Baert et al., 2012, Bamforth, 2004, Drost et al., 1990, Van Waesberghe et al., 2001) is recommended.

After mashing, during filtration (Figure 1.3C) the insoluble material (mainly husks and acrospire) is separated from the sweet wort. As discussed previously, when using coarse milling, the husks mostly stay intact and form the filter bed in the lauter tun. In lauter tun operations (Figure 1.3C1) compression of the filter cake is to be avoided. On the other hand, when using a thin-bed filter (Figure 1.3C2), the husks have to be finely milled and here the filter bed is compressed before sparging by an expandable membrane and again compressed after sparging (Andrews, 2004, De Rouck et al., 2013a, De Rouck et al., 2013b, Evans et al., 1998, Menger, 2006). The combination of fine milling and thin bed filters have been discussed to be a faster wort filtration with an increased extract

yield (Andrews, 2004, De Rouck et al., 2013a, De Rouck et al., 2013b, Menger, 2006). Whether using a lauter tun or a mash filter, sparging with water aids to remove the remaining sugars in the spent grains, but when using a mash filter the sparging rate (amount of liquor used for sparging) is lower (Andrews, 2004).

After the wort is separated from the spent grains, it is directed to a kettle to be boiled with hop additions (Figure 1.3D). The bittering hops are usually added at the beginning of the boiling process. During boiling the isomerisation of the hop acid precursors α-acids to the bitter-tasting derivatives (iso-α acids) occurs (Hudson and Birtwistle, 1966). In the last 15 minutes of the boil brewers usually add extra aroma hops for their flavour, a process known as late hopping (Hieronymus, 2012). During boiling, the sweet wort is sterilised, simultaneously proteins and sparingly soluble materials are precipitated (aiding the colloidal stability of the finished beer), and unwanted flavour or aroma compounds are stripped off. Very important is the efficient degradation of the DMS precursor S-methyl methionine to DMS and the volatilisation of the latter (Bamforth, 2014). Wort boiling should not be performed longer than necessary, because increased heat load on wort can impair final beer quality and flavour stability (De Rouck et al., 2010, De Schutter, 2008, Ditrych et al., 2019, Li, 2009, Malfliet et al., 2008). It is also energy intensive and thus costly. Consequently, brewhouse constructors nowadays try to operate at minimal heat load without compromising evaporation of unwanted flavours (De Rouck et al., 2010). The hot sterile wort is then separated from the trub by using a whirlpool system (Figure 1.3E), centrifuge or even through decantation in a combination vessel (boiling kettle and decanter). Subsequently, the wort is cooled (Figure 1.3F), (sterile) aerated and transferred into the fermentation vessel (Figure 1.3G). Usually either a culture of lager (*Saccharomyces pastorianus*) or ale yeast (*Saccharomyces cerevisiae*) is pitched to the cooled wort to start fermentation and wort is kept at the optimal fermentation temperature of 10-13°C and 18-20°C for lager or ale yeast, respectively. (White and Zainasheff, 2010). By adding the yeast, the sugars in the wort will be fermented through the yeast metabolism into ethanol and carbon dioxide. The formation of by-products has an important effect on the taste, aroma and mouthfeel of the final beer and are strongly dependent on yeast strain used, fermentation parameter and wort composition (Boulton and Quain, 2006, Pires et al., 2014). When the main fermentation is nearly completed, the yeast growth slows down

and the yeast enters a stationary phase of growth. The beer ('green' or 'immature' beer) is held for a period of maturation or secondary fermentation. During this time the flavour of the beer is refined (e.g. yeast reabsorbs diacetyl; hydrogen sulphide escapes through fermentation gases). After maturation, many beers (depending on the beer style) are chilled and filtered to remove residual yeast and maintain the brightness (Figure 1.3I). The CO_2 content of the beer is adjusted, and the beer can be submitted to a quick pasteurisation treatment before or after being bottled or transferred to cans and other types of packaging.

Throughout the whole brewing process, oxygen pick-up to the product stream must be avoided - except for sterile wort aeration before fermentation which supplies the oxygen required by yeast for synthesis of sterols and unsaturated fatty acids, which are essential components of the cell membrane. Oxygen levels should be < 300 g/O_2 per ton of malt at hydration (Van Waesberghe et al., 2001) and by all means oxygen pick up needs to be controlled during malting (Bamforth, 1999c, Schwarz and Pyler, 1984), avoided during wort production (De Buck et al., 1997) and wort separation (Drost et al., 1990) to avoid flavour deteriorations.

1.4 Introduction to beer flavour (in)stability

Due to globalisation and evolving consumer demand for fresh and traditional 'original' beers, solely in 2017, 8.7 billion litres of beer brewed in the EU travelled around the globe (Brewers of Europe, 2018). Both Germany and Belgium – Europe's biggest beer exporter – shipped 1.5 billion litres each abroad, over one third to countries beyond the EU. The overall goal of every brewer(y) is to provide the retail units, shops and most importantly the customer with consistently good beer at consistent quality. However, during storage, beer changes its chemical composition and thus, so called off-flavours appear, while desirable fresh beer aromas disappear. Light exposure, oxygen ingression (around the crown cork), vibrations during transportation (Jaskula-Goiris et al., 2019, Paternoster et al., 2019) and elevated temperatures were identified as primary factors to prompt beer flavour deterioration (Fratianni, 2001, Kaneda et al., 1997, Pankoke, 2015, Vanderhaegen et al., 2006). Previous research (Bamforth, 1999a) showed, that if beers are stored at 0-4°C signs of oxidation were greatly reduced, even after several months of storage. Unfortunately, due to increasing costs or lack of cooling storage

capacity in supermarkets or during transportation, beers are hardly ever stored cooled. It was shown (Pankoke, 2015) that during cargo shipping, beers can even experience temperatures above 40°C. Furthermore, the composition of the beer itself is also very important, as pH level, the presence of antioxidants (e.g. sulphites), as well as pro-oxidants (e.g. transition metal ions), but also oxygen already dissolved in the beer can significantly impact beer stability (Andersen and Skibsted, 1998, Lund et al., 2015).

Dalgliesh (Dalgliesh, 1977), Meilgaard (Meilgaard et al., 1979) and later updated by Zufall et al. (Zufall et al., 2005) created a guideline of the main flavour changes of pale lager beer during storage at 28°C, which is displayed in Figure 1.4. However, different flavour profiles can appear depending on the beer style and ageing conditions (e.g. temperature) (Lehnhardt et al., 2019). Off-flavours are perceived and can be identified as e.g. berry-like aroma, cardboard flavour (Drost et al., 1990, Narziss, 1986), "sunstruck" flavour (Drost et al., 1990, Gunst and Verzele, 1978), bread-like, sweet-toffee-like, sherry-like flavours, etc. (Dalgliesh, 1977, Drost et al., 1990). On the other hand, desirable sulphur, ester and floral aromas decline, bitterness quality diminishes. (Vanderhaegen et al., 2006)

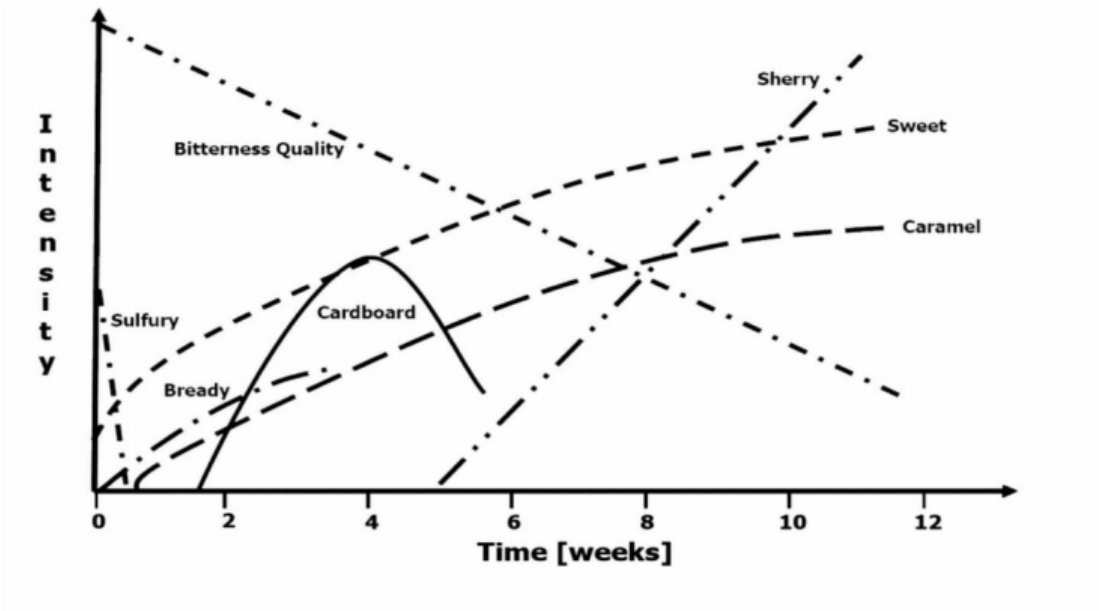


Figure 1.4: Changes in beer flavour during storage at 28°C (Zufall et al., 2005)

If the product does not meet the consumer's expectation, the product, beer style or brand could be rejected. Therefore, strategies to diminish unwanted flavour changes in beer are a primary target in the malting and brewing industry and researchers try to gain further knowledge about the factors and biochemical mechanisms causing beer ageing. In the last decades, many authors (Baert et al., 2018, Bustillo Trueba et al., 2018, De Clippeleer et al., 2010a, De Rouck et al., 2013a, Gastl et al., 2006, Malfliet et al., 2008, Wietstock et al., 2016) reported and suggested biochemical pathways for flavour instability, however, its complexity is not yet fully understood.

Most of the flavour active, staling related substances can be assigned to the chemical class of aldehydes, ketones, heterocyclic compounds, lactones, ethyl-esters and sulphuric compounds (Vanderhaegen et al., 2006). However, researchers provided evidence that the carbonyl compounds – aldehydes - are considered major contributors to beer staling, due to their very low flavour thresholds (Meilgaard, 1975a, Meilgaard, 1975b, Saison et al., 2009b) and the ability for being involved in synergistic interplay. Therefore, these carbonyl compounds, their origin and evolution have been a major focus for researchers over the past decade. The progression of beer staling and thus the appearance of off-flavours past their sub-threshold level are often linked to oxygen and transition metal ions present in bottled beers. Especially, Strecker aldehydes (Section 1.4.1.3) were shown to increase at higher oxygen levels (Narziss et al., 1999).

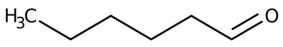
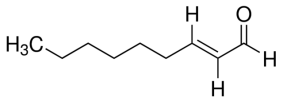
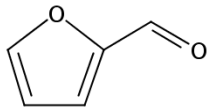
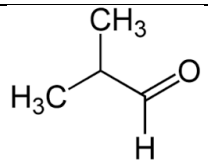
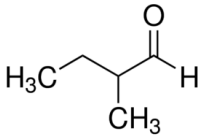
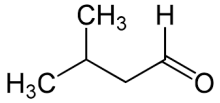
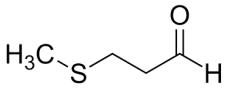
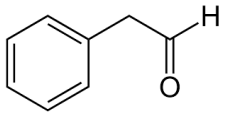
Previous studies (De Clippeleer et al., 2010b, Ditrych et al., 2019, Gastl et al., 2006, Guido et al., 2007) suggested that malt is the major source for staling precursors, such as amino acids, lipids, and flavour-active aldehydes in free or bound form. Thus, malt has a great impact on the beer flavour as well as beer flavour (in)stability. Jaskula et al. (Jaskula-Goiris et al., 2015) showed that the rate of beer ageing is positively correlated with FAN, Kohlbach Index and heat load (TBI) and free aldehyde content of the malt. Additionally, the generation of free radicals in malt was shown to increase with increasing heat load and thus Maillard reaction products present in malt (Cortés et al., 2010, Kunz et al., 2012a).

Naturally, flavour (in)stability and the origin of staling compounds is very complex. The following section seeks to address the most common pathways yielding the most commonly investigated 'marker' aldehydes.

1.4.1 Aldehydes and beer ageing

Already in the 60s, researchers (Hashimoto, 1966, Hashimoto and Kuroiwa, 1975) identified that an increase in stale flavour in beer is related to an increase of small volatile carbonyls - aldehydes. Fresh beer contains very low levels of these 'staling' aldehydes whereas the concentration rapidly increases during ageing (Baert et al., 2012, Jaskula-Goiris et al., 2011, Malfliet et al., 2008). Aldehydes are considered major contributors to beer staling, due to their very low flavour thresholds at ppb levels (Meilgaard, 1975a, Meilgaard, 1975b, Saison et al., 2009b). Moreover, due to synergistic effects, for example, if two or three appear at subthreshold levels, they can have a perceivable effect. In general, aldehyde levels in finished beer can increase through i) *de novo* formation and ii) release from bound-state. Amongst the potential pathways for *de novo* formation, the oxidation of unsaturated fatty acids, Maillard reactions and Strecker degradation, as well as direct oxidation of amino acids are considered the most common pathways (Baert et al., 2012, Kobayashi et al., 1994, Rakete et al., 2014, Vanderhaegen et al., 2006, Wietstock et al., 2016). The Strecker degradation is often further categorised as Maillard reaction, which include e.g. reactions of α-unsaturated carbonyls, α-dicarbonyls or Amadori compounds with amino acids (Baert et al., 2012). Additionally, the staling aldehydes can occur in bound form, by binding to compounds such as bisulphite (Dufour et al., 1999, Kaneda et al., 1994), cysteine (Baert et al., 2018, Baert et al., 2015a, Baert et al., 2015b, Bustillo Trueba et al., 2019) or other amino acids (forming imines) (Lermusieau et al., 1999, Liégeois et al., 2002). As per current theory, during ageing they can be gradually released, depending on factors like temperature, pH binding strength and thermodynamic stability (Baert et al., 2012, Bustillo Trueba et al., 2018, Lehnhardt et al., 2019, Liégeois et al., 2002). Several aldehydes were selected as beer flavour instability markers, i.e. 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, methional, benzaldehyde, phenylacetaldehyde, furfural, hexanal and *trans*-2-nonenal (Baert et al., 2012, Jaskula-Goiris et al., 2011, Malfliet et al., 2008, Saison et al., 2010b, Vesely et al., 2003). Table 1.1 displays the chemical structure, flavour threshold and boiling points of the identified marker aldehydes.

Table 1.1: Boiling points (O'Neil et al., 2006), flavour thresholds and flavour descriptors (Saison et al., 2009b). As previously summarised by Baert et al. (Baert et al., 2012).

aldehyde	BP	FT (µg/L)	description [a]	molecular structure
Fatty acid oxidation				
hexanal	131	88 [a], 350 [b]	bitter, winey	
trans-2-nonenal	101[16], 89[12]	0.03 [a], 0.11[b]	cardboard, cucumber	
Maillard reaction				
furfural	161.8	15157* [a], 150000 [b]	caramel, bready	
Strecker degradation				
2-methylpropanal	64	86* [a], 1000[b]	grainy, varnish, fruity	
2-methylbutanal	90-92	45 [a], 1250 [b]	almond, apple-like, malty	
3-methylbutanal	92.5	56* [a], 600 [b]	malty, chocolate, cherry,	
methional	62[11]	4.2 [a], 250 [b]	cooked potatoes	
phenylacetaldehyde	195	105 [a], 1600 [b]	hyacinth, flowery, roses	

The temperatures presented for boiling points are the values at which the liquid phase is in equilibrium with the vapour at a pressure of 760 mmHg (if available) (Lidel, 1999). Boiling points (BP) reported at different pressure are indicated in superscript (mmHg); asterisk *indicates odour thresholds. FT = flavor threshold, [a](Saison et al., 2009b), [b] (Meilgaard, 1975a)

1.4.1.1 Enzymatic oxidation

The theory of lipid oxidation has been thoroughly discussed in previous research papers (Baxter, 1982, Guido et al., 2005, Kobayashi et al., 2000b, Kuroda et al., 2002, Liégeois et al., 2002, Wackerbauer and Meyna, 2002a, Wackerbauer and Meyna, 2002b,

Wackerbauer et al., 2003, Yang and Schwarz, 1995, Yang et al., 1993) and reviews (Baert et al., 2012, Bamforth and Lentini, 2009, Vanderhaegen et al., 2006) and is an ongoing topic of research regarding the flavour stability of beer. In principle, a distinction is made in the peroxidation of fats between light-assisted photo-oxidation, enzymatic oxidation and finally radical autoxidation, although the products of all three reactions are partially similar or even of the same nature. Enzymatic lipid oxidation pathways are initiated by lipoxygenases (LOX). Lipoxygenase is an enzyme found in malt, especially in green malt, but residual activity can still be found in pale kilned malt (Hirota et al., 2005, Skadhauge et al., 2005). The oxidative activity of LOX is not limited to the free fatty acids, thus if LOX is present it can also oxidise the esterified fatty acids of the triacylglycerols and form hydroperoxides (Figure 1.5), ultimately resulting in hydroperoxy fatty acids (Kobayashi et al., 1994, Wackerbauer et al., 2003).

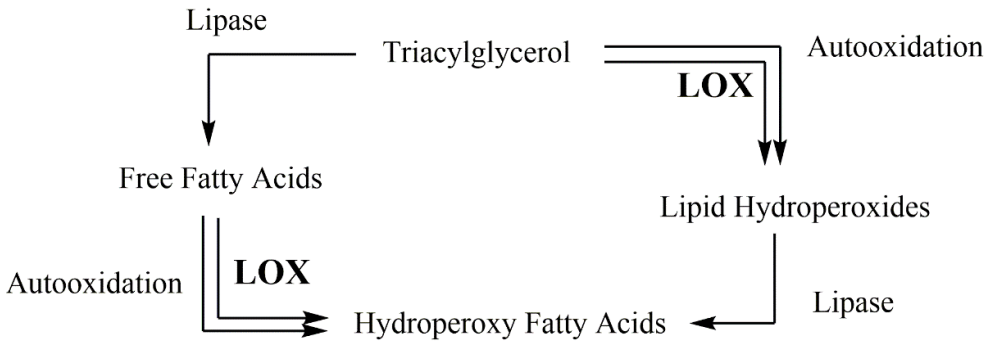


Figure 1.5: The formation of hydroperoxyl fatty acids through enzymatic lipidoxidation (lipase, lipoxygenase (LOX), and autooxidation (Baert et al., 2012, Kobayashi et al., 1994).

The hydroperoxy fatty acids can undergo further degradations to mono-/di-trihydroxy fatty acids through several pathways (Baert et al., 2012). The hydroxy fatty acids remain present in the beer (Kobayashi et al., 2000a) or can, in the presence of oxygen, be further degraded non-enzymatically to secondary metabolites known as ageing carbonyls; e.g. hexanal**,** *trans*-2-nonenal, which contribute to the staling of beer (Kobayashi et al., 1994, Liégeois et al., 2002). Figure 1.6 displays an overview of pathways of the enzymatic breakdown of linoleic acid initiated by lipoxygenase. The oxidation of unsaturated fatty acids is a major concern when brewing with green malt, thus this section will be further discussed in Section 1.8.4.

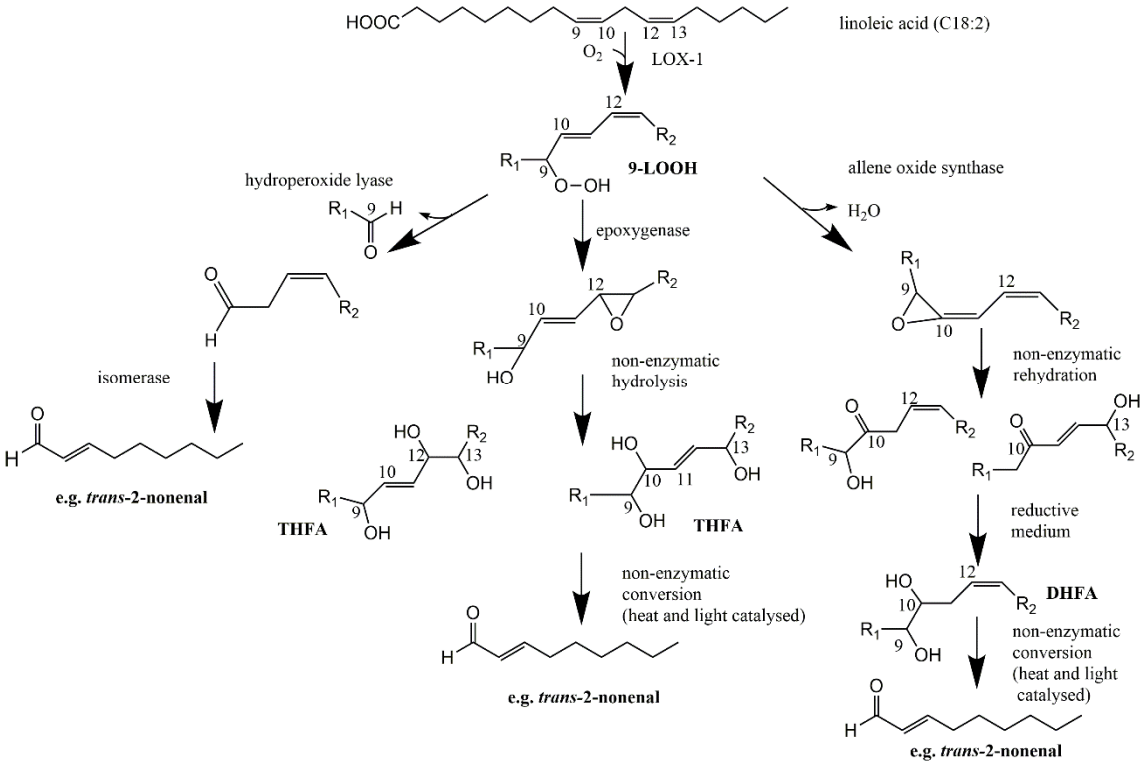


Figure 1.6: Overview of relevant pathways of the enzymatic breakdown of linoleic acids according to Baert et al. (Baert et al., 2012); THFA, trihydroxy fatty acids

1.4.1.2 Maillard reactions

The Maillard reaction, or nonenzymatic browning, is the chemical reaction between an amino acid, amine, peptide or protein and a reducing sugar (Figure 1.7). Reactions proceed rapidly at high temperatures, but can start at 50°C at a pH of 4-7 and their outcoming products usually increase the colour during wort production (Coghe et al., 2006).

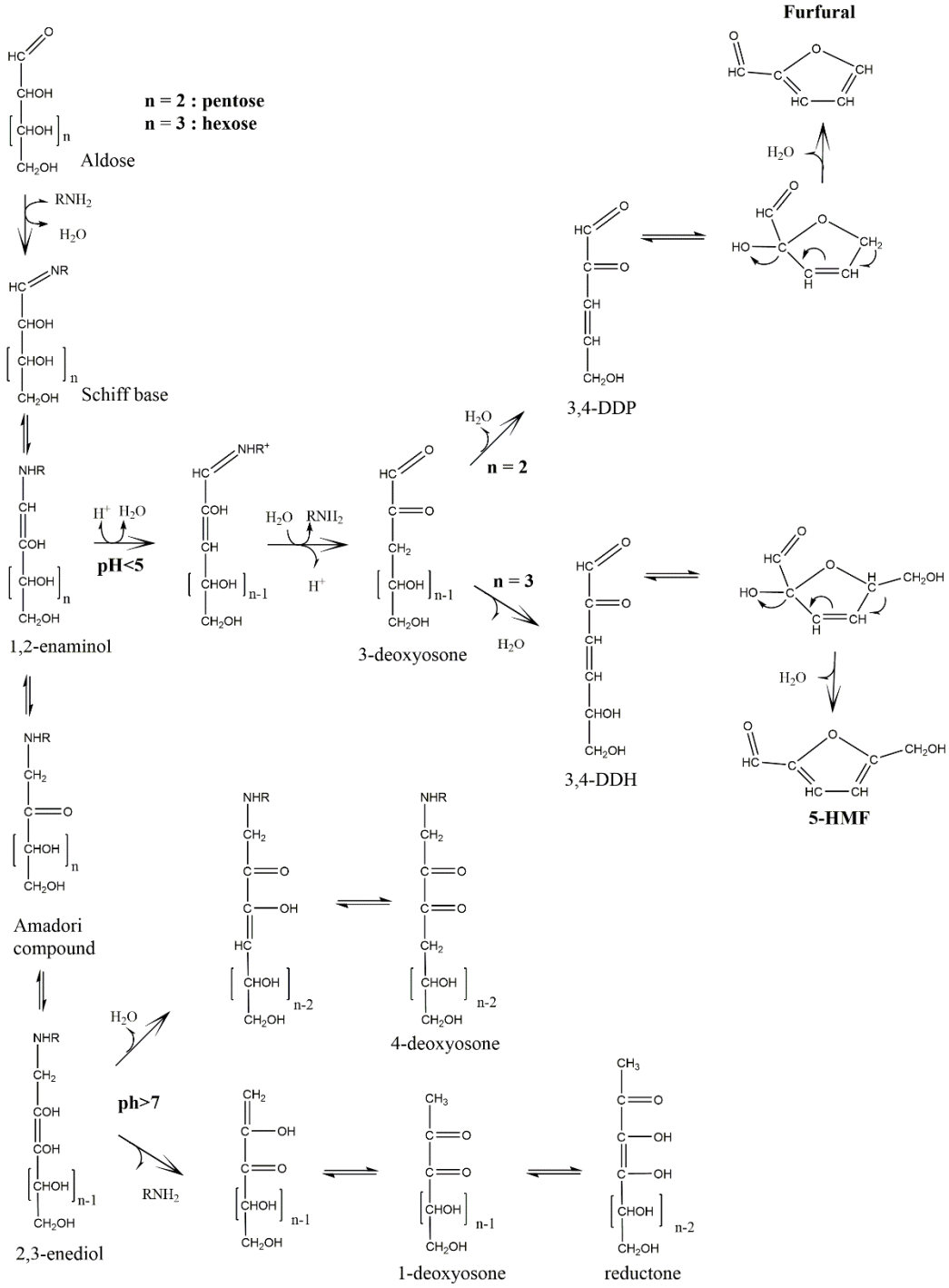


Figure 1.7: Overview of the Maillard reactions yielding 5-hydroxymethylfurfural (5-HMF) and furfural according to Baert et al. (Baert et al., 2012); 3,4-DDP, 3,4-dideoxypentosulose-3-ene; 3,4-DDH, 3,4-dideoxyhexosulose-3-ene; 5-HMF = 5-hydroxymethylfurfural

Many kinds of amino acids can react with various different sugars, thus numerous Maillard products can result from this reaction and were already identified in beer.

However, quantitatively furfural and 5-hydroxymethylfurfural (5-HMF) are the most relevant Maillard products in beer (Li, 2009, Madigan et al., 1998, Malfliet et al., 2008, Shimizu et al., 2001). Thus, the main focus in this review is on the Maillard reaction yielding these two aldehydes, which is displayed in Figure 1.7. Both, 5-HMF and furfural, are considered indicators of heat load experienced during malt, wort or beer production and can be determined through a standard analytical assay- the thiobarbituric acid assay (results expressed as TB-Index) (Herrmann et al., 2010, Li, 2009, Madigan et al., 1998, Malfliet et al., 2008). Chemically, furfural and 5-HMF are formed through nucleophilic addition of an amino group to the reducing end of a pentose or hexose (respectively) in open form, forming a Schiff base (imine). At standard wort or beer pH, the sugars are mostly in closed-chain form and amino acids are not reactive (loss of nucleophilic character, due to pK_a levels of ≥9). Therefore, the formation of the Schiff base and thus initiation of the Maillard reactions are accelerated at an alkaline environment (Baert et al., 2012, De Schutter, 2008, Ge and Lee, 1997). The formed imine is not stable and thus an Amadori compound is formed through the so called Amadori rearrangement. The subsequent degradation of the Amadori product is pH dependent. Under acidic conditions (pH<5), a 3-deoxyosone (α-dicarbonyl) is formed through the release of an amine. The 3- deoxyosone can subsequently yield furfural or 5-HMF through cyclisation (Shimizu et al., 2001). During ageing Maillard products increase at a linear rate (Madigan et al., 1998). Due to their high flavour threshold (Saison et al., 2009b) they are discussed to not play an important role in the flavour profile of a beer. However, in recent findings (De Clippeleer et al., 2011), spiking of furfural resulted in a sharper, harsher bitterness and increased astringency even when present in a sub-threshold flavour concentration of 400 µg/L.

1.4.1.3 Strecker degradation

The Strecker degradation is a transamination between an amino acid and an α-dicarbonyl, which is displayed in Figure 1.8. The Strecker degradation is sometimes categorised as Maillard reaction because various α-dicarbonyls are produced through Maillard reactions (Baert et al., 2012, Rizzi, 2008, Vanderhaegen et al., 2006, Yaylayan, 2003), which was shown before (Figure 1.7).

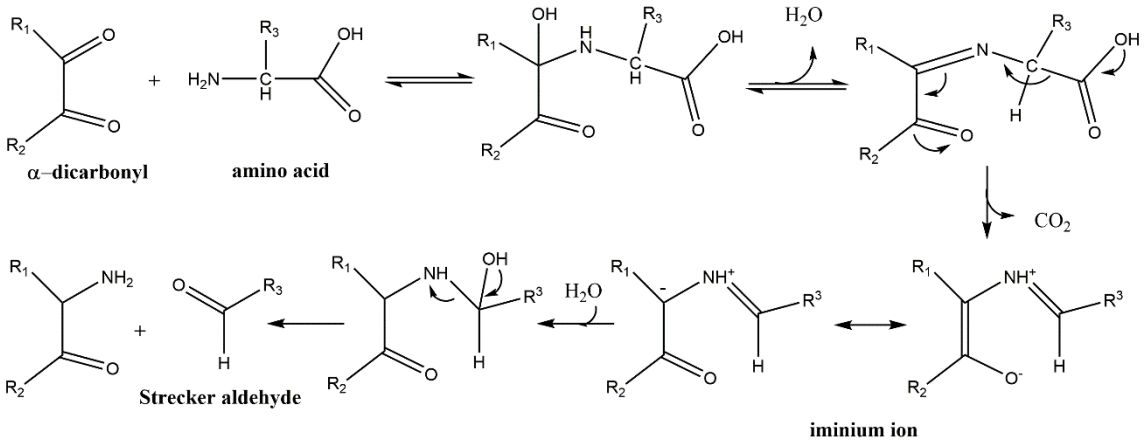


Figure 1.8: Formation of a Strecker aldehyde according to Baert et al. (Baert et al., 2012)

The Strecker degradation is initiated by the formation of an unstable hemiaminal via nucleophilic addition of an unprotonated amino group to an α-dicarbonyl. Subsequently, a zwitterion is formed through reversibly splitting water and irreversible decarboxylation. Via the addition of water an unstable amino alcohol is formed, which decomposes to an α-ketoamine and a Strecker aldehyde (Baert et al., 2012, Rizzi, 2008, Vanderhaegen et al., 2006, Yaylayan, 2003). Many amino acids are related to Strecker degradation, however, the most relevant due to the concentration of the amino acid in beer and the low flavour threshold of the resulting aldehyde are valine, isoleucine, leucine, methionine and phenylalanine. These amino acids can result in the Strecker aldehydes 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, methional and phenylacetaldehyde, respectively (Baert et al., 2012). The formed Strecker aldehydes contain one carbon atom less than the 'original' amino acid. Additionally, benzaldehyde is counted to this list, even though it is formed indirectly from phenylacetaldehyde (Chu and Yaylayan, 2008).

Furthermore, if an amino acid reacts with an α-unsaturated carbonyl compound, e.g. *trans*-2-nonenal, furfural or benzaldehyde (Rizzi, 2008), this reaction is considered a 'Strecker-like' reaction. The reactions start very similarly to the Strecker degradation by splitting water and decarboxylation. To the resulting imine zwitterion water is added and subsequently, the unstable amino alcohol is degraded to a Strecker aldehyde, but

also to a saturated aldehyde (for example nonanal from *trans*-2-nonenal) after the release of ammonia.

1.5 Analytical measurements to determine beer quality and flavour stability

The most common way for brewers to record how quickly or slowly beer changes in flavour is through so called forced ageing tests. These tests enable researchers to predict the flavour and colloidal (in)stability of the beers, without having to wait for several months to obtain the results. Forced ageing of beers is not performed after a standardised protocol, even small alterations in the 'ageing' conditions (temperature, time) can have a significant impact on the overall 'ageing' profile. The temperatures applied usually start at 28 up to 60°C, applied for several hours, days or even months (Lehnhardt et al., 2019). Based on the Arrhenius law, as a rule of thumb, an increase of 10°C at least doubles the reaction rate for many chemical and physical reactions. However, due to their different activation energies, chemical reaction rates do not increase equally in response to increasing temperature and this can result in very different aroma profiles during storage (Lermusieau et al., 1999). Even though it is very discriminative, as different ageing conditions can lead to a very different sensory profile, nevertheless forced ageing is a state-of-the-art technique to predict the flavour stability of a beer (Lehnhardt et al., 2019). The resulting 'stale' beer might then be compared to the fresh corresponding beer, or different beer styles treated equally.

In the literature, many analytically detectable indicators or 'ageing markers' are discussed. However, it should not be neglected that beer ageing is a very complex process, and numerous pathways can lead to the formation of unwanted 'staling' compounds or decrease of desirable 'fresh' beer compounds. Thus, it is not recommendable to base flavour (in)stability conclusions only on individual predictors of staling (Bamforth, 1999a). A combination of different methods might provide a more accurate picture of the beer quality. Apart from the more advanced analytical methods, such as gas chromatographic flavour profile determination, standard analytical parameters, such as colour, haze formation, or foam stability are relevant to evaluate beer quality.

1.5.1 Determination of aldehydes

As already highlighted previously, the carbonyl compounds - aldehydes - are considered major contributors to beer staling, due to their very low flavour thresholds (Meilgaard, 1975a, Meilgaard, 1975b, Saison et al., 2009b) and the ability to be perceived even at subthreshold level due to synergistic interplay of two or three aldehydes. Several aldehydes were selected as analytical indicators for beer flavour instability (Baert et al., 2012, Jaskula-Goiris et al., 2011, Malfliet et al., 2008, Saison et al., 2010b, Vesely et al., 2003), i.e. 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, methional, phenylacetaldehyde, furfural, hexanal and *trans*-2-nonenal. The state of the art method to determine and quantify free aldehydes in malt (Filipowska et al., 2020), wort and beer (Baert, 2015, Ditrych et al., 2019, Vesely et al., 2003) is by performing headspace-solid phase microextraction (HS-SPME) with on-fibre PFBHA (*o*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine) derivatisation. Solid phase microextraction is a common technique used for the extraction and analysis of volatile analytes. Hereby, a fiber coated with an extracting phase is exposed to the headspace of the sample. Subsequently, the fibre is transferred to the injector of a separating instrument, e.g. gas chromatography (GC), and subsequently the separated volatile compounds are captured and identified via mass spectrometry (MS). For a more precise measurement, a derivatisation agent, PFBHA, aids to derivatise the carbonyl group of the aldehydes and thus improve the selectivity. The amino group of PFBHA reacts with the carbonyl group of the respective aldehyde, forming very stable pentafluorobenzyloximes (PFBO's). This reaction is performed at ambient temperature in aqueous solutions and can proceed over a wide pH range (Baert, 2015).

Previous research has paid huge attention to the impact of LOX activity present in malt on beer flavour stability (Section 1.8.4). Thus, the concept of the 'nonenal potential' (Drost et al., 1990) was developed. This method enables to determine the amount of *trans*-2-nonenal formed and reversibly bound (adduct form) during the brewing process (Liégeois et al., 2002). During beer ageing, the *trans*-2-nonenal may dissociate and the free form would cause the cardboard flavour in the beer. The 'nonenal potential' method is performed by force ageing (100°C, 2 h) a wort sample under beer conditions (oxygen limited conditions, pH 4.0). The nonenal released is then extracted by liquid-

liquid extraction and quantified using gas chromatography- mass spectrometry (GC-MS).

1.5.2 Determination of the oxidative stability – ESR analysis

Oxygen in beer can cause a fast deterioration of beer flavour, thus oxygen pick-up is avoided wherever possible throughout the brewing and packaging processes. However, there is still a finite amount of dissolved oxygen (~ 0.05 mg/L) content in beer, as well as in the headspace when packaged (total packaged oxygen < 0.5 mg/L) (O'Rourke, 2002) - even with industry best practice. Additionally, packaged beer is not a perfectly closed system, thus some oxygen ingress for example through the crown cork during storage might occur. At this stage, the beer composition can determine the stability against oxidation. Certain antioxidants (e.g. sulphites) can hinder the formation of radicals, while pro-oxidants, such as transition metal ions, drive the formation of reactive oxygen species (Andersen et al., 2000, Andersen and Skibsted, 1998, Lund et al., 2015). Electron Spin Resonance (ESR) spectroscopy analysis provides information about the oxidative stability of the final product, by detecting and quantifying unpaired electrons in atoms and radicals (intermediates in oxidative reactions) in beer or wort. However, the formed radicals, in wort and beer are not stable and thus difficult to detect directly. Therefore, so-called “spin traps”, chemical compounds that can bind to radicals in solution, can stabilise the radical and make it easily detectable (Figure 1.9).

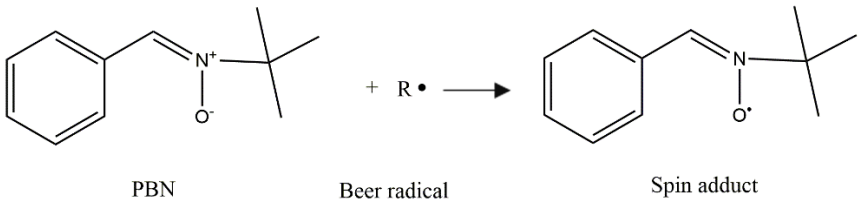


Figure 1.9: Reaction of PBN with a beer radical.

The most prevalent radical in beer is the 1-hydroxyethyl radical (Andersen and Skibsted, 2017, Huvaere and Andersen, 2008). PBN (N-tert-Butyl-α-phenylnitrone) (Andersen et al., 2000) or POBN (α-(4-pyridyl-1-oxide)-N-tert-butylnitrone) (Jenkins et al., 2018, Kunz et al., 2012a, Kunz et al., 2012b) are the most commonly used spin traps. POBN was

discussed to be a superior spin trap, partly because it does not alter the pH during the assay, unlike PBN (Kunz et al., 2012b). ESR spectrometers measure the absorption of electromagnetic radiation. Samples are placed between two electromagnets and subsequently irradiated with microwaves at constant frequency. The unpaired spins in the sample can then switch at characteristic magnetic fields to their high-energy state while absorbing microwaves in this process. An absorption spectrum will appear, similar to the one displayed in Figure 1.10.

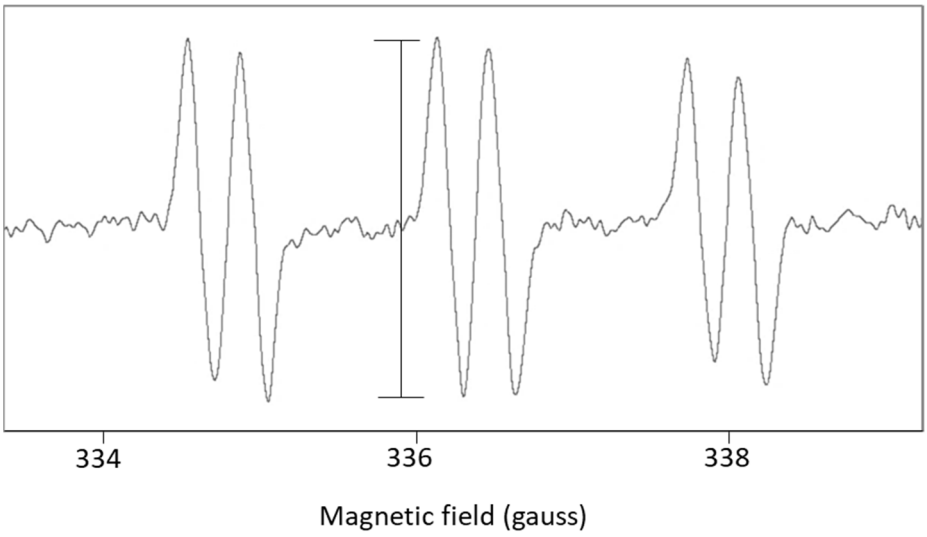


Figure 1.10: ESR spectrum of a PBN spin adduct. The amplitude of the third peak is recorded.

Subsequently, this absorption (amplitude) is measured and correlated to the number of free radicals in the system. The PBN spin adduct spectrum is evaluated either by determining the signal intensity at the first double peak (Figure 1.10), or alternatively by determining the average of the signal intensity of two different peaks. Usually, ESR analysis is performed by force ageing (60°C) a wort or (degassed) beer sample containing PBN or POBN as a spin trap (dissolved in ethanol) in a closed bottle under atmospheric oxygen to exhaust the natural antioxidants present (Uchida et al., 1996). Normally, the data are plotted over time from the beginning of the trial, throughout lag time, until radical formation reached a stationary phase (Uchida et al., 1996). The comparison of the endogenous antioxidative potential is usually enabled by comparison of the lag time (the time until notable amounts of radicals are generated) (Figure 1.11).

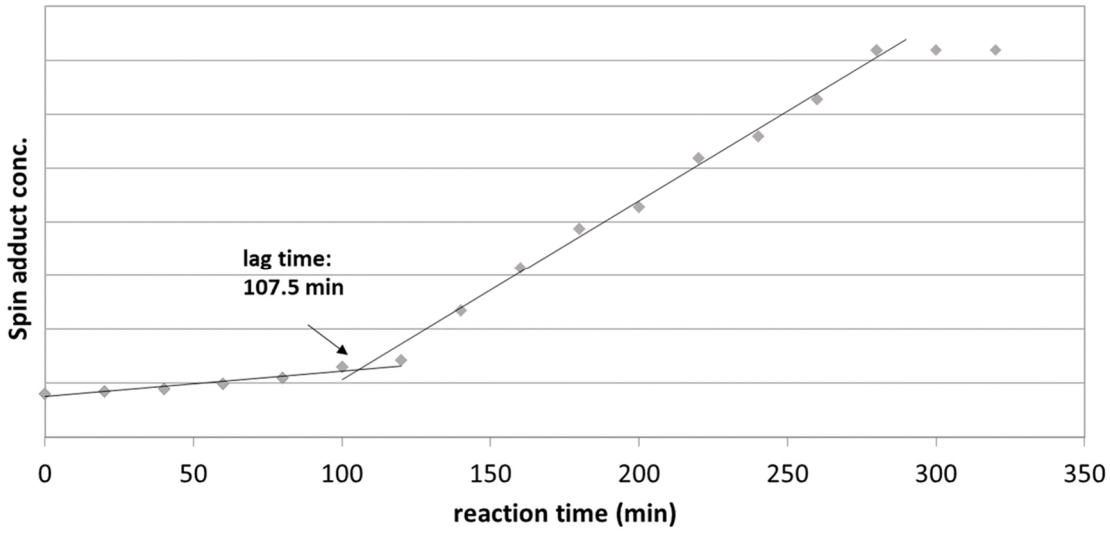


Figure 1.11: Change of signal intensity (concentration of PBN-spin adducts) during oxidative forced ageing at 60°C and determination of lag-time through the intersection of two regression lines.

Long lag times are associated with improved flavour stability and are related to levels of antioxidants present in beer (Andersen et al., 2000, Hashimoto, 1966). Additionally, particularly if no lag time is observed (e.g. wort samples), the number of radicals generated at a definite time (e.g. 120, 150 or 300 min) can be compared (Tx value).

1.6 Sustainable malting and brewing initiatives

Annually, European maltsters produce around 9.7 million tonnes of malt - and this trend is increasing (Euromalt, 2017). Malting barley production accounts for about 241 kg CO_2eq/t; malting itself adds 217 kg CO_2eq/t – doubling the total malt carbon footprint (Muntons, 2019). In the UK alone, emissions of more than 300,000 tonnes CO_2 per year are produced through the manufacturing of more than 1.6 million t of malt (CarbonTrust, 2011, Euromalt, 2017) – this corresponds to annual CO_2 emissions of about 14,851 British households (Buchs and Sylke, 2013). Thus, the malting industry is constantly exploring ways to improve its energy efficiency. To date, around 6-15% of the cost per ton of malt can be attributed to energy usage (CarbonTrust, 2011). Moreover, a number of governments impose national-level energy or carbon taxes, calculated based on the carbon content. Hence, diminishing energy usage is not just an environmental driver but also a financial driver. While some research has been carried

out on energy efficient malting (CarbonTrust, 2011, Davies, 2010, Ferrari-John et al., 2017, Huang et al., 2004, Jones et al., 2002, Müller et al., 2013), to our knowledge only a few studies (Cook and Hudson, 1964, Duff, 1963, Leclercq, 2020, MacWilliam, 1972, MacWilliam et al., 1963, Moir, 1992) focused on wort and beer production using green (germinated, undried) malt.

The most dominant contributors to the carbon footprint of the malting process are gas (or other process fuels) and electricity (CarbonTrust, 2011, Davies, 2010, Doug, 2010). A UK Carbon Trust report (CarbonTrust, 2011) demonstrated that fuel use accounted for about 68% and electricity about 32% of the malting sector's CO_2 emissions. Electricity usage is spread over all process steps, whereas gas and coal are primarily used in kilning (Doug, 2010). During the drying process, removal of free moisture from green malt is relatively easy until the grain reaches a moisture content of approximately 12%. To remove the remaining water in bound form a lot of energy is required. A review of the UK malting sector (CarbonTrust, 2011) reported average specific energy usage to be ca. 1,200 kWh/t malt and up to 80% of this energy was used in drying of malt (kilning). Hence, kilning is the most dominant user of heat and electricity, making it the main contributor to the carbon footprint associated with malting. Numerous previous studies have focused on ways in which to reduce the energy needed for kilning (Brudzynski and Roginski, 1969, CarbonTrust, 2011, Davies, 2010, Doug, 2010, Ferrari-John et al., 2017, Mauthner et al., 2014). The Carbon Trust's report evaluated different technologies, including kiln energy recovery, heat pumps or biomass burners as replacements for the heat energy used for kilning with regard to carbon emission reduction and payback periods for the industry. Although various technologies significantly reduce emission rates, not all of them are cost effective. Another option is the application of alternative heating methods, such as: electromagnetic heating (Ferrari-John et al., 2017), microwave drying (Jones et al., 2002), drying with supercritical CO_2 (Djekic et al., 2018), or freeze-drying (Brudzynski and Roginski, 1969, Ratti, 2001). In spite of this research and technology innovations that have been implemented to reduce specific energy usage across malting, there is still a demand to explore cost-effective methodologies to reduce the environmental footprint associated with malting and brewing.

1.6.1 Brewing with green malt – a feasible alternative?

Omitting the kilning process, thus producing beer with green malt, is one potential route to reduce the energy inputs required for malting. Additionally, de-carbonising and the reduction of primary energy usage through e.g. biomass CHP or hydrogen power could sit alongside the adoption of green malt in future strategy to meet environmental targets. Green malt differs from kilned malt in a number of respects. Green malt is not coloured green, it is a term used to refer to undried germinating malt. Apart from being a dominant consumer of heat and electricity, the kilning process has many beneficial impacts on malt quality, which must be considered if it is to be omitted. These include reduction of lipoxygenase activity (De Buck et al., 1995, De Buck et al., 1997, Doderer et al., 1992, Huang et al., 2016, Hugues et al., 1994, Kuroda et al., 2003, Yang and Schwarz, 1995, Yang et al., 1993, Ye et al., 2014), regulation of S-methyl methionine (SMM) levels (Anness and Bamforth, 1982, White and Wainwright, 1976a, White and Wainwright, 1976b, White and Wainwright, 1977, Yang et al., 1998), facilitating rootlet removal, diminishing unwanted "raw grain" characteristics (Moir, 1992) and, most importantly, developing the characteristic colour and flavours which malt imparts to beer. Furthermore, green malt, having a moisture content between 38-46%, is unstable and cannot be stored for prolonged periods. On the other hand, green malt is rich in β-glucanase (Bamforth and Martin, 1983, Barber et al., 1994, Hämäläinen and Reinikainen, 2007) and diastatic enzyme activity (Hämäläinen and Reinikainen, 2007, Schroeder and MacGregor, 1998, Sopanen and Laurière, 1989), hence it can very efficiently convert the starch of unmalted grains into fermentable sugars (Duff, 1963, MacWilliam et al., 1963). Additionally, by removing the kilning process, the thermal heat load on malt is substantially reduced. As a consequence, green malt, is free of DMSO (Anness et al., 1979, Yang et al., 1998), and contains lower concentrations of thermally generated compounds such as Maillard compounds or Strecker aldehydes, which are key agents in beer flavour change through shelf-life (Baert et al., 2012, De Clippeleer et al., 2010a, Drost et al., 1990, Gastl et al., 2006, Jaskula-Goiris et al., 2015, Malfliet et al., 2008, Vanderhaegen et al., 2006). On this basis, potential benefits regarding beer staling can be expected in beers brewed from green malt.

Well germinated green malt usually has a moisture content of 41-48%, depending on the malting procedure. The high moisture content of green malt is a perfect

environment for microorganisms, making the grain unstable. The microbial growth on malt accelerates when stored at warm temperatures, for example the doubling time of the filamentous fungus *Geotrichum candidum* was shown to be as fast as 1.7 h at 25°C and just 1.1 h at 30°C (Trinci, 1972). Furthermore, moisture contents > 8% make the abrasion of rootlets difficult (Briggs et al., 1981b). Malt rootlets are considered to impair the flavour of beer, therefore maltsters try to avoid excessive rootlet growth during germination and remove the rootlets, by abrading them after kilning. Thus, they form a malting loss of around 4%, usually sold as animal feed or organic fertilizer (Briggs, 1998a, Kunze, 2014). Rootlets are low in phytic acid and polyphenols, but contain a high amount of fatty acids, tocopherols (Vit. E), B-vitamins and proteins (10-35%) (Briggs, 1998a, Salama et al., 1997), calcium (19.9 g/kg), as well as DMS precursor (White and Wainwright, 1976a) and lipoxygenase (Yang et al., 1993). Rootlets of kilned malt are highly hygroscopic, due to their fibre content of up to 15% (Briggs, 1998a, Salama et al., 1997). Apart from the high water absorption they also highly absorb oil and have emulsification capacities (Kunze, 2014). However, malt rootlets (as analysed in kilned malts) show a high antioxidant potential (Bonnely et al., 2000, Meng et al., 2009, Peyrat-Maillard et al., 2001). The natural antioxidant phenolic compounds could potentially reduce the formation of free radicals, thus favouring wort and beer flavour stability. In the absence of a facile technique for rootlet removal from green malt, they are most easily included in the brewing grist. Whilst this will lower malting losses, the foregoing quality issues associated with rootlet usage need to be addressed, or better evaluated, across a range of beer styles.

1.7 Processing green malt

1.7.1 Microbiological stability

The brewing process presents numerous hurdles to the survival of microorganisms, of which mashing, wort boiling and the addition of hops, are considered the most effective. In addition, the composition of beer, mainly the presence of alcohol and CO_2, a low level of O_2 and the acidic pH, provides a very hostile environment for the growth of spoilage and pathogenic microorganisms (Vaughan et al., 2005, Vriesekoop et al., 2012). Wort, however, is a nutrient rich medium and represents an excellent environment for the growth and proliferation of microorganisms. The microbiology of malting and brewing

is a complex topic, and little is known about the microbial stability of wort and beer made of green malt. Most of the microflora on barley in the field consists of bacteria (predominantly), wild yeast and filamentous fungi originating from the air and soil (Briggs, 1978, Flannigan, 1996). Microbial colonisation of the grain is generally restricted to the outer layers, namely husk and between the husk and pericarp, although penetration into the endosperm does occur (Schwarz et al., 2002b). Green malts are covered in a complex microflora, with viable counts of various organisms that are 85-700 fold higher than measured on the original barley (Douglas and Flannigan, 1988, O'Sullivan et al., 1999, Petters et al., 1988, Sheneman and Hollenbeck, 1960), their growth stimulated by dissolved nutrients, moisture, warmth, and aeration (Briggs and McGuinness, 1993, O'Sullivan et al., 1999, Petters et al., 1988). Before drying, an average of tens of thousands of fungi, hundreds of thousands of yeasts and millions of bacteria can be measured in just one gram of malting barley (Briggs, 1998a, Petters et al., 1988). Douglas and Flannigan (Douglas and Flannigan, 1988) detected especially the yeast-like mould, *Geotrichum candidum*, in green malt. Usually, after kilning, the majority of microorganisms are destroyed (Douglas and Flannigan, 1988, Flannigan et al., 1982, O'Sullivan et al., 1999, Petters et al., 1988). Mostly lactobacilli (O'Sullivan et al., 1999) and aerobic heterotrophic bacteria (Petters et al., 1988) are still being detected afterwards and counts further decrease steadily during mashing, with only thermotolerant microbes, such as homofermentative lactic acid bacteria (Briggs and McGuinness, 1993), persisting. Table 1.2 compares representative numbers of microbes on barley, green malt and kilned malt according to Petters et al. (Petters et al., 1988).

Table 1.2
Representative numbers of microbes in/on barley, green malt and kilned malt (Petters et al., 1988).

	barley (dry)	**green malt (5 days)**	**kilned malt**
Aerobic heterotrophic bacteria/ kernel or ml	1.8×10^6	5.7×10^7	5.6×10^6
Lactobacilli/ kernel or ml	2.0×10^2	8.7×10^6	1.6×10^5
Filamentous fungi/kernel or ml	2.0×10^2	1.5×10^2	2.0×10^2
Yeasts/ kernel or mL	4.7×10^3	3.9×10^6	3.2×10^4

Whilst the hurdles presented by the brewing process would be expected to overcome this higher initial presence of microbes and still present a sterile wort for colonisation with yeast, it is unclear whether the increased microbial loading of green malt would have any negative impacts on wort or beer quality. However, we do know that green malt as-is, is not microbiologically stable, hence it needs to be either processed directly, by mashing-in immediately or by reducing its moisture content to a microbiologically safe level (kilning, freeze-drying, electromagnetic heating) or alternative technologies (Ferrari-John et al., 2017, Jones et al., 2002, Peterreins and Van Waesberghe, 2003). In general, it is recommended to avoid making malts from barley that is heavily infected with fungi – this applies especially to green malt brewing. It is recommended to store green malt cold and dry to reduce microbial activity and stabilise enzymatic activity until the grain is further processed – the sooner the better. Longer storage periods of green malt and the associated microbes present could greatly affect malt quality and thus impact beer quality (Bokulich and Bamforth, 2013, Justé et al., 2011, Scott, 1996). Of particular concern are mycotoxins present on poor malts, which might survive into the final beer (Scott, 1996). Alternatively, it was suggested (Leclercq, 2020) to mix proportions of 10-20% green malt with kilned malt or unmalted cereals, as the moisture content of the mixture would allow longer storage periods than green malt alone. Furthermore, Peterreins and Van Waesberghe (Peterreins and Van Waesberghe, 2003) proposed two methods that could be used to stabilise green malt; either by exposing the green malt to a brief heat shock by applying water vapour or stabilising it with lactic acid bacteria at 45°C. Lactic acid bacteria and associated antimicrobial metabolites could potentially inhibit the growth of bacteria or fungi (Benthin and Villadsen, 1995, Caplice and Fitzgerald, 1999). These preserving effects may be due not only to the end products of their fermentative activity, such as lactic acid, but also to the formation of small, heat stable inhibitory peptides referred to as bacteriocins (Ross et al., 2002).

1.7.2 Milling

One of the first challenges when handling green malt is its sensitivity to the milling procedure, due to the high moisture content of green malt, hence it cannot be milled as for kilned malt. Previous studies (Cook and Hudson, 1964, Duff, 1963, MacWilliam, 1972, MacWilliam et al., 1963) already proved that extracts of green malt with equal quality

to kilned malt can be achieved, provided a suitable mill is used. However, there is no general consensus on what is the most suitable milling technique. S.R. Duff (Duff, 1963) stated that higher extract yields are achievable by finely grinding the green malt. Furthermore, better yields were obtained by using a hammer mill rather than a roller mill. Unfortunately, no further information was given on the exact model or design of the hammer mill used in the study. In a standard hammer mill steel beaters rotate at speeds between 60-100 m/s, to form small particles which fall through holes in a sieve (Kunze, 2014). Therefore, this approach seems not well suited for green malt at a moisture content above 40%, as it would likely block the sieves. In general crushing rollers could be the most suitable option for handling green malt. Pre-soaking of barley or green malt before milling increased yield of extracts and facilitated the milling (Cook and Hudson, 1964, MacWilliam et al., 1963), indicating that a wet milling system is a suitable technique for processing green malt. About 20 years ago, Meura (Belgium) developed in collaboration with Castle Malting (Belgium) the 'hydromill'; a disc mill which finely mills malt underwater, designed to process malts with a high moisture content (Leclercq, 2020, Meura). Green malt used as 100% grist material still poses a technical challenge for present day brewhouse designs. Alternatively, standard kitchen meat grinders pose a more affordable option for homebrewers who would like to experiment with this 'novel' grist material. Lars Marius Garshol (Garshol, 2020) recently published a book on ancient brewing traditions and techniques; amongst them, brewing with homemade green (undried) rye malt, milled with an ordinary kitchen meat grinder and used as 100% grist material for mashing.

1.7.3 Mashing

As already highlighted previously, the malting and brewing process can be considered as a sequential adding and removing of water. If omitting the kilning process, thus brewing with green malt, the brewer could take advantage of the increased moisture content in the grain, hence less brewing water will be needed for mashing. However, brewing water adjustments are necessary to compensate for the increased water content in green malt. This would imply a temperature increase of the brewing liquor used for mashing, as well as lactic acid (pH regulation) and brewing salt addition (water hardness).

1.7.4 Filterability

Castle malting's research team has shown (Leclercq, 2020) that by replacing 20% of pilsner malt with green malt to a cereal recipe containing 30% unmalted barley, can significantly improve filterability. The resultant mash was filtered twice as efficiently. Therefore, green malt has the potential to compensate for the relatively high β-glucan levels of unmalted cereals such as barley (Briggs, 1998a), or poor malts (>250 mg/L β-glucan in the mash (Leclercq, 2020)), and thus, improve filterability.

1.8 Green malt's (bio)chemistry – the good and the bad

1.8.1 Diastatic enzyme activity

During malting, starch degrading enzymes, are formed which play a key role in brewing during the mashing process, where the starch is gelatinised to enable access of the starch hydrolysing enzymes. The enzymatic breakdown of starch into simpler sugars provides the major energy source for the fermentation process. α-amylase is more thermostable than the other diastatic enzymes (Hämäläinen and Reinikainen, 2007, Henson et al., 2014, Preece, 1948). According to previous research (Hämäläinen and Reinikainen, 2007, Sissons et al., 1995), kilning (80°C curing temperature) caused a significant loss of diastatic power (15%), limit dextrinase activity (25%), while α-amylase decreased only about 4% upon kilning. The thermosensitive β-amylase, on the other hand, suffered losses up to even 46% of initial activity during malt kilning (Evans et al., 1997). Modern day breeding programs have ensured that elite barley cultivars are rarely deficient in diastatic enzyme potential when malted and used as the main grist component in brewing. However, the extra diastatic potential of green malt could favour the degradation of starch of unmalted adjuncts into fermentable sugars, novel malted cereals lacking diastatic enzyme activity (e.g. malted lentils (Trummer et al., 2019)) or potentially be suitable for shortening the total mashing time, and thus further decreasing the total heat load.

1.8.2 β-glucanase activity

In addition to the diastatic enzymes, kilning causes significant losses of total β-glucanase activity (Bamforth and Martin, 1983, Barber et al., 1994, Hämäläinen and Reinikainen, 2007, Sissons et al., 1995). Previous research (Hämäläinen and Reinikainen, 2007,

Sissons et al., 1995) highlighted that β-glucanase was reduced by 43-44% when curing at 80°C, an inactivation that already started at the onset of kilning at a relatively low kilning temperature. Increasing the curing temperatures, as expected, further caused enzymatic activity losses (Hämäläinen and Reinikainen, 2007). Most of the necessary cytolytic degradation of barley (1,3)(1,4)-β-D-glucans via β-glucanases occurs during malting. Thus finished malts should contain low levels of β-glucan for a satisfactory brewing performance and to avoid the process problems associated with the elevated content of β-glucan (poor lautering performance (Bamforth and Martin, 1981, Jin et al., 2004) and colloidal stability of the finished beer (Bamforth, 1999b, Speers et al., 2003)). However, the increased β-glucanase content of green malt can be advantageous when unmalted adjuncts form part of the grist material used for brewing. Despite the enzymes heat-sensitivity and the recommendation to mash-in at >62°C to avoid LOX related off-flavours (Section 1.8.4), when using green malt as part of the grist material, previous research (Bamforth and Martin, 1981, Bamforth and Martin, 1983) has demonstrated that significant quantities of β-glucanase can survive infusion mashing at 65°C. β-glucanases were discussed to be protected from heat by high concentrations of protein, the association with particles of malt or sugars (Back et al., 1979), or reduced glutathione (Bamforth and Martin, 1983). Additionally, thick mashes can offer protection to more fragile enzymes (De Rouck et al., 2013b). Thus, when β-glucan is continuously released from its binding to protein through the activity of the more heat stable β-glucan solubilase (inactivation temp. 73°C, (O'Rourke, 2015)) during mashing, the malt β-glucanase can break down the β-glucan structure.

1.8.3 Anthocyanogenase

Claims have been made that beer produced from green malt and steeped barley has special advantages in connection with haze stability (Briggs et al., 1981b, Griffin et al., 1968, MacWilliam et al., 1963). MacWilliam et al. (MacWilliam et al., 1963) reported higher concentrations of anthocyanogens in wort prepared from kilned malt than from green malt. Anthocyanogens are polyphenolic compounds which play a role in the formation of chill haze in beer (Wettstein et al., 1980). Green malt appears to contain the enzyme anthocyanogenase which will hydrolyse or degrade the anthocyanogens

into simpler, less haze-inducing compounds (Curtis, 1966). Thus, the use of green malt for brewing might have beneficial effects on colloidal stability and beer shelf life.

1.8.4 Lipoxygenase

One of the key quality concerns when handling green malt is enzymatic lipid oxidation (Section 1.4.1.1). Enzymatic lipid oxidation pathways are initiated by lipoxygenases (LOX, which can eventually result in secondary metabolites known as ageing carbonyls (e.g. hexanal, *trans*-2-nonenal), which contribute to the staling of beer. In particular, one aldehyde is intensively investigated: *trans*-2-nonenal (Baert et al., 2012, De Buck et al., 1997, Guido et al., 2005, Liégeois et al., 2002, Santos et al., 2003, Van Waesberghe et al., 2001). *Trans*-2-nonenal is a major component for cardboard stale flavours in beer (De Buck et al., 1997, Liégeois et al., 2002, Meilgaard et al., 1979) and has a very low flavour threshold in the low ppb range (0.035 µg/L) (Jamieson and Van Gheluwe, 1970, Liégeois et al., 2002). Furthermore, the amount of hexanal, which is correlated with green/grassy scent and a bitter, winey flavour (Saison et al., 2010b), decreases with increasing malt colour (Coghe et al., 2004). In pilsner malt worts it was found in concentrations up to 50% higher than in wort made of dark malt samples (Coghe et al., 2004).

1.8.4.1 Barley lipoxygenases

In barley, around 3-4% of the dry matter is lipid, of which almost 60% is linoleic acid (C 18:2), making it the major substrate for lipoxygenases (Anness, 1984). However, the amount of free fatty acids in malt and barley is quite low. Linoleic and linolenic acid constitute around 6% of the total fatty acid content in barley (Anness, 1984), around 70% are found as triglycerides and 20% as polar lipids (phospholipids and glycolipids) (Anness and Reed, 1985).

The lipoxygenase activity in germinating malt is contributed by two LOX isoenzymes: LOX-1 and LOX-2 (Baxter, 1982, Doderer et al., 1992, Kobayashi et al., 1994, Yabuuchi, 1976, Yang and Schwarz, 1995, Yang et al., 1993). LOX-1 mainly oxidizes linoleic acid to 9-hydroperoxyoctadeca-10-12-dienoic acid (9-HPOD), whereas LOX-2 mainly forms 13-hydroperoxyoctadeca-9-11-dienoic acid (13-HPOD) from linoleic acid (Doderer et al., 1992, Holtman et al., 1997, Van Mechelen et al., 1999, Yabuuchi, 1976, Yang et al., 1993). LOX-1 is already present in barley and increases in activity during germination,

whereas LOX-2 is solely formed during germination (Franke and Frehse, 1953, Yabuuchi, 1976, Yang and Schwarz, 1995, Yang et al., 1993). During germination, both isoenzymes develop in the newly synthesised rootlets (only LOX-2) and acrospire (both isoenzymes) tissue (Yang et al., 1993). LOX is relatively unstable to thermal processing and the activity remaining after kilning is due to the somewhat more heat-stable LOX-1 which is then transferred into the wort (Kuroda et al., 2003, Yang and Schwarz, 1995)'.

Despite numerous efforts, little is known specifically about LOX from barley and malt, compared to LOX from other plants. As early as 1953, activities in different cereals including barley were measured (Franke and Frehse, 1953). The paper by Franke and Frehse showed that the activity from soybean far exceeds all other activities. Barley contains only 1.2% LOX activity compared with the activity in soybean. This membrane-bound enzyme had some similarities to LOX-1, such as its optimum pH, size, and preference to produce 13-hydroperoxy linoleic acid (Fornaroli et al., 1999). BLAST (Basic Local Alignment Search tool) alignment of amino acid sequences of $LOX_{Soybean}$ and $LOX_{Barley,}$ showed a degree of sequence similarity of 52.6% (Fenzl and Schönberger, 2018). Although there is (to the best of our knowledge) no paper which explicitly proves the presence of bound state LOX in malt, it is reasonable to assume that plant cells contain both soluble and membrane bound lipoxygenases (Boudnitskaya and Borisova, 1972, Braidot et al., 2004, Fornaroli et al., 1999).

1.8.4.2 Lipoxygenase activities in green malt

Huge attention has been paid as to the role of LOX in beer flavour stability. However, these studies focused mainly on the LOX activity present in kilned malt, which is mainly contributed by the more heat stable isoenzyme, LOX-1 (Kuroda et al., 2003, Yang and Schwarz, 1995). Kilning reduces - depending on the drying protocol and intensity - the lipoxygenase enzyme activity by 96% of the initial activity found in green malt (Schwarz and Pyler, 1984). Hence, omitting the kilning step will result in significantly higher lipoxygenase activities (De Buck et al., 1997, Schwarz and Pyler, 1984, Wackerbauer and Meyna, 2002b, Yang et al., 1993) and the usage of green malt in conventional brewing processes requires alternative techniques to reduce total LOX activity. An increase of LOX could result in elevated “rancidity” in the final beer, caused both by LOX-1 but also LOX-2. Furthermore, lipoxygenase worsens the foam stability of beer, possibly due to

the production of trihydroxy octadecenoic acid (THOD), which is detrimental to foam stability (Hirota et al., 2006, Yu et al., 2014). If LOX activity can be minimized at source, through adequate malting and/or mashing conditions, significant off-flavours in beer made from green malt could be avoided. Therefore, it is important to understand the origin, development and activity of LOX.

1.8.4.3 Considerations when brewing with green malt

As already discussed, LOX is primarily destroyed during kilning. However, LOX is also proven to be pH sensitive and requires oxygen as a substrate. Researchers do not agree on the exact pH optima of both isoenzymes, but the general consensus is that the pH-optimum is on the alkaline side of typical wort pH. The pH-optima for LOX-1 were reported to be around 6.3-6.5 (Doderer et al., 1992, Yang et al., 1993) and even 7.5 (Yabuuchi, 1976); 6.5 (Doderer et al., 1992) and pH 7.0-7.5 (Yabuuchi, 1976) for LOX-2. LOX-1 shows only 50% activity remaining at a pH of 5 whereas LOX-2 shows an activity rate close to zero, suggesting that LOX-2 is more pH sensitive than LOX-1 (De Buck et al., 1995, De Buck et al., 1997, Yang and Schwarz, 1995). Another important criterion to avoid LOX-related side effects is to perform the brewing process under oxygen-free conditions. Oxygen is a substrate of LOX, hence oxygen and oxygen pick up should be avoided by all means when brewing with green malt, especially during the mashing step. An important factor when brewing with green malt, is to consider the high lipoxygenase activity from the beginning of the process, meaning that milling and mashing need to occur in lipoxygenase hostile environments: e.g. mashing in at > 62 °C, pH: 5.3, under oxygen-free conditions (Bamforth, 1999c, De Buck et al., 1997, Drost et al., 1990, Schwarz and Pyler, 1984, Van Waesberghe et al., 2001).

Even though there are a number of process controlling methods, when brewing with green malt, Null-LOX (Hirota et al., 2005, Skadhauge et al., 2005) or low-LOX (Hirota et al., 2006, Hoki et al., 2018, Hoki et al., 2013, Yu et al., 2014) barley cultivars offer a further possible solution. However, low-LOX cultivars need to be differentiated, because the term is principally used to refer to low-LOX-1 cultivars, since LOX-1 activity is the main problem in kilned malt. However, regarding green malt brewing, the activity of LOX-2 should not be neglected. Beers made with a (kilned) lipoxygenase-1-less (LOX-less) malting barley variety had reduced levels of beer-deteriorating substances, such as

trans-2-nonenal and THOD compared to beers made with the control malt (Hirota et al., 2006, Hoki et al., 2018, Hoki et al., 2013, Yu et al., 2014). The sensory evaluation results indicated that LOX-less barley variety CDC PolarStar improved flavour stability without affecting other beer characteristics. Carlsberg's research in partnership with Heineken has shown that brewing beer using Null-LOX barley minimises negative beer-staling components, provides stable, quality foam with no aged off-flavours, and keeps its fresh flavour for longer. Although lipoxygenases can have adverse impacts on beer flavor stability, the products of the LOX pathway play an important role in the plant itself. The physiological function of LOX is associated with growth and development, mainly with lipid mobilisation (mainly via LOX-2) during seed germination (Garbe et al., 2006), wound-induced or pathogen infection signaling for the local defense reaction (Prasad et al., 2017) and participation in plant senescence (Rosahl, 1996). However, since Null-LOX barley varieties are already in commercial production without any reported adverse effects during plant growth, it might be suggested that these pathways are not insurmountable. Recent research confirmed (Vahamidis et al., 2017) that a total loss of LOX-1 and LOX-2 function did not cause any obvious disadvantages for Null-LOX cultivars over the traditional malt barley cultivar, in terms of grain yield, yield components, grain size, grain protein content and water use efficiency.

1.8.5 DMS and S-methyl methionine

Dimethyl Sulphide (DMS) is a highly volatile sulphur compound, with a boiling point of only 38°C. It has a characteristic flavour and odour usually described by brewers as cooked corn or cabbage-like. Although its odour plays an important role in some cooked vegetables, or contributes to the typical aroma of many lager style beers (Anderson et al., 1975), in most other styles, or at an excessive level, DMS gives beer an undesirable flavour (Anness and Bamforth, 1982, Kavanagh et al., 1976, Kavanagh et al., 1975). The flavour threshold is approximately 30 µg/L; however, the overall liking and acceptance of customers depends strongly on personal preferences. DMS originates from two possible precursors, S-Methyl Methionine (SMM) (White and Wainwright, 1976b, White and Wainwright, 1977) and DMSO (Anness et al., 1979). During germination, SMM, the thermal precursor of DMS, is produced from L-methionine and S-adenosyl-L-methionine catalyzed by L-methionine S-methyltransferase (MMT) (Pimenta et al., 1998, White and

Wainwright, 1976b). During barley germination, both the specific activity and the amount of MMT protein increase. SMM decomposes upon heating to yield free volatile DMS, and as a result, levels in malt are strongly regulated by the kilning stage which first breaks down SMM and then strips DMS into the exhaust gases (Anness and Bamforth, 1982, Dickenson, 1979, White and Wainwright, 1976a, White and Wainwright, 1976b). Besides SMM, as the thermal precursor of DMS, yeast can enzymatically reduce DMSO to DMS (Anness et al., 1979). However, through this pathway DMS cannot be readily removed and a high proportion remains in the finished beer. DMSO can be formed by oxidation of DMS during kilning and concentrations increase at higher kilning temperature (Anness, 1980, Anness, 1981).

Green malt is rich in SMM (White and Wainwright, 1977, Yang et al., 1998), not in DMSO (Anness et al., 1979, Yang et al., 1998), therefore the main focus when brewing with green malt is on the SMM pathway. Interestingly, according to a study by White and Wainwright (White and Wainwright, 1977), beers brewed from green malt had low levels of DMS, despite the significantly higher DMS potential, indicated by the high SMM levels in malt and wort (Section 1.9). Hence, DMS levels in the pitching wort can be controlled, provided that there is a sufficient removal of DMS via evaporation during wort boiling and elimination through fermentation gases. Even though those study outcomes seem very promising regarding DMS in beer made of green malt, the control of SMM-levels from the grist and throughout the process remains a significant issue to control the potential for DMS formation. Precursor levels vary with the barley variety and depend on malting parameters used for steeping and germination as well as the kilning regime. As summarised by Bamforth (Anness and Bamforth, 1982) an enhanced germination, via higher temperatures or by the aid of gibberellic acid result in increased SMM levels. On the other hand, inhibitors of germination, e.g. potassium bromate (outlawed in foodstuffs in most countries), reduce embryo development and rootlet growth, hence lower SMM levels in green malt (White and Parsons, 1975). Interestingly the half-life of SMM at 100°C is 38 min at a pH of 5.2, whereas a half-life of 32.5 min is reported at a pH of 5.5, indicating that the chemical decomposition of SMM is not solely temperature but also pH-sensitive (Dickenson, 1979). Furthermore, the use of a wort stripper could help to remove excessive DMS, but also purge other undesired volatiles (Bamforth, 2013). Additionally, attention should be paid to the origin and localisation of

SMM in grist materials. SMM is mainly located in the seedling which is why wort production after fine milling gives higher SMM levels than after coarse milling since the seedling remains more intact after coarse milling of the malt. However, at the end of wort boiling, no differences in levels of DMS precursor and free DMS were found between fine milled-thin bed mash filter operations and coarse milled-lauter tun operations (De Rouck et al., 2010). Additionally Heineken and Carlsberg described barley plants with combined traits of Null-LOX-1, Null-LOX-2 and Null-MMT (L-methionine S-methyltransferase) within one plant (Knudsen et al., 2011). Publications on brewing with green malt derived from malting the double-Null-LOX-null-MMT cultivar would be highly interesting regarding flavour and flavour stability.

1.8.6 Flavour and aroma compounds

Apart from being an abundant source of starch and enzymes, malt delivers a wide range of flavour and aroma components, such as aldehydes, ketones, alcohols, organic acids and furans, to the wort and final beer (Bettenhausen et al., 2018, Ditrych et al., 2019, Dong et al., 2013, Filipowska et al., 2020, Moir, 1992, Yahya et al., 2014). The formation of flavour active compounds is largely promoted through thermally driven processes, such as Strecker degradation, Maillard reaction or caramelisation. Thus, if omitting the kilning process, the brewer would introduce a grist material with a dissimilar mixture of volatile and non-volatile constituents compared to kilned malt. Moreover, green malt will most certainly induce subtle flavour changes in beers principally through its different chemical composition (compared to pale kilned malt), acting as a feedstock for yeast metabolism. As apparent in Table 1.3, hot water extracts of green malt contained a series of lipid-derived aldehydes and alkenols as well as sulphur compounds, while concentrations in worts prepared from lightly kilned malt, were much lower (Moir, 1992). Furthermore, traces of 4-vinylphenol and 4-vinylguaiacol were identified in a vacuum distillate of green malt. The final concentrations in beer were, however, far below those produced from phenolic off-flavour (POF+) producing yeasts.

Table 1.3: Lipid derived aldehydes, alkenols, sulphur compounds and phenols from malts indicated as relative amounts in each malt (Moir, 1992). GM = green malt, KM = kilned malt

Lipid-derived aldehydes			**Alkenols**			**Sulphur compounds**			**Phenols**		
	GM	KM		GM	KM		GM	KM		GM	KM
hexanal	+++	+	3-hexen-1-ol	+++	++	dimethyl sulphide (DMS)	+++	+	4-vinylguaiacol	++	++
2,4-decadienal	+++	++	2-nonen-1-ol	+++		2-methylthioacetaldehyde	++	+	4-vinylphenol	+	
2-hexenal	++	+	1-penten-3-ol	++		methional	++	+	phenol		
heptanal	++		2-penten-1-ol	++		4-methylthio-2-butanone		+	o-cresol		
2,4 heptadienal	++		2-hepten-1-ol	++		3-methylthiohexanal			p-cresol		
2-octenal	++		2,4-decadien-1ol	++					2-ethylphenol		
nonanal	++		2-hexen-1-ol	+					4-ethylphenol		
2-nonenal	++	++	1-octen-3-ol	+					4-ethylguaiacol		
2,6- nonedienal	++		2-octen-1-ol	+					eugenol		
2-butenal	+								isoeugenol		
2-heptenal	+	++									
2,4-nonadienal	+	+									

Moreover, malt is a major source of aldehydes, as well as aldehyde precursors and intermediate products (e.g. amino acids, peptides, Schiff bases, bound state aldehydes, *etc.*) (De Clippeleer et al., 2010a, De Clippeleer et al., 2010b, Ditrych et al., 2019, Dong et al., 2013), which were identified as contributors for stale flavour formation during beer ageing. Aldehydes were shown to increase in concentration during sprouting (Dong et al., 2013), dependent on germination time and temperature, according to (Herrmann et al., 2007). Higher green malt moisture significantly increased the formation of malt volatiles, whereas high germination temperatures, on the other hand, lowered them (Herrmann et al., 2007). Kilning or roasting, on the other hand greatly promotes the formation of Maillard compounds and Strecker aldehydes (Baert et al., 2012, Bamforth, 1999a, Beal and Mottram, 1994, De Clippeleer et al., 2010a, Dong et al., 2013, Drost et al., 1990, Gastl et al., 2006, Guido et al., 2005, Huang et al., 2016, Schwarz and Pyler, 1984, Vanderhaegen et al., 2006). A wide range of Maillard compounds were determined in kilned or roasted malts, with only furfural, 1-acetylfuran and furfuryl alcohol identified in green malt (Moir, 1992). LOX enzymes, on the other hand, are thermally inactivated, thus a reduction in enzymatic oxidation of unsaturated fatty acids is expected particularly at the onset of the wort production process. However, during malting, LOX activity was shown previously (Kaukovirta-Norja et al., 1998) to increase mainly in the first 2 – 6 hours of kilning, thus the risk to oxidise lipids remains at moderate kilning temperatures. Dong et al. (Dong et al., 2013), for example, showed that the *trans*-2-nonenal concentrations greatly increased when producing crystal malts compared to the corresponding green malt. Thus, it might be suggested that less *trans*-2-nonenal is introduced into the brewing process when using green malt. Hexanal and 2-hexenal, which are correlated with green/grassy scent and a bitter, winey flavour (Saison et al., 2010b), on the other hand, were shown to be present in increased levels in green malt and decreased with increasing malt colour (Coghe et al., 2004, Dong et al., 2013). Further research is needed to define which of the flavour characteristics of green malt (in comparison to pale kilned malt) survives up- and downstream processing and has a direct (positive or negative) effect on the flavour and flavour stability of the finished beers.

1.9 Characteristics of wort and beer made from green malt

Early research on brewing with green malt (Cook and Hudson, 1964, Duff, 1963, MacWilliam, 1972, Moir, 1992) reported that worts and beers from 100% green malt were perfectly normal in their analytical and physical characteristics. Unfortunately, in these papers no detailed brewing protocol or assessment of the resulting beer flavour or its' stability were published. Since most of the literature available on brewing with green malt dates back as early as the 1960's, there was great interest for the scientific community for new research, particularly as analytical techniques have greatly improved since then.

1.9.1 Wort characteristics

According to Macwilliam et al. (MacWilliam et al., 1963) the worts from green malt were more fermentable than those from kilned malt. This was related to the higher activities of α- and β- amylase in addition to the increased levels of limit dextrinase associated with green malt. Analysis of the wort carbohydrates further confirmed very high values for maltose and maltotriose at the expense of dextrins. Furthermore, proteolysis proceeded further when using green malt mashing than with conventional malts, which explained the high values for both soluble and amino nitrogen (Table 1.4). The anthocyanogen content was significantly lower in wort prepared from green malt than the control wort, possibly due to the still functioning anthocyanogenase in green malt (MacWilliam et al., 1963). The relatively high colour of the green malt wort (8-10 EBC, (MacWilliam et al., 1963)) compared to the reference (3 EBC) was associated with this increased concentration of amino acids, which caused increased formation of melanoidins during wort production. Table 1.4 displays a summary of some characteristics of worts derived from green malt in comparison to kilned malt wort. Interestingly, previous research (White and Wainwright, 1977) concluded that worts from green malt resulted in (expected) elevated DMS precursor levels, but surprisingly low levels of DMS in the pitching worts. White and Wainwright (White and Wainwright, 1976a), on the other hand, reported DMS levels up to 500 µg/L in green malt pitching wort (no precursor levels reported). Furthermore, it was found that the use of undried malts can have benefits in terms of hop economy. According to previous research (Griffin et al., 1968, Maule, 1966), the amount of humulone and isohumulone absorbed

on the break of 'unkilned' malt wort was considerably less than on that of kilned malt wort.

Table 1.4
Representative analysis of worts prepared from green malt and pale kilned malt.

	green malt	kilned malt
Specific gravity[a]	1028	1028
ph wort	5.75-6.0[a], 5.32[b]	5.6[a], 5.12[b]
Colour	8-10[a]	3[a]
Nitrogen (mg/100 ml) wort [a]	60-75	40
Amino nitrogen (% of total)[a]	40	33
Fermentability[a]	86	75
Carbohydrate recovery (%)[a]	99	98
Anthocyanogen (unit not defined)[a]	0.05-0.15	0.45
Attenuation limit corrected to 1055 in wort[b]	1011.6.	1011.0
DMS (µg/L)	150-236[c], 100-500[d]	78-260[c], 6-16[d]
DMS-P (µg/L)	822-1022[c]	271-656[c]

[a] MacWilliam et al. (MacWilliam et al., 1963), [b] Duff SR (Duff, 1963), [c] White and Wainwright (White and Wainwright, 1977), [d] White and Wainwright (White and Wainwright, 1976a)

1.9.2 Beer characteristics

Table 1.5. displays a summary of some characteristics of beers derived from green malt in comparison to kilned malt beers. Previous work has shown that the EBC colour was not reduced as much as might have been predicted for green malt brews (MacWilliam, 1972). The origin of the unique pigments, hue and chroma yet has to be determined. Furthermore, due to the high dimethyl sulphide (DMS) potential, overall DMS levels were expected to be higher compared to the control. Previous research (White and Wainwright, 1976a, White and Wainwright, 1977) indicated that wort of green malt contains high concentrations of the DMS-precursor S-methyl methionine; however, DMS levels in final beers made of green malt were not higher than in beers prepared from pale kilned malt (White and Wainwright, 1976a, White and Wainwright, 1977). It is remarkable to note that the elevated DMS levels (up to 500 µg/L) in worts as reported by White and Wainwright (White and Wainwright, 1977) were gradually eliminated with

the fermentation gases and resulted in DMS levels in beers closely matched to the control.

Table 1.5
Selected analytical data for beers brewed from green malt and pale kilned malt.

	green malt	kilned malt
pH	4.01[a]	3.98[a]
Colour (EBC)	11[b]*, 12[b$]	9[b]*,15[b$]
Specific gravity[b]	3.03*, 3.01[$]	4.38*, 4.04[$]
Head retention (half life, sec.)[b]	89*, 92**, 91[$]	98*, 81**, 106[$]
Limiting attenuation[a]	1011.8 – 1013.0	1011.5
Total carbohydrate[b]	13.3	16.7
Residual fermentable sugars (g/L)[b]	1.1	2.8
Non-fermentable carbohydrate (g/L)[b]	12.2	13.9
Nitrogen (g/L)	0.72-0.80[a], 0.54[b] *, 0.53[b $]	0.68[a], 0.62[b]*, 0.65[b$]
DMS (µg/L)	31-38[c], 30-70[d]	61-84[c], 50-95[d]

[a] Duff (Duff, 1963), limiting attenuation and nitrogen content corrected to an original gravity of 1055, [b] MacWilliam et al. (MacWilliam, 1972) * = at bottling, **5 weeks after bottling, [$] draught, [c] White and Wainwright (White and Wainwright, 1977), [d] White and Wainwright (White and Wainwright, 1976a)

Even though these results seem very promising for the successful brewing of green malt there were still some substantial flavour differences that yet must be defined. Early studies (Cook and Hudson, 1964, MacWilliam, 1972) who described 100% green malt beer, rather vaguely described their beer brewed from green malt 'green-malt-like'. The intensity of 'green flavour' increasing with increasing malt germination time. The flavour was further described as 'unpredictable', meaning that it was sometimes clean and other times 'green' (Cook and Hudson, 1964). The precise nature and sensory stimuli causing this reported 'green' sensation in green malt beers were not defined, but it is likely that these 'green' flavour is related to lipid-derived aldehydes abundantly present in green malt, as discussed previously (Moir, 1992). Moir et al. (Moir, 1992) detected a grassy, beany taste in beers made of green malt, and proposed that this could have been a result of elevated levels of lipid-derived aldehydes. Although yeast is able to remove these aldehydes by reducing them to their saturated alcohol counterparts, the green grassy, pea-like character remained to some extent in green malt beer, with elevated

amounts of 1-hexanol (reduction of hexanal and 2-hexanal) being detected (Moir, 1992). Another possible route to explain the green flavours are the high number of alkenols found in green malt wort, formed by the action of a thermo-labile reductase. Yeast is not able to reduce the alkenols, which then remain in the beer (Moir, 1992).

1.9.3 Beer flavour (in)stability factors

Without the high temperatures from kilning, the heat load of the malt and future mash, wort and beer will be significantly lower. Additionally, the extra enzymatic potential of green malt, could potentially be suitable for reducing overall mashing process time, and thus implies additional reduction in total heat load. Heat load, expressed as TB-index, was correlated with the formation of beer staling compounds and reduced beer freshness (Baert et al., 2012, Gastl et al., 2006, Huang et al., 2016, Jaskula-Goiris et al., 2015). Thus, as discussed previously (Section 1.8.6) from the perspective of green malt brewing, less aldehydes are introduced into the brewing process (except hexanal). It is generally accepted that these aldehydes are greatly evaporated throughout wort production (except of furfural)(Ditrych et al., 2019) and yeast metabolism can reduce aldehydes in the wort to their corresponding alcohols (Debourg et al., 1994, Peppard and Halsey, 1981). However, aldehydes can bind to compounds such as bisulphites (Kaneda et al., 1994, Lermusieau et al., 1999) amino acids (formation of imines) (Lermusieau et al., 1999, Liégeois et al., 2002) or cysteine (Baert et al., 2018, Baert et al., 2015a, Baert et al., 2015b, Bustillo Trueba et al., 2019) during the wort production process, forming non-volatile 'bound-state aldehydes'. The current theory is that during beer storage, under specific conditions (temperature, pH value, redox potential, binding strength, thermodynamic stability), adducts may dissociate and release aldehydes in the free form (Baert et al., 2018, Bustillo Trueba et al., 2018, Debourg et al., 1994, Drost et al., 1990, Kaneda et al., 1994, Lermusieau et al., 1999, Liégeois et al., 2002, Perpète and Collin, 2000, Saison et al., 2010a). Naturally, the formation of bound-state aldehydes is very complex, but in principle, fewer aldehydes might be available for adduct formation during the wort production process when using green malt, and thus, an improved endogenous ageing potential in green malt beers, may be hypothesised.

Furthermore, several authors (Cortés et al., 2010, Furukawa Suárez et al., 2011, Kunz et al., 2013) suggested that an increased formation of Maillard reaction products was

associated with an acceleration of oxidative processes, and thus lower oxidative stability. Kunz et al. showed (Kunz et al., 2012a), that when using unmalted barley (lower total heat load) a lower content of specific Maillard reaction products led to a lower radical generation and thus better oxidative stability in the worts and beers measured. Green malt contains fewer Maillard reaction products due to the omission of the heating step, thus better oxidative stability might be expected in green malt wort and beer according to this hypothesis. Moreover, the pool of natural antioxidants which is enhanced in green malt could (Özcan et al., 2018) potentially reduce the formation of free radicals. Hence, from this perspective, green malt wort could have a better oxidative stability than kilned malt wort, provided lipoxygenase activity can be controlled.

Transition metal ions, such as iron, copper and manganese, were identified to play a key role in the oxidative degradation of wort and beer, as they drive formation of reactive oxygen species (ROS) in the absence of antioxidants (Andersen and Skibsted, 1998, Lund et al., 2015). Increased heat load on malt through kilning or roasting was shown to impact the content of transition metals with prooxidative effects in the wort (Hoff et al., 2012, Jenkins et al., 2018, Pagenstecher et al., 2020, Poreda et al., 2015). Thus, it would be very interesting to further investigate the ionic composition and thus influence on oxidative stability when using green malt as the grist bill.

Furthermore, lower heat loads during brewing have been associated with improved free amino acid (FAN) assimilation during fermentation (De Rouck et al., 2007), resulting in lower residual FAN levels in finished beers, which was associated with an improved beer flavour stability.

Overall, green malt appears to be a very promising grist material to produce wort and beer with enhanced flavour stability metrics - provided lipoxygenase activity is controlled.

1.10 Overview of thesis content

The overall aim of this research project was to demonstrate the feasibility of brewing with 100% green (germinated, but not dried) malt and propose solutions to some perceived technical and biochemical (flavour) barriers. Likewise, the focus was to determine the impacts of brewing with the addition of green malt on beer flavour

stability. This is important to establish as such a process will either improve flavour stability (reduced heat load should reduce the pool of staling aldehydes) or worsen it (since lipoxygenase activity and DMS potential can be regulated by heat treatment during kilning). The introduction (Chapter 1) focused on the main outline of the malting and the brewing process, as well as the main challenges, but also benefits of brewing with green malt. The experimental part of this thesis is separated into four parts. The project started (Chapter 2) by investigating the key quality concerns when brewing with green malt: i) DMS potential, ii) Lipoxygenase activity and iii) rootlet removal. The ultimate goal was to find preliminary solutions to overcome and avoid lipoxygenase related off-flavours in subsequent pilot scale brewing trials. In Chapter 3, pilot scale trials were performed to determine the quality of wort and beer using green malt as a raw material. Furthermore, a series of analytical techniques were performed to identify the standard analytical parameters, as well as quality indicators of wort and beer produced from green malt. The results were compared to wort and beer produced from a kilned malt control (pilsner style). In Chapter 4, a special focus was set on the flavour (in)stability markers of the beers produced and described in the Chapter 3. Furthermore, the influence of heat load on malt was investigated in relation to beer flavour instability. Hereby a focus was set on aldehydes from malt to the finished fresh beer. Additionally, the oxidative stability was determined in the worts and beers produced. The last experimental part, Chapter 5, was designed to identify beer chemistry changes in green malt as well as the corresponding control beers during forced ageing at 30°C for 30, 60 and 90 days. The overall conclusions and suggestions for further work are presented in Chapter 6.

The study presented in Chapter 2 has been published and corresponds to:
Dugulin CA, Clegg SC, De Rouck G, Cook DJ. 2020. Overcoming technical barriers to brewing with green (non-kilned) malt: a feasibility study. J Inst Brew 126:24-34. https://doi.org/10.1002/jib.602.

The study presented in Chapter 3 has been accepted at the Journal of the Institute of Brewing and corresponds to:
Dugulin CA, Acuña Muñoz LM, Buyse J, De Rouck G, Bolat I, Cook DJ. 2020. Brewing with 100 % green malt – process development and key quality indicators. J Inst Brew 126:343-353. https://doi.org/10.1002/jib.620

The study presented in Chapter 4 and 5 is written as paper manuscript but has yet to be published.

In all cases, the first author conducted, analysed and drafted the published manuscript under the guidance and with critical input from the co-authors.

2 Overcoming technical barriers to brewing with green (non-kilned) malt: a feasibility study.

2.1 Introduction

If omitting the kilning process entirely, the brewer must brew with freshly germinated (green) malt, which introduces new technical challenges, but offers the reward of significantly lower energy and water usage. However, apart from being a dominant consumer of heat and electricity (Davies, 2010), the kilning process has many beneficial quality impacts on malt quality, such as reduction of lipoxygenase activity (De Buck et al., 1997, Doderer et al., 1992, Yang and Schwarz, 1995, Yang et al., 1993), regulation of S-methyl methionine (SMM) levels (White and Wainwright, 1976a, White and Wainwright, 1976b, White and Wainwright, 1977), facilitating rootlet removal and most importantly in developing the characteristic colour and flavours which malt imparts to beer. On the other hand, green malt, rich in diastatic enzyme activity, can very efficiently convert the starch of unmalted grain into fermentable sugars (Duff, 1963, MacWilliam et al., 1963).

One major quality concern when handling green malt is the elevated activities of both lipoxygenase isoenzymes (LOX-1 and LOX-2; (De Buck et al., 1997, Yang and Schwarz, 1995)). Even relatively low activities of lipoxygenase in kilned malt are known to significantly influence flavour stability via enzymatic lipid oxidation (Hirota et al., 2005, Skadhauge et al., 2005). LOX enzymes can oxidise the unsaturated fatty acids, principally linoleic acid in barley, to hydroperoxy acids in the presence of oxygen. Hydroperoxy acids can be further transformed via several enzymatic pathways (Baert et al., 2012) to mono-, di-and trihydroxy fatty acids and can eventually be degraded non-enzymatically into flavour active carbonyls, such as *trans*-2-nonenal or hexanal, which are examples of beer staling compounds (Kuroda et al., 2003, Schwarz and Pyler, 1984, Yang and Schwarz, 1995). Furthermore, lipoxygenase worsens the foam stability of beer, possibly due to the production of trihydroxy octadecenoic acid (THOD), which is detrimental to foam stability (Hirota et al., 2006, Yu et al., 2014).

Furthermore, one important factor has been neglected in prior research – the rootlets of green malt. The rootlets of green malt are particularly rich in lipoxygenase (Schwarz and Pyler, 1984, Yang et al., 1993) and SMM (White and Wainwright, 1976a). Rootlets of kilned malt are hygroscopic, due to their fibre content of up to 15%, can highly absorb oil and have emulsification capacities (Salama et al., 1997). However, the antioxidant capacity of rootlets obtained from kilned malts has also been investigated (Bonnely et al., 2000, De-Jing et al., 2009, Peyrat-Maillard et al., 2001). The antioxidant potential, due to the high content of antioxidant phenolic compounds, could potentially reduce the formation of free radicals, thus becoming a source for natural antioxidants, favouring wort and beer flavour stability. Nevertheless, malt rootlets are considered to impair the flavour of beer, hence maltsters try to avoid excessive rootlet growth during germination (in order to minimise malting losses) and remove the rootlets, by abrading them after kilning, with an associated malting loss of around 4%. The rootlets are then usually sold as animal feed or organic fertiliser. Adequate removal of rootlets from green malt is problematic due to the high moisture content, meaning they will not form a malting loss and if untreated remain on the grain. Thus, rootlet composition needs to be considered before starting to brew with green malt.

To avoid an increased staling potential in the final beer a minimum requirement when brewing with green malt is that mashing needs to occur in a lipoxygenase hostile environment, mashing in at > 63 °C, at a pH in the region of 5.3 under oxygen-limited conditions (Baert et al., 2012, Bamforth, 2004, Drost et al., 1990, Van Waesberghe et al., 2001). Most certainly the usage of green malt for conventional brewing processes requires alternative techniques to reduce total LOX activity.

The research reported in this chapter aims to evaluate the feasibility of brewing using freshly germinated (green) malt, with omission of the kilning step. Here, the laboratory scale development of such a process is reported to enable evaluation of the significant quality impacts on the brewing process and finished beer. Attention was first directed to control lipoxygenase activities through its limiting factors: heat-sensitivity, pH-sensitivity (De Buck et al., 1995, De Buck et al., 1997, Doderer et al., 1992) and the availability of oxygen as a substrate. Those “weaknesses” could help to control LOX when brewing with green malt. Additionally, the quality of wort has been evaluated and

compared to wort produced from kilned malt, with a special focus on SMM levels. This knowledge will enhance the understanding of key quality concerns as well as potential benefits of using green malt and will form the basis for subsequent pilot-scale brewing trials.

2.2 Materials and methods

2.2.1 Malt samples

Barley variety Flagon (2-row, winter sown) was sourced from Crisp Malting Company, UK. High-purity water from a Water Purification Systems (SUEZ Water, Thame, UK) was used for all chemical analysis and for glassware washing.

2.2.2 Chemicals and reagents

Lipoxygenase activity: sodium acetate, sodium chloride, orthoboric acid and dibasic sodium phosphate were acquired from Sigma-Aldrich (Dorset, UK). Polyoxyethylenesorbitan monolaurate (Tween 20), linoleic acid (> 99%), Brij 99 (polyoxyethylene(20)-oleyl-ether) and sodium dihydrogenphosphate dihydrate were obtained from Fisher Scientific (Loughborough, UK). Acetic acid (glacial), sodium hydroxide and hydrogen chloride were obtained from VWR (UK). *Nonenal potential*: carbon disulphide (anhydrous > 99%), *trans*-2-nonenal (> 97%), hexanal (98%), 3-heptanone (> 98.5%), orthophosphoric acid (85%) were purchase from Sigma-Aldrich (Dorset, UK). *Dimethyl sulphide (DMS):* dimethyl sulphide (≥ 99%) and ethyl methyl sulphide (96%) were obtained from Sigma-Aldrich (Dorset, UK).

2.2.3 Malt and wort preparation methodology

Barley (500 g) was screened over a 2.2 mm sieve and put into a micro malting cage and malted in a Custom Lab micromaltings K steep germinator and kiln (Curio Malting, Milton Keynes, UK). Typical process parameters were as follows: Barley was steeped at 16°C using an automated program of alternating wet (immersed) and air rests designed to reach a steep-out moisture content of 46%. A '3-wet' steep cycle was used with the following cycle times (43 h in total): 7 h wet stand, 12 h air rest, 8 h wet stand, 12 h air rest and 4 h wet stand. Germination was conducted for 5 days at 12°C with automatic turning of the sample cages set at 1 min in every 10 min. Kilning: The air-on temperature

during drying was programmed as follows: 55°C for 12h, 72°C for 4 h and 80°C for 4 h. Malt rootlets were removed using a benchtop deculmer (Curio Malting, Milton Keynes, UK).

2.2.3.1 Production of 'endosperm-rich' extracts of green malt

To further investigate the properties of green malt rootlets, well-germinated malt was separated into an endosperm-rich and husk/rootlet fraction, with only the endosperm-rich fraction being used for conventional mashing. To use the same amount of green malt as in the standard mash beaker using 50 g of kilned malt, an adjustment for the higher moisture content in green malt was made. Approximately 10 g of green malt were weighed, and both the fractions (%) of rootlets and corn were determined by manually removing the rootlets from the corn. Based on a kilned malt value of 5% moisture 50 g would have a dry weight content of 47.5 g.

$$\frac{47.5}{dry\ weight\ of\ corn\ fraction} * 100 = total\ corn\ fraction\ wet\ weight$$

$$\frac{(total\ corn\ fraction\ wet\ weight)}{fraction\ corn\ (\%)} * 100 = total\ green\ malt\ weight$$

To separate the endosperm-rich fraction from the husk/rootlet fraction, the green malt was passed through an automated pasta roller (Marcato s.p.a., Atlas Motor, Italy) and gently squeezed into a Duran bottle (500 mL, Thermo Fisher Scientific, UK) to which 100 mL of water (20°C) was added. The bottle was sealed and placed on a roller bed (Bibby Scientific™Stuart™ Digital Tube Roller, UK) set at maximum speed for 15 minutes. The extract was filtered through a muslin cloth filter, and the grain residue washed with 100 mL RO water and placed again on the roller bed. This washing step was performed 4 times in total for 15 min with a total RO water volume of 400 mL. After the last wash the grain residues were poured into the muslin cloth filter and squeezed using the cafetière; the extract being used for mashing.

2.2.3.2 Procedure for mashing using kilned malt, green malt and extracts of green malt

For laboratory mashing trials the amounts of malt used for each of the different samples were matched on a dry weight basis to compensate for their widely differing moisture contents. Each mash beaker and the mashing liquor was preheated in a water bath for 10 to 15 minutes. The endosperm-rich extract of green malt (400 mL, 20°C) was transferred to a mash beaker and placed in a mash bath (1-Cube s.r.o, Czech Republic), 10 min prior to starting the mash-in protocol, to equilibrate the temperature to the same mash-in temperature of 63°C, as in the other samples. Kilned malt was milled using a laboratory DFLU disc mill (Buehler Miag, Uzwil - Switzerland); green malt was milled using a coffee grinder (De'Longhi KG49 Coffee Grinder, Hampshire, UK). A 'lipoxygenase hostile' mash schedule was performed under conditions designed to minimise LOX activity: Mashing in at 63°C, pH: 5.2 using de-aerated liquor (achieved by purging the water used for mashing with nitrogen prior to processing). Detailed mashing scheme was as follows: 63°C (30 min), 72°C (20 min) 78°C (1 min); rise in temperature at 1°C/min. The weight of the content of the beaker was adjusted to 450 ± 0.2 g by addition of water and filtered using filter paper (Whatman, grade 2555 ½ prepleated 320mm, Sigma-Aldrich, UK). The first filtrate of 100 mL was returned to the funnel in order to establish the filter bed.

2.2.4 Malt analysis

The moisture content of malt samples was measured by mass loss on drying according to Analytica EBC method 4.2.

2.2.4.1 Determination of Alpha- and Beta- amylase activity

Malt samples were analysed to determine the activity of α- and β-amylase, the two key diastase enzymes required to break down starch in subsequent brewing processes. Malt α-amylase was measured using the Ceralpha Megazyme kit (Megazyme, Bray, Ireland), and reported as Ceralpha Units. Malt β-amylase was determined using the Betamyl-3 kit (Megazyme, Bray, Ireland) with results expressed in betamyl units (BU). Results are reported throughout on a dry weight basis.

2.2.4.2 Determination of the total lipoxygenase activity in malt

In this study the LOX activity was determined by a spectrophotometric technique based on a combination of the methods of Guido et al. (Guido et al., 2005) and De Buck et al. (De Buck et al., 1995, De Buck et al., 1997). The oxidation of linoleic acid by LOX increases the production of conjugated diene which absorb at 234 nm. Milled barley or malt (5 g) was dispersed in acetate buffer (0.1 M, pH 5), containing the non-ionic detergent Brij 99 (0.1%) and stirred for 30 min. The homogenate was centrifuged (9632 x *g*, 5 minutes, 4°C) and the total LOX activity was determined spectrophotometrically using the supernatant as crude extract. To prepare substrate solution, 250 µL linoleic acid was dispersed by homogenisation in 5 mL borate buffer (25 mM, pH 9.0) with Tween-20 (0.25% v/v), NaOH (1 M, 0.65 mL) and cold RO water (3.85 mL) to facilitate dispersion. The total LOX activity was determined spectrophotometrically by adding 50 µL of the enzyme extract to 50 µL of the air-saturated substrate solution in 2.90 mL of sodium phosphate buffer (0.1 M, pH 6.8), equilibrated at 30 °C in a total volume of 3 mL in UV-cuvettes (Plastibrand disposable Macro plastic 2.5 mL, Fisher scientific, UK). The formation of a conjugated diene of the hydroperoxide as a result of LOX oxidation of linoleic acid was determined by measuring the absorption at 234 nm, using a UV/Vis Spectrophotometer (7315 UV/visible Spectrophotometer, Jenway, UK) and absorption was measured exactly 2 minutes after the addition of the enzyme, and then after 6 minutes. For the reference cell, the enzyme solution was replaced by buffer (2950 µL buffer and 50 µL substrate solution). The LOX activity correlates to the absorbance and is expressed in enzyme U/per gram of malt on a dry basis (U/g d.b.).

The equation used to determine the lipoxygenase activity was

$$Lipoxygenase\ activity\ \left(\frac{\mu mol}{min}\right):$$

$$\frac{Abs}{min} \times \frac{V \times 10^6 \times \mathrm{F} \times \mathrm{V'}}{\mathrm{V''} \times \Sigma \times \mathrm{d} \times 1000 \times \mathrm{M})} \times \frac{100}{(100 - H)}$$

in which ABS/min = slope variation of absorbance during time (between 2 and 6 min)
V = volume of the cell (3 mL)
F = dilution factor
V′ = volume of extraction buffer (50 mL)
V″ = volume of enzymatic extract (0.05 ml)
Σ = molar absorption coefficient (28,000 L/ (mol x cm)

d = light path (1 cm)
M = sample weight of milled barley, kilned malt or germinating malt (g)
H = moisture of malt (%)

2.2.4.3 Determination of DMS in grain samples

DMS was determined in grain samples by headspace SPME using a SCION 456-GC (Bruker, UK) fitted with a Combi PAL autosampler and controlled with Compass CDS software. The GC was equipped with a PTV injector and a pulsed flame photometric detector operated in sulphur mode. The column used was ZB-1MS (60m x 0.25 mm (I.D) – 1.00 µm film thickness; Phenomenex, USA) and nitrogen (BOC, UK) was used as a carrier gas at 1.0 mL/min. The inlet temperature was set at 250°C. The oven temperature was kept at 40°C for 7 min, then raised to 110°C at 7°C/min then raised to 190°C at 11.0°C/min and then to 235°C at 22°C/min and held for 6 minutes. The PFPD detector was set at 210°C and 600 V with air 1 flow at 17 mL/min, air 2 flow at 10 mL/min and hydrogen flow at 13.0 mL/min.

Extraction: Kilned and green malt samples (5 g) were extracted based on the ASBC method (Malt-14). Results are based on the weight used per dry weight. After sample preparation, the vial was pre-equilibrated for 10 min at 35°C. The SPME needle was then inserted through the PTFE/silicone septum (1.3 mm) and the PDMS/DVB fiber (Stableflex, 65 µm, Supelco, USA), previously conditioned for 2 min at 300°C, was exposed to the headspace for 10 min with agitation at 250 rpm. Quantification was performed by running an external calibration series (0.1, 0.5, 1, 2.5, 5, 10 µg/L) and the use of ethyl methyl sulphide (EMS, 1 µg/L) as internal standard. If necessary, samples were diluted by an appropriate dilution factor to allow for quantification within the linear range of the calibration curve.

2.2.4.4 Determination of S-methyl methionine in grain

The SMM determination in this paper was based on the original method proposed by White and Wainwright (White and Wainwright, 1976b) following the altered protocol described by De Rouck et al. (De Rouck et al., 2010) without the usage of NaOH to avoid side formation of oxidised products such as DMSO and $DMSO_2$. Since SMM is heat labile, its content in malt is commonly measured by the subtraction of free DMS from total DMS. During heating the non-volatile DMS precursor was converted into DMS which

allows this indirect quantification. Additionally, in contrast to the proposed ASBC method, the internal standard was added after (rather than before) heat treatment. Preliminary tests indicated a loss of almost 50% in EMS peak area, which did not occur when EMS was heated in water, suggesting that heating EMS in wort leads to side reactions.

2.2.5 Wort analysis

Wort specific gravity and density were analysed using an Anton Paar DMA 4500 (UK). Extract yield was calculated according to Analytica EBC Method 4.4).

2.2.5.1 Determination of free amino nitrogen in wort

The free amino nitrogen content (FAN) in wort was determined using colourimetry with ninhydrin based on the EBC method-8.10. The colour reagent was prepared by mixing disodium hydrogen phosphate (10 g, $Na_2HPO_4 \times 12\ H_2O$), potassium dihydrogen phosphate (6 g, KH_2PO_4), ninhydrin (0.5 g) and fructose (0.3 g) in 100 mL water. The pH was adjusted to 6.7 ± 0.1. This mixture was protected from light while stirring until completely dissolved.

Wort and degassed beer sample (20 µL) were mixed with 1.98 mL of mQ water. The blank was prepared by using only water (2.0 mL). Additionally, one sample was prepared using 20 µL of a glycine stock (1.072 g/L) to 1.98 mL mQ water. After dilution, 1 mL of colour reagent was added to the test tubes and vortexed briefly. The test tubes were placed in a dry heater for exactly 16 minutes. Afterwards the tubes were briefly vortexed and cooled down in a cold water bath for 20 minutes. Subsequently, 5 mL of dilution buffer (2 g of KIO_3 in 600 mL of mQ water and 400 mL of 96% ethanol) were added and vortexed. The absorbance was determined at 570 nm. The concentration of FAN in mg/L was calculated based on the absorbance of the test solution (Abs_{sample}), the amount of free amino nitrogen in the glycine standard solution (2 mg/L) and the dilution factor (100), relative to the measured absorbance of glycine ($Abs_{glycine}$).

2.2.5.2 Determination of DMS and S-methyl methionine in wort

DMS and indirectly SMM in wort were determined according to the above SPME-GC-PFPD methodology for malt analysis. Samples were prepared in a total volume of 5 mL in a headspace vial (20 mL; Agilent, UK), using an appropriate dilution factor to remain within the calibration curve. DMS concentrations were determined based on the

external calibration series (0.1 - 10 µg/L) and the internal standard, ethyl methyl sulphide (EMS, 1 µg/L).

2.2.5.3 Determination of the nonenal potential

The nonenal potential, an indicator of how a beer will release *trans-2-nonenal* during storage, was determined using gas chromatography–mass spectrometry (GC-MS) based on the method of Drost et al. (Drost et al., 1990) and the protocol described by Guido et al. (Guido et al., 2005). Filtered wort (150 mL, adjusted to pH 4, using 5% orthophosphoric acid) was purged for 5 min with nitrogen (99.5%, Air Liquide, BOC, UK) to reduce the oxygen level. The sample was subsequently heated at 100°C for 2 h under constant nitrogen purging and then placed on ice to cool down. The liquid–liquid extraction of nonenal was performed on a 70 mL aliquot of the wort, using carbon disulphide (3 mL), as well as 7 g of NaCl for a salt-induced phase separation. The mixtures were shaken for 30 min on a rotary action shaker. Then the sample was placed in ice for around 10 minutes to condense the carbon disulphide, subsequently transferred into a separating funnel (100 mL, Fisher Scientific, UK), and set aside to allow for the complete separation of the two immiscible solvent phases for 15 minutes. The lower (solvent) layer was separated into a 10 mL glass vial with cap, placed into a 50 mL falcon tube and centrifuged at 4704 x *g* for 10 min. The resultant carbon disulphide extract was removed using a glass syringe and analysed using an ISQ 7000 GC-MS system (Thermo Fisher Scientific, UK), fitted with an instant Connect SSL Injector for TRACE 1300 GC Series (Thermo Scientific) and a ZB-wax polar column (Phenomenex, Macclesfield, UK; 30 m x 0.25 mm ID with a 1 µm film thickness) was used. The carrier gas was helium (BOC, UK) at a set pressure of 18 psi. The mass data were collected in full scan mode with a scan range from m/z 35 to m/z 250. Compounds were analysed using selected ion mode and quantified by comparing the peak area of the selected compounds with the peak area of the internal standard 3-heptanone, as well as an external standard series run for *trans*-2-nonenal and hexanal (0.01 – 10 mg/L). The selected ions were as follows: *trans*-2-nonenal m/z 70, 96, 111; 3-heptanone m/z 57, 114; and additionally, hexanal m/z 56, 82.

2.2.6 Statistical analysis

All samples were analysed in at least three biological replicates with 2-4 technical replicates. The experimental design software used was Design-Expert, a statistical software package from Stat-Ease (Stat-Ease Inc., USA). SPSS Statistics software version 24 (IBM Corp.) was used for statistical analysis. Statistical significance of the data obtained was established with analysis of variance (ANOVA), a p-value below 0.05 was considered as statistically significant.

2.3 Results and discussion

2.3.1 Grain analysis

As a result of water removal during kilning the rootlets became brittle and could be removed using the desktop deculmer. Rootlets from green malt, however, did not form part of the malting loss. As displayed in Table 2.1, well-germinated (120 h) green malt consisted of about 14% rootlets (fresh weight), and the rootlets had a moisture content of around 66%. Malt rootlets, are considered to impair the flavour of beer, mainly due to their high content of lipoxygenase isoenzyme 2 (Yang et al., 1993) and their SMM content (White and Wainwright, 1976a). Therefore, in subsequent experiments the relative merits of mashing with or without rootlets present were investigated, by developing a laboratory protocol for preparing endosperm-rich extracts of green malt, separated from the husk and rootlet fraction (Section 2.2.3.1).

Table 2.1: Proportions by mass of kernel and rootlets and the moisture contents of each in green malt after 120 h germination.

Sample	**weight (% of green malt)**	**MC (%)**
green malt	100	44.7 ± 1.6
kernel	86.0 ± 0.5	39.2 ± 0.1
rootlets	14.0 ± 0.5	66.1 ± 3.6

Data are the mean ±SD of 3 biological with each 2 technical replicate measurements.

2.3.2 Enzymatic activity in well germinated green malt

The development of lipoxygenase activity (Figure 2.1) and SMM levels (Figure 2.2) were monitored across the malting process. In a parallel experiment under identical malting conditions, a sample of germinating malt was taken each day and the developing rootlets were excised by hand prior to analysis so that the impacts of the rootlets on development of LOX activity and SMM levels in the germinating grain could be ascertained. Figure 2.1 illustrates that incoming barley had a total lipoxygenase activity of 4.8 ± 0.3 U/g d.b., which relates to the activity of LOX-1, which is already present in unmalted barley (Kaukovirta-Norja et al., 1993, Yang and Schwarz, 1995, Yang et al., 1993).

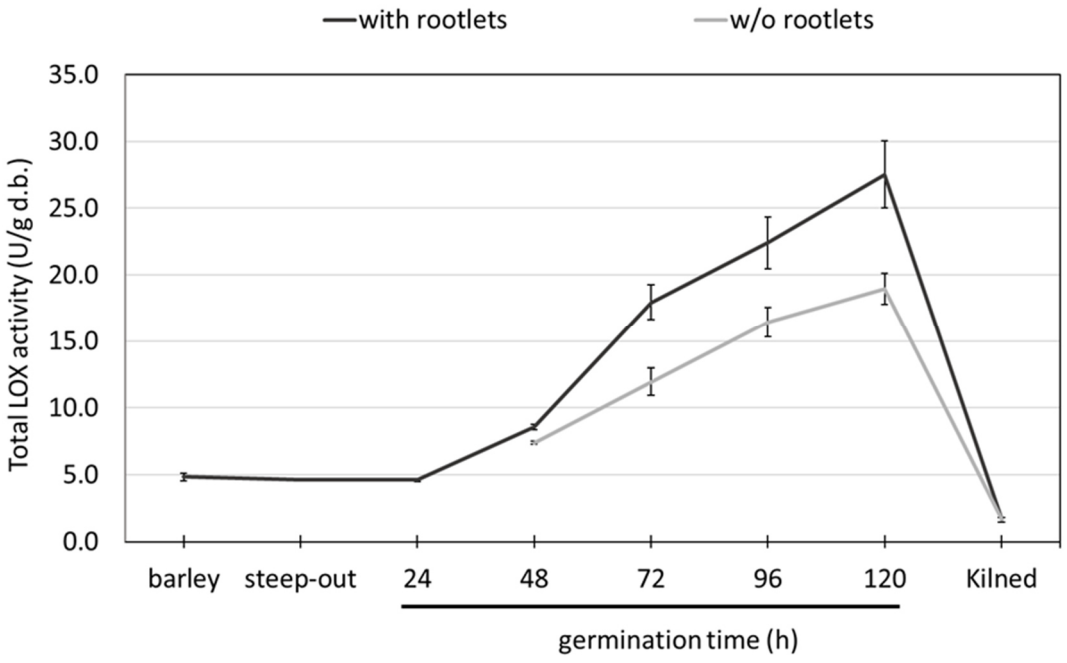


Figure 2.1: Lipoxygenase activity monitored during germination in the malting barley variety Flagon with/without rootlets. Data are the mean ±SD of 3 biological and 3 technical replicate measurements.

During malting the lipoxygenase activity started to increase significantly after 24 h of germination. Both isoenzymes are known to increase in activity during germination (Edward et al., 1981, Schwarz and Pyler, 1984). After 120 h of germination a total LOX activity of 27.5 ± 2.5 U/g d.b. was determined, which was reduced by kilning to 1.6 ± 0.2

U/g d.b. After removing the rootlets of the well germinated (120 h germination time) green malt (18.9 ± 1.2 U/g d.b) a significant reduction of about 30% in lipoxygenase activity was measured. According to previous research (Yang and Schwarz, 1995, Yang et al., 1993), only LOX-2 is in the malt rootlets, thus a large proportion of LOX-2 would be removed with the rootlets before mashing if such a procedure was applied.

A similar pattern was observed when monitoring the SMM development (Figure 2.2). The amount of SMM, (expressed as DMS equivalents), increased significantly between 24 h (2.5 ± 0.2 µg/g d.b.) and 48 h (11.1 ± 1.1 µg/g d.b.) of germination. Levels further increased up to 12.6 ± 2.5 µg/g after 120 h of germination and significantly dropped after kilning (2.6 ± 0.5 µg/g d.b.).

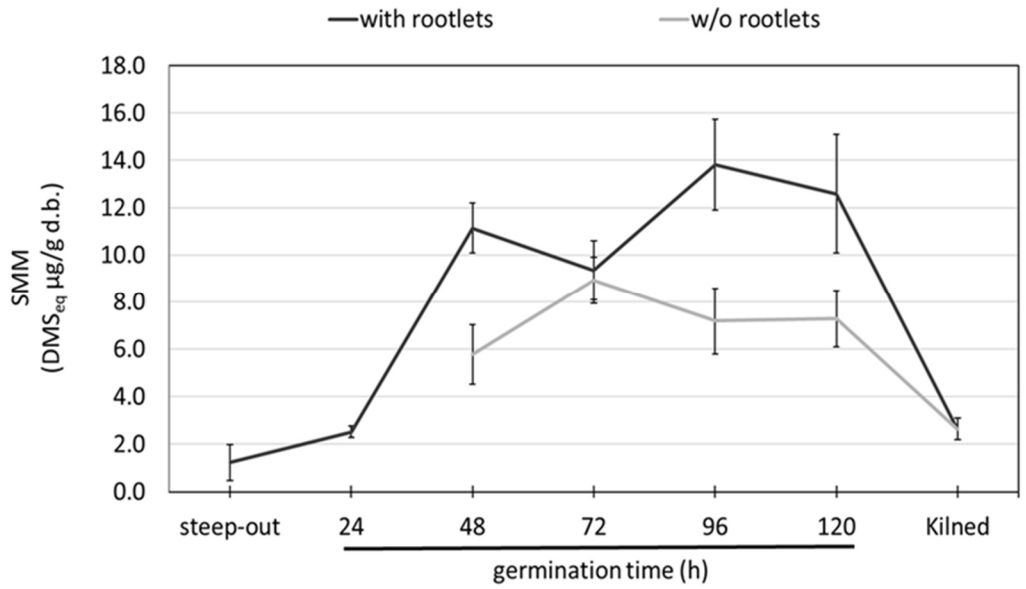


Figure 2.2: S-methyl methionine, expressed as DMS equivalents (µg/g d.b.) monitored during germination in the malting barley variety Flagon with/without rootlets. Data are the mean ±SD of 3 biological with each 3 technical replicate measurements.

When removing the rootlets, the DMS precursor in green malt significantly reduced to 7.3 ± 1.2 µg/g, an average decline of about 40% by removing the rootlets. These data for LOX and SMM, suggest that rootlets are a major concern when brewing with green malt and that their influence on quality needs to be further investigated.

Diastatic enzyme activities are of key concern to the brewer. Figure 2.3 displays the development of α- and β-amylase activities through the same micromalting process. β-amylase, which is present in bound form in unmalted barley, suffered a considerable loss during kilning from 15.3 ± 0.3 BU. to 9.7 ± 0.5 Units after kilning, whereas α-Amylase, in accordance with the literature (Hämäläinen and Reinikainen, 2007) was more thermostable without considerable enzyme activity loss across kilning.

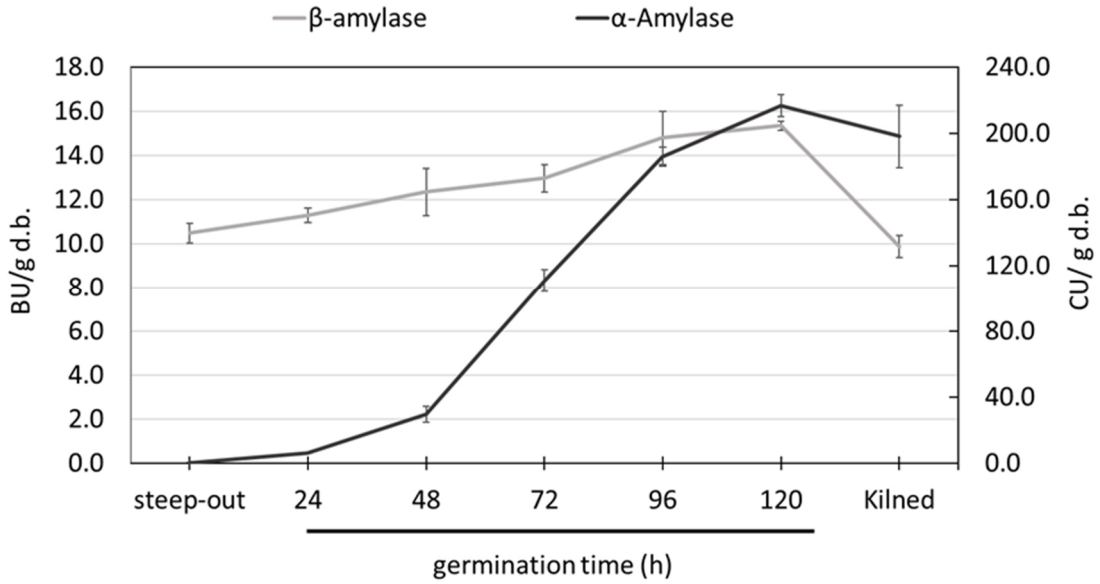


Figure 2.3: α- and β-amylase activity monitored during germination in the malting barley variety Flagon with rootlets; β-amylase (Betamyl-3®Units; BU) is displayed on primary axis whereas α-amylase (Ceralpha-Units; CU) is displayed on the secondary axis. Data are the mean ±SD of 3 biological and 2 technical replicate measurements.

These results support the hypothesis (Cook and Hudson, 1964, Duff, 1963, MacWilliam et al., 1963), that there is a good potential to generate highly fermentable worts using green malt.

2.3.3 Approaches to limit the lipoxygenase activity in green malt

Whilst the LOX activity in kilned malt is already low, previous research indicated that even this residual activity accelerated beer staling (Skadhauge et al., 2005). The increased LOX activity in green malt, as illustrated in Figure 2.1, is a primary concern in terms of beer flavour and flavour (in)stability. Thus, the usage of green malt for conventional brewing processes requires alternative techniques to reduce total LOX activity. If LOX activity can be minimised at source, through adequate malting and/or

mashing conditions, significant off-flavours in the resultant beer could be avoided. The hypothesis was to control lipoxygenase via its limiting factors: heat sensitivity, pH sensitivity, oxygen availability.

2.3.3.1 Short heat treatment of green malt

The high moisture content of green malt in combination with low heating temperatures (30-40°C) at the onset of kilning are promoting lipoxygenase activity (Guido et al., 2005). With increasing temperatures, the thermal stability of LOX is greatly reduced (Schwarz and Pyler, 1984, Yang and Schwarz, 1995). Preliminary lab-scale trials have identified heat treatments of malt at temperatures of 65°C or higher to be promising in reducing lipoxygenase activity, even after very short heating periods of maximum 10 minutes. Experimental design software (Design-Expert v 11, Statease, Minneapolis, MN, USA), was used to produce a response surface design with two numerical variables (time, 5-60 min; temperature, 65°C-90°C). The design consisted of 18 heat treatments of green malt, arranged into 3 blocks (according to different batches of green malt). Green malt was subjected to short heat treatments in a convection oven according to the 18 different combinations of time and temperature within the above time/ temperature ranges and as determined using the experimental design software. The run order was fully randomized within the design. Resulting data for LOX activity, α- and β-amylase were modelled across the design space resulting in either quadratic or two factor interaction (2FI) predictive models. Contour plots of these models are shown in Figure 2.4. The corresponding data points can be found in the Appendix.

Figure 2.4A shows a 2D contour plot of the derived model for lipoxygenase activity as a function of time (5-60 min) and temperature (65°C -90°C). Both heating time and temperature were significant factors in the model for LOX activity across the design space ($p < 0.0001$). Not surprisingly the LOX activity decreased as both temperature and the time of heat treatment of the green malt increased. Figure 2.4B and 5C indicate the potential to likewise reduce diastatic enzyme activities, suggesting that care must be taken not to destroy desirable enzyme activities by using excessive temperatures.

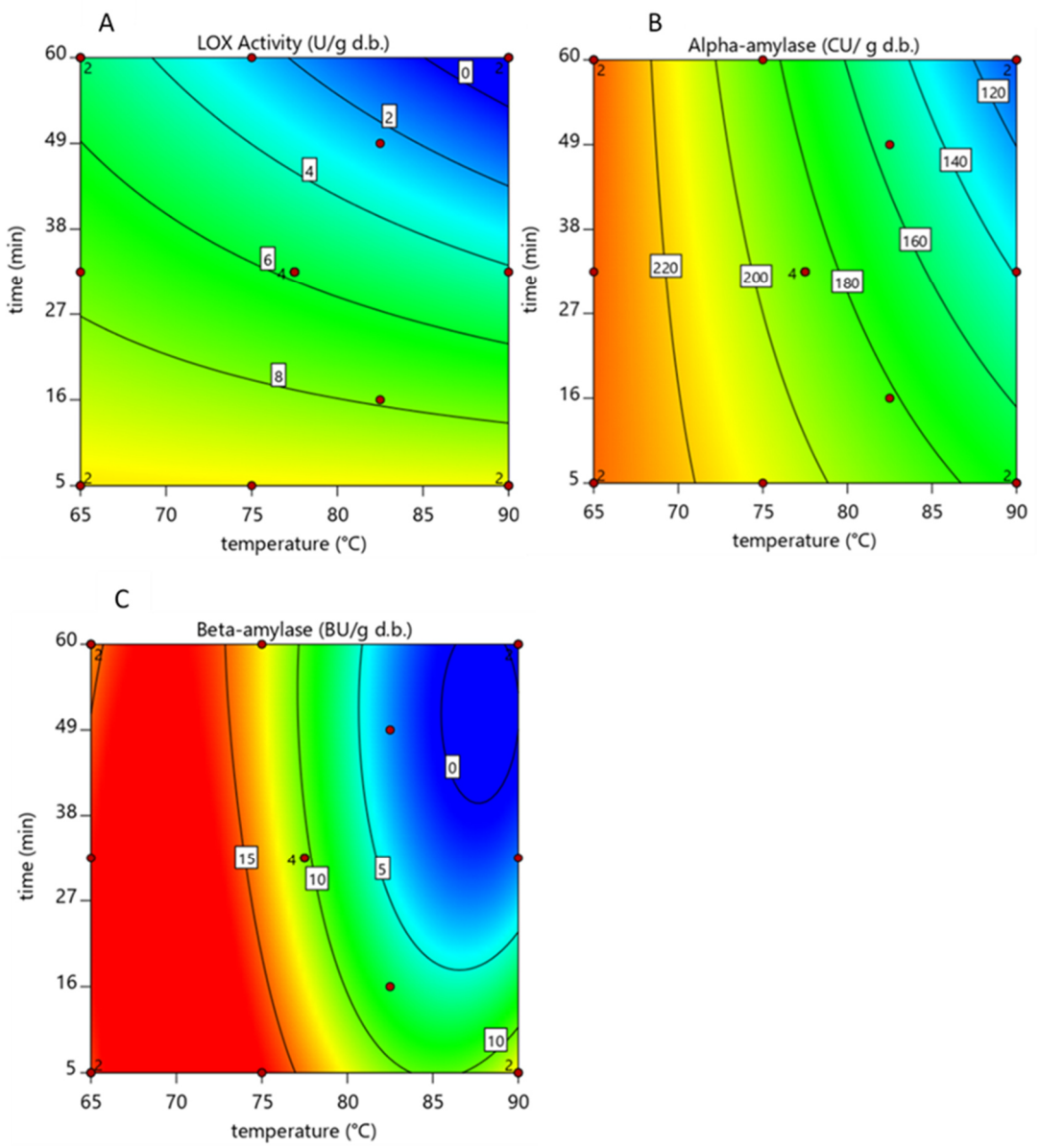


Figure 2.4:Contour plots modelling the influence of a short (5-60 min) heat treatment (65-90 °C) on the activities of (A) lipoxygenase (0-12.9 U/g d.b.), (B) α-amylase (96.7 – 251.0 CU/ g d.b.) and (C) β-amylase (0.4 – 16.5 BU/ g d.b.) in green malt. Colour legend: red (high) – blue (low). Plots show the predictive models fitted to data from 18 data points (red dots) across each design space. Model fit statistics: α-amylase: $p<0.0001$; $R^2=0.8205$; LOX: $p<0.0001$; $R^2=0.8715$; β-amylase: $p < 0.0001$; $R^2=0.8544$.

As summarised in Figure 2.5, LOX activity decreased after just 5 minutes at 65°C, further decreasing when heated for 60 minutes. Apart from avoiding any additional energy intensive heat load on the malt samples, most importantly diastatic enzyme activities were not substantially affected by this heat treatment. Heat treatments at higher

temperatures, on the other hand, reduced diastatic enzyme activity, particularly β-amylase activity (Figure 2.4B). Therefore, heat treatments at a more moderate temperature of 65°C, was considered the most promising result.

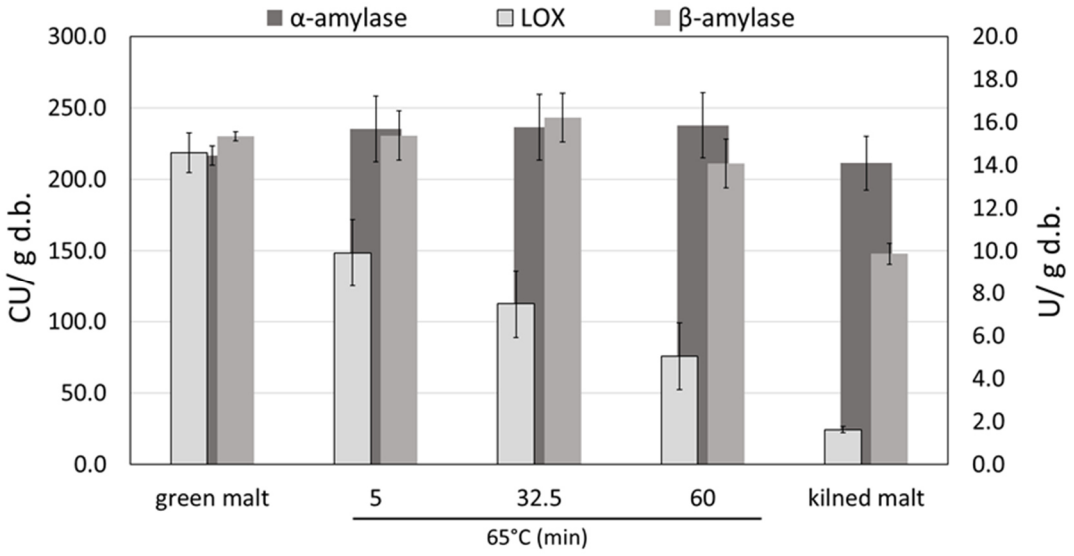


Figure 2.5: Model data showing the impacts of heating green malt at 65 °C for periods of up to 1 h. The primary axis displays α-amylase activity (Ceralpha units/ g d.b).; the secondary axis displays β-amylase (Betamyl-3® Unit/ g d.b.) and lipoxygenase activity (U/g d.b).; Data are predicted responses given by the model fitted to experimental results.

Additionally, after only 60 minutes the moisture content of the grain (including rootlets) was reduced to 29% and rootlets could be removed from the grain using a benchtop deculmer. Thus, this procedure could offer the added benefit of rootlet removal, should it prove scaleable.

2.3.3.2 Impacts of re-steeping green malt (oxygen limitation) at different pH values on LOX activity

Oxygen levels and oxygen pick-up need to be controlled throughout the malting and brewing process, to avoid LOX initiated enzymatic oxidations (Bamforth, 1999c, De Buck et al., 1995, Schwarz and Pyler, 1984, Van Waesberghe et al., 2001). Previous studies (Schwarz and Pyler, 1984) showed a decline in LOX activity during steeping, which was related to the dissolved oxygen in the steep water being used up quickly if the water is not aerated sufficiently. Consequently, the aim was to control lipoxygenase by removal

of its substrate, opting to re-steep the well-germinated green malt in deaerated water (grist:water, 1:3) for one hour. Furthermore, the pH of the water used for re-steeping was varied within the range pH 4 – 7. The optimum pH for LOX activity has been reported to be 6.5, with LOX-2 being more pH sensitive than LOX-1 (De Buck et al., 1995, De Buck et al., 1997, Yang and Schwarz, 1995). The most striking result (Figure 2.6), is that by re-steeping the grain for 1 h lipoxygenase activity decreased by around 50%.

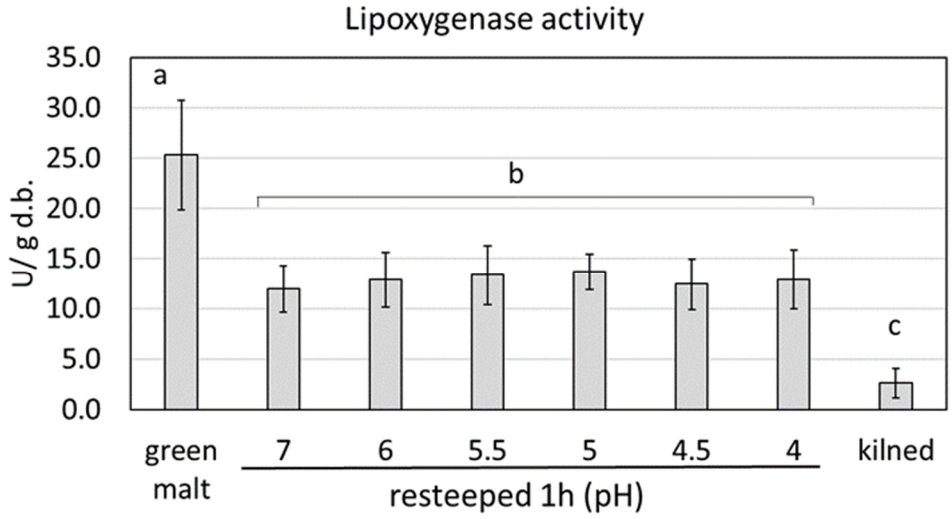


Figure 2.6: Influence of re-steeping at different water pH on the lipoxygenase activity in green malt. One-way ANOVA with Holm-Sidak post hoc test. Results are presented as mean values ±SD; n = 3, tr = 3.

Contrary to expectations, the pH of the water used for re-steeping did not influence the resulting lipoxygenase activity. However, it should be noted that the lipoxygenase activity is assayed in a buffer solution at a pH of 6.8 (Section 2.2.4.2). Thus, the results need to be interpreted with care. LOX-1 showed in previous studies only 50% activity remaining at a pH of 5 whereas LOX-2 shows an activity rate close to zero (De Buck et al., 1995, De Buck et al., 1997, Doderer et al., 1992, Kobayashi et al., 2000a), suggesting that LOX might have been inactive at an acidic pH, however, reactivated in the buffer solution. Further analysis will be necessary to obtain clearer information on lipoxygenase activity after re-steeping at different water pH values. Moreover, residual water samples after re-steeping were analysed to help understand the reasons for loss

of LOX activity. It was also important to know how much LOX activity was transferred to the re-steep water as it would be important for water use efficiency to be able to re-use this water elsewhere in the process. About 13.4 Units/ g d.b. in activity were lost by re-steeping the green malt, however, only 2.2 Units (16.7%) could be measured in the re-steep water. The remaining activity loss could at this stage not be further explained and requires more research but could be related to the onset of grain asphyxiation. The moisture content after re-steeping increased from the average 44.7% to 52.7%, which needs to be considered in terms of the subsequent brewing protocol. Re-steeping experiments (1 hour) demonstrated that LOX activity could be decreased by around 50%, whilst simultaneously preserving amylase activities (Figure 2.7).

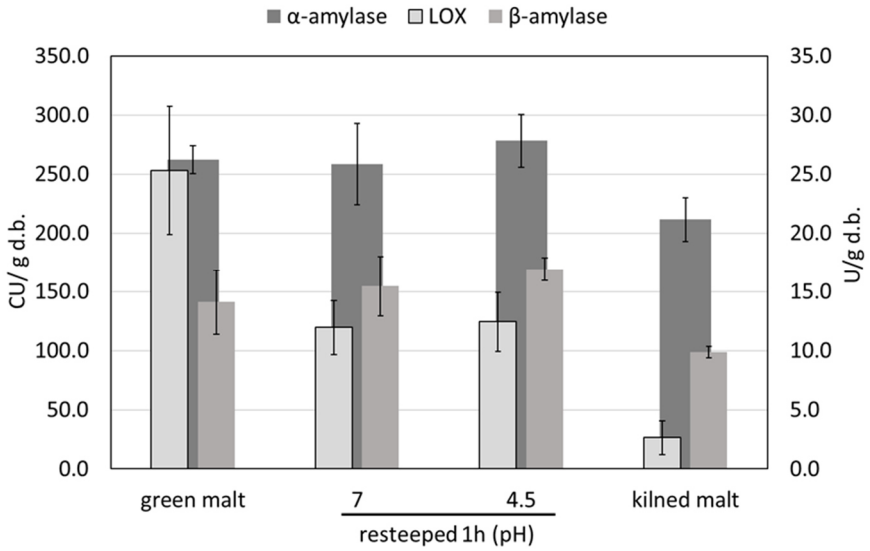


Figure 2.7: Lipoxygenase and amylase activities in green malt, re-steeped (at pH 7 or pH 4.5) as well as kilned malt. The primary axis displays α-amylase activity (Ceralpha units/ g d.b).; the secondary axis displays β-amylase (Betamyl-3® Unit/ g d.b.) and lipoxygenase activity (U/g d.b.). Data are the mean ±SD of 3 biological and 2 technical replicate measurements.

Table 2.2 provides a summary of the impacts of the main treatments developed to minimise green malt LOX activity, on the LOX and diastatic enzyme activities as well as levels of SMM. Reference values for kilned malt and for green malt with and without rootlets are provided by way of comparison.

Table 2.2: Enzymatic activities and S-methyl methionine concentration in malt; with or without pre-treatment.

Sample	**α – amylase*** **(CU/ g d.b.)**	**β-amylase*** ** **(BU/ g d.b.)**	**SMM[†] *** ** **(µg/g d.b.)**	**LOX*** ** **(U/ g d.b.)**
kilned malt	211.4± 18.7 [a]	9.9 ± 0.5[a]	2.6 ± 0.5[a]	1.6 ± 1.2[a]
green malt (including rootlets)	262.3 ± 11.8 [b]	14.1 ± 2.72 [b]	12.6 ± 2.5[c]	27.5 ± 2.5[e]
green malt w/o rootlets	n.m.	n. m	7.3 ± 1.2 [b]	18.9 ± 0.2[d]
re-steeped (pH 7)	258.0 ± 34.4 [a,b]	15.5 ± 2.5 [b]	11.0 ± 0.8[c]	11.9 ± 2.3[c]
re-steeped (pH 4.5)	278.2 ± 22.4 [b]	13.6 ± 3.3 [b]	9.6 ± 0.7[c]	12.4 ± 2.5 [c]
heated at 65 °C, 1h	239.8 ± 15.9 [b]	14.9 ± 0.5 [b]	6.3 ± 1.6[b]	5.2 ± 0.3 [b]

[†]Indirect determination of S-methyl methionine from (Total DMS – DMS); [a-e] Superscripts represent the ANOVA post-hoc groupings. In each column treatments differed significantly from one another if they have a different ANOVA group letter. Data are the mean ±SD of 3 biological and 2-3 technical replicate measurements. Statistics: One-Way ANOVA with Fisher's LSD posthoc test. Asterisks represent the p-value significance * $p<0.05$; ** $p<0.01$; *** $p<0.001$; n.m. = not measured; d.b. = dry basis

Either re-steeping or a heat treatment at 65°C for periods of 1 hour were effective in reducing LOX activity by around 50% in the green malt. However, residual levels were still 3-8 fold higher than in finished kilned malt. Furthermore, the SMM levels were elevated 2.5-4.5 fold relative to kilned malt and were only significantly reduced by the short heat treatment, but unaffected by re-steeping.

2.3.4 Wort analysis

The main treatments developed in terms of lipoxygenase control (Table 2.2) were then compared in terms of the resultant wort quality following laboratory mashing (Table 2.3). This table also features comparable results for mashing with the endosperm-rich extract of green malt so that the potential impacts of rootlet inclusion or removal are apparent. Wort was prepared under lipoxygenase hostile conditions (Bamforth, 2004, Bamforth, 1999c, Van Waesberghe et al., 2001, Wackerbauer et al., 2003): mashing-in temperature 63°C, at low mash pH (e.g., pH 5.2), under oxygen-limited conditions achieved by purging the water used for mashing with nitrogen prior to processing. Data were compared with mashing of kilned pale malt (equivalent mass on a dry weight basis) made from the same green malt. The extract yields of all 'intact' green malt mashes (90.0 ± 2.9%), were greater than that for kilned malt (82.3 ± 3.3) and wort FAN values were equivalent to or greater than the 188 mg/L in kilned malt wort. The endosperm rich extract yield was significantly lower, although extract values up to 71% as-is were achieved.

Additionally, colour and FAN levels were significantly lower in worts prepared from the endosperm rich extract. Based on the present data it is not possible to definitively explain the reason for the lower FAN levels in these worts. However, it is logical to suggest that the endosperm extraction process left behind some of the aleurone and sub-aleurone tissues associated with the outer layers of the barley grain and that these layers contain a significant proportion of grain nitrogen and proteolytic activity. Additionally, SMM levels in worts prepared from green malt were relatively high and were not significantly altered by our proposed processing techniques. Hence, due to the increased DMS potential, special care needs to be taken during wort boiling to remove the DMS deriving from its precursor.

Table 2.3: Analytical measures of wort of kilned malt or green malt – with or without pre-treatment.

Type of malt	**Colour*** **(EBC)**	**specific gravity*****	**Density***** **(kg/m^3)**	**Extract$^{\$}$***** **(%)**	**FAN***** **(mg/l)**	**SMM***** **(µg/L) DMS$_{eq}$)**	**DMS$^{n.s.}$** **(µg/L)**
kilned malt	3.43 ± 0.36^{a}	1.0339^{a}	1.0318^{a}	82.3 ± 3.3^{a}	188.39 ± 28.04^{a}	587.5 ± 45.6^{a}	88.11 ± 39.20
green malt (including rootlets)	3.26 ± 0.46^{a}	1.0390^{a}	1.0371^{a}	90.0 ± 2.9^{b}	224.46 ± 20.98^{a}	1082.8 ± 88.2^{b}	40.50 ± 10.50
green malt endosperm-rich extract	1.33 ± 0.45^{b}	1.0271^{b}	1.0258^{b}	67.2 ± 4.6^{c}	125.37 ± 8.06^{b}	897.3 ± 21.7^{b}	31.36 ± 6.06
re-steeped (pH 7)	2.64 ± 0.59^{a}	1.0364^{a}	1.0331^{a}	84.3 ± 5.7^{a,b}	189.27 ± 11.30^{a}	911.9 ± 50.3^{b}	40.92 ± 23.45
re-steeped (pH 4.5)	2.83 ± 0.48^{a}	1.0361^{a}	1.0345^{a}	83.5 ± 4.4^{a,b}	183.50 ± 4.83^{a}	858.1 ± 44.8^{b}	45.15 ± 20.68
heated at 65°C ,1 h	2.85 ± 0.83^{a}	1.0376^{a}	1.0357^{a}	86.9 ± 2.1^{a,b}	215.29 ± 23.76^{a}	858.0 ± 62.5^{b}	25.90 ± 11.42

Indirect determination of S-methyl methionine from: (Total DMS – DMS); $^{\$}$ extract corrected for weight used; $^{a\text{-}e}$ superscripts represent the ANOVA post-hoc groupings. In each column treatments differed significantly from one another if they have a different ANOVA group letter. Data are the mean ±SD of 3 biological and 3-4 technical replicate measurements. Statistics: One-Way ANOVA with Fisher's LSD post-hoc test. Asterisks represent the p-value significance *p<0.05; **p<0.01; ***p<0.001; n.s. = not significant.

Therefore, further research and brewing trials are required to evaluate the quality implications of the elevated precursor levels.

2.3.5 Nonenal potential

Increased *trans*-2-nonenal levels, as a result of enzymatic lipid oxidation, can become a major concern when using green malt for the brewing process. *trans*-2-nonenal is an unsaturated aldehyde which is known to contribute cardboard stale flavours to beer. It has a very low flavour threshold in the low ppb range (0.035 µg/L; (De Buck et al., 1997, Liégeois et al., 2002, Meilgaard et al., 1979). The nonenal potential was determined to further investigate if the proposed malt treatments, combined with lipoxygenase hostile mashing parameters, successfully controlled LOX activities and thus the formation of *trans*-2-nonenal in wort. Hexanal was also monitored as a marker of lipid oxidation. Hexanal is perceived as a green type odour and this flavour note decreases with increasing malt colour (Coghe et al., 2004). It can be found in pale malt worts at up to 50% higher concentrations than in wort made from dark malts.

Evaluation of the *trans*-2-nonenal and hexanal potentials of the worts revealed (Figure 2.8) that the separation technique used to prepare the endosperm-rich green malt extract caused increased *trans*-2-nonenal potential relative to all other treatments. This could result from the cold-water extraction, meaning that mashing in temperatures initially were lower than 63°C. Thus, enzymatic lipid oxidation could proceed until the required temperatures to destroy the enzyme were reached, which emphasizes the importance of temperature control during mashing. Re-steeping treatments resulted in *trans*-2-nonenal potentials which were not significantly different to that of kilned malt, but with an elevated hexanal potential. Furthermore, there was no difference between re-steeping the grain at pH 4 versus pH 7, indicating that the pH of re-steeping did not significantly impact on the *trans*-2-nonenal or hexanal potentials. Heating the green malt at 65°C for one hour decreased hexanal concentrations relative to those in wort prepared from kilned malt, but increased *trans*-2-nonenal levels.

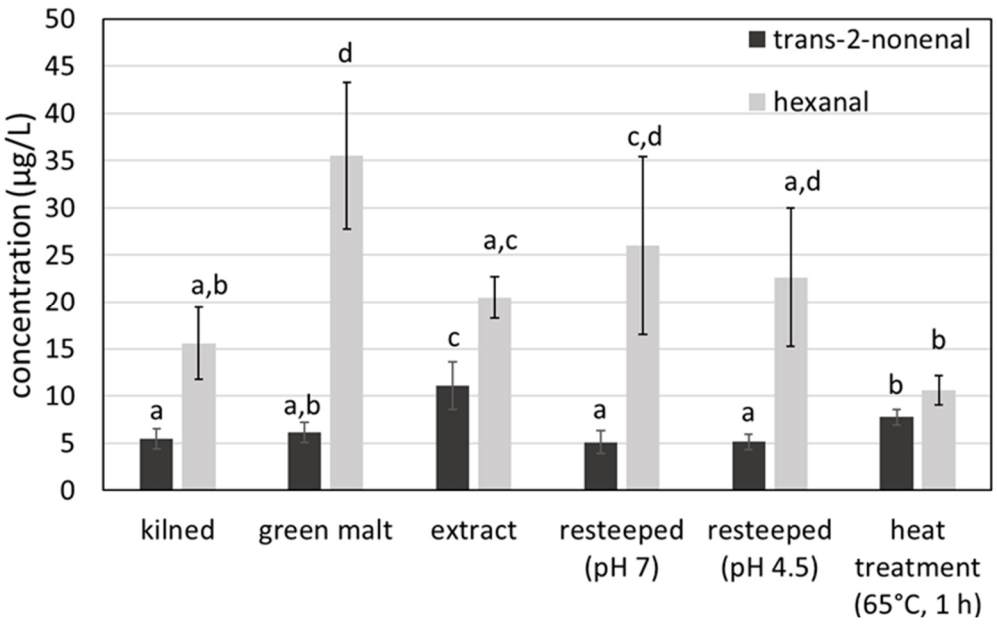


Figure 2.8: *trans*-2-nonenal and hexanal potentials (µg/L) after forced aging of wort samples. Data are the mean ±SD of 4 biological and 2-4 technical replicate measurements. Statistics: One-Way ANOVA on the ranks with Dunn's posthoc test.

Interestingly, the *trans*-2-nonenal potential of the green malt wort without treatment was not significantly higher than for kilned malt, indicating that by mashing in under LOX hostile conditions, lipid oxidation in the mash was already controlled to a sufficient extent. However, hexanal levels remained a concern for the untreated green malt mash and would need to be regulated through wort boiling or stripping. The coefficient of variation (CV) of the *trans*-2-nonenal measurements was considerably lower than that for hexanal. This appeared to arise from variations between biological replicates, since the CV for technical replicates (instrumental analysis) was of the order of just 5.5%.

2.4 Conclusions

Development of processes to brew directly with green malt would represent disruptive technology and this approach is unlikely to be widely implemented in present day breweries. This forward-looking project aims to develop proof-of-principle and enabling technology, with the potential to influence designs for the 'brewery of the future' when presumably operational and environmental pressures will prevail and force the malting and brewing chain to implement more energy-efficient processes. Green malt production is a sustainable way of developing diastatic enzyme activities without moving

to brewing with the use of unmalted grist materials and exogenous enzyme cocktails. Furthermore, proportions of green malt might be used in a mash to digest unmalted cereal adjuncts, rather than envisaging a process using solely green malt.

Lipoxygenase activity and SMM levels in green malt represent major concerns for the manufacture of pale lager beers. The results from this study indicated that controlling LOX activity by mashing in at 63°C at pH 5.2 in deaerated liquor resulted in a *trans*-2-nonenal potential for wort prepared from green malt without any pre-treatment which was not significantly higher than when using kilned malt. However, hexanal potential was significantly higher for the green malt process. Furthermore, this work has revealed two potential methods to lower the LOX activity in green malt without adversely affecting the diastatic enzyme levels, namely: i) re-steeping the grain (1 h) before mashing in or ii) a short heat treatment at 65ºC for up to an hour. The resultant brewing process would need to be optimised to deal with the elevated levels of SMM and hexanal in green malt worts.

In order to gain further information on the quality of wort and beer made from green malt, pilot scale brewing trials are required and will form the next stage of this study. This will enable the sensory impacts of mashing with rootlets on to be evaluated and techniques for dealing with the elevated SMM and hexanal levels in wort to be optimised. Sensory acceptability of green malt beers will most likely determine their ultimate feasibility.

3 Pilot scale brewing trials with 100% green malt – technical feasibility and key quality indicators.

3.1 Introduction

Brewing with green malt will most certainly introduce both technical (milling, etc.) and biochemical (flavour) challenges. Previous research (Cook and Hudson, 1964, Duff, 1963, MacWilliam et al., 1963), dating back to the 1960's, reported that wort and beer of acceptable quality could be produced from green malt, provided a suitable mill was used. However, to date, most breweries are not equipped to process grain with very high moisture contents, let alone green malt with a moisture content exceeding 40%. Unfortunately, in these papers (Cook and Hudson, 1964, Duff, 1963, MacWilliam et al., 1963) no detailed brewing protocol or assessment of the resulting beer flavour or its' stability were published. Additionally, the beer style utilised (stout), could potentially have masked flavour defects (Duff, 1963), so was not the most demanding test of the impacts of brewing with unkilned green malt on beer flavour quality. Hence, there is high interest in providing new and detailed knowledge on this novel method to produce beer using green malt, thus, saving the substantial energy input associated with kilning and conserving the water contained in the green malt.

Two major quality concerns when brewing with green malt – elevated LOX activity and DMS potential – were clearly demonstrated in the first experiments reported in Chapter 2. Furthermore, it was evident that rootlets contributed substantially to this problem. Past research has already shown that even low residual lipoxygenase activities in pale kilned malt can cause serious flavour deteriorations in the final beer (Hirota et al., 2005, Skadhauge et al., 2005). The elevated lipoxygenase activities in green malt (17-fold higher than in the reference kilned malt), as proven in our laboratory-scale trials, would, in theory, cause major flavour defects, as well as poor foam stability, in green malt beers. Potentially, LOX-less (Hoki et al., 2018, Yu et al., 2014) or Null-LOX barley (Skadhauge et al., 2005) varieties might have to be selected when brewing with green malt. Additionally, elevated S-methyl methionine concentrations in green malt, as discussed in Chapter 2, as well as the sensory impacts of mashing with rootlets, must be evaluated and most certainly regulated in upcoming brewing trials. Furthermore, green malt is not

microbiologically stable, hence it needs to be either processed directly, by mashing-in immediately, or by reducing its moisture content to a microbiologically safe level.

On the other hand, as shown in Chapter 2, green malt is rich in α- and β-amylase (diastase enzymes), with great capacity, for example, to convert the starch into fermentable sugars (Duff, 1963, MacWilliam et al., 1963). Additionally, the extra enzymatic potential of green malt could potentially be suitable for mashing in less time. The heat load of the malt and thus, in summary, the total head load of the future mash, wort and beer made of green malt is also significantly lower, implying a decrease in Maillard reactions and Strecker aldehyde formation, which should favour beer flavour stability (De Clippeleer et al., 2010a, Gastl et al., 2006, Jaskula-Goiris et al., 2015, Malfliet et al., 2008). Furthermore, higher heat loads during brewing have been associated with a decrease in free amino nitrogen (FAN) assimilation during fermentation (De Rouck et al., 2007). Thus, reducing heat load might improve FAN assimilation and thereby lower residual FAN levels after fermentation, leading to an improved beer flavour stability. Lastly, unlike kilned malt, green malt does not contain DMSO (Yang et al., 1998, Anness et al. 1979), which can be reduced to DMS by yeast during fermentation.

The main objectives of the research presented in this chapter were to evaluate the technical feasibility of pilot-scale brewing using germinated green malt and to facilitate a comparison between key quality parameters of beers made from green malt and kilned malts prepared from the same batches of green malt. Additionally, three further pairs of beers were brewed whereby the green malt was pre-steeped under de-aerated water for 1 hour; as this procedure had previously been shown (Chapter 2) to lower LOX activity in green malt. Attention was first directed to the technical challenges when using green malt, as well as standard wort and beer quality. Furthermore, particular attention was then paid to trihydroxy fatty acid (THFA) levels which can result from LOX activity, as well as DMS and S-methyl methionine levels. The data reported will help to define the future challenges and potential benefits of implementing beer production using green malt.

3.2 Material and methods

3.2.1 Malt samples

The French malting barley variety Etincel was sourced from Boortmalt, Antwerp. Samples (green malt and the corresponding kilned pilsner style malt) were collected at the equivalent time-points (final day of germination and off-kiln respectively) during six industrial malting cycles. The green malt, which had a moisture content of 40.7 ± 1.1% was not microbiologically stable and could not be stored for extended periods. Therefore, the brewing trials using green malt were started at the earliest possible time point, about 1.5 - 2 hours after malt collection. No further information of the commercial malting procedure is available.

3.2.2 Chemicals and reagents

Ultrapure type-1 grade (mQ, 18.2 MΩcm at 25°C) water obtained from a Synergy 185 system from Milipore S.A. (Molsheim, France) was used for chemical analysis and glassware washing. The determination of esters and higher alcohols was performed at the University of Nottingham; the high-purity water was obtained from a Water Purification Systems (SUEZ Water, Thame, UK).

Free Amino Nitrogen (FAN): Sodium phosphate dibasic dodecahydrate ($Na_2HPO_4 \times 12$ H_2O), potassium dihydrogen phosphate (KH_2PO_4), ninhydrin, fructose and glycine were ordered from Sigma-Aldrich (St. Louis, USA). *Thiobarbituric acid index (TBI)*: Thiobarbituric acid was obtained from Merck KGaA (Darmstadt, Germany). Acetic acid (glacial) was purchased from Sigma-Aldrich (St. Louis, USA). *Total Polyphenol*: Carboxymethylcellulose (CMC) and ethylenediamine tetraacetic acid (EDTA), ammonia and ammonium iron(III) citrate were all purchased from Sigma-Aldrich and of analytical grade. *Flavanoids*: p-dimethylaminocinnamaldehyde (98%) and hydrochloric acid were purchased from Sigma-Aldrich (St. Louis, USA). Methanol (anhydrous) was purchased from Fisher Scientific. *Proanthocyanidin*: 1-butanol (99%) was purchased from Fisher Scientific. *Dimethyl sulphide (DMS):* dimethyl sulphide (≥ 99%) and ethyl methyl sulphide (96%) were obtained from Sigma-Aldrich (Dorset, UK). *Trihydroxy fatty acid*: (S)-lactic acid was purchased from Merck KGaA (Darmstadt, Germany). Heneicosan (98%), hexane (anhydrous, 95%) and pyridine (98%) were purchased from Sigma-Aldrich. The silylation reagent (Silyl-991) was purchased from Machery-Nagel (Germany) and diethyl ether

(extra pure) from Fisher Scientific. *Amino acids (beer):* Solid 5-sulphosalicylic acid (SSA, ≥ 99%) and DL-norleucine were purchased from Sigma-Aldrich (UK). *Esters and higher alcohols*: 3-methyl-1-butanol (99%), ethyl acetate (99%), isobutanol (99%), 1-propanol (> 99%); isoamyl acetate (> 99%), isobutyl acetate (98%); ethyl hexanoate (99%), ethyl octanoate (> 99%); ethyl butyrate (99%) were purchased from Fisher Scientific (UK). Acetaldehyde (≥99.5%) was purchased from Sigma- Aldrich (UK).

3.2.3 Wort production and fermentation

Beers were prepared using 100% green malt (n = 3), green malt re-steeped before mashing (n = 3) or the corresponding reference kilned malt (pilsner malt, n = 6), utilising the 5 hL pilot brewing plant at KU Leuven, Technology Campus Ghent (Figure 3.1), brewing at 50% total capacity (2.5 hL). A thick mash was produced using a grist:liquor ratio of 1:2.2. Samples were collected throughout the brewing process and compared with wort and beer samples from brews produced using conventional pale lager malt, brewed under the same conditions (other than the amount of brewing liquor). Temperature, calcium and lactic acid additions were adjusted to compensate for the higher moisture content in green malt such that mash conditions were consistent across the trials.

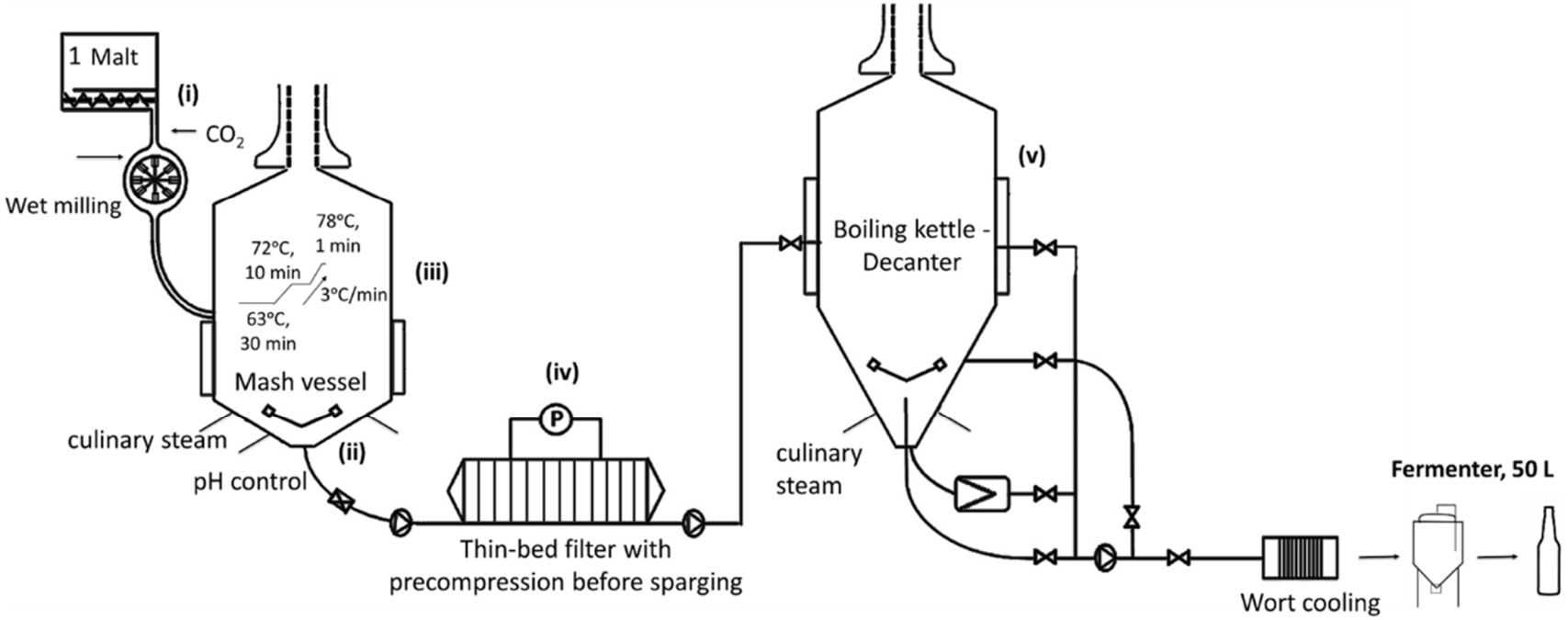


Figure 3.1: Process outline of the KU Leuven brewery; Points i-v indicate critical points when brewing with green malt. i) wet milling; ii) mash agitation; iii) mash conversion vessel; iv) membrane mash filter; v) kettle-decanter.

3.2.4 Wort production using green malt

Milled green malt (68.9 kg, 40% moisture content; wet disc mill, Hydromill, Meura, Belgium) was mixed with 70.4 kg (85°C) of deaerated, reverse osmosis brewing water enriched with 109 mg/L Ca^{2+} in the form of $CaCl_2$ (calcium chloride dehydrate, Merck KGaA, Darmstadt, Germany). CO_2 was injected in the mill inlet, increasing the protection against oxidation. Mashing conditions were selected to minimise lipoxygenase activity: pH 5.2 (1.4 mL/hL lactic acid; pH adjustment with 30% (v/v) lactic acid from 90% (v/v) (S)-lactic acid, Merck KGaA, Darmstadt Germany), mashing in at 63°C under oxygen limited conditions. The following mashing protocol was applied: 63°C (30 min), 72°C (15 min) 78°C (1 min) – temp rise 3°C/ min. Wort was filtered using a membrane assisted thin bed filter (Meura 2001, Meura, Belgium) with a weak-worts cut-off point of 1.5°P. At onset of boiling, the sweet wort was adjusted to 13°P. Additionally, $ZnCl_2$ was added to give free Zn^{2+} ions at 0.2 mg/L. Wort was boiled for 60 min (atmospheric boiling) and hopping applied in pellet form: first hop – Magnum (13.0% (w/w) α- acids; 50.5 g/hL); late hop – Tettnanger (3.0% (w/w) α- acids; 100 g/hl) and Saaz (2.5% (w/w) α- acids; 120 g/hL) aiming for 29 mg iso-α-acids/L in the final beer. Wort clarification was performed by decantation in the combination vessel (wort settling) with a duration of 15 min. Samples for analysis in each batch were collected at onset of mashing, end of mashing, mash filtration, first wort collection, onset of boiling, end of boiling, end of clarification and end of cooling (pitching wort; after wort aeration).

3.2.5 Wort production – re-steeping of green malt before mashing

A total of 68.9 kg of green malt was re-steeped (1 h, re-immersed in water after germination) in 70.4 kg water (deaerated, reversed osmosis brewing water enriched with 1.4 mL/hL lactic acid and 109 mg/L Ca^{2+} in the form of $CaCl_2$). Afterwards, the green malt was separated from the brewing liquor using a fine-meshed net. In order to remain water efficient, the water used for re-steeping was re-used for mashing. The used re-steep water was heated to 85°C using a mobile immersion heater prior to use. Subsequently, the same brewing parameters were applied.

3.2.6 Wort production using reference malt (kilned, pilsner style)

For the kilned pilsner style malt, the same brewing parameters (apart from the brewing liquor) were applied. Pilsner malt: 44 kg of malt were used and mixed with 96.6 kg (69°C) of deaerated water containing 80 mg/L Ca^{2+} ($CaCl_2$) and lactic acid 1.0 mL/hL.

3.2.7 Fermentation, filtration and bottling

All worts were pitched with 10^7 yeast cells/mL (S-O4, Fermentis, top-fermenting strain). Fermentation was performed in a cylindroconical vessel (50 L) at 24°C. After fermentation, beer was submitted to 14 days of maturation at 0°C in 50 L kegs. Matured beer was filtered using a plate filter (BECOPAD Eaton 350). All the batches received carbonation up to 5.6 g CO_2 per litre. Beer samples were bottled using a six-head counter pressure filler with double pre-evacuation with intermediate CO_2 rinsing and over-foaming with hot water injection before capping (Monobloc, CIME, Italy). Bottled beers were stored at 0°C prior to analysis.

3.2.8 Malt, wort and beer analysis: Standard analysis

The moisture content of malt samples was measured by mass loss on drying according to Analytica EBC method 4.2. Wort specific gravity and density, as well as alcohol content of the beer, were analysed using an Anton Paar Alcolyser with a DMA 5000 density measurement device (Anton Paar Benelux, Gentbrugge, Belgium). Extract yield was calculated according to Analytica EBC Method 4.4. Wort and beer colour were determined based on EBC method 9.1 by measuring the absorption at 430 nm using a spectrophotometer (Varian Cary 100, Agilent Technologies Inc., Australia). The CO_2 content of beers were measured by the Haffmans INPACK TPO/CO_2 METER (Haffmanns c-TPO) and foam stability using the NIBEM-T Meter (Haffmans, Venlo, Netherlands). Cold haze (analysis of the turbidity of beer kept for a minimum of 24 h at 0°C) and permanent haze (analysis of turbidity of chilled beer kept for 24 h at 20°C) were determined using the Haffmans VOS ROTA 90 Turbidity meter, 90° light scatter.

3.2.9 Determination of free amino nitrogen in wort and beer

The free amino nitrogen content (FAN) in wort and beer was determined using colourimetry with ninhydrin based on the EBC method-9.10 (8.10 for wort). The full procedure of the assay is as previously presented in Section 2.2.5.1.

3.2.10 Determination of the thiobarbituric acid index (TBI)

The thiobarbituric acid–index (TBI) is used as an indicator of the thermal stress of wort and beer and was determined according to the method described by Thalacker and Böβendörfer (Thalacker and Böβendörfer, 2005). Hereby, mainly Maillard reaction products, especially 5-hydroxymethylfurfural, but also other organic compounds are measured. Wort or degassed beer sample (1 mL) was mixed with mQ water (9 mL) and 5 mL of thiobarbituric acid (2.88 g/L in 90% acetic acid). The test samples were heated in a glycol bath for 70 min at 70°C; the blank samples were not heated. After cooling down in an ice water bath and subsequent mixing, the absorbance was measured at 448 nm. The TBI index was expressed as TBI for 100 mL of wort or beer.

3.2.11 Determination of total polyphenol content in wort and beer

The total polyphenol concentration in wort and beer was determined based on the EBC method (9.11). The polyphenols present in beer or wort react with iron ions in alkaline medium, forming a red compound with maximum absorbance at 600 nm.

Beer at room temperature was degassed by sonification. In a test tube, 5 mL of wort or degassed beer were mixed with 3 mL mQ water, 4 mL CMC/EDTA reagent (2 g/L EDTA, 10 g/L CMC), followed by 0.25 mL of ammonium hydroxide (25% (v/v) in mQ water) and 0.25 mL of the colour reagent (35 g/L ammonium ferric citrate in mQ water). The blank samples were prepared by mixing 5 mL of wort or beer with 3.25 mL of mQ water, 4mL of CMC/EDTA reagent and 0.25 mL of the ammonia solution – but no colour reagent was added. The test tubes were sealed with a screw cap and mixed thoroughly. Subsequently, the mixture was transferred to cuvettes and the absorbance was measured after a minimum of 10 minutes at 600 nm against the blank. The absorbance was multiplied by 820 to get the total polyphenolic concentration in mg/L.

3.2.12 Determination of flavanoids in wort and beer

The flavanoid content in wort and beer was determined based on the EBC method (9.12). The flavanoids (e.g. (+)-catechin) present in beer or wort react with p-dimethylaminocinnamaldehyde in an acidic medium, forming a coloured complex with maximum absorbance at 640 nm. To prepare the colour reagent 100 mg of p-dimethylaminocinnamaldehyde (98%) were added to a 100 mL volumetric flask and filled with 25% HCl (v/v)/methanol solution. Immediately after dissolving the resultant

chromogen solution (1g/L) was protected from light. Beer at about 20°C was degassed by sonification. The beer or wort samples (1 mL) were mixed with 9 mL of mQ water in a test tube. Subsequently, 0.5 mL of this dilution was added to a cuvette and 2.5 mL of the colour reagent were added and carefully mixed. To obtain a blank, 0.5 mL of water was used instead. After 10 minutes, the absorbance was measured at 640 nm using a spectrophotometer (Varian Cary 100, Agilent Technologies Inc., Australia). The results are multiplied by 335 and expressed as catechin equivalents.

3.2.13 Determination of proanthocyanidins in wort and beer

Determination of proanthocyanidins was performed by measuring the red coloured cyanidin complex formed with HCl/1-butanol using the method according to Bate-Smith (Bate-Smith, 1973). Beer at room temperature was degassed by sonification. The beer or wort samples (0.5 mL) were mixed with 3 mL of 5% HCl (v/v)/1-butanol in a test tube and sealed with a screw cap, mixed and placed in a dry heater set to 100°C for 2 hours and vortexed again afterwards. After the tubes were cooled down, the absorbance was measured at 550 nm using a spectrophotometer (Varian Cary 100, Agilent Technologies Inc., Australia). To obtain the blank sample, 3 ml of water were added to 0.5 ml of beer; the blank was not heated. The concentration was based on the molecular mass of the red cyanidin chloride complex formed (322.7 g/mol). The equation used to determine the proanthocyanidin concentration was

$$Proanthocyanidin\ (\frac{mg}{L}) = \left(\frac{E}{15000 \times \mathrm{l}}\right) \times df \times 322.7 \times 1000$$

in which E = Extinction (absorbance) of the sample; Ɛ = molar extinction coefficient (15000 $L \cdot mol^{-1} \cdot cm^{-1}$); l = pathlength (1 cm); df = dilution factor

3.2.14 Determination of DMS and S-methyl methionine in wort and beer

Headspace SPME GC-PFPD was used to quantitatively determine DMS and also indirectly S-methyl methionine (SMM) in wort and beer using the Thermo Finnigan TraceGC Ultra system (Interscience, Louvain-la-Neuve, Belgium). The GC system was equipped with a CTC CombiPAL autosampler, an S/SL injector with narrow bore glass inlet liner, an RTX-1 fused silica capillary column (30 m × 0.32 mm i.d., 3 µm film thickness, Restek), and a

pulsed flame photometric detector (PFPD 5380, OI Analytical, Texas, USA) operating in sulphur mode. Helium was used as carrier gas (1.2 mL/min). The inlet temperature was set at 250°C and injection was carried out in the split mode (split ratio 10:1). The oven temperature was kept at 35°C for 3 min, then raised to 250°C at 5°C/min and held at 250°C for 5 min. The PFPD was set at 250°C and 560 V with air 1 and air 2 at 10 mL/min and hydrogen at 12.5 mL/min. Data processing was performed using Chromcard 2.3.2 (Thermo Electron Corporation, Milan, Italy) and WinPulse 32 2.0 (OI Analytical). After sample preparation, the vial was pre-equilibrated for two minutes at 30°C. The SPME needle was conditioned for 2 min at 300°C and then inserted through the septum. The Carboxen™/Polydimethylsiloxane (CAR/PDMS light blue) fiber (Stableflex, 85 µm, Supelco, Bellefonte, USA) was exposed to the headspace for 15 min, agitating at 250 rpm. The SPME fibre was thermally desorbed into the injection port of the GC for 3 min and subsequently post-conditioned for 2 min at 300°C. The quantification of the DMS content in the unknown sample (wort, beer) is based on a calibration curve with standards of a known concentration of DMS (0.1-10 µg/L) and EMS (1 µg/L) as internal standard. The standard samples (calibration curve) prepared for the beer samples were adjusted for ethanol content. The ratio of the area of the DMS to the surface of the EMS peak is correlated with the ratio of the DMS/EMS concentration. If necessary, samples were diluted by an appropriate dilution factor to allow for quantification within the linear range of the calibration curve.

The indirect quantification of the DMS precursor, S-methyl methionine, was based on the original method proposed by White and Wainwright (White and Wainwright, 1976b) following a modified protocol by De Rouck et al. (De Rouck et al., 2010), without the utilisation of NaOH to avoid possible side formations of oxidised products (DMSO and $DMSO_2$). The sample is prepared and placed at 100°C for 160 min. Due to this thermal treatment, the non-volatile SMM in the sample is converted to DMS. The difference between the content of DMS in the vial subjected to thermal treatment and the content of DMS in the non-heated vial is taken as the SMM concentration in the unknown sample and expressed as DMS equivalents.

3.2.15 UPLC determination of amino acids in wort

The Acquity Ultra Performance Liquid Chromatography (UPLC) separation system from Waters was used to quantify individual free amino acids in wort. At first, the wort sample (1 mL), 20 µL Carrez I reagent (106 g potassium ferrocyanide trihydrate ($K_4Fe(CN)_6$ x $3H_2O$ in 1 L water) and 20 µL Carrez II reagent (220 g zinc acetate dihydrate and 30 mL acetic acid, filled with mQ water to a total volume of 1 L) were mixed, to remove the proteins. Afterwards, the samples were pipetted into an Eppendorf microtube (2 mL), to precipitate the protein. Subsequently, the sample derivatisation was performed using the Waters AccQ-Tag Ultra Chemistry Package and amino acids were separated on the Acquity UPLC (Waters, Milford, USA), equipped with a PDA detector, column heater, sample manager, binary solvent delivery system and an AccQ-TagTM Ultra column (2.1 i.d. × 100 mm; Waters, USA). Data were processed using the Empower 2 Chromatography Data Software (Waters, USA). The gradient elution was performed according to the described Waters AccQ-Tag Ultra method using the AccQ-Tag Ultra Eluent A Concentrate (10 times diluted, Waters, Milford, USA) and AccQ-Tag Ultra Eluent B (Waters, Milford, USA). Total analysis time was 9.5 min at a constant flow rate of 0.7 mL/min at 60°C.

3.2.16 Determination of amino acids in beer

Amino acids were isolated from beer and derivatised using the EZ:Faast^{TM} amino acid kit (Phenomenex, Macclesfield, UK). A conical centrifuge tube containing 60 mg of solid 5-sulphosalicylic acid (SSA, for deproteinisation) was cooled to 4°C. Beer (980 µl) and 20 µL of the internal standard norleucine (10 µmol/L) were added to the conical centrifuge tube, mixed with the SSA and allowed to stand for 1 hour at 4°C. The mixture was centrifuged for 15 min at 4°C using a centrifuge to spin down the precipitate. The supernatant was removed and filtered through a 0.2 µm filter (Millipore Cat No. GSWP02500). The amino acid standard solution was treated the same way. Subsequently, 20 µL of treated standards or sample was injected to an Amino Acid Analyser (Biochrom 20 Plus) equipped with an ion-exchange column and UV detector for analysis. The concentration of amino acid was calculated by the EZChrom Elite software (EZChrom Elite, Chromatography Data System).

3.2.17 Determination of volatiles in beer

Volatiles in beer were determined via GC-HS-FID method, using a SCION 456-GC (Bruker, UK) fitted with a Combi PAL autosampler and controlled with Compass CDS software. Degassed, cold beer sample (4°C, 10 mL) was pipetted into a 20 mL headspace vial (Fisher Scientific, UK), internal standard (1-butanol; 50 mg/L) was added followed by sodium chloride (3.5 g). The vial was sealed with a crimp cap lined with a PTFE/silicone septa (Sigma-Aldrich, UK). The sample was equilibrated at 60°C for 20 min, agitated at 500 rpm. The GC was equipped with an S/SL injector at 150°C (Split ratio: 1:20). Volatiles were separated on a ZB Wax column (60 m x 0.25 i.d., 0.5 µm film thickness) using helium carrier gas (BOC, UK, 15 psi, constant pressure). The oven temperature was kept at 85°C for 10 min, then raised (25°C/min) to 110°C for 13 minutes and held at 200°C for 13.25 min (8°C/min). The FID detector was set at 250°C with air flow at 300 mL/min, helium flow at 25 mL/min and hydrogen flow at 30 mL/min. Signal response was corrected against the internal standard and the compounds quantified using an external standard series. The standard series was prepared in the following concentrations: 3-methyl-1-butanol, acetaldehyde, ethyl acetate (10-100 mg/L); isobutanol, propanol (5-50 mg/L); isoamyl acetate, isobutyl acetate (0.05 – 0.5 mg/L); ethyl hexanoate, ethyl octanoate (0.1 – 1 mg/L); ethyl butyrate (0.025 – 0.25 mg/L).

3.2.18 Determination of trihydroxy fatty acids in grain, wort and beer

Gas chromatographic analysis of trihydroxy fatty acids (THFA) in beer samples was based on the published procedures of Moeller-Hergt et al. (Möller-Hergt et al., 2001) and Wackerbauer and Meyna (Wackerbauer and Meyna, 2001). Extraction of THFA in malt samples was conducted by using 50.0 ± 0.05 g of malt with 390 mL mQ water, 10 mL of Brewtan (6 g/L) and 1 mL lactic acid (9%), preheated to 70°C. The mix was mashed for 10 min at 70°C. Afterwards, the weight of the content of the beaker was adjusted to 450 ± 0.2 g by addition of reverse osmosis water and filtered on ice using filter paper (Whatman, grade 2555 ½ prepleated 320 mm, Sigma-Aldrich, UK). The first 20 mL of the filtrate was transferred to a small glass bottle and immediately frozen until further liquid-liquid extraction.

The following liquid-liquid extraction was performed on a 5 mL aliquot of the (extracted) malt or wort sample, using 16 mL diethyl ether (extra pure grade, Fisher Scientific). The

mixture was shaken for 3 minutes and centrifuged at 9344 x *g* for 5 minutes (Hettich 320R, Germany). The upper layer was transferred to a new glass vial using a glass syringe. Subsequently, this diethyl ether layer was evaporated using nitrogen. The liquid-liquid extraction was repeated three times (on the same 5 mL aliquot). After the final evaporation 500 µL of the internal standard, heneicosan ($C_{21}H_{44}$) diluted in hexane, was added to the glass vial and evaporated. The internal standard used was prepared by diluting 91.40 mg heneicosan (exact weight noted for final calculation) in 250 ml clean hexan and further diluted 1:10. To avoid further variations, the same internal standard was stored and used for all analysis. For the derivatisation 300 µL of the silylation reagent (Silyl-991) and 100 µL pyridine were added. The samples were subsequently heated at 90°C for 1 h using a laboratory block heater (digital heat block, VWR). The liquid was transferred into HPLC vials and kept at -20°C until GC analysis. The equipment used was a GC-FID (ThermoQuest Trace GC 2000; Interscience, Louvain-la-Nueve, Belgium) equipped with a fused silica analytical capillary column (CP-Sil 5 CB Low BLEED/MS; 50 x 0.25 mm i.d., 0.25 µm and a cyano-phenyl-methyl deactivated retention gap (2.5 x 0.53 mm i.d., Varian, Netherlands). Samples (2 µL) were manually injected using a Hamilton syringe (10 µL, Model 701 N Syringe). The oven temperature was kept at 40°C for 5 min, then raised to 290°C at 6°C/min and held at 290°C for 20 min. Helium was used as a carrier gas at a flow rate of 1.0 mL/min. Data processing was performed by Chromcard software 1.07. The equation used to determine the trihydroxy fatty acid concentration was

$$THFA\ (\frac{mg}{L}) = \frac{mg\ IS\ x\ area\ THFA\ x\ 1000}{area\ IS\ x\ vol\ sample}$$

in which area IS = area of Internal Standard; area THFA = area of trihydroxy fatty acids; mg IS = internal standard derivatised (0.01828 mg); vol sample =volume of wort or beer sample (5 mL).

3.2.19 Statistical analysis

All samples were analysed in at least three biological replicates with 2-4 technical replicates. The statistical significance of the data obtained was established with analysis of variance (ANOVA), a p-value below 0.05 was considered as statistically significant.

3.3 Results and Discussion

3.3.1 Brewing performance and technical challenges

The commercially produced green malt was used as 100% of the grist in pilot scale brewing trials (2.5 hL). Six paired trials were conducted whereby beers were brewed first from a batch of green malt and subsequently from the kilned malt prepared from that green malt. Each pair of malts were sampled from a different batch, albeit produced using the same barley variety and industrial malting process. Beers were produced under the same brewing conditions, other than the amount of brewing liquor. To account for the higher moisture content (40%) in green malt, less water (as described in Section 3.2.4) needed to be added at the onset of mashing. Thus, a more water efficient process was achieved by brewing with green malt. A summary of the brewing performance of green malt (n = 3), re-steeped (prior to mashing) green malt (n = 3) and the corresponding reference pilsner malt (n = 6) is shown in Table 3.1.

The pilot brewery at KU Leuven (Figure 3.1) is equipped with a wet milling system (i), suitable for milling green malt. CO_2 was injected in the malt bin and the mill inlet, increasing the protection against oxidation, thus potentially favouring lipoxygenase control. The water flow during wet milling (considering the amount of water already in the grain) and the gap distance setting of the mill (19 kilned malt, 12 green malt, equipment specific units, Hydromill, Meura) were adjusted. Inappropriate setting of the disc gap (too fine or too coarse) led to blocking of the mash filter (iv) when brewing with green malt - as a result, the brews had to be stopped and discarded.

Table 3.1*:* Brewing performance of green malt, re-steeped green malt and the corresponding reference kilned (pilsner) malt.

Brew Nr.	**1**		**2**		**3**		**re-steeping trials**					
							4		**5**		**6**	
	GM	**KM**	**GM**	**KM**	**GM**	**KM**	**GM**	**KM**	**GM**	**KM**	**GM**	**KM**
Mash filter (MF) filling time (min)	8.0	3.7	9.6	5.4	11.3	4.4	12.0	5.0	5.0	4.0	4.0	4.0
MF filtration (min) before sparging (min)	32.0	20.2	27.7	20.7	50.9	22.4	26.0	22.0	19.0	28.0	39.0	39.0
MF sparging and final compression(min) time (min)	29.9	77.6	121.9	99.6	145.2	59.4	75.1	99.6	78.8	101.9	89.4	66.8
Boiling time (min)	60	60	60	60	60	60	60	60	60	60	60	60
Total wort volume (L)	190	220	180	240	200	199	190	230	200	220	180	184
Brewhouse yield (%)	61.8	72.5	55.5	80.3	44.0	67.0	64.2	73.9	65.8	69.2	57.3	62.1

GM = green malt; KM = kilned malt

The filtration process in the pilot brewing trials of green malt wort was found to be considerably slower than that of typical pale kilned malt worts (Table 3.1). In part, this might reflect a need for further optimisation of the milled particle size distribution using the wet disc mill, but also relates to the thickness of the mash. Additionally, the mash stirring device (ii) employed was not a conventional agitator, but a homogeniser allowing low shear, ideal for kilned malt mashes. However, it appears not to be optimal for mixing green malt mashes. The homogeniser, which sits in the bottom of the mash kettle (Figure 3.2B), could not cope with the thickness of the mash of green malt, therefore only 50% of the total mash kettle capacity could be used and the brews had to be scaled down to 2.5 hL.

Figure 3.2: Mash kettle; Inlet and thick (1:2.2) green malt 'mash' (A), homogeniser at the bottom of the mash kettle (B)

Temperature and pH control (iii) at the onset of mashing were difficult due to the noted sub-optimal mixing. To allow for a lipoxygenase hostile (Baert et al., 2012, Bamforth, 2004, Drost et al., 1990, Van Waesberghe et al., 2001) mashing temperature and pH, the mash-in water volume needed to be reduced to allow for the higher water content of

the green malt, meaning that liquor needed to contain more lactic acid and be heated to a higher temperature. Whilst these adjustments were calculated and applied, the pH and temperature proved very difficult to control accurately, which may have been due to insufficient mixing. Additionally, milling of kilned malt already causes friction which can increase the temperature and, in terms of pH, the composition of the steeping water used in the malting process was unknown. Filling of the mash filter (iv) took about twice as long (4.0 - 12.0 min) in all six green malt brews compared to the reference brews (3.7 – 5.4 min; Table 3.1). Total filtration time increased in all six green malt brews. This could probably be improved in future by optimisation of the milling process, use of a mash vessel equipped with a more suitable type of low shear stirring device, and fine tuning of the liquor to grist ratio. In general, the green malt brews had low flow rates and in consequence sparging times took longer than for the reference brews (Table 3.1). Poor sparging rate could be attributed to the spongy and cohesive structure of the green malt "cake", not allowing sparging water to sufficiently wash out the remaining sugars. Thus, brewing yield was lower in green malt brews than kilned malt brews.

In future trials, an optimised milling system is advised, in combination with a mash vessel equipped with a 'normal' mash agitator instead of the low shear homogeniser, used in the pilot brewery. Additionally, filtration and sparging operations need to be adjusted to cope with the structure of the green malt "cake". However, milling optimisations could potentially improve the composition of the grist, thus filterability and sparging rate. There were no technical issues during the boiling (v), clarification and cooling operations of the six green malt brews.

3.3.2 Wort characteristics

The characteristics of the cold pitching wort are shown in Table 3.2. Worts prepared from untreated green malt are compared to worts prepared from the kilned reference; similarly re-steeped green malt worts are compared to their corresponding reference brews. The colour of the worts prepared from untreated green malt and re-steeped green malt was significantly lower compared to worts prepared from the kilned malt control, however, a satisfactory yellow colour was still attained.

Table 3.2: Pitching wort characteristics prepared from green malt, re-steeped green malt or the corresponding reference kilned (pilsner) malt.

			re-steeping trials	
	GM	**KM**	**GM**	**KM**
pH ***	5.4 ± 0.2 [a]	5.2 ± 0.1 [b]	5.4 ± 0.1 [a]	5.2 ± 0.1 [b]
Colour (EBC) ***	8.1 ± 1.9 [a]	10.9 ± 1.3 [b]	7.2 ± 0.5 [c]	10.9 ± 1.4 [d]
Density (g/cm^3) n.s.	1.0478	1.0439	1.0500	1.0481
Original extract (° Plato) n.s.	12.4 ± 0.5	11.8 ± 1.3	12.9 ± 0.3	12.4 ± 1.3
FAN (mg/L) ***	220.9 ± 41.2 [a]	287.5 ± 35.8 [b]	269.5 ± 19.9 [c]	259.7 ± 47.8 [c]
Total polyphenols (mg/L) ***	311.9 ± 33.6 [a]	379.0 ± 47.1 [b]	372.3 ± 36.7 [b,c]	363.0 ± 43.2 [c]
Flavanoids ((+)-catechin eq. mg/L) ***	50.3 ± 3.7 [a]	54.6 ± 1.7 [a]	75.0 ± 2.5 [b]	66.1 ± 5.4 [c]
Proanthocyanidins (mg/L) ***	59.0 ± 7.2 [a]	74.6 ± 20.1 [b]	71.4 ± 13.6 [c]	84.5 ± 19.0 [d]
Thiobarbituric acid index***	15.4 ± 1.5 [b]	45.1 ± 4.7 [a]	20.6 ± 1.5 [c]	51.2 ± 7.9 [d]
DMS (µg/L) ***	106.1 ± 41.9 [a]	97.4± 22.3 [a]	139.0 ± 27.9 [b]	56.9 ± 27.9 [c]
SMM (mg/L)† ***	0.54; 0.23; 0.38 [a]	0.61; 0.05; 0.24 [b]	0.26; 0.46; 0.26 [a,b]	0.13; 0.05; 0.09 [c]
THFA (mg/L) ***	3.8 ± 1.5 [a]	7.8 ± 0.9 [b]	7.4 ± 0.5 [b]	6.0 ± 0.9 [c]

†Indirect determination of SMM from (Total DMS – DMS), expressed as DMS equivalents

a-d superscripts represent the ANOVA post-hoc groupings. In each row, treatments differed significantly from one another if they have a different ANOVA group letter. Asterisks represent the p-value significance * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Data are the mean ±SD of 2-3 technical replicate measurements, Statistics: One-Way ANOVA with Fisher's LSD post-hoc test. GM = green malt; KM = kilned malt

This supports previous findings reported by MacWilliam et al. (MacWilliam et al., 1963). Kilned malt imparts characteristic colour compounds to beer, formed mainly via Maillard reactions initiated between reducing sugars and amino-compounds during kilning. Nevertheless, the yellow colour in green malt pitching wort might originate from natural yellow pigments, such as polyphenols or the water-soluble vitamin riboflavin. Riboflavin is a yellow colouring matter, present in malt (1.2 – 5.0 µg/g) (Briggs, 1998a). The precise origins of the colour contributed by green malt should be further investigated.

Contrary to expectations, the free amino nitrogen content of worts prepared from green malt (n = 3) were significantly lower compared to levels of their corresponding reference worts (Table 3.2). However, reported minimum levels (140 mg/L (Kunze, 2014)), as nutrition for the yeast during fermentation, were easily achieved in all worts. Green malt is known to have a higher proteolytic activity than kilned malt. As shown in previous research, proteases seem to be protected at very thick mashing conditions even when mashing in at an elevated temperature of 63°C (De Rouck et al., 2013b). One possible reason for the decreased FAN levels could be the presence of proteolytic inhibitors in green malt. Previous research (Jones, 2005) found endogenous proteins in both barley and malt that have the ability to inhibit the enzymatic activities of proteases. FAN levels measured in worts prepared from re-steeped green malt, on the other hand, did not differ significantly from the relevant control worts. When re-steeping green malt those inhibitors might have been removed, or proteolytic activity increased through some mechanism. Certainly, this observation requires further investigation.

Significantly lower concentrations of polyphenols (311.9 ± 33.6 mg/L) were measured in worts prepared from untreated green malt compared to their reference worts (379.0 ± 47.1 mg/L). These results further support the idea that especially the kilning step increases total polyphenol levels (Chandra et al., 2001), as well as polyphenol solubilisation (Narziss, 1976). However, polyphenol levels differed greatly between the individual brews, presumably due to the difficulties that occurred during sparging of green malt, affecting retention of polyphenols. Hence, a more technically consistent process is necessary to gain further information on the factors which determine total polyphenol levels in green malt wort. Flavanoid levels in wort did not differ significantly whether the wort was prepared from green malt (50.3 ± 3.7 mg/L) or kilned malt (54.6 ± 1.7 mg/L). Proanthocyanidins, the main haze active polyphenols, were significantly

reduced in worts prepared from green malt (59.0 ± 7.2 mg/L) compared to the controls (74.6 ± 20.1 mg/L), which is consistent with the observations made by MacWilliam et al. (MacWilliam et al., 1963) who reported much lower anthocyanogen contents in green malt wort. Re-steeping, on the other hand, appeared to affect polyphenol solubilisation. The total polyphenol concentration of the re-steeped GM brews did not differ from the control brews (Table 3.2), while flavanoid levels were elevated (75.0 ± 2.5 mg/L). Additionally, re-steeping increased proanthocyanidin levels (71.4 ± 13.6 mg/L), compared to worts prepared from untreated green malt. These results, which potentially impact beer colloidal stability, are discussed in the following sections.

The thiobarbituric acid index (TB-Index) is traditionally used as an indicator for evaluating heat load during wort production and determines the 5-hydroxymethylfurfural (5-HMF) potential of wort and beer. The omission of the kilning process dramatically decreased the heat load of the malt, which resulted in a decreased TBI level in the wort (Table 3.2). Hence, the lower TBI of green malt wort (15.4 ± 1.53) and re-steeped green malt wort (20.6 ± 1.5), compared to the corresponding reference wort (45.1 ± 4.7 and 51.2 ± 7.9, respectively), could potentially benefit the flavour stability of the beer (Gastl et al., 2006, Malfliet et al., 2008). Further process optimisation, so as to reduce the total mash filtration times of green malt brews, could even further decrease the total heat load and thus improve flavour stability.

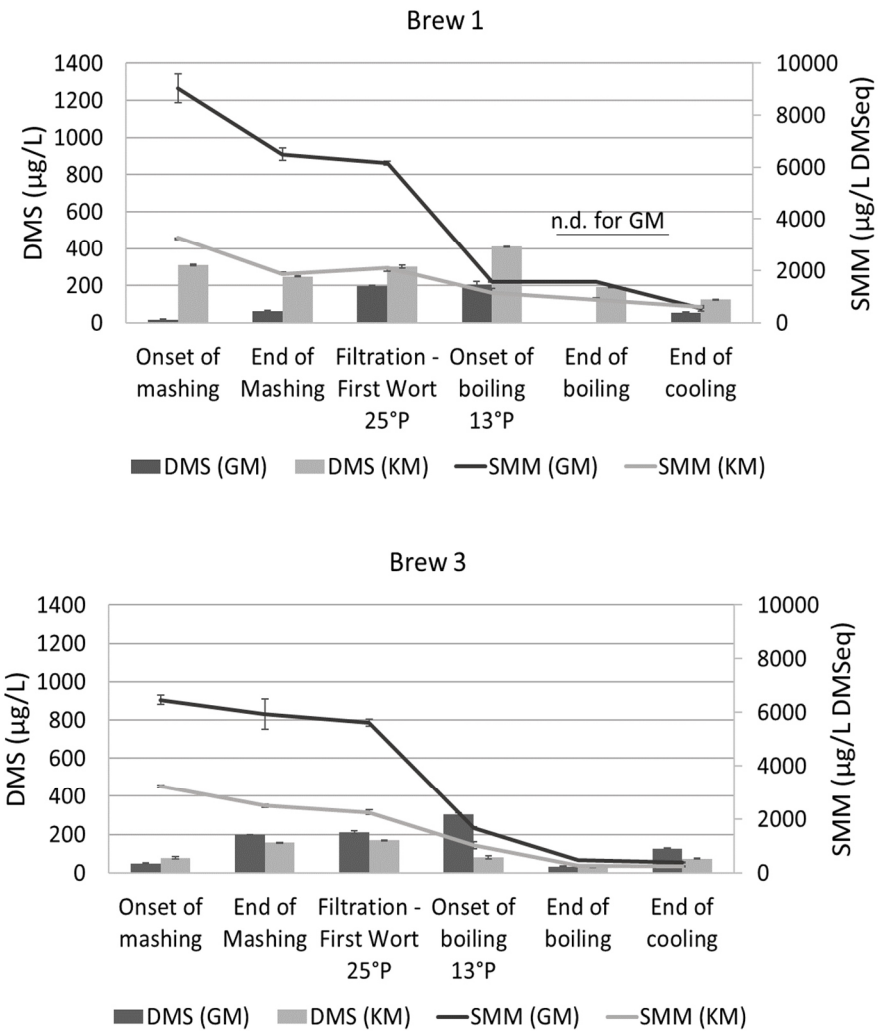
3.3.3 DMS and S-methyl methionine – determination in wort

DMS and (indirectly) S-methyl methionine levels (SMM) were measured in all pitching worts. Data were compared with worts prepared from their kilned malt control, prepared from the same green malt. Green malt is rich in the DMS-precursor SMM (White and Wainwright, 1977), therefore overall DMS levels were expected to be higher compared to the control. S-methyl methionine levels were determined throughout the brewing process of the three untreated green malt samples and compared to the reference brews. Figure 3.3 illustrates the DMS and SMM levels from the onset of mashing to the pitching wort. It is noticeable that in all three brews the SMM levels were 2-3 times higher (7.3 ± 1.3 mg/L) at mashing-in compared to the respective reference brew (3.0 ± 0.4 mg/L). SMM is being transformed into free volatile DMS for both kilned malt and green malt brews. As expected, DMS levels were rising (prior to boiling), while

the amount of SMM was declining; the individual measured concentrations varied substantially between the different brews. This appeared to arise from variations between biological malt replicates (since there was variation in SMM levels already at onset of mashing). Errors due to sampling and analysis were likely smaller, as wort samples were taken at the same time-point in each case and immediately put on ice prior to analysis.

For example, the first and second brew indicated significantly higher DMS levels until the onset of boiling in the green malt brews. In the second brew, the DMS concentration was even double that of the kilned malt brews. In contrast, the third brew indicated higher DMS levels in the kilned malt wort. Although the analysis did not reveal a clear uniform pattern on DMS levels, overall the results show that a major part of the precursor already gets converted during mashing and filtration, and not solely during wort boiling (100°C, 60 min).

As already shown in previous studies (Anness et al., 1979, Yang et al., 1998), during malt kilning, SMM already decomposes (pH-dependent) at temperatures above 70°C to DMS and L-homoserine. Any remaining DMS was satisfactorily evaporated during boiling, leaving worts of green malt brews with higher S-methyl methionine levels, but acceptable DMS concentrations. Additionally, DMS and SMM levels were determined in the pitching wort prepared from re-steeped green malt and the corresponding reference malt, again resulting in acceptable DMS levels (Table 3.2). On average, all six brews using green malt as the raw material resulted in elevated SMM level, but acceptable DMS levels (122.6 ± 36.1 µg/L DMS v 77.15 ± 30.6 µg/L DMS). It appears that DMS levels in pitching wort can be controlled even when using green malt, given a sufficient removal of DMS via evaporation during wort boiling.



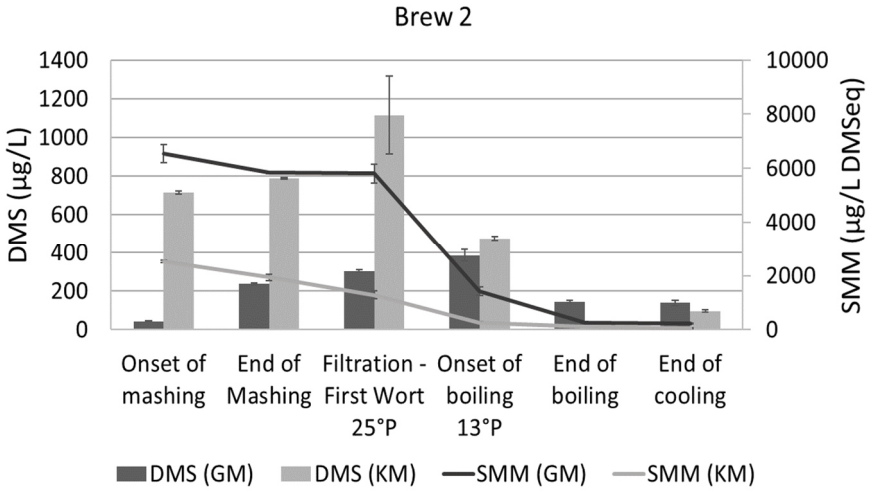


Figure 3.3: DMS and S-methyl methionine (expressed as DMS equivalents, µg/L) monitored in three individual brewing processes using green malt and the corresponding reference (pale) kilned malt. Data are the mean ±SD of 2 technical replicates.

3.3.4 Trihydroxy fatty acids – determination in wort

The malts used for the preparation of the beers were analysed for trihydroxy fatty acid (THFA) levels in the raw materials themselves. Clearly, green malt has a higher lipoxygenase (LOX) activity compared to kilned malt (as shown in Chapter 2) which poses a major threat for beer flavour and stability. The determined contents of THFA in the malts used for this study were significantly lower in kilned malt (39.6 ± 9.9 mg/kg, dry basis) compared to green malt (68.3 ± 4.5 mg/kg, d.b.). Interestingly, however, the THFA concentration measured at onset of mashing was significantly lower in all three brews using green malt (n = 3, Figure 3.4).

This suggests a rapid breakdown of THFA to degradation products during wet milling and entry to the mash vessel. Similarly, significantly lower THFA levels were detected in all three pitching worts of green malt (3.8 ± 1.5 mg/L) compared to their kilned malt reference (7.8 ± 0.9 mg/L). There was a clear THFA increase across mashing in kilned malt brews (Figure 3.4), whereas in green malt brews levels were more or less stable throughout the brewhouse operations.

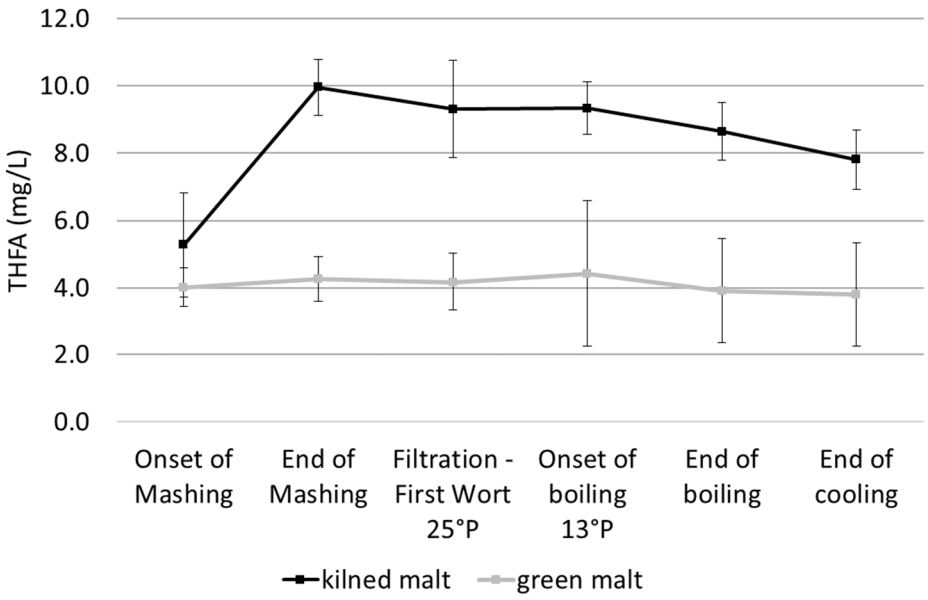


Figure 3.4: Trihydroxy fatty acid (THFA, mg/L) monitored in three individual brewing processes using green malt or its corresponding reference (pilsner) kilned malt. Data are the mean ±SD of 3 biological with each 2 technical replicate measurements.

The formation of trihydroxy fatty acids from hydroperoxy fatty acids can occur through several enzymatic pathways (Baert et al., 2012). Green malt shows higher LOX activity than kilned malt (Chapter 2), however, it is likely that the oxygen-boosted drying of green malt, triggers the oxidation of unsaturated fatty acid, giving rise to both THFA (as intermediate) and aldehydes, such as hexanal and *trans*-2-nonenal. Consequently, the increased extraction of THFA during mashing when using kilned malt (Figure 3.4). Subsequent determination of the fatty acid oxidation aldehydes, *trans*-2-nonenal and hexanal, in both green and kilned malts further confirmed this hypothesis (Section 4.3.1). Thus, the evolution of free staling aldehydes was further investigated and discussed in Chapter 4.

Prior research (Chapter 2) had indicated that re-steeping of green malt in water for an hour was an effective means to reduce the LOX activity of green malt by around 50%. Therefore, the quality impacts of this putative process at pilot scale were assessed, including re-use of the re-steep water as mashing liquor in the green malt brewing process in order to minimise overall water usage in the chain. Contrary to expectations, brewing with re-steeped malt almost doubled (7.4 ± 0.5 mg/L) THFA levels compared to the use of untreated green malt (3.8 ± 1.5 mg/L) and it did significantly differ from its kilned malt control (6.0 ± 0.9 mg/L; Table 3.2). This suggests that not all appropriate mashing conditions were fulfilled to control unwanted LOX reactions. Possibly by re-heating the steep water and not de-aerating it prior to mashing, oxygen pick-up may have occurred (Figure 3.5).

By keeping lipoxygenase hostile mashing parameters (63°C, pH 5.2 and oxygen-free), LOX-related reactions can be kept under control. However, considering that temperature and pH control were challenging in green malt brews due to the noted incompatibility of the mash homogeniser, these findings suggest that oxygen exclusion is a key criterion to avoid THFA formation.

Overall, the main conclusion of this part of the study was that LOX activity was sufficiently controlled in the original green malt brewing process, such that the potential advantage in LOX activity reduction offered by re-steeping was not realised. Future studies should be directed to the oxidation of unsaturated fatty acids to further elucidate why THFA increased when brewing with kilned malt, but contrary to expectations not when using green malt.

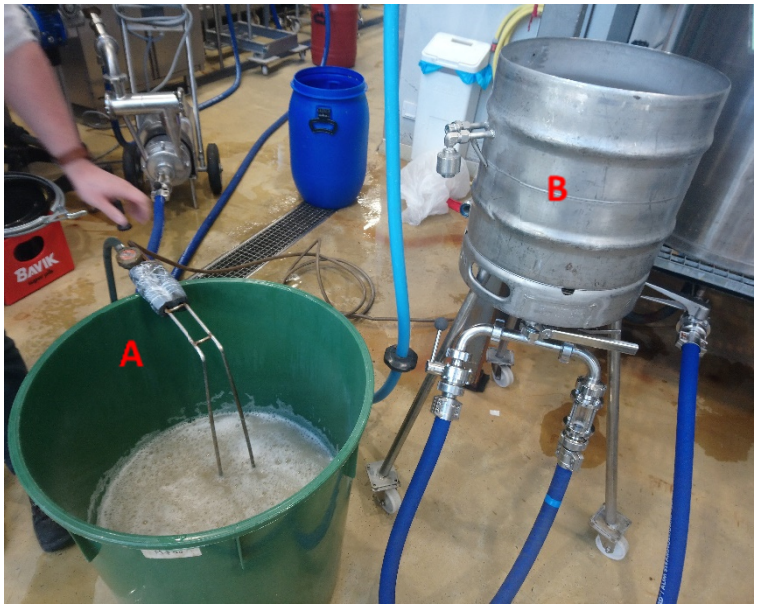


Figure 3.5: Heating of re-steeping water using an immersion heater (A); water tank connected to mash kettle (B)

3.3.5 Fermentation performance

Fermentation progression was similar across kilned malt and green malt worts and reached the stationary phase three days after wort pitching. The pH dropped from 5.4 ± 0.1 to 4.3 ± 0.1 in green malt brews, and 5.2 ± 0.1 to 4.4 ± 0.1 in kilned malt brews. Final pH in the beer did not significantly differ across treatments, and all beers reached typical finished beer pH values (4.2-4.5; Table 3.3). The kilned malt control fermentations reached an alcohol level of 5.4 - 5.7% v/v, which was more consistent than the green malt fermentations 5.0 – 5.9% v/v (Table 3.3). However, statistically, all beers brewed were of similar alcohol content and degrees of fermentation, which did not significantly differ among the malts used.

Table 3.3: Fermentation performance and beer characteristics prepared from green malt or the corresponding reference kilned (pilsner) malt.

			re-steeping trials	
	GM	**KM**	**GM**	**KM**
pH	4.2 ± 0.0	4.4 ± 0.1	4.4 ± 0.1	4.3 ± 0.0
Alcohol by volume (% v/v)	5.5 ± 0.4	5.6 ± 0.1	5.7 ± 0.1	5.6 ± 0.2
Density (g/cm^3)	1.0054 ± 0.0018	1.0065 ± 0.0015	1.0059 ± 0.0020	1.0067 ± 0.0001
Specific gravity	1.0072 ± 0.0019	1.0083 ± 0.0015	1.0077± 0.0020	1.0085 ± 0.0001
Original gravity (°P)	12.1 ± 0.3	12.6 ± 0.5	12.7 ± 0.3	12.6 ± 0.3
Real extract%(w/w)	3.8 ± 0.3	4.1 ± 0.4	4.0 ± 0.5	4.2 ± 0.0
Real degree of fermentation (RDF)	69.7 ± 3.5	68.6 ± 1.9	69.8 ± 2.9	68.3 ± 0.5
Calories (kJ/100mL)	182.9 ± 4.9	189.9 ± 8.7	192.4 ± 5.2	191.0 ± 4.0

Data are the mean ±SD of 2-3 technical replicate measurements, GM = green malt; KM = kilned malt. Statistics: One-Way ANOVA with Fisher's LSD post-hoc test. There was no significant difference.

As illustrated in Figure 3.6, the FAN content of worts and beers prepared from untreated green malt (n = 3) were lower compared to levels of their corresponding reference wort/beer.

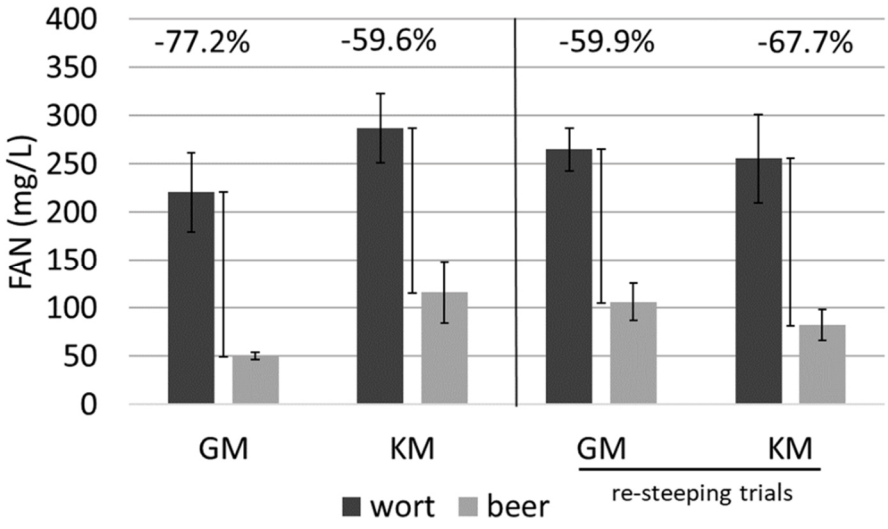


Figure 3.6: FAN levels (mg/L) in worts and beers prepared from green malt, re-steeped green malt and their corresponding reference malt. The average percentage uptake (%) from pitching wort to matured bottled beer is indicated. Data are the mean SD of 3 biological with each 3 technical replicate measurements; GM = green malt, KM = kilned malt.

Across fermentation, a higher proportion of FAN uptake (ranging between 70 – 82% FAN uptake) was observed relative to the corresponding kilned malt trials (52 – 66% FAN uptake), subsequently resulting in lower residual FAN in green malt beers compared to control. Previous studies suggested that higher heat loads in wort production led to lower FAN uptake, suggesting that heat related compounds reduce the assimilability of FAN by yeast (De Rouck et al., 2007). However, when brewing with re-steeped green malt the tendency was towards the opposite effect (ranging between 53 - 64% FAN uptake; Figure 3.6). High levels of FAN in the pitching wort, as found in the worts of kilned malt or re-steeped green malt, resulted in higher residual FAN in the final beer. High residual FAN in beer can result in elevated levels of Strecker aldehydes and consequently contribute to beer staling (De Rouck et al., 2007, Jaskula-Goiris et al., 2011).

To further understand and explain the assimilability of the FAN, the amino acid profile from both kilned malt and green malt pitching worts and beers were determined. The results are displayed in Figure 3.7.

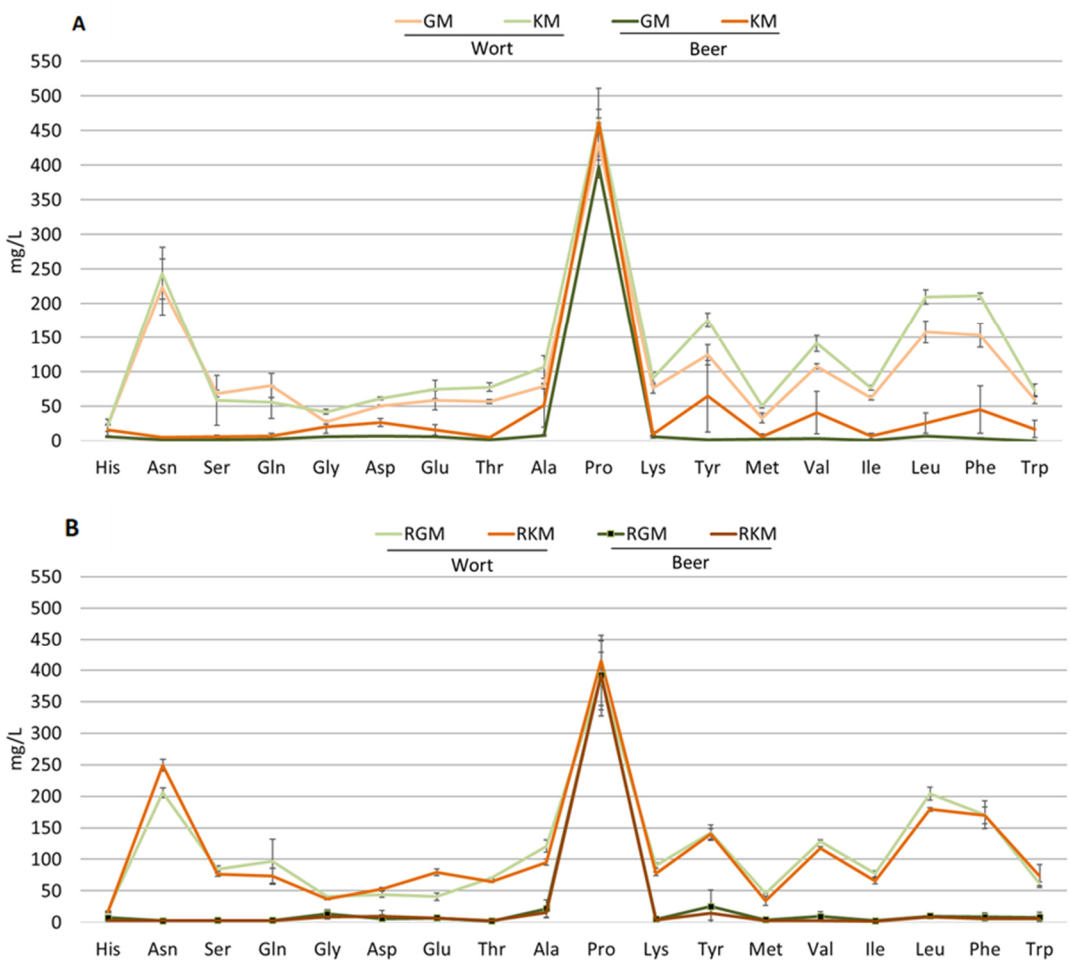


Figure 3.7: Amino acid profile of worts and beers produced from (A) green malt 'as is' and the corresponding control; (B) re-steeped green malt and the corresponding control. Data are the mean ±SD of 3 biological with each 2 technical replicate measurements; GM = green malt, KM = kilned malt, RGM= re-steeped green malt, RKM= kilned malt (control for re-steeping trials).

During fermentation, the yeast requires nitrogen sources for the synthesis of new metabolites, particularly proteins, peptides and nucleic acids. From the brewer's perspective, the composition of amino acids in wort is a very important factor determining the formation of beer aroma compounds as, for example, higher alcohols can be excreted as metabolic by-products of the protein metabolism (Ehrlich

mechanism). Additionally, changes in the profile of higher alcohols affect in turn the ester profile, as the higher alcohols have the secondary role of providing precursors for the ester synthesis (Boulton and Quain, 2006). The amino acids in wort have been categorised into four groups (A-D) based on the fermentation performance (Jones and Pierce, 1964), where amino acids in the first group (A) are assimilated immediately after yeast contact. In green malt wort significantly ($p < 0.001$) higher levels of threonine, tyrosine, valine, leucine and phenylalanine were detected, compared to the control wort (KM1-3, Figure 3.7A). Worts prepared from re-steeped green malt, on the other hand, contained lower levels of asparagine and glutamine compared to the control wort (KM4-6, Figure 3.7B). Overall, a clear decline of selected amino acids (except proline) was detected from all worts to the finished fresh beers, resulting, as expected, in very low residual amino acids compared to the levels determined in pitching wort. Proline, on the other hand, the sole member of Class D amino acids cannot be assimilated by most yeast strains. Thus, unsurprisingly, there was no uptake of proline for all the wort fermentations conducted ($n = 12$). The detailed amino acid composition of the beers is displayed in Table 3.4. The high standard deviations for some amino acids, especially in the control worts and beers KM1-3, indicated that there was some variance between the three biological replicate control brews. Therefore, the results are displayed as individual values in Table 3.4. Even though the amino acid levels in beers were very low compared to the initial concentrations in the wort, it is apparent that the highest total amino acid content was found in the control beers KM1 and KM2. The re-steeping trials and controls, on the other hand, resulted in very low levels of residual amino acids which did not significantly differ amongst malts used. For all beers, biological malt replicates, as well as the same yeast strain (top-fermenting, S-04; Fermentis) and fermentation protocol was used, thus it is very likely that the high variance in the control beers is related to fermentation performance of the yeast. Unfortunately, due to the high standard deviation of amino acids in the worts and beers prepared from the kilned malt (pilsner style) controls, further replicates are needed to explain if there is an impact of heat load on yeast assimilability. However, the present findings could help to gain further information on the flavour stability of the beers, as the amino acids valine, isoleucine, leucine, methionine, phenylalanine can be precursors of significant beer staling Strecker aldehydes (Chapter 5).

Table 3.4: Amino acid profile (mg/L) of beers produced from green malt 'as is' and the corresponding control; re-steeped green malt and the corresponding control.

					re-steeping trials	
	GM	**KM1**	**KM2**	**KM3**	**GM**	**KM**
His	6.2 ± 1.5	20.7	20.3	5.0	7.7 ± 2.6	3.2 ± 1.7
Asn	1.6 ± 0.3	5.3	6.1	2.6	2.6 ± 1.4	2.7 ± 0.7
Ser	1.2 ± 0.6	7.0	7.5	3.6	3.3 ± 1.6	3.3 ± 0.9
Gln	2.6 ± 0.5	8.2	9.8	3.0	3.3 ± 0.8	3.1 ± 0.6
Arg	7.4 ± 2.0	50.9	56.8	10.6	12.0 ± 4.9	18.3; 11.2; 5.8
Gly	6.3 ± 1.1	24.7	26.6	9.8	13.6 ± 6.7	8.8 ± 3.3
Asp	7.2 ± 0.2	23.2	33.4	23.6	13.1; 0.7; 3.7	10.1; n.d.; 19.1
Glu	6.4 ± 2.0	19.3	21.1	6.8	7.3 ± 0.7	7.3 ± 0.8
Thr	1.4 ± 0.7	4.8	5.8	3.9	2.0 ± 0.5	2.4 ± 0.7
Ala	7.7 ± 1.2	62.5	74.6	16.1	21.6 ± 14.2	15.8 ± 7.3
Pro	397.7 ± 9.8	486.3	493.4	405.3	391.9 ± 64.4	392.4 ± 55.0
Lys	5.8 ± 2.3	10.5	13.4	5.9	5.0 ± 2.3	4.2 ± 0.9
Tyr	1.2 ± 0.8	88.5	100.3	5.4	54.5; 18.1; 2.2	23.2; 17.9; 1.8
Met	2.1 ± 0.2	6.7	9.3	0.8	4.1 ± 0.6	3.4 ± 2.5
Val	3.3 ± 0.5	50.6	66.1	6.3	18.6; 5.3; 4.6	2.8 ± 2.7
Ile	0.9 ± 0.3	7.6	10.2	2.4	2.5 ± 1.0	2.1 ± 0.5
Leu	6.9 ± 0.1	28.3	38.5	9.7	10.2 ± 1.4	9.2 ± 1.2
Phe	3.5 ± 0.8	58.3	71.1	6.7	16.6; 5.1; 4.5	5.3 ±1.6
Trp	0.1 ± 0.1	22.9	25.4	2.5	8.0 ± 8.3	5.5 ± 3.7

Data are the mean ±SD of 3 biological with each 2 technical replicate measurements; GM = green malt, KM = kilned malt.

3.3.6 Characteristics of finished beers

The characteristics of the finished beers are presented in Table 3.5. All beers showed acceptable foam stability and low haze (chilled and permanent) formation. Haze formation in beer is caused mainly by interactions between haze active polypeptides and polyphenols (Bamforth, 1999b, Leiper et al., 2005, McMurrough et al., 1996, Siebert and Lynn, 1998, Siebert and Lynn, 2008). Polyphenols and flavanoid levels did not differ in beers prepared from green malt relative to the control beers (Table 3.5). The natural haze-active polyphenols in beer are mainly proanthocyanidins, because of their size and potential to cross-link haze active proteins or peptides. However, in contrast to the

lower proanthocyanidin levels reported in untreated green malt wort, there were no substantial differences noted in the fresh beer. Cold break haze is formed at 0°C and will dissolve at room temperature, as the polypeptides and polyphenols are non-covalently bound (Steiner et al., 2010). Upon beer ageing, covalent bonds will be formed, creating insoluble complexes that will not dissolve, resulting in permanent haze. Thus, haze formation needs to be further investigated in (forced) aged beers.

Unsurprisingly, the colour in the kilned malt control beers was higher than in the green malt beers. However, an acceptable colour was still attained (Figure 3.8).

Figure 3.8: SurGreen (left) made of 100% green malt, in comparison to the reference beer (right) brewed with 100% pilsner malt.

Interestingly, the beers prepared from re-steeped green malt were significantly lower in colour than the beers prepared from green malt 'as is'. Potentially, the natural yellow colour pigments in malt (as discussed previously) could have been washed out during re-steeping. This theory would support our previous suggestion, that the colour of 'green malt beers' results from natural colour pigments, such as polyphenols or riboflavin.

Table 3.5: Characteristics of beer prepared from green malt, re-steeped green malt or the corresponding reference kilned (pilsner) malt.

			re-steeping trials	
	GM	**KM**	**GM**	**KM**
colour (EBC) ***	7.3 ± 1.2 [a]	9.5 ± 2.1 [b]	5.3 ± 0.4 [c]	8.4 ± 1.7 [d]
CO_2 (g/L)	5.8 ± 0.3	5.9 ± 0.1	6.3 ± 0.5	6.4 ± 0.3
NIBEM foam stability (sec.) n.s.	176; 196; 115	139; 131; 119	178; 146; 151	141; 154; 164
chill haze (EBC 90°scatter) n.s.	1.32; 11.76; 7.72	6.21; 13.53; 1.86	6.3; 1.74; 2.97	6.52; 7.84; 4.61
permanent haze (EBC 90°scatter) n.s.	1.14; 7.94; 5.61	4.85; 10.65; 1.34	3.67; 1.33; 2.17	3.60; 4.93; 2.84
FAN (mg/L) ***	50.3 ± 4.0 [a]	116.2 ± 32.2 [b]	106.3 ± 19.1 [b]	82.5 ± 15.8 [c]
Total polyphenols (mg/L) n.s.	234.9 ± 31.7	250.9 ± 46.5	251.2 ± 7.7	268.5 ± 12.8
Flavanoids ((+)-catechin eq. mg/L) ***	63.6 ± 5.1 [a]	60.1 ± 12.5 [a]	70.7 ± 3.3 [b]	73.4 ± 4.0 [b]
Proanthocyanidins (mg/L)	39.4 ± 5.7 [a]	44.9 ± 5.3 [a]	34.6 ± 2.9 [b]	33.4 ± 2.8 [b]
Thiobarbituric acid index***	10.6 ± 0.9 [a]	33.6 ± 6.4 [b]	15.5 ± 0.8 [c]	40.4 ± 5.1 [d]

a-d superscripts represent the ANOVA post-hoc groupings. In each row treatments differed significantly from one another if they have a different ANOVA group letter. Asterisks represent the p-value significance * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Data are the mean ±SD of 2-3 technical replicate measurements, Statistics: One-Way ANOVA with Fisher's LSD post-hoc test. GM = green malt; KM = kilned malt

The TBI levels decreased from the pitching wort to the final beers, presumably due to the reducing power of yeasts, reducing aldehydes to alcohols. Nevertheless, the untreated green malt (10.6 ± 0.9) and re-steeped green malt (15.5 ± 0.8) beers still had a significantly lower TBI in the beer compared to the reference (33.6 ± 6.4; 40.4 ± 5.1, respectively), potentially benefitting beer flavour stability.

3.3.6.1 Flavour profile of beers made of green malt

DMS and SMM levels were measured in all beers. Analysis of finished beers revealed DMS levels of 23.8 ± 9.9 µg/L on average (n = 3) in beers prepared from green malt and levels of 10.9 ± 2.7 µg/L in beers prepared from re-steeped green malt, which did not significantly differ from their controls (Table 3.6). SMM levels in all green malt beers (untreated and re-steeped) remained higher than those for kilned malt beers, although fermentation significantly reduced SMM levels. This confirms previous findings by White and Wainwright (White and Wainwright, 1977). However, remaining SMM could potentially be decomposed to DMS during pasteurisation, which is detrimental to final beer flavour. Thus, further research was conducted to evaluate the impact of in-pack pasteurisation processes on finished beers (Section 3.3.7).

To further describe the flavour profile of the beers, Table 3.6 additionally presents analytical data for a selection of major flavour active volatiles formed by yeast during fermentation. During the course of fermentation and maturation, acetaldehyde, an intermediate in the formation of ethanol or acetate (green beer aroma) was successfully broken down and could not be detected in the finished beers (n = 12). Esters and higher alcohols can positively contribute to the fruity, fresh beer flavour. The esters isoamyl acetate (banana ester) and ethyl acetate (apple, fruity ester), as well as the higher alcohol 3-methyl-1-butanol, also commonly known as isoamyl alcohol, were detected in concentrations above their reported flavour threshold (Table 3.6) (Meilgaard, 1975a) and most likely imparted fruity flavour and aroma to the fresh beers. However, multiple flavour active compounds working together can create synergistic effects, meaning they can impact the flavour even when present below their individual threshold (Meilgaard, 1975b).

Table 3.6:The concentration of selected volatile compounds in the final beers.

					re-steeping trials	
mg/L (µg/L)	**Unit**	**FT**	**GM**	**KM**	**GM**	**KM**
DMS $^{n.s.}$	µg/L	30	23.8 ± 9.9	24.3 ± 11.0	10.9 ± 2.7	12.7 ± 3.0
SMM $^{\dagger ***}$	µg/L	-	136.4 ± 37.1 ^{a}	44.1 ± 13.0 ^{b}	104.4 ± 45.5 ^{c}	13.5 ± 6.6 ^{d}
Acetaldehyde	mg/L	25	n.d.	n.d.	n.d.	n.d.
Esters						
Ethyl acetate **	mg/L	25-30	51.6 ± 16.9 ^{b}	93.3 ± 27.1 ^{a}	73.2 ± 12.4 ^{a,b}	88.3 ± 12.4 ^{a}
Isoamyl acetate $^{n.s}$	mg/L	1.6	1.7± 0.5	2.4 ± 0.5	3.4 ± 0.2	3.0 ± 0.3
Isobutyl acetate **	µg/L	1600	83.9 ± 24.0 ^{b}	84.9 ± 17.1 ^{b}	122.9 ± 26.1 ^{a}	104.2 ± 7.4 ^{a,b}
Ethyl butyrate $^{n.s}$.	µg/L	400	104.0 ± 19.4	153.4 ± 51.8	129.7 ± 12.5	130.1 ± 16.6
Ethyl hexanoate $^{n.s}$.	µg/L	230	109.5 ± 26.9	156.2 ± 64.7	154.7 ± 5.7	157.9 ± 13.2
Ethyl octanoate $^{n.s}$.	µg/L	900	272.0 ± 120.3	425.4 ± 239.3	438.3 ± 32.5	513.1 ± 45.0
Higher alcohols						
1-Propanol $^{n.s}$.	mg/L	800	27.6 ± 4.4	30.0 ± 1.8	35.1 ± 4.2	38.4 ± 5.6
Isobutanol $^{n.s}$.	mg/L	200	60.2 ± 22.1	43.0 ± 12.1	54.7 ± 6.7	41.2 ± 0.7
3-Methyl-1-butanol *	mg/L	70	97.6 ± 22.3 ^{a,b}	89.9 ± 9.8 ^{a,b}	103.7 ± 4.6 ^{a}	82.8 ± 3.3 ^{b}

Results are the mean ± SD of three biological with each 2-3 technical replicate measurements. †Indirect determination of SMM from (Total DMS – DMS), expressed as DMS equivalents; a-d superscripts represent the ANOVA post-hoc groupings. In each row treatments differed significantly from one another if they have a different ANOVA group letter. Asterisks represent the p-value significance * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Data are the mean ±SD of 2-3 technical replicate measurements, Statistics: One-Way ANOVA with Fisher's LSD post-hoc test. GM = green malt; KM = kilned malt. Flavour thresholds (FT) in beer according to Meilgaard (Meilgaard, 1975a)

Whilst the esters and higher alcohols of the re-steeping trials resulted in very low standard deviations amongst the biological replicate beers (n = 3, Table 3.6); some variation was observed in the 'untreated green malt' and corresponding control replicate beers. This is most likely due to differences in the wort composition as, amongst other things, amino acids can affect the quantity and type of higher alcohols formed, which in turn affects the ester profile (Boulton and Quain, 2006). Furthermore, the availability of different sugars in wort can affect the formation of volatile compounds by yeast during fermentation (He et al., 2014); e.g. Verstrepen et al. (Verstrepen et al., 2004) reported that worts high in glucose can strongly increase the production of esters. Thus, it would be interesting to further investigate the sugar profile of green malt wort.

3.3.7 Influence of pasteurisation on DMS formation

Further research was undertaken to evaluate the potential quality implications of the elevated SMM levels during beer pasteurisation. In a parallel experiment, beers (n = 3) were pasteurised to different degrees (20, 40, 60 Pasteurisation units; PU) so that the impacts of elevated SMM in green malt beers could be ascertained. Typical process values for beer pasteurisation are about 14-15 PU, depending on beer style, alcohol content and the degree of contamination (Kunze, 2014). Hence, these data (Figure 3.9) suggest, that pasteurisation is not a major concern when brewing with green malt, provided that the initial DMS concentration is within an acceptable range.

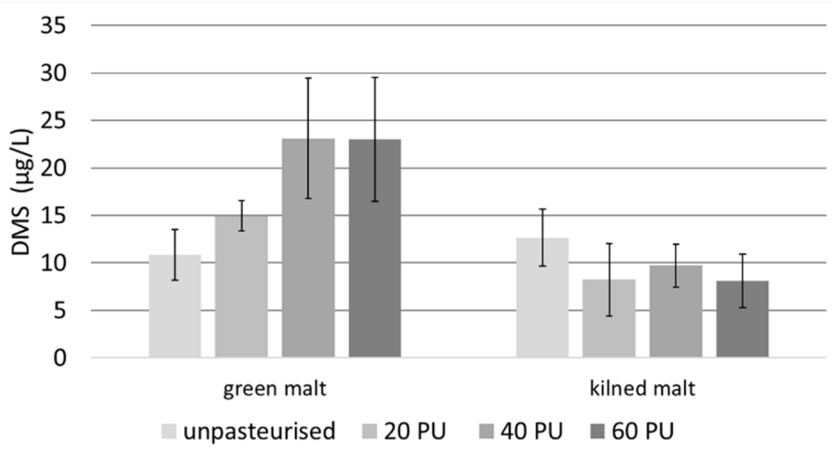


Figure 3.9: Influence of pasteurisation on DMS and SMM. Data are the mean ± SD of 3 biological with each 2 technical replicate measurements.

3.3.8 Analysis of re-steeping water

Because the re-steeping water was used for mashing (to minimise overall water usage in the chain), it was likewise analysed for selected parameters. Due to the turbidity of the re-steeping water, difficulties were experienced expressing the pale-yellow colour of the re-steeping water in numbers. However, these findings support the view that the colour of beer is not only influenced by Maillard products, but also by other water-soluble compounds in the grain. In the re-steeping water, polyphenols (23.8 ± 7.2 mg/L) and flavanoids (5.2 ± 1.5 mg/L) were detected, but no proanthocyanidins. Additionally, FAN (31.7 ± 7.2 mg/L), low levels of DMS (5.9 ± 3.7 µg/L), and a surprisingly high concentration of SMM (407.4 ± 81.3 µg/L) were detected. No THFA were detected in the re-steeping water. Heating of the re-steeping water to reach the required temperature for the onset of mashing did not influence the analytical results significantly.

3.4 Conclusions

The aim of this study was to evaluate the feasibility of brewing with 100% green malt with intact rootlets and to determine the quality of wort and beer made from green malt as compared to kilned malt brews processed from the same batch of malt. Even though further technological and process optimisations are undoubtedly required, we proved that an acceptable potable beer can be brewed using 100% green malt. No significant taints or obvious defects were detected in any of the beers prepared from green malt (untreated or re-steeped) as compared to the reference brews. The beers were tasted informally by expert tasters at both KU Leuven and the University of Nottingham, as well as a selection of visitors to our poster at the EBC Congress in Antwerp, 2019. The absence of any noted defects amongst 30-40 regular beer consumers is the basis for our conclusion that the green malt beers were ‘acceptable’ sensorially. Nevertheless, more detailed sensory evaluation of the organoleptic properties of green malt beers are required to evaluate their unique flavour profile and further understand how this might be complemented with the use of other grist materials to generate a more conventional kilned malt flavour in finished beer.

Since most breweries are set up to brew with kilned pale malt, adaptations are required when utilising green malt with a moisture content of more than 40%. Technical adaptations and milling optimisations are inevitable in order to avoid technical

difficulties and reduced brewing yields due to poor sparging efficiency. In the present research, the thickness of the mash (1:2.2) obtained at the beginning of the process proved problematic for the low shear homogeniser used in this study. Also, the complex structure of the spent grains bed formed during mash filtration ('spongy', cohesive structure), increased the likelihood of blockages and extended filtration and sparging periods. An optimised brewhouse process for wet milling, in combination with a normal (low shear) stirring device, instead of the low shear homogeniser used in the pilot brewery, is advised.

Increased SMM levels were measured in worts made from green malt, however DMS concentrations in the pitching wort were within an acceptable range. A further decline in SMM levels occurred across all fermentations. Tests carried out on the final beers, confirmed that DMS levels in beers made of green malt did not differ significantly from their reference brews. The presented data suggest that pasteurisation is not a major concern when brewing with green malt, provided that the initial DMS concentration is within an acceptable range. Finished beer specification was acceptable in terms of colour, pH, alcohol content and foam stability. The TBI was significantly lower in worts and beers prepared from green malt. It was interesting to note that the free amino nitrogen in green malt beer was considerably lower compared to kilned malt beers. Both of the prior factors should, in theory, be beneficial for the flavour stability of the aged beer.

Even though re-steeping seemed a promising technique by which to reduce LOX activity in green malt at laboratory scale, present results suggest that it was unnecessary. LOX was adequately controlled in the pilot plant process by wet milling in deaerated liquor under CO_2 and mashing-in at 63°C, pH 5.2 under oxygen free conditions. However, considering that temperature and pH control were challenging in green malt brews due to the noted incompatibility of the mash homogeniser, these findings suggest that oxygen exclusion is a key criterion to avoid THFA formation. Significantly, lower trihydroxy fatty acid levels were determined in worts prepared from untreated green malt, compared to the reference wort. Furthermore, our results demonstrate that brewing with green malt need not be limited to the use of LOX-free barley varieties, although the latter may be beneficial for breweries where strict LOX-hostile conditions

cannot be applied or who wish to avoid additional costs (and health and safety considerations) of CO_2 injection.

Overall, the results presented in Chapter 3, showed that an acceptable potable beer can be brewed using even 100% green malt. Furthermore, wort and beer analysis revealed promising indicators for flavour stability in untreated green malt beers. Therefore, further analysis was necessary to learn of the impacts of kilning on malt quality and subsequently worts and beers.

4 Evolution of free staling aldehydes and oxidative stability of green malt wort and beer

4.1 Introduction

Beer in pack is not in chemical equilibrium and is known to irreversibly change in flavour and aroma during storage. Increased beer export, higher consumer demands for new and fresh beers, an increasingly profound and conscious beer culture, as well as the craft beers' "taste revolution" (Aquilani et al., 2015, Kleban and Nickerson, 2012) are a great incentive to improve the flavour stability. Even though many important factors to avoid beer staling are widely understood by the brewing community, beer staling and controlling the sensory deterioration still poses a challenge to brewing chemists.

During beer ageing, the beer changes its flavour in numerous ways; fresh flavour and aroma characteristics can decline in intensity (e.g. pleasant bitterness), whereas undesired compounds may arise or increase in concentration and impart stale characteristics. Meilgaard (Meilgaard et al., 1979), Dalgliesh (Dalgliesh, 1977) and later Zufall et al. (Zufall et al., 2005) described those changes in detail for specific beers. However, stale beer flavour cannot be generalised across beer styles, or even for different brands of the same beer style. The changes which occur during storage depend on temperature, time, light exposure, pH-level, oxygen content and beer style (Vanderhaegen et al., 2006). Furthermore, recent work observed ageing reactions initiated by a combination of elevated temperatures and vibrations during beer transport (Jaskula-Goiris et al., 2019, Paternoster et al., 2019).

High levels of oxygen in packaged beer are associated with a fast deterioration of beer flavour, hence oxygen pick-up is avoided wherever possible throughout the brewing and packaging processes. However, the packaged beer (can, bottle, etc.) is not always a perfectly closed system. In many oxidative reactions, radicals are formed as intermediates, reacting with beer components and greatly catalysing beer deterioration (Uchida et al., 1996). Antioxidants such as sulphites, thiols, some vitamins (e.g. Vit. C and E) can compete with pro-oxidants (e.g. metal ions like iron or copper), hence inhibit the formation of radicals (Andersen and Skibsted, 1998, Lund et al., 2015). Interestingly some antioxidants can act beneficially as well as detrimentally on the oxidative beer

stability; e.g. Vitamin C can reduce transition metal ions back to their lower oxidation state, thus allowing them to act as pro-oxidants again (Belitz, 2009, Kunz et al., 2013). Furthermore, Bamforth, among others, highlighted that beer deterioration can occur even at very low oxygen levels, suggesting that some beer staling pathways are non-oxidative (Bamforth, 1999a).

Vanderhaegen et al. (Vanderhaegen et al., 2006) summarised in a review the main compound classes associated with beer staling as principally ketones, cyclic acetals, heterocyclic compounds, ethyl esters, lactones, sulphur-compounds and aldehydes. The latter are considered major contributors to beer staling, due to their very low flavour thresholds (Meilgaard, 1975a, Meilgaard, 1975b, Saison et al., 2009b). Therefore, aldehydes have been intensively investigated since the first report in the 1960s by Hashimoto et al (Hashimoto, 1966), who noted a major increase in concentrations of these volatile carbonyls during beer storage, which coincided with the appearance of off-flavours during beer ageing. Since then, several aldehydes have been proposed as beer flavour instability markers, including compounds such as 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, methional, benzaldehyde, phenylacetaldehyde, furfural, hexanal and *trans*-2-nonenal (Baert et al., 2012, Jaskula-Goiris et al., 2011, Malfliet et al., 2008, Saison et al., 2010b, Vesely et al., 2003). In general, aldehyde levels in finished beer can increase through i) *de novo* formation and ii) release from bound-state. Numerous pathways have been proposed in previous studies (Hashimoto and Kuroiwa, 1975, Kobayashi et al., 1994, Wietstock and Methner, 2013) and reviews (Baert et al., 2012, Vanderhaegen et al., 2006) on the origin of aldehydes formed *de novo*. Amongst the potential pathways, the oxidation of unsaturated fatty acids, Maillard reactions and Strecker degradation, as well as direct oxidation of amino acids are considered the most common pathways. Alternatively, aldehydes could be converted to non-volatile adducts, mainly by binding to compounds such as bisulphite (Dufour et al., 1999, Kaneda et al., 1994), cysteine (Baert et al., 2018, Baert et al., 2015a, Baert et al., 2015b, Bustillo Trueba et al., 2019) or other amino acids (forming imines) (Lermusieau et al., 1999, Liégeois et al., 2002). Up to now, scientists are still debating the contribution of bound-state forms on beer staling. Aldehydes in adduct form cannot be evaporated during the wort production process, due to their decreased volatility compared to the free forms (Ditrych et al., 2019). On the one hand, the interaction of free and bound

aldehydes is in chemical equilibrium – while free aldehydes are reduced during fermentation, bound-state aldehydes dissociate releasing free forms, which yet again can be reduced by yeast (Bamforth, 1999a). However, several researchers (Debourg et al., 1994, Drost et al., 1990, Perpète and Collin, 2000, Saison et al., 2010a) emphasised the complexity of aldehyde reducing systems; aldehydes can interact with numerous wort components making them non-reducible by the yeast during fermentation. Especially, the binding strength and dissociation rate is strongly affected by the pH (Baert et al., 2018, Bustillo Trueba et al., 2018, Kaneda et al., 1994), which changes substantially from malt (pH = 6), wort (pH = 5.2) up to the finished beer (pH = 4.3). Thus, the equilibrium between free and bound aldehydes might be insufficient for complete aldehyde removal and therefore bound-state aldehydes remain potential contributors to beer flavour deterioration.

Previous studies (De Clippeleer et al., 2010b, Ditrych et al., 2019, Gastl et al., 2006, Guido et al., 2007) suggested that malt is a major source of staling precursors, such as amino acids, lipids, and flavour-active aldehydes in free or bound form. In other studies, beer ageing has been positively correlated to free amino nitrogen (FAN) content, Kolbach Index and heat load (TB-Index) (Jaskula-Goiris et al., 2015, Jaskula-Goiris et al., 2011, Thalacker and Böβendörfer, 2005). Additionally, free radical formation increases with increasing malt colour, heat load and thus Maillard reaction products present in malt (Cortés et al., 2010, Kunz et al., 2012a).

From the perspective of green malt brewing, the lower amount of heat load applied (hence lower TBI values as discussed in Chapter 3), could potentially be favourable for beer flavour stability. This lower total head load might result in less occurrence of Maillard reactions and thus a decrease of Strecker aldehyde formation (De Clippeleer et al., 2010a, Gastl et al., 2006, Jaskula-Goiris et al., 2015, Malfliet et al., 2008). Additionally, the pool of natural antioxidants present in green malt, especially those within the rootlets (Bonnely et al., 2000, De-Jing et al., 2009, Peyrat-Maillard et al., 2001), could potentially reduce the formation of free radicals. However, the high lipoxygenase activity in green malt (Doderer et al., 1992) can lead to enzymatic lipid oxidation. In summary, green malt wort and beer could have a potential advantage in terms of flavour stability of the beer, provided lipoxygenase activity can be controlled

(De Clippeleer et al., 2010a, Gastl et al., 2006, Jaskula-Goiris et al., 2015, Malfliet et al., 2008).

Considering the preliminary results discussed in Chapter 3, which demonstrated promising indicators for flavour stability in worts and beers produced from green malt, the following trials intensively focused on the flavour and flavour stability. To the best of our knowledge, no prior studies have focused on the flavour stability of wort and beers made from green malt. Evaluating the flavour stability of green malt beers has the potential to improve understanding of the factors which influence flavour stabilities of regular kilned malt beers. Because there is substantially less heat load on the malts added to the brewing process this could result theoretically in less aldehyde formation. Therefore, the present study aims to evaluate the content of free aldehydes, classified as staling markers in green malt as well as the corresponding kilned malt control used for the previously discussed brewing trials (Section 3.2.1). Additionally, the evolution of staling aldehydes was monitored across the wort production process and subsequently in finished fresh beers. The oxidative stability of worts and beers produced from green malt have been evaluated using Electron Spin Resonance (ESR) spectroscopy. Levels of significant pro- or antioxidants in beer are reported, such as sulphites (able to remove hydrogen peroxide), free thiols, and transition metal ions. The results will enable us to further understand the impacts of brewing with green malt (thus lowering the heat load on raw materials inwards to the process) on these flavour stability indicators.

4.2 Material and methods

4.2.1 Chemicals and reagents

Ultrapure type-1 grade (milli-Q, 18.2 MΩcm at 25°C) water obtained from a Synergy 185 system from Milipore S.A. (Molsheim, France) was used for chemical analysis and glassware washing. Electron Spin Resonance analysis was performed at the University of Copenhagen and water was purified through a Milli-Q water purification system (Millipore, Billerica, USA). *Free Aldehydes:* The carbonyl compounds 2-methylpropanal (2MP ≥ 99%), 2-methylbutanal (2 MB, ≥ 95%), 3-methylbutanal (3 MB, 98%), hexanal (HEX, ≥ 98%), furfural (FUR, ≥99%), methional (MET, ≥ 95%), *trans*-2-nonenal (T2N, ≥95%)and phenylacetaldehyde (PHE, ≥ 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deuterated 2-methylbutanal (2MB-d_{10}) was requested from

MercaChem (Nijmegen, the Netherlands); deuterated benzaldehyde (benzaldehyde-d_6) was purchased from Sigma Aldrich (St. Louis, MO, USA). Ethanol absolute (≥ 99.5%) was purchased from Merck KGaA (Darmstadt, Germany). The derivatisation agent stock solution PFBHA (o-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride) was obtained from Sigma Aldrich (St. Louis, MO, USA). *Electron Spin Resonance (ESR)*: PBN (N-tert-Butyl-α-phenylnitrone) and TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl radical) were purchased of analytical grade from Sigma Aldrich (Steinheim, Germany). *Sulphite and free thiols:* ThioGlo1 fluorescent reagent was purchased from Berry & Associates Inc. (Dexter, MI, USA). Sodium sulphite (Na_2SO_3) was purchased from J.T. Baker (Deventer, The Netherlands) and N-acetylcysteine (NAC) from Sigma-Aldrich (St. Louis, MO, USA). *Metal analysis*: Nitric acid was purchased at trace metal grade (HNO_3, Fisher Scientific, Loughborough, UK).

4.2.2 Beer production

The malting barley variety Etincel was sourced from Boortmalt, Antwerp. Samples (green malt and the corresponding kilned pilsner style malt) were collected at the equivalent time-points (final day of germination and off-kiln respectively) during six industrial malting cycles. No further information about the commercial malting procedure is available.

The brewing, fermentation and filtration protocol used was described in detail in Chapter 3 (Section 3.2; Material and Methods). Samples were collected at the onset of mashing, end of mashing, first wort (mash filtration), onset of boiling, end of boiling, end of clarification and end of cooling (pitching wort). All wort samples were N_2 flushed and stored at -20°C immediately after the sample was taken. The samples taken at the onset and end of mashing were centrifuged to remove the suspended grist material before nitrogen flushing and subsequent freezing of the samples.

Beer samples were bottled using a six-head counter pressure filler with double pre-evacuation with intermediate CO_2 rinsing and over-foaming with hot water injection before capping (Monobloc, CIMEC, Italy). The resulting beers produced from green malt ‘as is’, re-steeped green malt and the corresponding reference malts were stored at 0°C to preserve freshness.

4.2.3 Determination of moisture content of malt

To recalculate the obtained aldehyde concentrations to the dry mass of malt samples, the moisture content was determined according to the EBC method (Analytica 4.2).

4.2.4 Extraction of aldehydes from malt

Free aldehydes in malt were determined, using authentic reference compounds according to the method described by Filipowska et al. (Filipowska et al., 2020). Finely milled malt (1 g) was mixed with 99 mL of Milli-Q water (N_2 flushed) under oxygen limited conditions. Samples were mixed for 15 min at ambient temperature. Subsequently, after sedimentation, 10 mL of the supernatant was transferred into an amber glass vial (20 mL), capped and subjected to aldehyde quantification.

4.2.5 HS-SPME-GC-MS determination of free aldehydes

Free aldehydes - 2-methylpropanal (2MP), 2-methylbutanal (2 MB), 3-methylbutanal (3 MB), hexanal (HEX), furfural (FUR), methional (MET), phenylacetaldehyde (PHE) and *trans*-2-nonenal (T2N) - were determined according to De Clippeleer (De Clippeleer, 2013) and Baert (Baert, 2015) following the protocol described by Ditrych et al. (Ditrych et al., 2019). The selected aldehydes were determined using headspace-solid phase microextraction (HS-SPME) with on-fibre PFBHA (*o*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine) derivatisation, followed by gas chromatography coupled with mass spectrometry. Samples, placed on a cooling tray (5°C) were transferred by the CombiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland) from the cooling tray to the agitator (30°C). Here the sample was spiked with a stable isotope-labelled internal standard (20 µg/L of 2-methylbutanal-d_{10} and 20 µg/L of benzaldehyde-d_6) combined in ethanol absolute and subsequently homogenised through shaking at 500 rpm for 2 minutes (5 s shaking, 2 s rest). The SPME fibre (65 µm, PDMS/DVB fibre, Stableflex/SS SPME Fibre Assembly, Supleco Analytical, Bellefonte, PA, USA) was first subjected to bake-out conditioning, then exposed to the headspace of 10 mL of freshly prepared aqueous PFBHA solution (derivatisation agent; 1g/L). The fibre was loaded with PFBHA for 10 min during its exposure to the headspace while being shaken at 250 rpm (5 sec shaking, 2 sec rest). Subsequently, the loaded fibre was exposed to the sample's headspace, extracting aldehydes for 30 min, while being shaken under the same conditions. Due to the interaction of aldehydes with PFBHA,

pentafluorobenzyloximes (PFBOs) are formed. Subsequently, the PFBOs were thermally desorbed from the solid phase by the introduction of the fibre into the injector of a Focus GC gas chromatograph (Thermo Fisher Scientific Inc., Waltham, MA, USA) for 3 minutes at 250°C. The GC was equipped with a split/ splitless injector with a narrow glass inlet liner (0.5 ml volume), and a RTX-1 Crossbond 100 % dimethyl polysiloxane capillary column (40-m length, 0.18-mm i.d., 0.20-µm film thickness, Restek Corporation, Bellafonte, PA, USA). Helium was used as a carrier gas and the flow rate was set to 0.8 mL/min. The inlet temperature was set at 250°C and injection was carried out in the split mode with a split flow of 10 mL/min and split ratio of 12. The oven temperature was kept at 50°C for 2 min, then raised to 250°C at 6°C/min up to 250°C and held at 250°C for 5 min.

The transfer line between the GC and the mass spectrometer was kept at 260 °C. Aldehyde detection was achieved using the ISQ Single Quadrupole (Thermo Fisher Scientific Inc., Waltham, MA, USA) with a chemical ionisation source type operated at 185°C. Methane was used as the reagent gas (1.5 mL/min). The mass spectrometer operated as follows: electron lens was set to 1.5 V, the electron energy to 70 eV, the emission current to 50 µA, and the detector grain to 3.00×10^5. Compounds were detected and quantified using selected ion mode (SIM), by choosing one characteristic ion with a negative charge per compound. The selected ions were as follows: 2MP m/z 247; 2MB and 3MB m/z 261; HEX m/z 275; FUR m/z 271; MET m/z 279; PHE m/z 295; T2N m/z 315; 2MB-d_{10} m/z 270; benzaldehyde-d_6 m/z 287. Data were processed with XCalibur™ (Thermo Electron Corporation, Waltham, MA, USA) and quantified based on the external calibration line prepared from authentic reference compounds (N_2 flushed milli-Q water was used as a matrix for calibration).

4.2.6 ESR analysis of wort and beer with PBN as the spin trap

The oxidative stability of wort or beer can be determined by measuring the formation of free radicals over time, due to forced ageing, using electron spin resonance spectroscopy (ESR). Samples were incubated in a water bath at 60°C based on the method described by Uchida et al. (Uchida et al., 1996). Before ageing, the beers were degassed by adding 1-octanol (10 µL). PBN (N-tert-Butyl-α-phenylnitrone) was used as a spin trap to detect the 1-hydroxyethyl radical in beer and wort. The spin trap (0.1 mL of

a 600 mM PBN solution in 96% ethanol) was dissolved in 1.9 mL samples of wort or beer. Subsequently, the wort or beer samples containing PBN (30 mM final concentration) were heated at 60°C in a closed bottle under atmospheric oxygen to exhaust the natural antioxidants present. Assays were run for at least 120 min with 12 samples taken during this time.

The ESR spectra were obtained using an ECS 106 spectrometer (Bruker, Rheinstetten, Germany) equipped with an ER 4103 TM cavity. The settings were as follows: microwave power, 20 mW; modulation amplitude, 1.0 G; modulation frequency, 100 kHz; conversion time, 164 ms; and time constant, 82 ms. The wort or beer samples were contained in a quartz flat aqueous cell (Wilmad Glass, Buena, NJ), and all spectra were recorded at room temperature. The response of the ESR instrument was checked daily by recording the spectrum of an aqueous solution of 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO). All data were corrected against the TEMPO standard signal.

4.2.7 Quantification of sulphites and free thiols

Quantification of sulphite and free thiols (R-SH) was performed according to the previously described methods by Abrahamsson et al. (Abrahamsson et al., 2012) and Hoff et al. (Hoff et al., 2013) based on the derivatisation of sulphite and free thiols with ThioGlo1 fluorescent reagent (2.6 mM) in water free acetonitrile followed by separation with reversed-phase high-performance liquid chromatography (RP-HPLC) and fluorescence detection. Standard addition curves were prepared with sulphite (Na_2SO_3) and N-acetylcysteine (NAC) in untreated beer samples. The column used was a Jupiter C18, fully porous silica column (LC columns 150 x 2. 0mm, 5 µm particle size, 300Å pore size, Phenomenex). The fluorescence detector was set to 242 nm excitation and 492 nm emission. Total run time was 16 min with 4 min post-run.

4.2.8 Multi element analysis by ICP-MS

Nitric acid (HNO_3, trace metal grade) was added to beer samples to a final concentration of 2% and let stand for 24 h. Quantification of a wide range of minerals in the fresh beers was achieved by using inductively coupled plasma–mass spectrometry (ICP–MS) (Thermo-Fisher iCAP-Q, Waltham, MA, U.S.A.) with a 'Flatopole collision cell' (charged with helium gas) upstream of the analytical quadrupole. Internal standards were introduced to the sample stream via a T-piece and included Sc (50 µg/L), Ge (20 µg/L)

Rh (10 µg/L), and Ir (5 µg/L) in the matrix of 2% HNO_3. External calibration standards were used for quantification. Samples were introduced via an autosampler (Cetac ASX-520; Thermo-Fisher Scientific, Waltham, MA, U.S.A.) through a venturi nebuliser (Thermo-Fisher Scientific). Sample processing was undertaken using Qtegra software (Thermo-Fisher Scientific, Waltham, MA, U.S.A).

4.2.9 Statistical analysis

All samples were analysed in at least three biological replicates with 2-4 technical replicates. Statistical analyses were performed using Microsoft Excel 2013/XLSTAT (XLSTAT version 2020.1.1.64347, Addinsoft, Life Science, New York, USA). An α-risk of 0.05 was set as the level of significance in all data analyses. The statistical significance of the data obtained was established with analysis of variance (ANOVA), Tukey's HSD test was performed as the post-hoc test. Principal component analysis (PCA) was performed to characterise variation and highlight strong patterns in the dataset. The Pearson correlation coefficient was established to determine linear relationships between two variables; the strength and direction of the relationship was reported as a Pearson correlation coefficient (r).

4.3 Results and discussion

4.3.1 Investigation of free staling aldehydes in malt

Malt is known to be a major source of aldehydes, the impact on beer quality and flavour has already been discussed in several studies (De Clippeleer et al., 2010b, Ditrych et al., 2019, Gastl et al., 2006, Guido et al., 2007). Kilning directly impacts the formation of aldehydes (e.g. by Strecker degradation), with an increased heat load positively correlating with aldehyde formation. From the perspective of green malt, the lower amount of heat load applied (low TB-Index), could result in lower free aldehyde levels. Thus, the malts used for the preparation of beers described in Chapter 3 were evaluated for their free aldehyde levels. Several aldehydes were selected as beer flavour instability markers (Baert et al., 2012, Jaskula-Goiris et al., 2011, Malfliet et al., 2008, Saison et al., 2010b, Vesely et al., 2003), namely: 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, methional, furfural, phenylacetaldehyde, hexanal and *trans*-2-nonenal.

As illustrated in Table 4.1, pilsner-style (kilned) malt contained significant concentrations of the measured free aldehydes. In germinated (green) malt, on the other hand, significantly lower ($p < 0.0001$) total aldehyde levels were detected compared to the respective control malt. Even though, the same malting barley variety was used and malted after the same malting scheme (according to the supplier), the free marker aldehydes concentrations differed substantially between the biological replicates of green malt (n = 3), re-steeped green malt (n = 3) or kilned malt (n = 6). The total marker aldehydes level in kilned malt samples ranged from 1.9 mg/kg d.b. (KM3) to 6.7 mg/kg d.b. (KM2). Potentially, the nature of the samples (industrial scale) could have caused this high variation in aldehyde levels. Additionally, it is unknown at which depth in the kiln bed the samples were taken. Previous studies (Guido et al., 2005, Müller et al., 2014) already showed, that the temperature and moisture conditions at the different kiln layers (upper, middle or bottom layer), impacted the chemical reactions occurring in the malt. Thus, malt sampled from different bed depths in a kiln can show a very different aldehyde profile (Guido et al., 2005).

Table 4.1: Aldehydes in malt expressed in µg/kg dry base (d.b.). GM = green malt, KM = kilned malt; 2MP = 2-methylpropanal; 2MB = 2-methylbutanal; 3MB = 3-methylbutanal, PHE = phenylacetaldehyde; MET = methional; FUR = furfural; HEX = hexanal; T2N = *trans*-2-nonenal

	Brew Nr.	µg/kg d.m.	2-MP	2-MB	3-MB	HEX	FUR	MET	PHE	T2N	Total
	1	GM	58.0	10.0	41.5	94.9	24.9	11.9	124.7	48.0	**413.9**
		±SD	4.1	1.5	0.4	18.9	1.7	2.0	6.9	5.8	41.3
		KM	187.7	145.0	398.2	245.9	190.8	61.1	317.1	456.2	**2002.1**
		±SD	47.6	33.6	35.0	9.0	5.8	1.3	24.6	71.4	228.3
	2	GM	225.0	27.1	84.1	86.8	23.6	14.9	118.2	30.4	**610.2**
		±SD	11.4	8.1	5.5	16.8	1.2	4.2	15.9	2.4	65.5
		KM	1098.1	885.7	2048.0	209.0	367.4	219.8	1024.3	809.2	**6661.4**
		±SD	19.0	2.5	11.1	9.5	56.9	10.3	17.3	27.8	154.3
	3	GM	103.9	8.5	33.8	77.2	28.9	15.8	139.2	38.2	**445.5**
		±SD	16.2	0.7	1.2	14.1	1.0	3.2	4.1	3.2	43.9
		KM	196.6	174.1	345.5	279.0	85.3	65.9	166.9	587.2	**1900.6**
		±SD	1.2	7.6	12.2	44.3	17.4	11.5	12.4	23.3	129.9
re-steeping trials	4	GM	81.2	9.7	34.8	441.9	26.2	17.3	96.5	35.3	**742.8**
		±SD	13.7	1.3	3.8	34.7	0.3	0.0	8.9	2.2	65.0
		KM	541.5	492.0	1252.5	183.1	329.5	165.6	675.2	509.4	**4148.8**
		±SD	85.5	87.9	202.2	30.0	22.5	2.2	78.4	64.6	573.3
	5	GM	111.9	13.6	43.5	301.8	33.5	14.8	178.6	29.6	**727.5**
		±SD	25.6	2.2	3.6	23.3	2.8	2.8	24.1	1.5	85.8
		KM	369.6	344.4	863.3	186.5	297.0	143.1	507.2	447.7	**3158.6**
		±SD	40.9	47.3	114.2	10.1	60.6	25.8	68.0	57.2	424.1
	6	GM	48.8	4.6	21.7	109.9	24.7	12.5	111.8	17.6	**351.5**
		±SD	3.5	0.3	0.1	20.0	1.1	2.7	14.1	0.2	42.2
		KM	770.9	681.4	1707.2	194.0	419.2	180.5	595.5	381.2	**4930.0**
		±SD	22.1	26.0	47.1	16.5	17.2	2.6	10.9	31.1	173.5

The concentrations of aldehydes in kilned malt are significantly higher compared to their corresponding green malt, with levels up to 36-fold higher (3-methylbutanal). These findings further confirm the influence of heat load on the aldehyde content of malt. In all kilned malt samples, 3-methylbutanal was present in the highest concentrations, which is in accordance with previous findings by Jaskula-Goiris et al. (Jaskula-Goiris et al., 2011). In green malt ('as is') clearly, phenylacetaldehyde and 2-methylpropanal dominated, whereas the hexanal levels significantly increased in two re-steeped green malt replicates. Figure 4.1 displays the Bi-plot principal components 1 and 2 resulting from PCA of the malts and aldehyde levels.

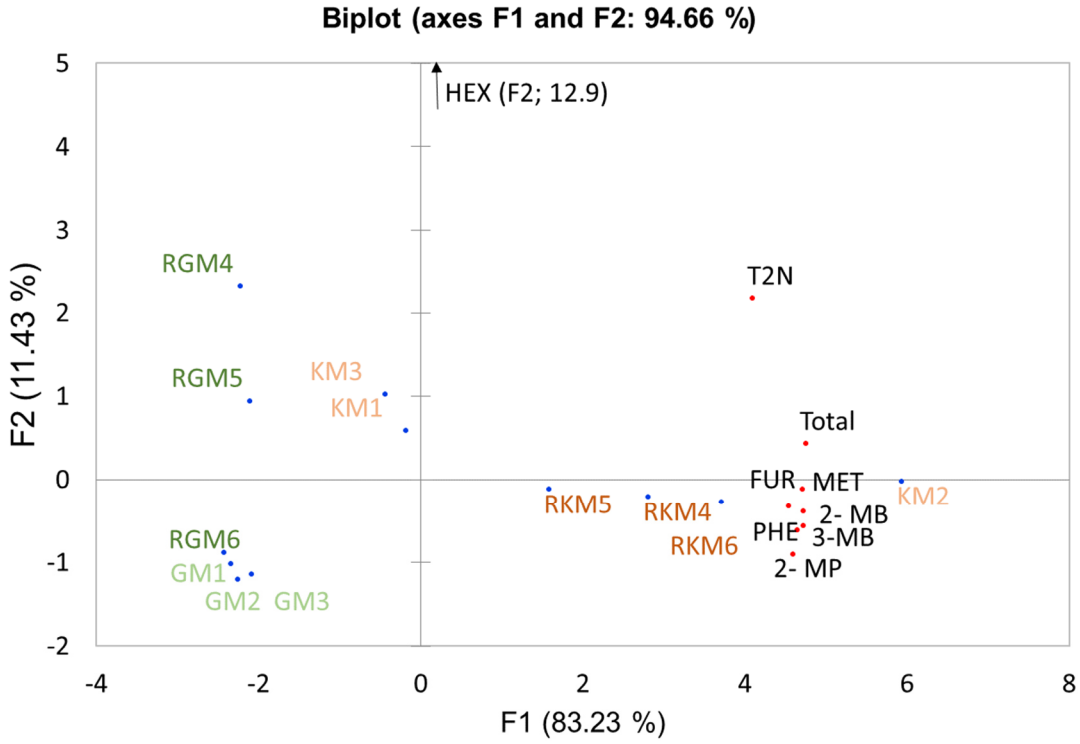


Figure 4.1: Bi-plot of PCA on the aldehyde levels obtained from malt. Green malt 'as is' (GM 1-3) and the corresponding reference malt (KM 1-3); as well as re-steeped RGM (4-6) and the corresponding reference malt (RKM 4-6). 2MP = 2-methylpropanal; 2MB = 2-methylbutanal; 3MB = 3-methylbutanal, PHE = phenylacetaldehyde; MET = methional; FUR = furfural; HEX = hexanal; T2N = *trans*-2-nonenal

Overall, the biplot of PC 1 and 2 accounts for about 95% of the variation in the data set. The negative loadings along PC1 (83% of variation) were associated with malts low in the selected free aldehyde markers, except for hexanal which was primarily loaded on PC2. The positive loadings in PC1 were associated with higher aldehyde levels and mainly

with the kilned malt samples. Furthermore, the resulting biplot highlighted the great inconsistency between the malt samples. In particular, kilned malt samples KM1, KM2 and KM3 differed greatly. Whilst the malt sample KM2 contained the highest amount of free aldehydes, KM1 and KM3 contained the lowest levels out of all six kilned malts selected for this study, even though all of these malt samples are supposedly biological replicates (same malting procedure and time-point of malt collection), as was discussed previously.

The loading plot separated hexanal (PC2, 11.4% of variation) clearly from the remaining marker aldehydes. This is most likely because hexanal is the only aldehyde that was present at higher concentrations in two of the re-steeped green malts (but not in trial RGM6), compared to their control kilned malts. Untreated green malts (GM1-3), on the other hand, contained significantly lower hexanal levels than the corresponding control kilned malts. The re-steeping process was shown (Chapter 2) to suppress LOX activity by 50% from its initial activity. Hexanal could have originated through enzymatic lipid oxidation due to residual LOX activity, autooxidation, or through dissociation of bound hexanal and thus release of free hexanal. However, the ambiguous results received did not allow to draw clear conclusions.

It is interesting to note, that even though lipoxygenase activity reduced significantly during kilning compared to the initial activity measured in the green malt (Huang et al., 2016, Schwarz and Pyler, 1984), concentration of *trans*-2-nonenal was lower in all green malt samples; untreated and re-steeped (18 – 48 µg/kg d.b., n = 6) compared to the control malts (381 – 809 µg/kg d.b.). As shown in a previous study, at the onset of kilning, mainly the first 2 – 6 hours, LOX activity can increase (Kaukovirta-Norja et al., 1998). These results indicate that the risk to oxidise lipids remains at moderate kilning temperatures. However, it remains true that LOX activity is significantly higher in green malt compared to finished kilned malt, thus it is necessary to further evaluate the *trans*-2-nonenal and hexanal levels during wort production.

4.3.2 Investigation of free staling aldehyde levels throughout the brewing process

Firstly, the aldehyde content determined in malt was compared with the corresponding levels at the onset of mashing. Considering the high variations of aldehydes in the malt, high deviations at the onset of the brewing process were observed. However, a strong

positive correlation ($r(10)= 0.9341$, $p < 0.001$; Figure 4.2) was found between the levels of aldehydes quantified in malt and samples taken at the onset of mashing, which is in agreement with earlier findings by Ditrych et al. (Ditrych et al., 2019).

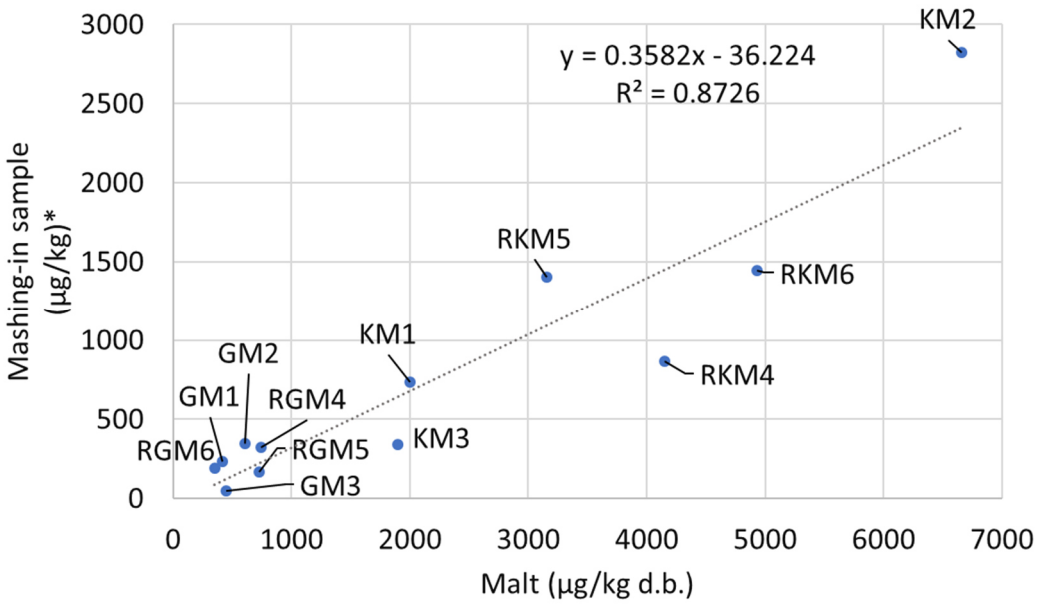


Figure 4.2: Correlation of total marker aldehyde concentration in malt (µg/kg d.b.) and the corresponding samples taken at the onset of mashing (µg/kg d.b.; *aldehyde concentration recalculated to 1 kg of malt).

Additionally, aldehyde levels were determined throughout all twelve brewing trials. Samples were collected at the onset of mashing, end of mashing, first wort (mash filtration), onset of boiling, end of boiling, end of clarification and end of cooling (pitching wort). As already observed in malt, also throughout the brewing process we experienced a high variation in total aldehyde levels, resulting in very high standard deviations when summarising the biological replicates. Nevertheless, the resulting data still reveal very important information about green malt worts. Thus, Figure 4.3 (A-D) displays the averages of the biological replicates; standard deviations are omitted for clarity. The full details of each individual brewing trial can be found in the appendix.

Comparing the averages of the aldehyde concentrations measured during the wort production cycles (Figure 4.3), the highest aldehyde levels (except for furfural) were observed at the onset of mashing, confirming (De Clippeleer et al., 2010b, Ditrych et al., 2019, Gastl et al., 2006, Guido et al., 2007) that malt is the major source of aldehydes

entering the wort production process. Hence, as expected, significantly lower levels of marker aldehydes were measured when mashing in with green malt, compared to the mash resulting from the reference kilned malts. To emphasise the quantitative difference the graphs plotted in Figure 4.3 were adjusted to similar axis scales. Naturally, the formation of bound-state aldehydes is very complex, but in principle, fewer aldehydes are available for adduct formation when using green malt, and thus, an improved endogenous ageing potential in green malt beers, may be hypothesised.

Due to the low boiling points of the smaller aldehydes, their levels dropped, especially during wort boiling, whilst furfural, a heat load indicator, clearly increased in concentration especially during wort boiling. All measured aldehydes derived from lipid oxidation or Strecker degradation declined significantly in concentration during the wort production process.

Another important trend that can be observed is that in all green malt brews (n = 6, Figure 4.3 and appendix) the *trans*-2-nonenal levels significantly decreased during mashing. This may indicate that the high LOX activity measured in green malt, was sufficiently controlled by the lipoxygenase hostile conditions applied at the onset of mashing. On the other hand, the mashing process was very efficient in all twelve brews to reduce the free *trans*-2-nonenal levels delivered to the brewing process by the malt. This is possibly due to binding of *trans*-2-nonenal to insoluble matter associated with the spent grains.

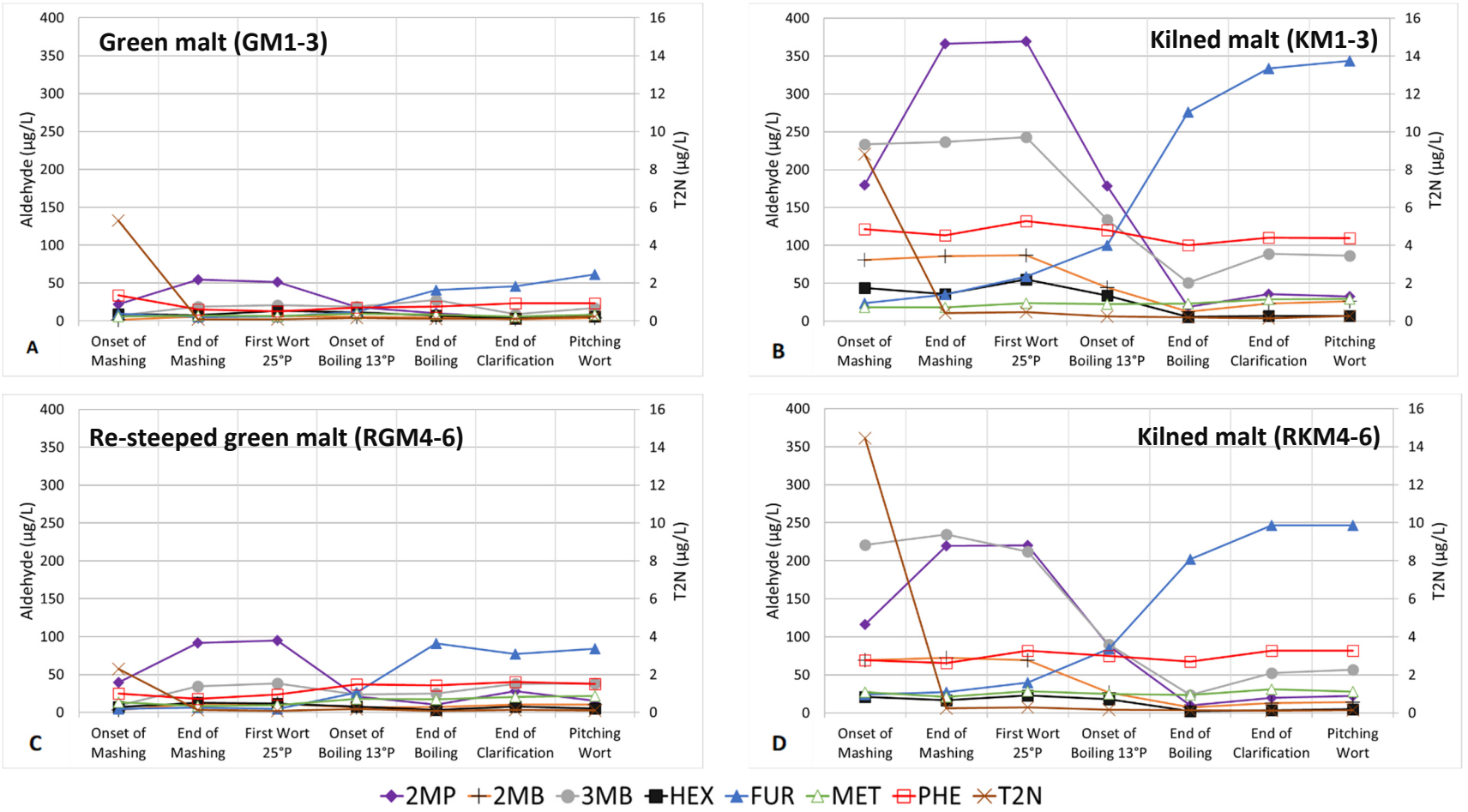


Figure 4.3: Change in selected aldehyde concentrations during wort production. Worts prepared from A) green malt (GM1-3), B) kilned malt (KM1-3), C) re-steeped green malt (RGM4-6), D) kilned malt (RKM4-6). Data represent the average of 3 biological replicates with each 2-3 technical replicate measurements.

Figure 4.4 presents the analysed concentrations of the free aldehydes in pitching wort. Kilning resulted in significantly higher concentrations of determined free aldehydes, except for the lipid oxidation products hexanal and *trans*-2-nonenal. Re-steeping resulted in significantly higher concentrations of free aldehydes (except hexanal and *trans*-2-nonenal) compared to GM 'as is'.

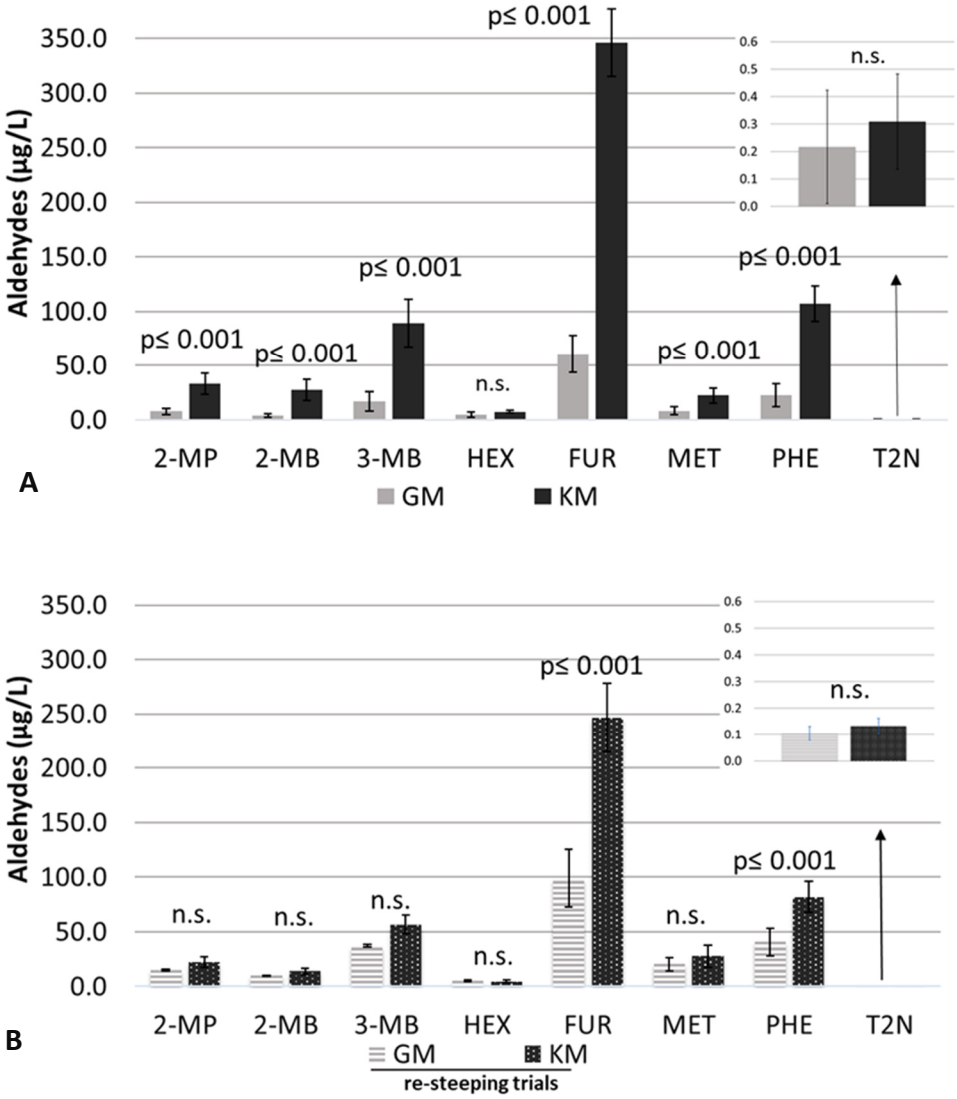


Figure 4.4: Aldehyde levels in cool clarified wort prepared from (A) green malt 'as is' and the corresponding control kilned malt (B) from re-steeped green malt and the corresponding control. Samples are presented as mean ±SD of 3 biological replicates with each 2 replicate measurements. Statistics: One-Way ANOVA

The furfural concentration was 5.5-fold higher in kilned malt wort compared to wort from green malt 'as is' and 2.5-fold higher in kilned malt compared to the wort prepared from re-steeped green malt. According to these results, significantly lower total free aldehyde levels can be expected in pitching worts produced from green malt 'as is' compared to all other brews. Those results once again suggest that green malt wort has very promising flavour stability metrics. Furthermore, it is interesting to note, that even though such a high variability was observed when comparing the biological replicates, the resulting wort showed aldehyde levels with low standard deviations. Between the concentrations of aldehydes in the malt and the resulting pitching wort we can still find a moderate positive correlation ($r(10) = 0.7468$, $p < 0.05$). Without furfural, which was formed in the highest concentrations during thermal processes throughout brewing and cannot be evaporated due to the high boiling point (161.7 at 760 mmHg 25°C (Lidel, 1999)), a slightly stronger correlation was achieved ($r(10) = 0.78$, $p < 0.05$).

4.3.3 Measurement of free staling aldehydes in fresh beer

In all fresh beer samples following fermentation, no significant differences were found in staling aldehyde concentrations regardless of whether they were prepared from green malt or kilned malt (Figure 4.5, $r(10)= 0.14$; n.s.). It is generally accepted that yeast metabolism can reduce aldehydes in the wort to their corresponding alcohols (Debourg et al., 1994, Peppard and Halsey, 1981).

Nevertheless, the immense reduction of free aldehydes throughout the brewing process and subsequently fermentation is remarkable, considering the enormous differences between these concentrations and corresponding aldehyde levels in the malts. These results (Figure 4.5) suggest that even though malt is the major source of aldehydes in the brewing process, the upstream brewing process and subsequent fermentation, are sufficient to reduce the free aldehydes to levels below the relative sensory threshold as reported by Saison et al. (Saison et al., 2009b).

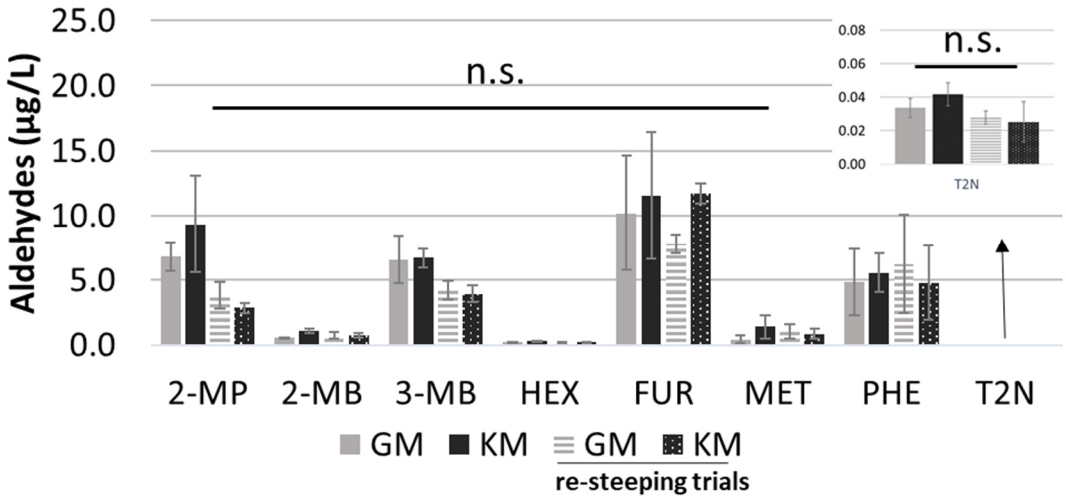


Figure 4.5: Free aldehyde levels measured in fresh beer (µg/L). Samples are presented as mean ±SD of 3 biological replicates with each 2 replicate measurements. GM = green malt; KM = kilned malt. Statistics: One-Way ANOVA

Thus, we cannot conclude at this stage that higher levels in the malt will result in higher levels in the fresh beer. However, it should be remembered that aldehydes may also be converted into adduct forms, due to their binding affinity with, e.g., bisulphite (Dufour et al., 1999, Kaneda et al., 1994), cysteine (Baert et al., 2018, Baert et al., 2015a, Baert et al., 2015b) or other amino acids (forming imines) (Lermusieau et al., 1999, Liégeois et al., 2002). The formation of adducts would reduce the volatility of free aldehydes, and thus impede evaporation during the brewing process. Additionally, it is not yet understood whether the chemical equilibrium between free and bounds forms enables a complete reduction of free aldehydes by yeast. Thus, to fully understand the implications of varying grist bill on the staling potential of beers it is necessary to investigate the aldehyde formation (and potential release) during beer ageing (Chapter 5).

4.3.4 Determination of oxidative stability of worts and fresh beers via ESR analysis

Electron Spin Resonance (ESR) analysis provides information about the oxidative stability of samples by detecting and quantifying radicals formed as intermediates in oxidative reactions induced by forcing the samples at 60°C. ESR measurements were performed on pitching worts and finished beers to determine the influence of green malt or kilned malt used for the brewing trials on the oxidative stability.

Several authors (Cortés et al., 2010, Furukawa Suárez et al., 2011, Kunz et al., 2013) suggested that an increased formation of Maillard reaction products was associated with an acceleration of oxidative processes, and thus lower oxidative stability. Kunz et al. showed (Kunz et al., 2012a), that when using unmalted barley (lower total heat load) a lower content of specific Maillard reaction products led to a lower radical generation and thus better oxidative stability in the worts and beers measured. Green malt contains fewer Maillard reaction products due to the omission of the heating step, thus better oxidative stability was expected in green malt wort and beer according to this hypothesis.

Figure 4.6 is an example of a typical ESR profile of the wort from green malt plotted in comparison to the control wort, prepared from the corresponding kilned malt. This graph highlights that the wort produced from green malt produced fewer radicals than the reference.

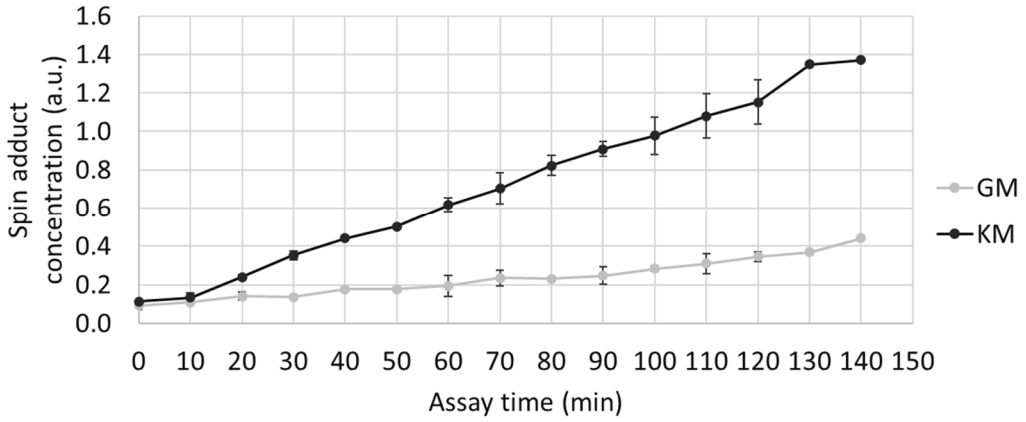


Figure 4.6: Electron Spin Resonance (ESR) measurements of free radical formation versus time of forced ageing at 60°C. Comparison between green malt wort (GM3) and its paired control (KM3). Data are presented as mean ±SD of 2 replicate measurements.

The T120 value indicates the ESR signal intensity (extent of radical formation) determined after force ageing (60°C) the sample for 120 min. Figure 4.7 summarises the T120 values of all worts prepared from green malts ‘as is’, re-steeped green malt and their respective controls.

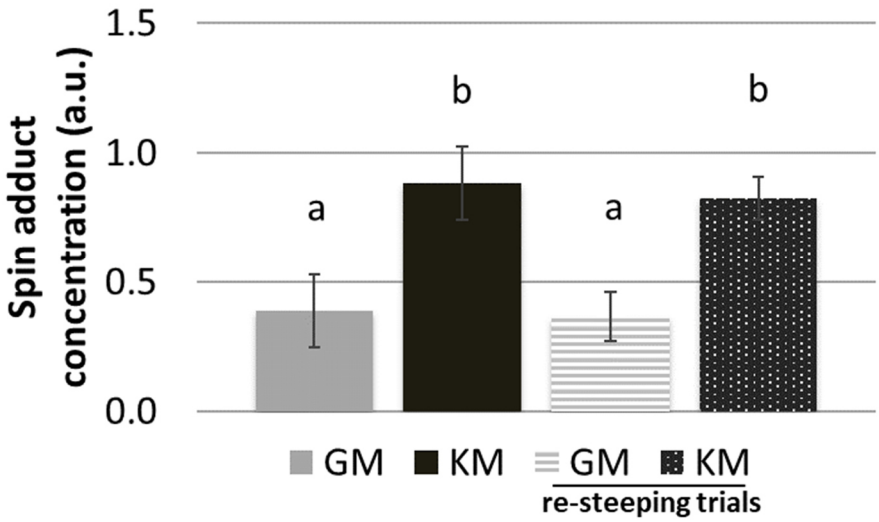


Figure 4.7: Formation of spin adducts in pitching wort samples measured by ESR. Wort samples were forced aged for 120 min at 60°C (T120 values) after the addition of PBN (30 mM final concentration). Data corrected against a TEMPO standard (10 µM) and presented as mean ±SD of 3 biological replicates with each 2 replicate measurements. Statistics: One-Way ANOVA with Tukey's post-hoc test. a-b superscripts represent the ANOVA post-hoc groupings.

Kilning, even at very moderate temperatures to produce this pilsner style malt, led to a higher radical generation and showed a stronger decrease in the oxidative stability of wort. Furthermore, it was interesting to note that re-steeping of green malt did not impact the radical formation in wort. Thus, the concentration of spin adducts formed in all green malt worts (untreated and re-steeped) were significantly lower compared to the control worts.

Additionally, the ESR profiles of all beers prepared from green malt 'as is', re-steeped green malt and their respective controls were determined. The aim was to assess the beer at the commencement of the trial, throughout lag time, until radical formation reached a stationary phase. Long lag times are associated with improved flavour stability and are related to levels of antioxidants present in beer (Andersen et al., 2000, Hashimoto, 1966). Pale beers ideally express long lag phases, indicating a very good oxidative stability (Jenkins et al., 2018). As an example, Andersen et al. (Andersen et al., 2000) reported lag phases in measured beers of up to 100 min to even 120 min (forced ageing at 55°C, PBN spin trap). Even though all beers prepared for this study were packaged with double pre-evacuation to guarantee very low oxygen levels in the final

beer, all beers produced for this research project (n = 12) almost instantly formed radicals (0 – 10 min), which were captured by the spin trap and visible in the ESR spectra. The dissolved oxygen levels determined in all fresh beers after bottling were below 0.075 mg/L dissolved oxygen (n = 12), however, oxygen ingress during transportation (ESR analysis was performed at the University of Copenhagen) cannot be excluded. Nevertheless, it is very likely that the lack of a long lag time suggests there were low SO_2 levels in these beers, which is consistent with the high temperature, vigorous fermentation employed (for further discussion of this aspect, see Section 4.3.5).

Figure 4.8 shows the spin adduct concentrations in beers after forced ageing for 90 min at 60°C.

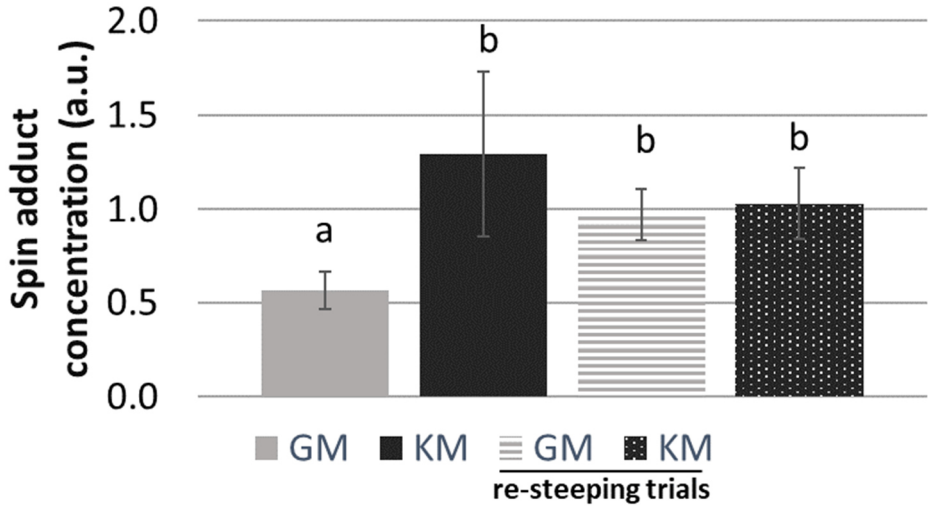


Figure 4.8: Formation of spin adducts in beers measured by ESR. Beer samples were forced aged for 90 min at 60°C (T90 values) after the addition of PBN (30 mM final concentration). Values corrected for TEMPO used (10 µm) and presented as mean ±SD of 3 biological replicates with each 2 replicate measurements. Statistics: One-Way ANOVA with Tukey's post-hoc test. a-b superscripts represent the ANOVA post-hoc groupings.

Green malt ('as is') showed similar trends to the results from the wort analysis – i.e. there was significantly lower radical formation in the green malt beers relative to the corresponding kilned malt control beers. However, no significant difference was observed between beers prepared from re-steeped green malt and the control beers.

Notwithstanding the lack of lag times, the green malt beers oxidised at a much lower rate. Another interesting finding of this research is that wort of the re-steeped green malt brews had an equivalent oxidative stability to worts prepared from untreated green malt. However, this observation was not preserved into finished beers, where the re-steeped green malt beers showed similar radical formation within the ESR study to the kilned control beers. Naturally, wort and beer differ substantially in their composition, yet it would be interesting to establish whether precursors in green malt wort can be metabolised by yeast to form an antioxidative species. When re-steeping, these precursors might be removed or altered, and thus, significantly more radicals were formed than in untreated green malt beers (Figure 4.8). Certainly, this theory requires further investigation.

4.3.5 Sulphites and free thiols

Sulphites are known to prolong the ESR lag-phase, and this is one way in which yeast can affect the oxidative stability of beer (Andersen et al., 2000, Saison et al., 2009a, Uchida et al., 1996). Thus, the sulphite content is necessary to help with the interpretation of the ESR data. As expected, no sulphites (limit of quantification 0.8 mg/L) were detected in either the green malt nor the reference beers, which could explain the almost immediate oxidation (or very short lag times) of the beers during forced ageing (60°C). Sulphites are secreted by yeast as an intermediate product of cysteine and methionine biosynthesis. According to the literature, sulphite excretion is amongst other dependent on yeast strain (lager strains produce more SO_2 than ale strains (Hysert and Morrison, 1976)) and fermentation temperature. According to the review by Baert et al. (Baert et al., 2012), higher fermentation temperatures can result in higher SO_2 contents. Unfortunately, the cited literature was either not accessible (proceedings or conference presentations), or no further information on the particular (higher) temperature ranges was available. Ilett et al. (Ilett and Simpson, 1995), on the other hand, summarised in a review the work from several researchers who reported that SO_2 levels increased if the fermentation temperature is reduced. Kaneda et al. (Kaneda et al., 1991) studied the flavour stability of beers brewed at several fermentation temperatures (6-30°C) in a pilot brewing plant, showing that sulphite contents in the fresh beers increased linearly with decreasing fermentation

temperatures using a brewing yeast strain (*Saccharomyces cerevisiae*, no further details). Therefore, it is very likely that the fermentation temperature applied in this study (24°C) was in general unfavourable for sulphite production. Furthermore, sulphite concentrations were most likely reduced, due to increased vigour of fermentation, stripping small volatile molecules like SO_2 (as was observed for DMS, Chapter 3).

Within the same experimental setup, the free thiol concentrations of beers were determined. The free thiol levels were significantly higher ($p < 0.001$) in all reference beers ($n = 6$; 18.0 ± 6.8 µM) compared to the untreated and re-steeped green malt beers (Table 4.2). Thiol groups are discussed to have antioxidant properties and play an important role, together with sulphite in complex antioxidative mechanisms.

Andersen et al. (Andersen et al., 2017) investigated the reactivity of thiols during early stages of oxidative degradation of beer. The researchers suggested that in pilsner style beers the thiol concentration (free thiols measured in standard pilsner beers ~ 25 µM) was too low to have any significant antioxidative effect. Thus, in the beers used for this study, at this concentration (Table 4.2), there might not be a significant antioxidative effect. Therefore, the present research should be repeated potentially by using a different yeast strain or fermentation protocol to get a better picture of the oxidative stability of green malt wort and beer.

Table 4.2: Metal ion contents, sulphites and free thiols in fresh beers.

				re-steeping trials	
		GM	KM	GM	KM
Na n.s.	mg/L	12.5 ± 1.4	12.6 ± 0.6	10.0 ± 0.5	9.8 ± 0.6
Mg n.s.		79.9 ± 8.8	93.2 ± 5.7	79.2 ± 3.4	79.5 ± 3.9
P ***		250.6 ± 18.6 ^{a}	325.6 ± 41.9 ^{b}	259.0 ± 28.0 ^{a}	249.9 ± 18.2 ^{a}
S **		70.9 ± 3.3 ^{a,b}	99.4 ± 9.8 ^{a}	81.1 ± 4.6 ^{b}	88.6 ± 6.5 ^{a,b}
K n.s.		574.6 ± 32.9	659.5 ± 70.3	617.3 ± 30.4	640.0 ± 24.6
Ca **		61.5 ± 17.1 ^{a}	63.5 ± 18.4 ^{a}	55.6 ± 4.6 ^{a}	37.7 ± 4.6 ^{b}
Mn n.s.	µg/L	219.3 ± 28.9	268.9 ± 71.2	235.2 ± 61.7	185.2 ± 16.6
Fe *		40.8 ± 20.4 ^{a,b}	53.6 ± 14.4 ^{a}	36.6 ± 4.4 ^{a,b}	33.3 ± 2.6 ^{b}
Ni n.s.		17.8 ± 5.1	29.6 ± 5.7	17.0 ± 1.8	23.7 ± 5.4
Cu ***		37.0 ± 11.9 ^{a}	74.4 ± 3.2 ^{b}	58.7 ± 10.5 ^{a,b}	46.7 ± 12.7 ^{a,b}
Zn n.s.		35.9 ± 9.6	52.3 ± 11.7	47.5 ± 6.3	38.3 ± 20.4
Rb ***		215.7 ± 19.4 ^{a}	230.9 ± 28.8 ^{a}	272.1 ± 7.7 ^{b}	277.0 ± 8.6 ^{b}
Sr ***		154.8 ± 21.9 ^{a,b}	195.8 ± 40.9 ^{a}	135.4 ± 7.8 ^{b}	101.0 ± 9.6 ^{b}
Ba ***		67.7 ± 11.9 ^{a,b}	91.7 ± 29.0 ^{b}	46.3 ± 10.8 ^{a,c}	33.4 ± 2.5 ^{c}
Sulphites		n.d.	n.d.	n.d.	n.d.
Free thiols ***	µM	7.0 ± 1.7 ^{a}	22.2 ± 6.8 ^{b}	10.0 ± 2.8 ^{c}	13.8 ± 3.7 ^{d}

a-d superscripts represent the ANOVA post-hoc groupings. In each row treatments differed significantly from one another if they have a different ANOVA group letter. Asterisks represent the p-value significance * p<0.05; **p<0.01; ***p<0.001. Data are the mean ±SD of 2 technical replicate measurements, Statistics: One-Way ANOVA with Tukey's post-hoc test. GM = green malt; KM = kilned malt.

4.3.6 Metal ion analysis

Transition metal ions, such as iron, copper and manganese, were identified to play a key role in the oxidative degradation of wort and beer, as they drive formation of reactive oxygen species (ROS) in the absence of antioxidants (Andersen and Skibsted, 1998, Lund et al., 2015). Those transition metals can convert molecular oxygen into superoxide radicals, peroxyl radicals and hydrogen peroxide. To further investigate the reasons for the better oxidative stability (ESR results) in green malt wort and beer, the transition metals were determined in the fresh beers using ICP-MS.

The transition metal ion contents of all beers produced for this study (n = 12) are summarised in Table 4.2. Moreover, the analysis performed provided further information about the minerals in the fresh beers, which is added to the table for additional information regarding the differences effected by brewing with green malt. The iron (Fe), or manganese (Mn) concentrations were not significantly different between the green malt or kilned malt beers. This was somewhat surprising, as increased heat load on malt and thus increased beer colour was expected to impact the transition metal content, as shown for example by means of mildly roasted malt (increased Fe (Hoff et al., 2012)), or the beer style stout (Jenkins et al., 2018). Copper (Cu), on the other hand, was significantly lower in the untreated green malt beers compared to the corresponding control, but not compared to the other beers (re-steeped and controls 4-6). Therefore, it is unlikely that the lower T90 level in green malt beers can be explained by the difference in copper content. Unfortunately, at this stage, no further wort samples were available to determine the metal concentrations in the wort to examine potential differences in metal ions induced by kilning.

It is evident that transition metal ions play a key role in beer staling, however, previous studies still revealed great differences in the contents of iron, copper, and manganese in beers. For example, a review (Pohl, 2008) of the literature on this area found iron in the range of 15– 1006 µg/L, copper 8–800 µg/L and manganese 31– 180 µg/L, which was discussed to be related to differences in raw materials, production conditions, but also detection methods. Overall, iron (24- 72 µg/L) and copper (23 - 78 µg/L) levels detected in the beers prepared for this study were comparable and in the lower range of the

concentrations reported in the literature (Pohl, 2008). Manganese was the metal present in the highest concentration in all 12 beers produced (160 – 340 µg/L).

The high manganese levels might be explained by the hopping applied, as this metal ion is present at very high concentration in hops and leached into beer (Porter and Bamforth, 2016). However, this does not explain the high variation of manganese found in the final beer.

Overall, the metal ion concentrations in themselves did not explain the improved oxidative stability of beers prepared from green malt. Future studies on the current topic are therefore required and should focus on the metal concentrations in the wort samples. Furthermore, previous studies (Pohl and Prusisz, 2010, Wietstock and Shellhammer, 2011) suggested that metals can be found in their free form, but also bound to phenolic or other organic compounds, which most likely affects oxidation properties. Therefore, it would be interesting to study the forms – free or bound - in which the transition metals are present in green malt wort and beer in order to better understand the improved oxidative stability compared to the kilned malt controls, but also re-steeped green malt beers.

4.4 Conclusion

This study aimed to evaluate the key quality parameters and flavour stability metrics of wort and beer produced from green malt. Even though kilned malt delivered significantly higher contents of free staling aldehydes into the brewing process, the brewing process (wort production and fermentation) significantly reduced these aldehydes to levels not different to beers prepared from green malt. However, the quality of pitching wort (i.e. the feedstock from which the yeast creates the character of the beer) is generally regarded by brewers as crucial and essential to the quality of beer. In addition to the promising indicators for flavour stability shown previously (Chapter 3), pitching worts from green malt ‘as is’ contained a significantly lower pool of staling aldehydes. Due to the potential binding of aldehydes throughout the brewing process into non-volatile adduct forms, these bound aldehydes could potentially be released during beer ageing. Considering the relatively low number of aldehydes in green malt, it would be expected that fewer aldehydes might be bound and released during ageing. However, additional research on the ageing behaviour of the trial beers is necessary.

Overall, *trans*-2-nonenal and hexanal, lipid oxidation products which are major concerns when using green malt for brewing, did not differ significantly in the resulting pitching worts or beer. In all beers (green malt and kilned malt) *trans*-2-nonenal and hexanal levels were the highest at the onset of mashing and then significantly declined during the mashing process. This again suggests that the lipoxygenase hostile conditions applied for mashing were sufficient to control LOX when using green malt as the raw material.

Furthermore, worts and beers produced from green malt 'as is' proved to have better oxidative stability compared to the reference beers. Pitching worts prepared from re-steeped green malt showed less radical formation during forced ageing than the control, however, this effect could not be observed in the finished beers. This confirms previous findings discussed in Chapter 3, that re-steeping is not necessary when using green malt for brewing.

In conclusion, green malt appears to be a very promising grist material to produce wort and beer with enhanced flavour stability metrics. However, further research on the flavour profile of aged beers is inevitably required to substantiate this theory and this will provide the focus of Chapter 5.

5 Flavour stability assessment of green malt beers

5.1 Introduction

During storage, various flavours or odours may (dis)appear, altering the sensory properties and thus the quality of the product. Subsequently, the appearance of so-called off-flavours (e.g. aldehydes) and disappearance of desired flavours (e.g. hop derived bitterness) can lead to the rejection of the brand. As already mentioned in Chapter 4, bottled beer is not a perfectly closed system. Exposure to light, elevated temperatures, vibrations during transport, as well as free radical and oxygen content have been identified as the primary causes for beer deterioration (Jaskula-Goiris et al., 2019, Paternoster et al., 2019, Vanderhaegen et al., 2006). While beers stored at 0 - 4°C did not show signs of oxidation even after several months of storage (Bamforth, 1999a), in supermarkets or during transportation, beers are hardly ever stored cooled (increasing costs, lack of cooling storage capacity). Previous research (Pankoke, 2015) has highlighted that beers can even experience temperatures above 40°C during cargo shipping. Lager beers, for example, are very susceptible to flavour change. Staling flavours can already be perceived when stored at 18°C for 3-6 months, as shown by Ilett and Simpson (Ilett and Simpson, 1995). Based on the Arrhenius law, as a rule of thumb, an increase of 10°C at least doubles the reaction rate for many chemical and physical reactions. However, due to their different activation energies, chemical reaction rates do not increase equally in response to increasing temperature and this can result in very different aroma profiles during storage (Lermusieau et al., 1999). While inadequate storage or transportation conditions can hardly be influenced by the maltster or brewer, a major goal is to produce a product which is robust against deterioration. However, researchers cannot wait several months to investigate the staling compounds formed, thus accelerated ageing studies (forced ageing at higher temperatures) are a common technique to predict flavour stability. Forced ageing is performed at different temperatures (usually 28 - 60°C) for several days or weeks and is proven to be a very useful, but discriminative technique to predict the flavour and colloidal stability (Lehnhardt et al., 2019). Since beer ageing is very temperature (Arrhenius law) and time dependent, a different ageing trial can lead to a very different sensory profile (Lehnhardt

et al., 2019). Nevertheless, forced ageing is still a state-of-the-art technique to predict the flavour stability of a beer (style).

The brewing process can significantly influence the stability of the resulting beer. From the perspective of the brewing process, the exposure of wort/beer to oxygen, the heat load applied during processing (e.g. wort boiling time) and the contact with transition metal ions, are critical factors (Bamforth and Lentini, 2009, Narziss, 1986, Wietstock et al., 2016). According to previous studies, the brewing protocol applied in this study (described in Chapter 3, Materials and Methods), was considerably optimised for the flavour stability of the beers. The thick mashing scheme (1:2.2) applied, lowers energy and water usage, while achieving a high extract yield, but also proved to reduce fatty acid oxidation (De Rouck et al., 2013b) and extraction of the staling aldehydes, hexanal and *trans*-2-nonenal (Ditrych et al., 2019). The latter was discussed previously (Ditrych et al., 2019) to be related to the highly hydrophobic nature of the fatty acid oxidation aldehydes. Additionally, the LOX hostile mashing conditions proved to be successful to control residual LOX activity during mashing when using kilned malt (Jaskula-Goiris et al., 2015), but also proved, as shown in Chapter 3 and 4, to control even the considerable LOX activity in green malt. The mash filter used for wort filtration allowed very quick mash filtration for the kilned malt brews, which has been reported to positively correlate with improved flavour stability (Narziss, 1986, Van Waesberghe, 1991). Unfortunately, due to the sparging difficulties when using green malt as the grist material, sparging took significantly longer. The time of wort boiling (60 min) and clarification (15 min) was of limited duration, i) to lower energy usage, ii) to reduce total head load – a factor associated with flavour instability, and iii) to reduce the *de novo* formation of staling aldehydes, such as furfural (as discussed in Chapter 4). On the other hand, sufficient boiling time was allowed to evaporate volatile compounds such as staling aldehydes, which are mostly imparted to the brewing process through malt (De Clippeleer et al., 2010b, Ditrych et al., 2019, Gastl et al., 2006, Guido et al., 2007). As the wort is still exposed to heat load and the separated hot trub contains significant amounts of aldehyde precursors (e.g. lipids or aldehydes bound to insoluble trub particles), wort clarification time was kept limited, and wort was pumped into the fermenter after 15 minutes total time of decantation (De Schutter et al., 2008, Masaaki Yano and Motoo,

2019, Van Waesberghe, 1991). Furthermore, oxygen was avoided (except sterile wort aeration before fermentation) throughout the brewing process by de-aerating the brewing liquor and injecting CO_2 into the malt bin, inlet, and mash kettle. In summary, the wort production process was performed using technical solutions provided by the brewing community to brew beer with a very promising flavour stability metrics.

As already mentioned in Chapter 4, malt is a major source of staling precursors (De Clippeleer et al., 2010b, Ditrych et al., 2019, Gastl et al., 2006, Guido et al., 2007). In this chapter, a great focus was on the flavour (in)stability of the green malt beers. Green malt contains very low concentrations of free staling aldehydes compared to kilned malt (Chapter 4). Additionally, wort and fresh beers from green malt have proven to have a very promising flavour stability metrics. As already discussed in Chapter 4, aldehyde levels in fresh beers did not differ significantly irrespective of whether beers were produced from green malt or kilned malt, even though significantly higher levels were observed in kilned malt worts up to the finished pitching wort. However, aldehydes cannot only be formed *de novo* but they can also bind to compounds such as bisulphites (Kaneda et al., 1994, Lermusieau et al., 1999) amino acids (formation of imines) (Lermusieau et al., 1999, Liégeois et al., 2002) or cysteine (Baert et al., 2018, Baert et al., 2015a, Baert et al., 2015b, Bustillo Trueba et al., 2019). The current theory is that during beer storage, under specific conditions (temperature, pH value, redox potential, binding strength, thermodynamic stability), adducts may dissociate and release aldehydes in the free form (Baert et al., 2012, Lehnhardt et al., 2019, Lermusieau et al., 1999, Liégeois et al., 2002). Thus, beer chemistry changes during staling may differ depending on raw materials used for the brewing process.

The current work aimed to evaluate the analytical changes of the six green malt beers (green malt 'as is' and re-steeped), as well as their corresponding reference (pilsner malt) beers regarding their flavour (in)stability. To identify the beer chemistry changes during staling, beers were subjected to ageing at 30°C for 30, 60 and 90 days. The hypothesis was that green malt used for the brewing process has the potential to significantly improve the flavour stability of the beers. Additionally, there is considerable academic interest for the malting and brewing community in learning of the impacts of kilning on malt quality and thus flavour (in)stability. Hence, the novelty of this green

malt brewing research should contribute knowledge useful to the quest for beer flavour stability.

5.2 Material and methods

5.2.1 Chemicals and reagents

Free Aldehydes: The carbonyl compounds 2-methylpropanal (≥ 99%), 2-methylbutanal (≥ 95%), 3-methylbutanal (98%), hexanal (≥ 98%), furfural (≥99%), methional (≥ 95%), *trans*-2-nonenal (≥95%) and phenylacetaldehyde (≥ 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deuterated 2-methylbutanal (2MB-d_{10}) was requested from MercaChem (Nijmegen, the Netherlands); deuterated benzaldehyde (benzaldehyde-d_6) was purchased from Sigma Aldrich (St. Louis, MO, USA). Ethanol absolute (≥ 99.5%) was purchased from Merck KGaA (Darmstadt, Germany). The derivatisation agent stock solution PFBHA (o-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride) was obtained from Sigma Aldrich (St. Louis, MO, USA). *Amino acids (beer):* Solid 5-sulphosalicylic acid (SSA, ≥ 99%) and DL-norleucine were purchased from Sigma-Aldrich (UK). *Esters and higher alcohols*: 3-methyl-1-butanol (99%), ethyl acetate (99%), isobutanol (99%), 1-propanol (> 99%), isoamyl acetate (> 99%), isobutyl acetate (98%), ethyl hexanoate (99%), ethyl octanoate (> 99%); ethyl butyrate (99%) were purchased from Fisher Scientific (UK). Acetaldehyde (≥ 99.5%) was purchased from Sigma- Aldrich (UK). *Determination of (reduced) iso-α-acids:* Dihydrogenphosphate was purchased from Merck (85% H_2PO_4, Darmstadt, Germany) and acetonitrile was purchased at HPLC-grade (CH_3CN, Novasol, Belgium).

5.2.2 Ageing of beer samples

Malt collection, wort production and fermentation were performed according to the protocol described in Section 3.2.3. All bottled beer samples were aged in the dark under forced conditions at 30°C in a thermostatically controlled room for 30, 60 and 90 days. Subsequently, they were cooled to 0°C until analysis.

5.2.3 Standard analysis of fresh and aged beers

Specific gravity, density and alcohol content of the beer were determined using the Anton Paar DMA 4500 and Alcolyzer Plus. The pH was measured using a pH meter

(Mettler Toledo). Wort and beer colour was determined based on EBC method 9.1 by measuring the absorption at 430 nm using a spectrophotometer (Varian Cary 100, Agilent Technologies Inc., Australia). Cold haze (analysis of the turbidity of beer kept for a minimum of 24 h at 0°C) and permanent haze (analysis of turbidity of chilled beer kept for 24 h at 20°C) were determined using the Haffmans VOS ROTA 90 Turbidity meter, 90° light scatter. The free amino nitrogen content (FAN) in beer was determined using colourimetry with ninhydrin based on the EBC method-9.10, as described in Section 2.2.5.1. The total polyphenol content and flavanoid content of beer was determined according to EBC Beer method 9.11 and 9.12. This method is as described in Section 3.2.11 and 3.2.12, respectively. Proanthocyanidins were determined by measuring the red coloured cyanidin complex formed with HCl/1-butanol according to the protocol described in Chapter 3 (Section 3.2.13).

5.2.4 Amino acid analysis in beer

Amino acids were isolated from beer and derivatised using the EZ:Faast^{TM} amino acid kit (Phenomenex, Macclesfield, UK) and subsequently injected to an Amino Acid Analyser (Biochrom 20 Plus) equipped with an ion-exchange column and UV detector for analysis. The full procedure of the assay is as previously presented in Section 3.2.16.

5.2.5 UPLC determination of (reduced) iso-α-acids

Ultra-Performance Liquid Chromatography (UPLC) separation of (reduced) iso-α-acids were performed using the Acquity UPLC (Waters, Milford, USA), consisting of a Photodiode Array Detector (PAD), column heater, sample manager, binary solvent delivery system and an Acquity UPLC HSS C18 1.8 µm column (2.1 i.d. x 150 mm; Waters, USA). Data reprocessing was done using the Empower 2 software (Waters, USA). The mobile phase consisted of a binary solvent system of (A) milli-Q water adjusted to pH 2.8 with dihydrogen phosphate (H_2PO_4, 85%) and (B) HPLC-grade acetonitrile (CH_3CN). The composition of the mobile phase was kept at isocratic elution mode, using 52% (v/v) solvent B and 48% (v/v) solvent A. The total analysis time was 12 minutes at a flow rate of 0.5 mL/min and a column temperature of 35°C. The sample's absorption was measured at 270 nm (iso-α acids) and 254 nm (tetrahydro-iso-α-acids) using a UV detector. The *trans/cis* iso-α-acids ratio (T/C ratio) was based on the concentrations of

trans- and *cis*-isocohumulone and *trans*- and *cis*-isohumulone and calculated according to the following equation:

$$\frac{T}{C}ratio = \frac{(trans - isocohumulone) + (trans - isohumulone)}{(cis - isocohumulone) + (cis - isohumulone)}$$

5.2.6 HS-SPME-GC-MS determination of free aldehydes

Free aldehydes - 2-methylpropanal (2MP), 2-methylbutanal (2 MB), 3-methylbutanal (3 MB), hexanal (HEX), furfural (FUR), methional (MET), phenylacetaldehyde (PHE) and *trans*-2-nonenal (T2N) - were determined according to the described according to De Clippeleer (De Clippeleer, 2013) and Baert (Baert, 2015). The full procedure of the assay is as previously described in Chapter 4 (Section 4.2.5).

5.2.7 Determination of beer volatiles

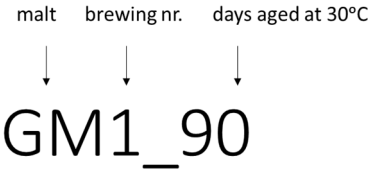
Volatiles in beer were determined via GC-HS-FID method, using a SCION 456-GC (Bruker, UK) fitted with a Combi PAL autosampler and controlled with Compass CDS software. The full procedure is as previously described in Chapter 3 (Section 3.2.17).

5.2.8 Statistical analysis

All samples were analysed in at least three biological replicates with 2-4 technical replicates. Statistical analyses were performed using Microsoft Excel 2013/XLSTAT (XLSTAT version 2020.1.1.64347, Addinsoft, Life Science, New York, USA). An α-risk of 0.05 was set as the level of significance in all data analyses. Principal component analysis (PCA) was performed to emphasise variation and highlight strong patterns in the dataset. Additionally, Pearson correlation coefficient was established to determine linear relationships between two variables; the strength and direction of the relationship was reported as Pearson correlation coefficient (r).

5.2.9 Abbreviations

In the following chapter all beers, fresh to aged, are abbreviated as follows:



GM = green malt; KM = kilned malt; RGM = re-steeped green malt; RKM = kilned malt (control for re-steeping trials)

5.3 Results and discussion

5.3.1 Analytical evaluation of fresh beer

Analytical data for the fresh beers prepared from green malt (n = 3), re-steeped green malt (n = 3) and the corresponding kilned malt (n = 6) were subjected to principal components analysis (PCA) to identify the main sources of variation in the data set. Even though the beers were prepared each in three biological replicates, substantial variation was evident in the sample set. The two PCA bi-plots shown in Figure 5.1A and B together accounted for about 80% variation within the analytical data set. In Figure 5.1A the positive loadings along PC1 (49% of variation) were associated with beers high in amino acids, free amino nitrogen, esters, pH and susceptibility to radical formation (ESR T90 value). The high variance between beers prepared from the control malt KM1-3 was mainly driven by the huge variance in residual amino acids/FAN content in the fresh beers (as discussed in Chapter 3). Especially the beers produced from the control malt KM1 and KM2 were located along the positive loadings of PC1, while the beers prepared from the biological replicate malt KM3 loaded negatively on this axis. Furthermore, it is important to highlight that amino acids known as precursors for Strecker aldehyde formation (valine, isoleucine, leucine, phenylalanine and methional) were increased in the fresh beers of KM1 and KM2 in comparison to all other beers. Thus, increased Strecker aldehyde formation during forced ageing might be observed, particularly in these beers.

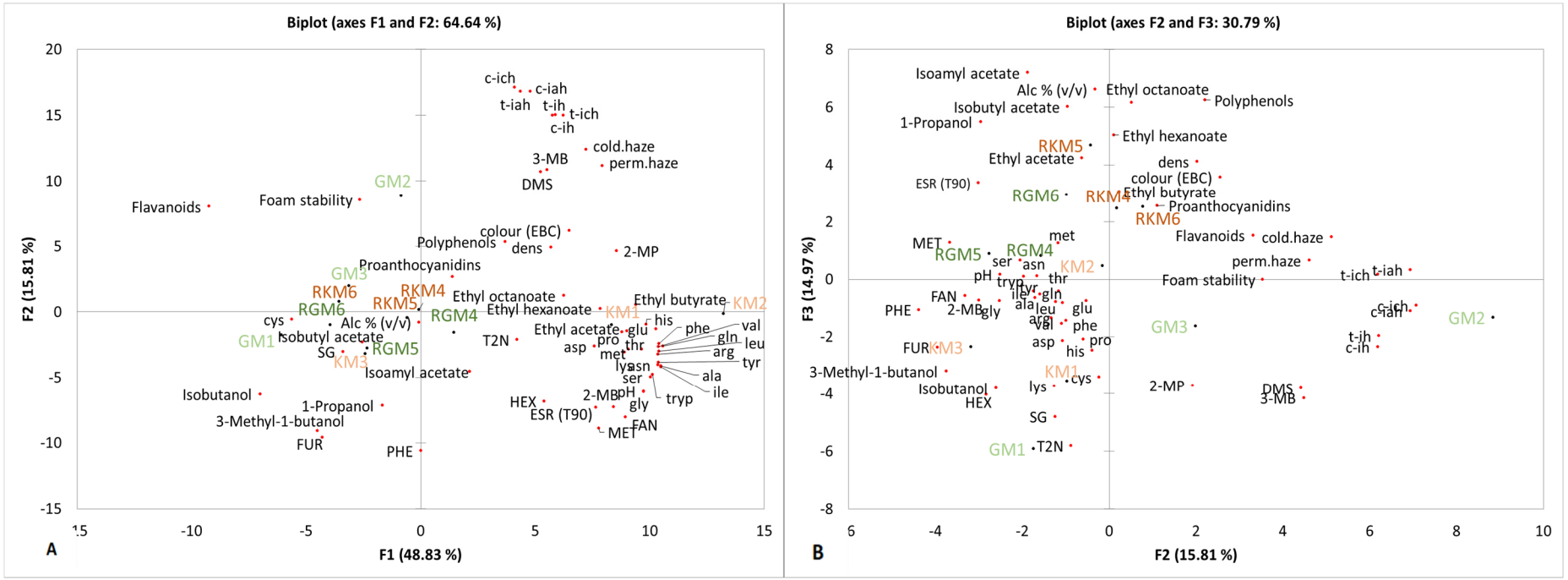


Figure 5.1: Bi-plot of the PCA of the analytical data obtained from fresh beers. Component identification: 2MP = 2-methylpropanal; 2MB = 2-methylbutanal; 3MB = 3-methylbutanal, PHE = phenylacetaldehyde; MET = methional; FUR = furfural; HEX = hexanal; T2N = *trans*-2-nonenal; t-ich: *trans*-isocohumulone; c-ich: *cis*-isocohumulone; t-ih: *trans*-isohumulone; c-ih: *cis*-isohumulone; t-iah: *trans*-isoadhumulone; c-iah: *cis*-isoadhumulone; DMS = dimethyl sulphide, ala: alanine; arg: arginine, asn: asparagine; cys: cysteine; his: histidine; gln: glutamine; gly: glycine; ile: isoleucine; leu: leucine; lys: lysine; met : methionine; phe: phenylalanine; pro: proline; ser: serine; thr: threonine; trp: tryptophan; val: valine

Additionally, while all control beers (KM1-6) were more susceptible to radical formation during ESR analysis compared to the untreated green malt beers (GM1-3), beers from KM1 and 2 formed the largest proportion of spin adducts (T90 levels, Chapter 4). Hence, beers brewed from KM1 and KM2 might provide additional information about staling precursors and consequent changes during beer ageing.

The positive loadings along PC2 (16%), identified beers high in flavanoids, iso-α-alpha acids, foam stability as well as increased haze formation (chill and permanent). Likewise, some variation was observed across the green malt replicate beers GM1-3. Beers prepared from GM2 showed longer head retention, thus better foam stability (Figure 5.1A). This is further highlighted in Figure 5.1B, which accounted for 31% of variation within the data set. Beers prepared from GM2, located on the positive axis of PC2, were correlated with high levels of iso-α-alpha acids, as well as increased haze formation (chill and permanent).

This is interesting as all worts were boiled and decanted for the same time using hops from the same batch, thus thermal isomerisation should have occurred at a similar rate. The increased amount of iso-acids, which were identified to play an important role for the quality of foam as they stabilize the head retention (Hughes, 2000, Smith et al., 1998) could thus explain the better foam stability in GM2 beers.

5.3.2 Analytical evaluation of aged beers

To determine characteristics associated with (in)stability, all twelve beers were aged at 30°C for 30, 60 and 90 days. Subsequently, standard analytical parameters such as gravity, pH, colour, haze (permanent and cold), as well as free amino nitrogen, total polyphenol, flavanoid and proanthocyanidin content were determined. Additionally, beer flavour characteristics like staling aldehydes, esters, higher alcohols and bitter acids were determined during beer ageing. The most striking analytical characteristics for each brewing trial are discussed in this section. All remaining data can be found in the Appendix.

To assist with summarising a complex data set, explorative analysis of the variation in the analytical data from the selected beers was performed using PCA. The bi-plot shown in Figure 5.2A displays about 49% variation within the analytical data set.

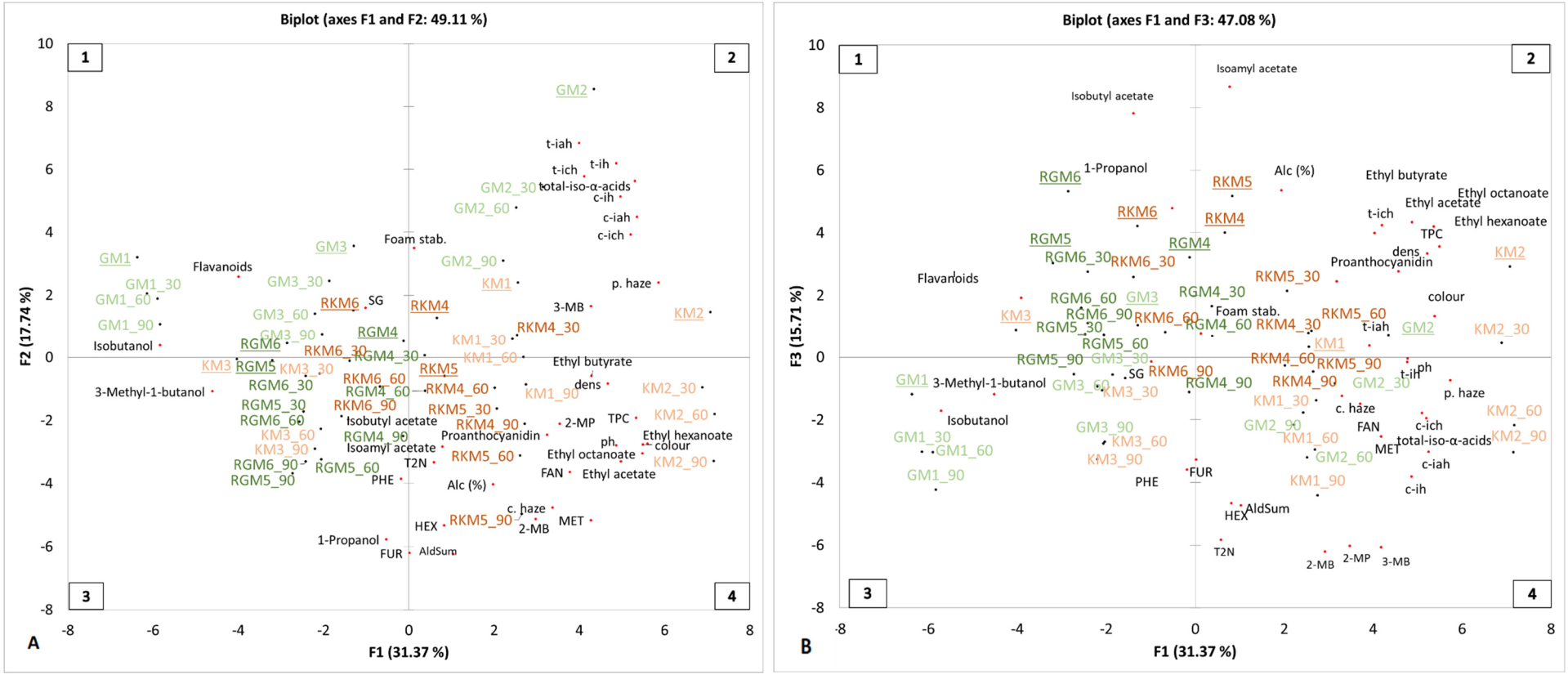


Figure 5.2: Bi-plot of PCA of analytical data obtained from fresh and aged green malt (GM1-3), control (KM1-3), re-steeped green malt (RGM4-6) and corresponding control (RKM4-6) beers; n = 3. Fresh beers are underlined. Component identification: AldSum: sum aldehyde markers; 2MP = 2-methylpropanal; 2MB = 2-methylbutanal; 3MB = 3-methylbutanal, PHE = phenylacetaldehyde; MET = methional; FUR = furfural; HEX = hexanal; T2N = *trans*-2-nonenal; t-ich: *trans*-isocohumulone; c-ich: *cis*-isocohumulone; t-ih: *trans*-isohumulone; c-ih: *cis*-isohumulone; t-iah: *trans*-isoadhumulone; c-iah: *cis*-isoadhumulone, TPC: total polyphenol content, SG: specific gravity

Upon beer ageing, each beer in the biplot shifted downwards towards the negative axis of PC2 (18% of variation in the data), which represented compounds that seem to be formed or increase during ageing. This shift was mostly driven by the formation of staling marker aldehydes and cold haze. Interestingly, 1-propanol, a higher alcohol formed as metabolic by-product by yeast during fermentation, appeared to cluster with this group and should thus be further investigated by comparing the individual data. On the other hand, the variables which loaded positively on PC2, such as the bitter acids were associated with the fresh beers and thus, decreased during forced ageing. PC1, accounting for 31% of the variation in the data, highlighted once more the analytical differences between the beers, as already described by means of the fresh beers displayed in Figure 5.1. Beers prepared from GM2, located in quadrant 2, were associated with increased concentrations of bitter acids whereas the two biological replicate beers from GM1 and GM3 were located in quadrant 1.

Likewise, fresh beers from KM1 and KM2 were located along the positive axis of PC1 (quadrant 2) and shifted towards quadrant 4 upon ageing; whereas the beers (fresh and aged) prepared from the reference kilned malt - KM3 - were located in quadrant 3. Thus, variance in the ageing behaviour of the three kilned malt control beers, KM1-3, was expected and further investigated. Interestingly, it can be noted that in Figure 5.2A the untreated green malt beers were separated from their corresponding control kilned malt beers. On the other hand, the re-steeped green malt beers did not separate as much from their reference beers.

The green malt beers remained on the positive axis of PC2 during ageing (quadrant 1 and 2), whereas the corresponding control beers moved towards the negative axis of PC2 along quadrant 3 and 4 approaching the staling marker aldehydes. Based, on the analytical data, aged beers clustering in quadrant 4 (KM1, KM2, RKM4, RKM5) can be described as the most ‘stale’, probably due to the highest increase in the levels of staling aldehydes. Beers located in quadrant 1 (GM1 and GM3), on the other hand, appeared to have formed the lowest number of aldehydes in the data set. The key drivers of the quadrant groupings were further confirmed on PC1 and PC3 which accounted for 47% of variation within the data set (Figure 5.2B). Additionally, the positive axis along PC3 (16% of variation) highlighted that isoamyl acetate and isobutyl acetate were found at higher concentrations in fresh beers, and thus appeared to decrease upon ageing. Thus,

the results summarised and displayed in this bi-plot suggested that there was some differences in the beers during beer ageing and that untreated green malt beers developed fewer (analytical) signals of ageing than their respective controls. Therefore, the following section aimed to evaluate the key differences between green malt beers and the corresponding kilned malt reference beers.

A detailed inspection of the data was conducted to get an overview of the analytical changes occurring during ageing. Figure 5.3A-E displays trends through ageing for some standard analytical measurements such as colour, FAN, total polyphenols, flavanoids and proanthocyanidins.

In all twelve beers, a small increase in colour after 60 days ageing is noticeable, irrespective of the malt used (Figure 5.3A). This is most likely due to the increase of coloured Maillard reaction compounds, or structural rearrangements of flavan-3-ol monomers affecting beer colour during beer storage (Callemien and Collin, 2007). The FAN (Figure 5.3B) and total polyphenol (Figure 5.3C) content did not substantially change in all twelve forced ageing trials. Polyphenols can be oxidised and cause haze during ageing. However, the assay for total polyphenols is a non-specific spectrophotometric technique, which determines the total number of phenol cores, hence insensitive to monitor oxidative polymerisation of polyphenols. However, the assay used to detect the flavanoids in beers, quantifies the number of flavanoid structures, expressed as catechin equivalents. Flavanoids are generally considered as very sensitive to oxidation, thus they oxidise faster than other polyphenol classes (Jaskula-Goiris et al., 2011, McMurrough et al., 1996). Figure 5.3D displays a small decrease of flavanoids in beers prepared from GM2 and RKM6 during ageing, while total polyphenols remained consistent (Figure 5.3C). Thus, the decrease of flavanoids suggested polymerisation due to oxidative mechanisms (Vanderhaegen et al., 2003) in beers prepared from GM2 and RKM6. The flavanoid content in the remaining beers was not affected by forced ageing, which could be interpreted as good oxidative stability or potentially no oxygen ingress into the bottle while forced ageing.

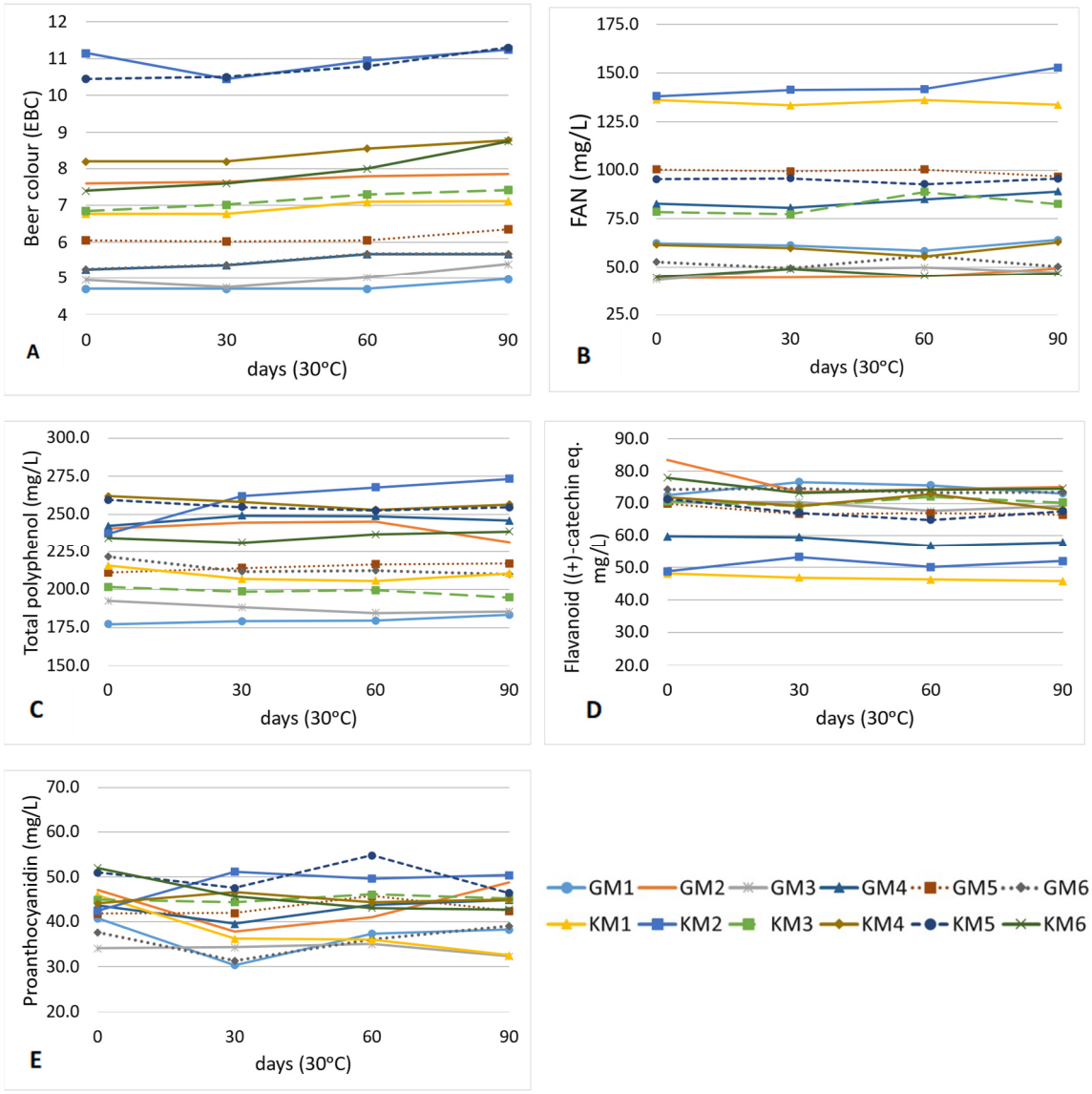


Figure 5.3: Evolution of A) beer colour, B) free amino nitrogen, C) total polyphenols, D) flavanoid and E) proanthocyanidin concentration during ageing of beer (30°C). Results are the mean of 3 technical replicate measurements.

Proanthocyanidins are known to be responsible for colloidal instability during beer storage, because of their size and potential to cross-link haze active proteins/ peptides (McMurrough et al., 1992). No clear pattern can be observed in the proanthocyanidin contents during ageing. In the majority of beers proanthocyanidin levels (Figure 5.3E) decreased after 30 days of ageing, however, a small increase is observed with progression of forced ageing. Proanthocyanidins are oligomers originating from catechin and epicatechin, which are soluble in water and form red cyanidin complexes, in amounts increasing with molecular size when heated with butanolic HCl (Bate-Smith,

1973). Even though it is a very fast and easy method to investigate the proanthocyanidin content in beers, however, higher levels of tetramer and higher oligomers can cause some cloudiness in the supernatant and thus, affect photometric absorption (Bate-Smith, 1973). For more precise measurement of proanthocyanidin it is thus recommended to perform phloroglucinolysis in conjunction with RP-HPLC coupled with diode array detection and electrospray ionisation mass spectrometry to reveal the flavan-3-ol subunits and to estimate the changes in mean degree of polymerisation (Aron and Shellhammer, 2017).

Haze formation in beer is caused mainly by interactions between haze active polypeptides and polyphenols. While these components are discussed to exist in equilibrium in beer, they manifest as haze when the polyphenol polymerises (Bamforth, 1999b). All beers showed a significant increase in chill haze formation, especially at beers aged for 60 and 90 days. However, at room temperature, the cold break haze disappeared even in beers aged for 90 days at 30°C, thus no statistically significant change in permanent haze formation was found from fresh to aged beers (Figure 5.4A, B). This was surprising, as permanent haze was expected to increase through shelf life but could be related to the rather ‘gentle’ ageing temperature (30°C) applied, in comparison to standard parameters (60°C) used to determine colloidal stability.

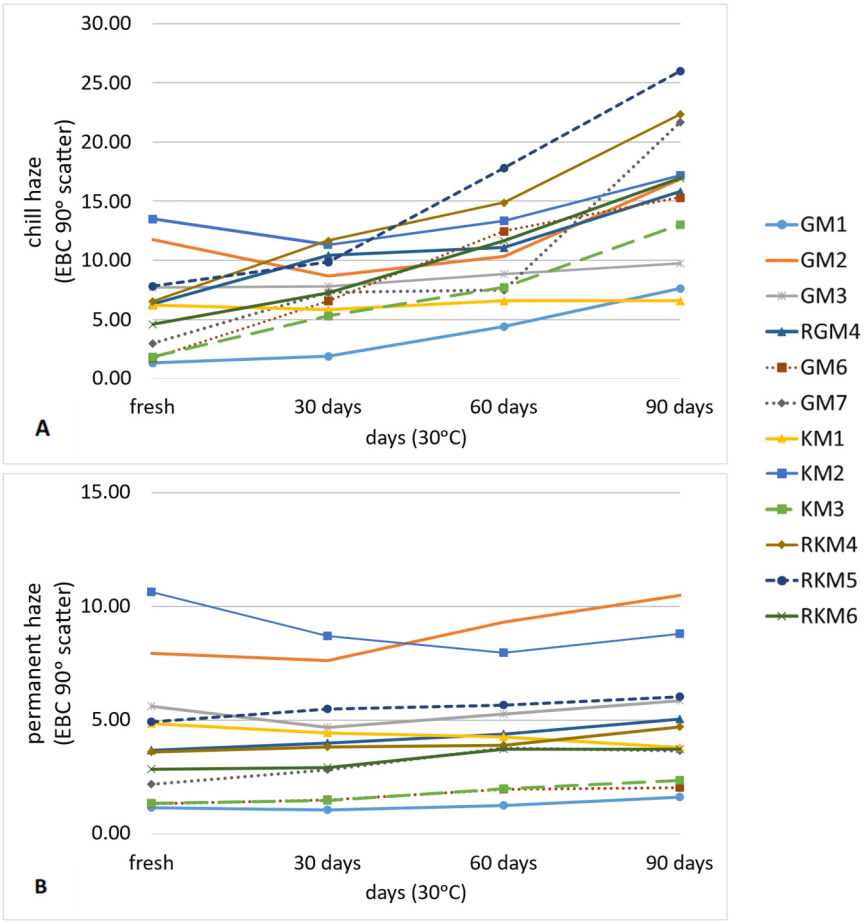


Figure 5.4: Evolution of A) chill haze and B) permanent haze during ageing of beer (30°C). Results are the mean of 3 technical replicate measurements.

Furthermore, UPLC determination of bitter acids revealed a decline in *trans*-iso-α-acids, in all beers, while only small changes were observed in the cis-counterparts. This is in agreement with previous literature, as the *cis*-iso-α-acids were identified to be more thermodynamically stable than the *trans*-counterparts (De Cooman et al., 2000, Jaskula-Goiris et al., 2011, Jaskula et al., 2007), which is due to entropic differences pertaining structural geometry favouring the *cis*-isomer (De Cooman et al., 2000). Overall, the *cis*-isomers were observed in greater quantities than the *trans*-isomers, as higher quantities of *cis*-isomers are formed as a result of the isomerisation reaction of α-acids into iso-α-acids. Furthermore, *trans*-isomers were identified to have a greater foam activity compared to *cis*-isomers (Hughes, 2000). The resulting decline of the *trans/cis* ratio is displayed in Figure 5.5. Thus, a decrease in bitterness can be expected in the aged beers.

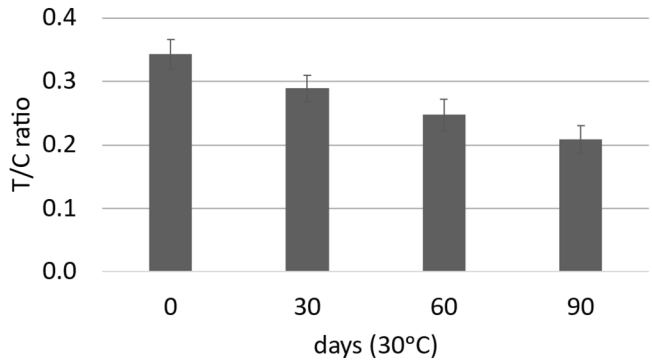


Figure 5.5: *Trans/cis* iso-α-acids ratio (T/C ratio) based on the concentrations of *trans*- and *cis*-isocohumulone and *trans*- and *cis*-isohumulone. Results are the mean ±SD of n = 12 with each 2 technical replicate measurements

In Chapter 3, several yeast flavour active metabolites (esters and higher alcohols) found in beer were described. Here the concentrations of the previously selected volatiles of the beers monitored during forced ageing are reported. According to Neven et al. (Neven et al., 1997), during ageing, both chemical hydrolysis or extracellular esterases secreted from the yeast may catalyse a breakdown of esters during storage, which causes a reduction of the fruity (fresh) flavour of beer during ageing. Table 5.1 displays the fold change (indicated as colour chart) of the determined esters and higher alcohols; yellow-red coloured cells represent compounds which changed substantially during forced ageing. Isobutyl acetate, isoamyl acetate and ethyl acetate were identified to give the beer a fruity flavour, which is very important for the flavour profile of the fresh beers. According to the results presented in Table 5.1, the acetate esters – isoamyl acetate and isobutyl acetate- decreased in all beers 20% to even 60% from the original concentration in the fresh beers. Furthermore, ethyl butyrate decreased in most of the beers (except GM5 and GM6), however, the highest decrease was noted in beers from GM1, GM4 and KM6. Interestingly, as previously discussed by means of Figure 5.2A, 1-propanol clustered with compounds that appeared to increase during beer ageing.

Table 5.1: Fold change of esters and higher alcohols from fresh beer to aged beers (30°C, 90 days); Numbers below 1.0 indicate a decrease (yellow-red). Green malt 'as is' (GM 1-3) and the corresponding reference malt (KM 1-3); as well as re-steeped GM (4-6) and the corresponding reference malt (KM 4-6).

			Esters						**Higher alcohols**		
			EAc	IsoAA	IsoBA	Ebut	Ehex	Eoct	1Prop	IsoB	3M1B
re-steeping trials	1	GM	1.0	0.7	0.8	0.2	0.8	0.7	1.0	1.0	1.0
		KM	0.9	0.5	0.6	0.9	0.9	1.0	1.0	1.0	1.0
	2	GM	0.9	0.7	0.8	0.9	0.9	0.9	1.0	1.0	1.0
		KM	0.7	0.4	0.6	0.6	1.0	1.0	1.0	1.0	1.0
	3	GM	0.8	0.7	0.8	0.9	0.9	0.9	1.0	1.0	1.0
		KM	0.8	0.9	0.8	0.9	1.0	1.0	1.0	1.0	1.0
	4	GM	1.0	0.7	0.8	0.3	0.7	0.7	1.0	1.0	1.0
		KM	0.9	0.7	0.8	0.9	1.0	1.0	1.0	1.0	1.0
	5	GM	1.0	0.8	0.9	1.0	0.9	0.8	1.0	1.0	1.0
		KM	0.9	0.8	0.9	0.9	1.0	1.0	1.0	1.0	1.0
	6	GM	1.0	0.7	0.8	1.0	0.8	0.8	1.0	1.0	1.0
		KM	1.0	0.7	0.7	0.3	0.7	0.6	1.0	1.0	1.0

EAc.= ethyl acetate, IsoAA = isoamyl acetate, IsoBA = isobutyl acetate, Ebut = ethyl butyrate, Ehex = = ethyl hexanoate, Eoct = ethyl octanoate, 1Prop = 1-propanol, IsoB = isobutanol, 3M1B= 3-methyl-1-butanol

However, the higher alcohols measured in this study did not change during ageing (Table 5.1). Thus, a closer look at the individual data (Appendix 3) revealed that this cluster is due to brewing trials Nr. 4 and 5 having the highest amount of 1-propanol rather than an increase during forced ageing. Overall, due to the decrease of the desirable flavours (esters, bitter acids), the elevated concentrations of undesirable compounds such as staling aldehydes will tend to become more dominant in terms of the overall flavour profile of the beer.

5.3.2.1 Aldehyde formation in forced aged beers

Furthermore, chemical instability was investigated by determining the free aldehyde concentrations in aged beers. Hereby, a great focus was on the difference between the heat load experienced by malt (green malt vs kilned malt) and the resulting aldehyde content in aged beers. The investigation of free aldehydes throughout the brewing process, which was discussed in Chapter 4, highlighted that fresh beers from kilned (pilsner-style) malt resulted in similar concentrations of free staling aldehydes compared to green malt beers – despite the high aldehyde concentrations in the kilned malt and

particularly at the onset of the brewing process. However, prior to the current studies, there have been no published data on aldehyde formation in green malt beers during ageing. Therefore, the selected aldehydes of the fresh and aged beers were compared based on PCA analysis to visualise the main differences among the beers (Figure 5.6).

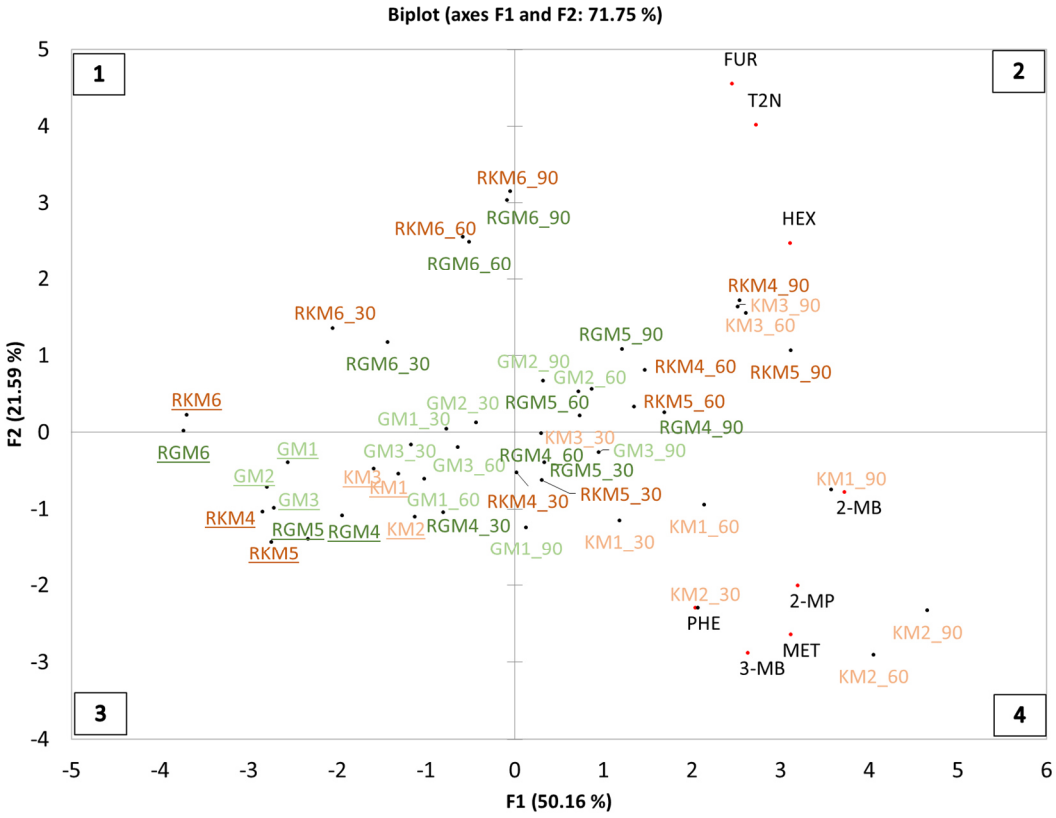


Figure 5.6: Bi-plot of PCA on the aldehyde levels in fresh and aged beers. Fresh beers are underlined. 2MP = 2-methylpropanal; 2MB = 2-methylbutanal; 3MB = 3-methylbutanal, PHE = phenylacetaldehyde; MET = methional; FUR = furfural; HEX = hexanal; T2N = *trans*-2-nonenal sum = total of selected staling marker aldehydes

This resulted in two major principal components, PC1 and PC2, that described 50% and 22% of the variation, respectively. In Figure 5.6 the negative loadings along PC1 identified beers low in the marker aldehydes. Thus, the fresh beers clustered mostly in quadrant 3 or at the border to quadrant 1 (RKM6, RGM6). During beer ageing the beers shifted towards the positive axis of PC1, however, clearly separated alongside PC2. The positive loadings along PC2, in quadrant 2 were associated with beers high in furfural, hexanal and *trans*-2-nonenal. Interestingly, the beers from KM1 and KM2 moved

towards quadrant 4 as they aged, towards the negative loadings along PC2, which correlated with beers high in the Strecker aldehydes phenylacetaldehyde, methional, 2-methylbutanal, 3-methylbutanal and 2-methylpropanal. This is specifically interesting because the fresh beers of KM1 and KM2 were identified previously to contain the highest amount of residual amino acids (Figure 5.1) and will be further investigated in the following Section 5.3.2.2.

Upon beer ageing for 90 days, the lowest increase in the aldehyde markers was observed in the untreated green malt beers, in comparison to all control beers (KM1-6). Furthermore, beers prepared from re-steeped green malt RGM4 and RGM5 developed significantly lower total concentration of aldehyde markers after 90 days ageing at 30°C, compared to the corresponding control beers RKM4 and RKM5, respectively. However, according to the resulting bi-plot, displayed in Figure 5.6, beers prepared from RGM6 and RKM6 (third biological replicate of the re-steeping trials) moved similarly towards quadrant 2 on ageing. This suggested similar aldehyde formation when beers were subjected to ageing. A more detailed investigation into the dataset revealed that the total concentration of aldehyde markers after 90 days ageing (30°C) only differed slightly between RGM6 and RKM6; 576 µg/L vs 613 µg/L, respectively (Figure 5.7).

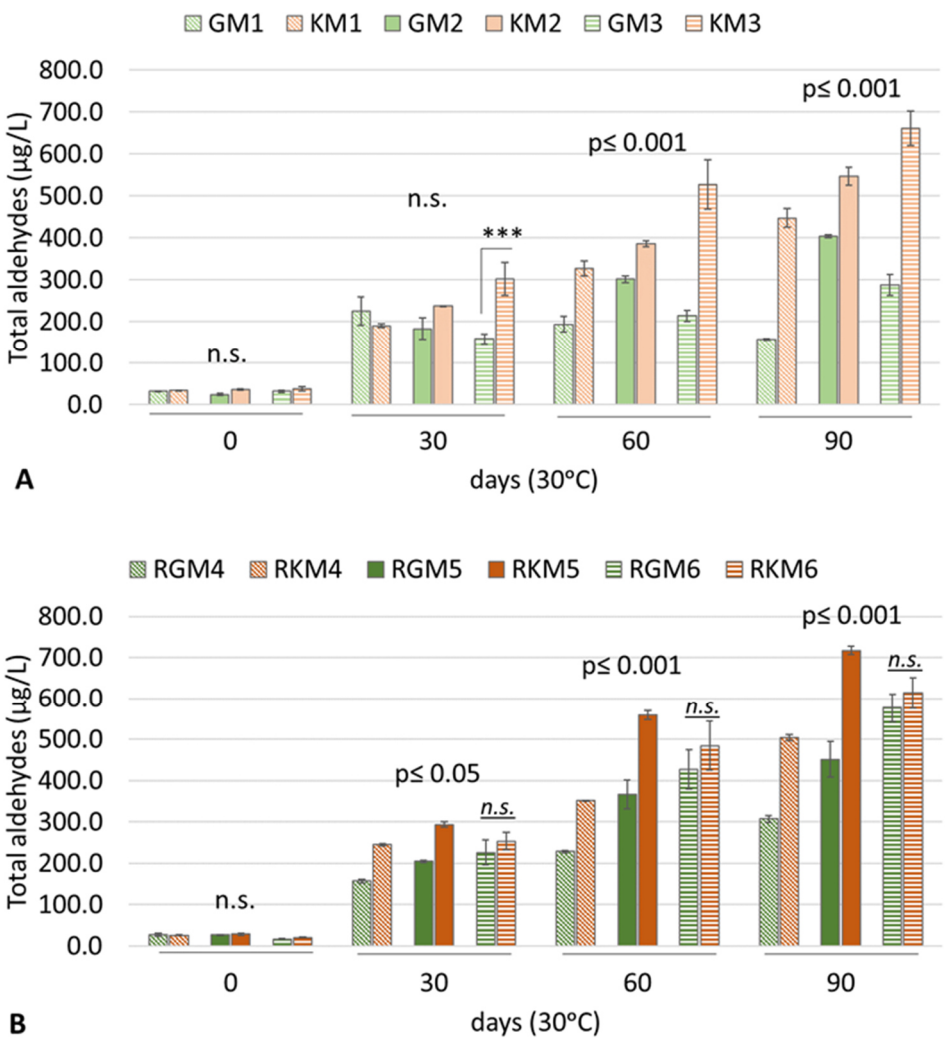


Figure 5.7: Total selected aldehyde levels in beers prepared from (A) green malt 'as is' and the corresponding control kilned malt (B) from re-steeped green malt and the corresponding control. Samples are presented as mean ±SD of 2-3 replicate measurements. Statistics: One-Way ANOVA; Student's t-test GM3_30 vs KM3_30, significant differences indicated by ***: p< 0.001; Student's t-test RGM6 vs RKM6 *n.s.*: not significant

Even though overall all six green malt beers (untreated and re-steeped) in sum showed lower levels of total marker aldehydes, the relative differences (comparison of biological replicates) between the green malt beers and the control differed substantially. Beer ageing is a very complex process involving many reaction mechanisms and pathways; several factors are involved in aldehyde formation, not solely aldehyde levels from the grist material. Thus, it was not surprising that the simplistic attempt to correlate free aldehydes in malt with the corresponding free aldehydes determined in all beers aged

at 30°C for 30 days (r = 0.4871, n.s.), 60 days (r = 0.4654, n.s.) or 90 days (r = 0.5396, n.s.), was not successful.

Nevertheless, due to the potential binding of aldehydes throughout the brewing process into non-volatile adduct forms, these bound aldehydes could potentially be released during ageing. Thus, the concentration found in the grist used for brewing could still be an important parameter for the aldehyde release during ageing. Considering the low number of aldehydes in green malt, potentially fewer aldehydes are available for binding and release during ageing. However, the adduct formation is very complex and aldehydes can react with numerous wort components, depending on several factors (temperature, pH value, redox potential, binding strength, thermodynamic stability) (Baert et al., 2012, Bustillo Trueba et al., 2018, Lehnhardt et al., 2019, Lermusieau et al., 1999, Liégeois et al., 2002).

In this instant, furfural might serve as an example for potential adduct formation and release during storage. Furfural was significantly lower in five aged green malt beers (except GM2) compared to the corresponding kilned malt controls, even though fresh beer levels were similar and ageing parameters (temperature, time) were identical (Figure 5.8A). This suggests that furfural leached out from the grist material into the wort, was bound, and subsequently released during ageing. Interestingly, beers prepared from KM1 and KM2 developed significantly lower levels of furfural (up to 50% less) during forced ageing, compared to the other reference (kilned malt) beers. However, similarly, these two beers contained the highest Strecker aldehyde (Figure 5.8C) levels, compared to the other reference brews.

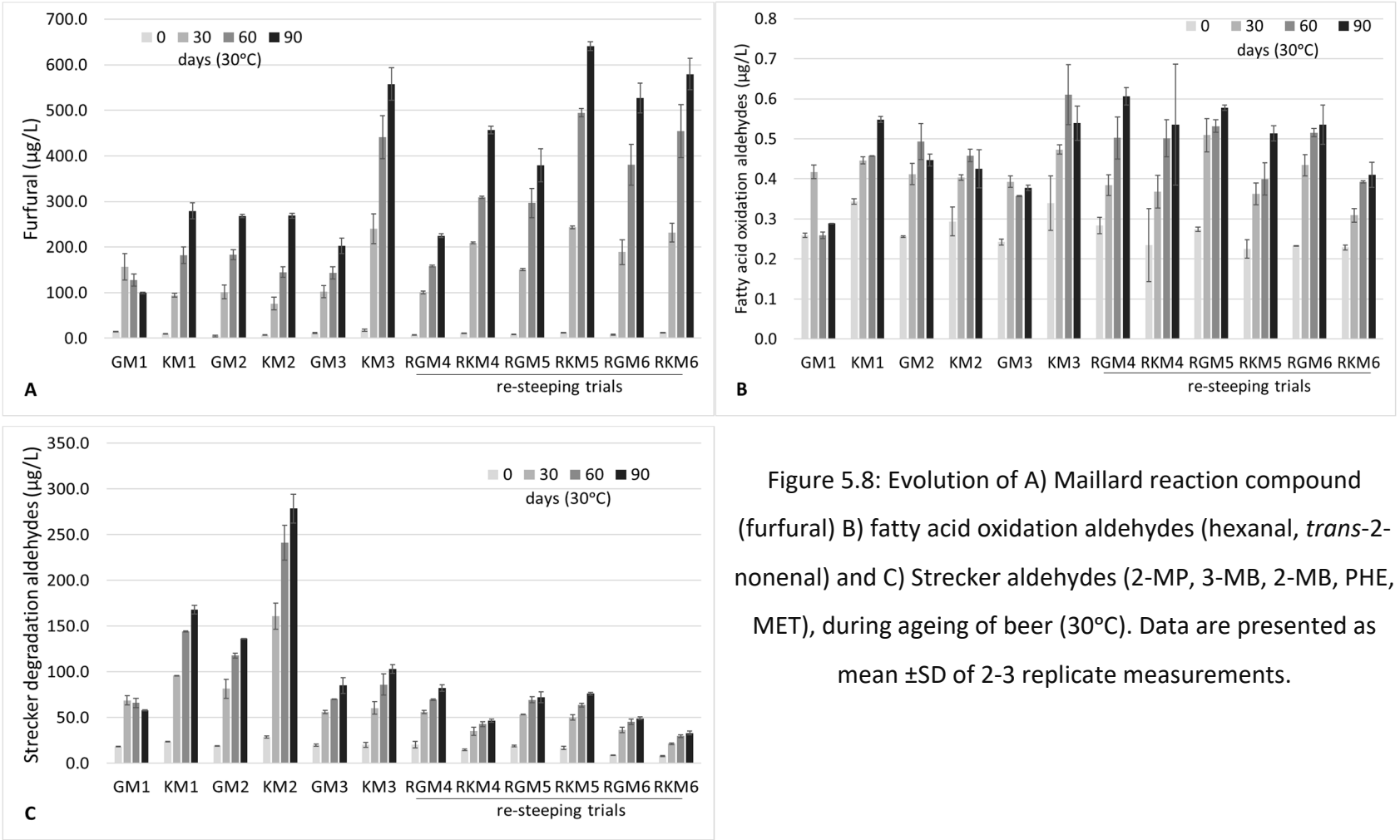


Figure 5.8: Evolution of A) Maillard reaction compound (furfural) B) fatty acid oxidation aldehydes (hexanal, *trans*-2-nonenal) and C) Strecker aldehydes (2-MP, 3-MB, 2-MB, PHE, MET), during ageing of beer (30°C). Data are presented as mean ±SD of 2-3 replicate measurements.

Furthermore, only modest changes of *trans*-2-nonenal and hexanal were observed during ageing (Figure 5.8B). While hexanal levels were clearly below the flavour threshold of 350 µg/L, *trans*-2-nonenal, which is discussed to be perceived at 0.11 µg/L (Meilgaard, 1975a) or even 0.03 µg/L (Saison et al., 2009b), might still impact the flavour. Previous research (Noël et al., 1999b) suggested that *trans*-2-nonenal and hexanal formation are not influenced by beer storage. In this study (Noël et al., 1999b), researchers injected the stable oxygen isotope $^{18}O_2$ into the headspace of the beer just before ageing. Their research concluded that sulphites, polyphenols and isohumulones were oxidised, however, oxygen was not incorporated into the carbonyl fraction. Thus, the cardboard flavour in beer, caused by *trans*-2-nonenal is not due to lipid oxidation during ageing, but formed during wort production, hence bound to wort components and subsequently released during beer ageing. (Coghe et al., 2004, Lermusieau et al., 1999, Liégeois et al., 2002, Noël et al., 1999a). The evidence from prior studies, point towards the idea, that during wort production (described in Chapter 3), some hexanal and *trans*-2-nonenal was bound, hence, the small but not insignificant increase observed in the forced aged beers (Figure 5.8B). Considering brewing with green malt, it is very important to highlight that in summary, considering all data collected and discussed in Chapters 3,4 and 5, it can be confirmed that lipoxygenase activity was sufficiently controlled by applying lipoxygenase hostile brewing conditions. Thus, lipoxygenase can be controlled even at activity levels 17-fold higher than a pale kilned malt (Chapter 2).
Naturally, the complexity of beer ageing should not be neglected, thus, the higher aldehyde concentration might not be solely related to adduct formation and subsequent release during ageing. However, it is highly recommended for future studies to further investigate the origin of free and bound aldehydes in green (undried) malt beer, which could add substantially to our understanding of malt kilning on flavour (in)stability.
In general, all aldehyde concentrations reached after 90 days ageing were well below the sensory threshold levels. However, due to the synergistic effects and the complexity of ageing (other compounds increase as well), they can still affect and impair the beer flavour. Table 5.2 aims to summarise the fold change of the selected staling aldehydes during storage. Quantitatively the highest change was observed in furfural and 2-methylpropanal, up to 81.6- and 18.6-fold increase, respectively. By comparing the biological replicates substantial variances are noticeable, mainly when comparing the

fold change of furfural (especial brewing trial 1-3). Even though furfural quantitatively increased the most the levels are still under the proposed flavour (150 000 µg/L (Meilgaard, 1975a)) or odour threshold (15157 µg/L (Saison et al., 2009b)). However, as shown by to De Clippeleer et al. (De Clippeleer et al., 2011), spiking of furfural resulted in a sharper, harsher bitterness and increased astringency. Nevertheless, the highest furfural concentration detected in the beers used for this study was 590 µg/L (RKM6_90), thus it can be assumed that furfural will not affect the beer flavour. The aldehyde levels determined in fresh and aged beers and reported flavour thresholds found in the literature are summarised in Appendix 2.

Table 5.2: Fold-change of aldehydes from fresh beer to aged beers (30°C, 90 days); Green malt 'as is' (GM 1-3) and the corresponding reference malt (KM 1-3); as well as re-steeped RGM (4-6) and the corresponding reference malt (RKM 4-6).

			2-MP	2-MB	3-MB	HEX	FUR	MET	PHE	T2N	Sum
re-steeping trials	1	GM	6.3	3.6	1.5	1.1	7.0	2.2	1.5	1.1	4.8
		KM	16.1	2.1	1.4	1.6	26.8	2.8	1.4	1.3	13.1
	2	GM	15.1	2.4	0.9	1.8	54.6	6.8	1.7	1.6	16.5
		KM	18.6	3.0	1.4	1.5	39.2	1.6	1.8	1.2	15.3
	3	GM	10.5	4.6	1.3	1.5	17.6	1.6	1.3	1.8	9.1
		KM	6.2	2.0	0.9	1.9	81.3	0.6	2.1	1.6	18.4
	4	GM	12.4	1.7	1.3	2.2	31.2	1.9	1.0	1.6	11.1
		KM	9.2	2.2	1.5	2.2	41.5	2.9	1.5	3.4	19.4
	5	GM	16.5	2.1	1.2	2.1	45.4	1.4	0.9	2.2	16.5
		KM	17.4	3.2	1.8	2.2	54.6	2.3	1.1	2.9	24.8
	6	GM	13.7	2.3	1.3	2.4	66.4	1.4	1.2	2.0	33.7
		KM	9.9	2.8	1.2	1.8	46.8	1.6	2.2	2.0	29.8

2MP = 2-methylpropanal; 2MB = 2-methylbutanal; 3MB = 3-methylbutanal, PHE = phenylacetaldehyde; MET = methional; FUR = furfural; HEX = hexanal; T2N = *trans*-2-nonenal sum = total of selected staling marker aldehydes

5.3.2.2 Correlation between residual amino acids in beer and Strecker aldehyde formation

As already highlighted in Figure 5.1, fresh beers from KM1 and KM2 varied substantially regarding their residual amino acid levels in the fresh beers, compared to the other beers (n = 10, green malt, re-steeped green malt and kilned malt). The following section

will more closely examine the amino acid concentrations, specifically the Strecker aldehyde precursors. It is interesting to note that out of the 12 measured beers (Figure 5.9), the beers prepared from green malt 'as-is' contained very low levels of amino acids (valine, isoleucine, leucine, methionine, phenylalanine) known as Strecker aldehyde precursors (sum = 16.7 ± 1.2 mg/L, n = 3). Beers from KM1 and 2 measured levels of 151.5 mg/L and 195.2 mg/L, respectively, whereas beers prepared from the controls KM3 – KM6 contain solely 23.5 ± 6.8 mg/L on average. Hence, beers brewed from KM1 and KM2 theoretically have a greater capacity to form Strecker aldehydes upon beer ageing (by direct Strecker degradation in-pack) compared to the other beers (green malt and control beers).

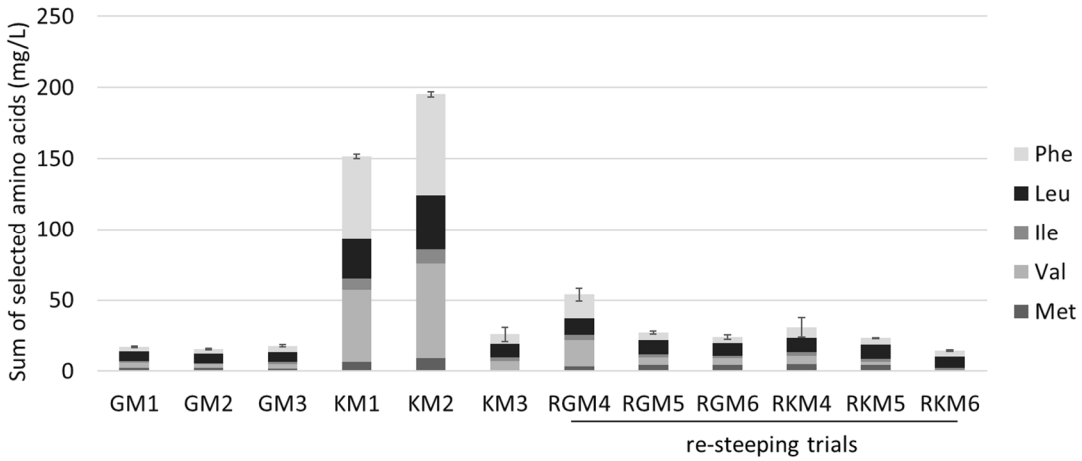


Figure 5.9: Sum of selected amino acids (valine, isoleucine, leucine, methionine, phenylalanine) in fresh beers prepared from green malt 'as is' (GM1-3) and the corresponding control (KM1-3); re-steeped green malt (RGM4-6) and the corresponding control (RKM4-6). Data are the mean ± SD of 2 technical replicate measurements; GM = green malt, KM = kilned

To further investigate this correlation, the formed Strecker aldehydes in aged beers (30°C, 90 days) were compared to the corresponding amino acids determined in the fresh beers. Indeed, the beers prepared from KM1 and KM2 formed the highest amount of Strecker aldehydes during forced ageing, with 167.9 ± 5.0 and 278.6 ± 15.8 µg/L, respectively, formed after 90 days at 30°C. The sum of selected amino acids and corresponding aldehydes in aged beers were found to be strongly correlated (Figure

5.10; $r(10) = 0.88$, $p < 0.001$). However, this correlation is driven greatly by the beers KM1 and KM2, the other beers clustered and did not obey this correlation (Figure 5.10).

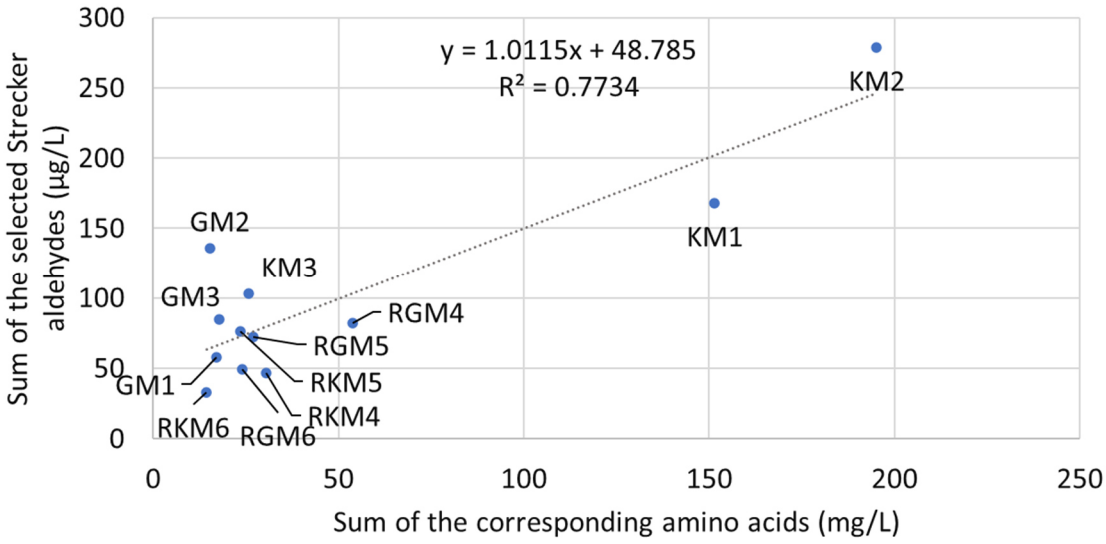


Figure 5.10: Concentrations of the sum of selected Strecker aldehydes (2-methylpropanal, 2-methylbutanal, 3-methylbutanal, methional and phenylacetaldehyde) measured in aged beers (30°C, 90 days), plotted as a function of the corresponding amino acids (valine, isoleucine, leucine, methionine and phenylalanine, respectively) in the fresh beers.

A similar (statistically significant) pattern was observed for high concentrations of valine, isoleucine and methionine and the increase of the Strecker aldehydes 2-MP, 2-MB and methional, respectively. However, similarly to Figure 5.10, these correlations were mostly driven by the kilned malt beers KM1 and KM2. Leucine moderately correlated with 3-MB formation; however, no correlation was found between phenylalanine and phenylacetaldehyde. Phenylacetaldehyde was found to be the most effective precursor of benzaldehyde (Chu and Yaylayan, 2008). Hence, after 90 days of ageing, the majority of phenylacetaldehyde might have already been converted to benzaldehyde, which was not measured within this experiment. However, no correlation was found between phenylalanine measured in the fresh beer and the phenylacetaldehyde levels determined at 30- or 60-days ageing. Additionally, phenylacetaldehyde concentration only changed minimally during beer ageing, with concentrations reported between 1.8 – 9.0 µg/L in aged beers and thus way below the flavour threshold (1600 µg/L).

According to the presented data, it appears that beers with high levels of amino acids can accelerate Strecker aldehyde formation during ageing, however, residual amino acids in beer are not the sole source of Strecker aldehyde formation. The remaining question is why KM1 and KM2 had such high wort FAN relative to KM3. The FAN level determined in the malts used for the presented brewing trials did not differ significantly amongst the biological replicates, with an average FAN concentration of 115.1 ± 9.6 mg/100g determined in KM1-3 (n = 3, tr = 3); in comparison GM1-3 measured 117.2 ± 13.7 mg/100g. Thus, it could be assumed that more FAN was extracted from the malt during mashing, even though mashing parameters chosen for all brewing trials were identical. Further research on the amino acid pattern and the relation to the Strecker aldehyde formation during ageing is required.

Interestingly, the third highest Strecker aldehyde concentration was found in beers prepared from the untreated green malt beer GM2. The bi-plot of the fresh beers shown in Figure 5.1, separated fresh GM2 beer from the other beers based on the high concentrations of bitter acids compared to the other beers. The degradation of bitter acids throughout beer ageing and the resulting formation of a myriad of volatile compounds, amongst them the Strecker aldehydes 2-methylpropanal, 2-methylbutanal and 3-methylbutanal, has been controversially discussed in the past. While research from Hashimoto et al. (Hashimoto and Eshima, 1979) reported that beer without hop addition developed fewer characteristic staling markers during ageing, however, this was in turn contradicted by recent research (De Clippeleer et al., 2010a). De Clippeleer et al. (De Clippeleer et al., 2010a) could not link the formation of 2-MP, 3-MB and 2-MB to the hop product degradation. Thus, it was suggested, that aldehyde formation through bitter acid degradation is of minor importance.

5.3.2.3 The influence of beer pH on the aldehyde formation during forced ageing

During beer ageing, the FAN levels and the pH did not change, but a higher FAN content in the beer resulted in a higher pH ($r(46) = 0.86$; $p < 0.001$, Figure 5.11), presumably due to buffering effects.

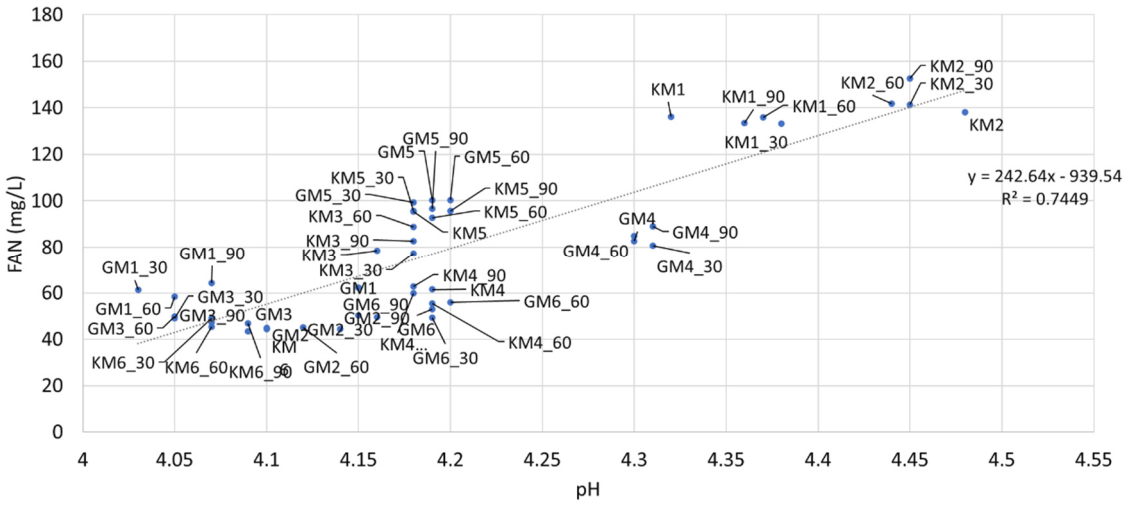


Figure 5.11: Correlation plot between beer pH and FAN (mg/L) in fresh and aged beers.

However, previous research by Kaneda et al. (Kaneda et al., 1997) suggested that beer ageing is accelerated at lower beer pH values. They reported that lowering the pH even slightly from 4.3 to 4.1 significantly increased stale flavour after ageing. These findings were related to an acceleration of oxidative degradation reactions (Kaneda et al., 1997) and the release of volatile carbonyls from Maillard intermediates (De Schutter et al., 2008) at lower pH. Additionally, *trans*-2-nonenal release from the bound state (imine form) was discussed to be enhanced at lower pH (Lermusieau et al., 1999). However, in the present study, a higher pH measured in beer was strongly positively correlated with the increase of the sum of Strecker degradation aldehydes during ageing ($r(10) = 0.8024$, $p < 0.0016$, Figure 5.12) for beers aged 90 days. However, according to the graph, this is again driven by the anomalously high FAN contents of KM1 and KM2.

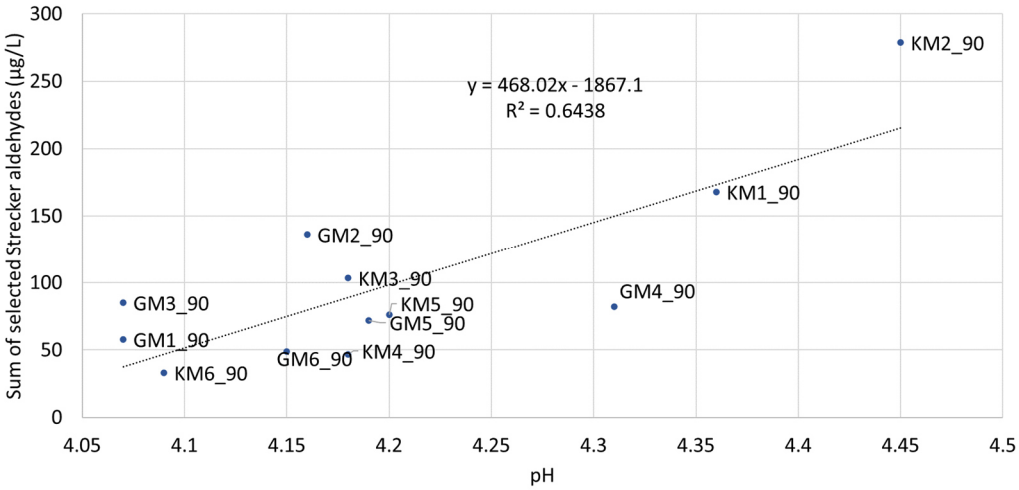


Figure 5.12: Concentrations of the sum of selected Strecker aldehydes (2-Methylpropanal, 2-methylbutanal, 3-methylbutanal, methional and phenylacetaldehyde) measured in aged beers (30°C, 90 days), plotted as a function of the corresponding pH in the beers.

There was no correlation between the amount of lipid oxidation aldehydes and the pH in aged beers ($r(10) = 0.3403$, n.s.), which is in contrast to finding by Jaskula-Goiris et al. (Jaskula-Goiris et al., 2011). Also, no correlation was found between furfural formation and pH.

5.4 Conclusion

This study aimed to determine the analytical changes of beers produced from green malt, with the focus being on staling aldehyde development. Additionally, this research helped to further investigate the impact of heat load applied on malt, a major source of staling precursors, on the flavour (in)stability of beers.

In general, all aldehyde concentrations reached after 90 days ageing at 30°C were well below the reported sensory threshold levels. However, due to the synergistic effects and the complexity of ageing, they can still affect and impair the beer flavour. All beers were tasted informally by expert tasters at both KU Leuven and University of Nottingham. Green malt (untreated and re-steeped) beers were found to develop fewer off-flavours during ageing than the corresponding reference beer. Particularly interesting was the comparison of beers aged for 30 days at 30°C; while the reference kilned malt beers already developed typical stale flavours (e.g. honey), only subtle changes were noted in

the green malt beers. Moreover, these changes in the green malt beers even added some positive flavour attributes to the beers - likely some typical 'kilned malt' flavours that might have been still missing in the fresh 100% green malt beers. Even though all kilned malt beers were prepared according to the 'best practice' standards to diminish beer staling, the beers prepared from untreated and re-steeped green malt (except RGM6) formed lower amounts of total marker aldehydes compared to the control beers measured after 30, 60 and 90 days ageing. Thus, the present data suggest that the heat load applied to the raw materials used for brewing is an important parameter for beer ageing. Unfortunately, the high variations even in the control kilned malts and subsequent beers (which should be very similar), impede derivation of clear correlations.

Furfural was present at significantly lower concentrations in green malt beers compared to the kilned malt controls, even though fresh beer levels were similar and ageing parameters (temperature, time) were identical. This suggests that a pool of furfural from the malts delivered to the brewing process was bound to as yet unspecified wort components and subsequently released during ageing. Thus, further research on adduct formation and subsequent release of aldehydes during ageing is recommended. Alternatively, furfural intermediates pre-formed during malting and brewing (e.g. 3-deoxyosone derived from pentose, Section 1.4.1.2), could end up in finished fresh beer and thus further converted into furfural during beer ageing.

Furthermore, it is important to highlight, that lipoxygenase activity was sufficiently controlled by applying lipoxygenase hostile brewing conditions, even when using green malt. The resulting data further suggest that high residual amino acid concentrations in beer can accelerate Strecker aldehyde formation during ageing. However, no linear relationship was found between residual fresh beer amino acids and Strecker aldehydes in aged beers. Thus, residual amino acids were not the sole precursor of Strecker aldehydes formed during ageing, with release from bound forms hypothesised to be the other major source. Intermediates of Strecker degradation of amino acids (pre-formed during malting and brewing) might have ended up in finished beer and would thus be converted into Strecker degradation aldehydes during beer ageing.

The results presented in this chapter emphasise the complexity of beer ageing. However, substantial knowledge is added to our understanding of the flavour

(in)stability of beer through the ability to directly compare kilned with green malt beers. Nevertheless, further brewing trials with green malt with the usage of different yeast strains under a range of different fermentation conditions, as well as altered ageing conditions are necessary to fully understand the multi-factorial flavour stability of green malt beers. Brewing with green malt has the potential to advance current knowledge on beer staling, particularly the impact of malt kilning. Therefore, it is recommended to add 'green malt beers' to future beer stability investigations.

6 Major findings and future work

The overall goal of this doctoral thesis was to develop underpinning biochemical and technical knowledge for the development of brewing processes based on green (germinated, but not dried) malt. Such a process would enable a substantial saving in energy input associated with kilning, similarly, making the process more water efficient by conserving the water contained in the green malt. Likewise, the aim was to assess whether acceptable wort and beer without major quality defects can be brewed using even 100% green malt. Subsequently, the focus was on the impacts on beer flavour stability, which was important to establish because reduced heat load could either improve flavour stability (reduced pool of staling aldehydes) or worsen it (since e.g. lipoxygenase activity is regulated by heat treatment during kilning).

Considering the paucity of literature (Cook and Hudson, 1964, Duff, 1963, Leclercq, 2020, MacWilliam, 1972, MacWilliam et al., 1963, Moir, 1992) available on brewing with green malt the primary task was to examine the major quality concerns associated with green malt. Therefore, lipoxygenase (LOX) activity and S-methyl methionine (SMM) levels were monitored through a micromalting cycle both with and without rootlets. When removing the rootlets, LOX activity and the concentration of the DMS precursor declined by 30% and 40%, respectively. These results for LOX and SMM confirmed (White and Wainwright, 1976a, Yang et al., 1993) that rootlets are a major concern when brewing with green malt and that their influence on quality needs to be further investigated. However, even after rootlet removal the relative activity or concentration was still significantly higher compared to the kilned malt control. Therefore, attempts were made to reduce lipoxygenase activity by taking advantages of its heat- and pH-sensitivity, as well as through limiting the availability of oxygen as a substrate (Bamforth, 1999c, De Buck et al., 1997, Drost et al., 1990, Schwarz and Pyler, 1984, Van Waesberghe et al., 2001). Overall, this work has revealed two potential methods to lower the LOX activity in green malt without adversely affecting the diastatic enzyme levels: (1) re-steeping the grain for one hour before mashing-in or (2) a heat treatment at 65°C for up to an hour. Green malt worts were then prepared from i) whole green malt immediately post-germination; ii) heat-treated green malt (65°C x 1 h) and iii) re-steeped green malt.

Naturally, both methods can be easily performed on laboratory scale but would require technical adaptations to be applicable on industrial scale. As an example, malt drying at 65ºC must be performed uniformly, as it was shown previously (Guido et al., 2005, Müller et al., 2014) that inhomogeneity (temperature and moisture conditions) in the kiln can impact the chemical reactions occurring in the malt. Furthermore, the water used for re-steeping must be re-used for mashing, to remain water efficient. To further investigate the properties of green malt rootlets, a novel technique to produce wort without the husk/rootlet fraction was developed. With the aid of a pasta roller, the grain was crushed and subsequently sparged with water and filtered using a muslin cloth filter. The resultant iv) endosperm rich extract was then used for wort production. Laboratory mashing was performed using a 'LOX-hostile' mash schedule (mashing-in at 63°C, pH 5.2, oxygen-limited conditions). Data were compared with mashing of kilned pale malt made from the same green malt, as a reference point. The results from this study indicated that controlling LOX activity by mashing in at 63°C, pH 5.2 in deaerated liquor resulted in a *trans*-2-nonenal potential for wort prepared from green malt, without any pre-treatment, which was not significantly higher than when using kilned malt. Conversely, hexanal potential was significantly higher for the green malt process, most likely due to the high hexanal levels found in green malts, usually reduced through thermal processing (kilning or roasting) (Coghe et al., 2004, Dong et al., 2013, Moir, 1992). Overall results indicated that the resultant brewing process would need to be optimised to deal with the elevated levels of SMM and hexanal in green malt worts.

This work presented in Chapter 2 built the basis for subsequent pilot brewing trials, discussed in Chapter 3. The aim was to evaluate the wort/beer quality, as well as the feasibility of brewing with 100% green malt (in a pale lager wort, and a top fermented beer, with no masking of flavour defects). Beers were prepared using 100% green malt (n = 3) or kilned pilsner malt (n = 3) prepared from the same batch in each case utilising the pilot brewery at KU Leuven, brewing at 50% total capacity (2.5 hL). One major focus at this part of the study was to evaluate the main quality concerns observed in Chapter 2. Previous work (Hirota et al., 2005, Skadhauge et al., 2005) has shown that even low residual lipoxygenase activities in pale kilned malt can cause serious flavour deteriorations in the final beer. The elevated lipoxygenase activities in green malt (17-

fold higher than in the reference kilned malt), as proven in the laboratory scale trials, could (in theory) cause major flavour defects in green malt beers. Therefore, three further pairs of beers were brewed whereby the green malt was pre-steeped under de-aerated water for 1 hour; as this procedure had previously been shown (Chapter 2) to lower LOX activity in green malt.

The brewery at the Technology Campus Ghent, KU Leuven is equipped with a wet milling system by Meura, which is advertised to efficiently grind malts with high moisture content (Leclercq, 2020, Meura). Moreover, the injection of CO_2 both into the malt inlet and bin has the potential to improve oxidative stability and thus provided a very promising outline for successfully brewing with green malt. Further technological and process optimisations are undoubtedly required, as the complex structure of the spent grains bed formed during mash filtration ('spongy, cohesive structure) increased the likelihood of blockages and extended filtration and sparging periods. Most likely, some technical optimisation of the mill design, and/or fine tuning of the liquor to grist (2.2:1) ratio might be sufficient to enable successful brewing with 100% green malt without significant losses in extract. However, in total six green malt beers (untreated and re-steeped) were brewed with acceptable specifications in terms of pH, alcohol content, foam stability and colour. The characteristic colour compounds to beer are mostly formed via Maillard reactions between reducing sugars and amino-compounds during kilning. Thus, it was surprising that EBC colour was not reduced as much as might have been predicted for green malt brews. The distinctive colour, might arise from the prevalence of different natural yellow colour pigments present in malt (e.g polyphenols or water-soluble riboflavin (Briggs, 1998a)) relative to the melanoidins contributed by kilned malts. This theory was further supported as the re-steeped green malt beers were significantly lower in colour than the beers prepared from green malt 'as is', suggesting that these water-soluble natural yellow pigments were washed out during re-steeping (and potentially retained during filtration in the fine-meshed net). Overall, no significant taints or obvious defects were detected in 100% green malt beers with intact rootlets, compared to their reference kilned malt beers. The organoleptic testing performed alongside the study was not performed to a publishable standard (~35 informal/expert taster comments). Based on this limited sensory evaluation it was not appropriate to comment beyond the fact that the beers were 'acceptable'. Therefore, more detailed

sensory evaluation of the organoleptic properties of green malt beers are required to evaluate their unique flavour profile. Nevertheless, the presented research demonstrates that three of the major quality concerns when brewing with green malt (rootlets, LOX activity, and DMS potential) were not insurmountable problems. Even though increased S-methyl methionine levels were detected in malts (Chapter 2) and measured in worts and beers made from green malt (Chapter 3), however, DMS concentrations in the finished beers did not differ significantly from the reference brews. This is remarkable considering that the DMS potential determined at the onset of mashing was 2-3 times higher than in the reference brews. Considering the results from this study and previous research (White and Wainwright, 1976a, White and Wainwright, 1977), it appears that DMS levels in pitching wort and finished beers can be controlled even when using green malt, given a sufficient removal of DMS via evaporation during wort boiling and through elimination via fermentation gases. Due to the high LOX activity in green malt, an elevated 'rancidity' in the final beer was expected. However, no significant (LOX) related taints were detected in worts and beers prepared from green malt. Furthermore, an increased extraction of trihydroxy fatty acids (THFA levels) - intermediates of the LOX pathway - were measured when brewing with kilned malt. It is very likely, that the oxygen-boosted drying of green malt, triggers the oxidation of unsaturated fatty acids, giving rise to both intermediates, such as THFA and fatty acid oxidation aldehydes – *trans*-2nonenal and hexanal. The latter was further confirmed by investigating the free staling aldehyde contents in the malts used for these brewing trials (Chapter 4). Both, *trans*-2-nonenal and hexanal levels were found in significantly lower concentrations in the green malts compared to the corresponding kilned malts. Using re-steep water in green malt brewing (for reasons of water economy), however, increased THFA levels, possibly because oxygen uptake was not adequately controlled at this step. Further technical optimisations could improve 're-steeping' and thus limit oxygen uptake even on pilot scale, but will cause extra effort and costs. However, re-steeping proved to be unnecessary as LOX could be adequately controlled in the pilot plant process by wet milling in deaerated liquor under CO_2 and mashing-in at 63°C, pH 5.3 and once again under oxygen-limited conditions. Considering that temperature and pH control were challenging in green malt brews due to the thickness of the mash and the noted incompatibility of the mash homogeniser, these findings suggest that oxygen

exclusion is a key criterion to limit LOX activity. Thus, it is possible to brew with green malt on the condition that mashing-in occurs under LOX hostile conditions, or by using Null-LOX barley (Hirota et al., 2005, Skadhauge et al., 2005) varieties. The latter may be beneficial for breweries where strict LOX-hostile conditions cannot be applied or who wish to avoid additional costs (and health and safety considerations) of CO_2 injection. Additionally, there is no risk for precursor formation during malting when using Null-LOX varieties. Overall, the results presented in Chapter 3 demonstrated very promising indicators for flavour stability, such as reduced TBI (Gastl et al., 2006, Jaskula-Goiris et al., 2015), lower residual FAN (De Rouck et al., 2007, Jaskula-Goiris et al., 2015) and trihydroxy fatty acid (THFA) levels (Baert et al., 2012, Gastl et al., 2006, Kobayashi et al., 1993, Kuroda et al., 2002, Vanderhaegen et al., 2006) in brews using untreated green malt. Therefore, the focus of the study presented in Chapter 4 was to further elucidate the flavour stability impacts of brewing with green malt. Malt is a major source of staling precursors, especially aldehydes (De Clippeleer et al., 2010b, Ditrych et al., 2019, Gastl et al., 2006, Guido et al., 2007). Therefore, the green malt, re-steeped green malt and the corresponding kilned malt, used for the brewing trials described in Chapter 3, were evaluated for their free staling aldehyde content. Subsequently, the free staling marker aldehydes, as well as the oxidative stability of worts and beers prepared from green malt and re-steeped green malt were evaluated and compared to equivalent data for the corresponding control beers. All green malts (untreated and re-steeped) contained significantly lower free aldehyde levels compared to the control malts, emphasising the effect of thermal treatment on free aldehyde formation. Despite the elevated LOX activities in green malt, the *trans*-2-nonenal and hexanal levels significantly decreased during mashing. These results indicated that lipoxygenase hostile mashing conditions were sufficient to avoid negative effects associated with this enzyme even at the very high activity levels measured in green malt. Even though kilned malt contained significantly higher levels of free aldehydes than green malt, the wort production process and subsequent fermentation reduced the aldehydes to levels not significantly different from those for the fresh green malt beer, which is remarkable considering the enormous differences determined between the green and corresponding control malt (4.3-14.0 fold higher). However, these aldehydes might bind to other compounds forming non-volatile adducts (Baert et al., 2018, Baert et al., 2015a, Baert et al., 2015b,

Bustillo Trueba et al., 2019, Debourg et al., 1994, Kaneda et al., 1994, Lermusieau et al., 1999, Peppard and Halsey, 1981), thus the aldehyde content needed to be investigated further during beer ageing (Chapter 5). Additionally, the oxidative stability of the wort and beers prepared was evaluated using ESR analysis. The worts and beers produced from untreated green malt showed significantly lower radical formation relative to the corresponding kilned malt control wort or beer samples. Pitching worts prepared from re-steeped green malt showed less radical formation during forced ageing than the control, however, this effect could not be observed in the finished beers.

None of the twelve beers expressed any lag time (the time until notable amounts of radicals are generated), due to the lack of SO_2 present in the finished beers, which was related to the high temperature and vigorous fermentation (24°C) employed (Ilett and Simpson, 1995, Kaneda et al., 1991), as well as the choice of yeast strain (as ale strains are known to produce less SO_2 than lager strains (Hysert and Morrison, 1976)). To further investigate the reasons for the better oxidative stability (ESR results) in green malt wort and beer, the transition metals were determined in the fresh beers using ICP-MS. Increased heat load on malt through kilning or roasting was shown to impact the transition metal content and thus the ionic composition of the wort (Hoff et al., 2012, Jenkins et al., 2018, Pagenstecher et al., 2020, Poreda et al., 2015). Overall, the metal ion concentrations measured in the beers did not explain the improved oxidative stability of beers prepared from green malt. Therefore, further research and sampling during the wort production process is required to get a better picture of the oxidative stability of green malt worts and beer.

The work presented in Chapter 5 aimed to elucidate if green malt used as the grist material for brewing (and thus a significantly reduced heat load applied to malt) can be beneficial for beer flavour stability. The previously described beers prepared from green malt (n = 3), re-steeped green malt (n = 3) and corresponding reference (pilsner style) malt (n = 6) (as described in Chapter 3) were aged at 30°C for 30, 60 and 90 days. To evaluate the ageing and consequent changes in beer, standard analytical parameters, as well as flavour compounds (aldehydes, esters and higher alcohols) were analysed across the storage trial. Overall, an increase in concentrations of undesirable staling aldehydes and a decrease in concentrations of desirable compounds (acetate esters, bitter acids) was noted. Even though, the same malting barley variety was used and malted after the

same malting scheme (according to the supplier), the free marker aldehydes concentrations differed substantially between all biological replicates. This was potentially due to the nature (industrial scale) of the samples, as previous studies (Guido et al., 2005, Müller et al., 2014) have highlighted that malt sampled from different bed depths can impact the chemical reactions occurring in malt. The resulting variations in wort composition can greatly affect and determine the formation of beer aroma compounds. Nevertheless, the resulting data still revealed very important information about green malt worts and beers. The results showed that green malt beers were less susceptible to beer ageing flavour change than kilned malt beers, due to a lower formation or release of staling aldehydes. Considering the low amount of aldehydes in green malt, potentially fewer aldehydes are available for binding and released during ageing. Furfural, for example, increased significantly in control beers compared to green malt beers even though fresh beer levels were similar and ageing conditions (temperature, time) were identical. Furthermore, the cardboard flavour in beer, caused by *trans*-2-nonenal was discussed to not be due to lipid oxidation during ageing, but formed during wort production, hence bound to wort components and subsequently released during beer ageing. (Coghe et al., 2004, Lermusieau et al., 1999, Liégeois et al., 2002, Noël et al., 1999a). This points towards the idea, that during wort production (described in Chapter 3), some hexanal and *trans*-2-nonenal was bound, hence, the small but not insignificant increase observed in all forced aged beers. Considering the elevated lipoxygenase activity measured in green malt, hereby it was confirmed that lipoxygenase hostile conditions during wort production are sufficient to avoid negative effects on flavour stability associated with this enzyme. Naturally, beer ageing and aldehyde adduct formation in particular is very complex. Aldehydes can react with numerous wort components, depending on several factors (temperature, pH value, redox potential, binding strength, thermodynamic stability) (Baert et al., 2012, Lehnhardt et al., 2019, Lermusieau et al., 1999, Liégeois et al., 2002). However, considering the presented data it is highly recommended for future studies to further investigate the origin of free and bound aldehydes in green (undried) malt beer, which could add substantially to our understanding of malt kilning on flavour (in)stability. Furthermore, it was shown that the two beers with the highest amount of residual amino acids also formed the highest amount of Strecker aldehydes during forced ageing.

However, as the other beers did not follow this pattern of behaviour, Strecker aldehyde formation cannot solely be related to residual amino acids in fresh beers.

Brewing with green malt is a disruptive technology and the process needs to be further optimised before it could be implemented in present day breweries. However, returning to the question posed at the beginning of this study, it is now possible to state that green malt with intact rootlets can be used to brew wort and beer without any flavour defects, with superior flavour stability metrics, and decreased aldehyde formation during shelf-life.

6.1 General principles of brewing with green malt

This research project aimed to provide the scientific community, brewers and maltsters with technical and (bio)chemical guidelines to successfully brew green malt beers. Therefore, general principles, which could serve as a base for future studies on successfully brewing with green malt, are highlighted here, as follows:

- Green malt should be stored cold and dry to reduce microbial activity and stabilise enzymatic activity until the grain is further processed – the sooner the better. It is either necessary to prepare malt/wort extract or to process rapidly by having a brewery and maltings co-located (transport of the high moisture commodity is not feasible). Longer storage periods of green malt and the associated microbes present could greatly affect malt quality and thus impact beer quality (Bokulich and Bamforth, 2013, Justé et al., 2011, Scott, 1996).
- If omitting the kilning process, the brewer could take advantage of the increased moisture content in the grain, hence less brewing water will be needed for mashing. E.g. in the presented (Chapter 3) pilot scale brewing trials the total volume of the mash water for the brews using kilned malt was 97 L, compared to 70.5 L when using green malt at 40% moisture content. Additionally, the brewing liquor used for mashing needs to be appropriately adjusted (e.g. adjustment of temperature, calcium and lactic acid addition) to account for the extra water in the grain.
- A wet milling system seems to be a good choice to mill green malt, however, the design should be improved and the liquor:grist ratio adjusted so that an optimal

grain bed forms during filtration and for sparging when brewing with 100% green malt. This will enable brewers to take full advantage of the highly fermentable worts of green malt.

- The malt and the milling installation should be sparged with CO_2 or N_2 to suppress enzymatic oxidation via LOX enzymes throughout the wet milling process.
- Lipoxygenase activity should be controlled in order to avoid an increase in the staling potential of the final beer (De Buck et al., 1997, Yang and Schwarz, 1995), as well as impaired foam stability (Hirota et al., 2006, Yu et al., 2014). Thus, mashing should be performed at a LOX hostile mash environment: > 62 °C, pH: 5.3, oxygen-free (Bamforth, 1999c, De Buck et al., 1997, Drost et al., 1990, Schwarz and Pyler, 1984, Van Waesberghe et al., 2001).
- LOX-less (Hirota et al., 2006, Hoki et al., 2018, Hoki et al., 2013, Yu et al., 2014) or Null-LOX (Hirota et al., 2005, Skadhauge et al., 2005) barley varieties might offer advantages for brewing with green malt. Particularly in breweries where LOX-hostile conditions cannot be applied or to avoid additional costs for CO_2 injection.
- Green malt is very rich in diastatic enzymes (Hämäläinen and Reinikainen, 2007, Schroeder and MacGregor, 1998, Sopanen and Laurière, 1989), thus, when brewing with 100% green malt (under optimal technical conditions), it might be necessary to reduce the times spent at the ~62°C (mashing) rests to strike the proper balance between fermentable and non-fermentable sugars. This could help to build a little more body to the beer.
- EBC colour is a relatively simplistic indicator to define wort or beer 'colour'. In general, the EBC colour of beers produced from green malt was not reduced as much as might have been predicted for green malt brews. However, green malt beers had their own pigments, hue and chroma, which was better demonstrated by means of Figure 3.8 (Chapter 3). The presented figure supports that the appearance was not unattractive or deficient as might be feared if one assumed that the colour of a conventional 100% malt lager beer principally derives from

kilning. Nevertheless, adjustments can be easily made by adding small amounts of coloured (kilned or roasted) malts.

- It is important to maintain control of the high SMM levels in green malt, to avoid ending up with excessive DMS levels in the finished fresh beers. The brewhouse design (over-dimensioned chimneys with condensate traps) and the increased fermentation temperature (24°C) used for the brewing trials reported in this thesis was most certainly advantageous for DMS removal. The finished beer DMS was closely matched between green and kilned malt beers, which is remarkable considering the elevated precursor levels in green malt (5-fold higher compared to the corresponding kilned counterpart), as proven in the laboratory-scale trials (Chapter 2). In case DMS problems occur, higher fermentation temperatures or the use of a wort stripper (Bamforth, 2013) could help to remove excessive amounts of DMS. Additionally, Heineken and Carlsberg described barley plants with combined traits of Null-LOX-1, Null-LOX-2 and null-MMT (L-methionine S-methyltransferase) within one plant (Knudsen et al., 2011). This barley variety avoids both LOX related off-flavour development but also suppresses DMS formation.
- In addition, an oversized chimney with condensate trap supports the removal and similarly prevents re-entrance of unwanted volatiles (e.g. DMS) during wort boiling (De Rouck et al., 2010).

6.2 Recommendations for future work and potential industrial application of green malt

Brewing with green malt represents a disruptive technology and multiple factors need to be considered to successfully brew with this 'novel' grist material. The findings from this thesis suggest the following directions for future research:

- Most importantly, future research should focus on maximising the efficiency of brewing operations with green malt and the production of beers which are palatable for the consumer. This implies firstly the development of optimal processing of green malt, without any yield losses during filtration and sparging of the green malt 'cake'. Potentially, by technical optimisation of the milling system, or adjustment of the liquor to grist ratio, thus achieving a mash with

optimal composition for the mash filter, these challenges can be overcome, and yield can be maximised.

- Replacing 20% of pilsner malt with green malt to a cereal recipe containing 30% unmalted barley, was shown (Leclercq, 2020) to significantly improve filterability and would thus be very interesting to investigate in future studies.
- Some organoleptic studies of the wort, fresh beers and corresponding aged beers were performed alongside the experimental work for this thesis, however, the sensory work was not performed to a publishable standard (informal/ expert taster comments). Green malt (untreated and re-steeped) beers were found to develop fewer off-flavours during ageing than the corresponding reference beer. Based on this limited sensory evaluation it was not appropriate to comment beyond the fact that the beers were 'acceptable'. Particularly interesting was the comparison of beers aged for 30 days at 30°C; while the reference kilned malt beers already developed honey, and sherry flavours, only subtle changes were noted in the green malt beers. Moreover, these changes in the green malt beers even added some positive flavour attributes to the beers - likely some typical 'kilned malt' flavours that might have been still missing in the fresh 100% green malt beers. Unfortunately, due to the complexity of the ageing process, numerous compounds decline or arise, thus, differences were not captured to a full extend by solely comparing the analytical data. Therefore, it is highly recommended for future studies to repeat the brewing trials using an improved brewing protocol and extending the list of analytical measurements to get a broader picture of the differences. Furthermore, the resulting finished fresh and forced aged beers should be subjected to detailed sensory analysis to collect flavour descriptors from a professional sensory panel.
- On a broader level, research is needed to determine the impacts of brewing with the addition of green malt to different ratios of kilned malts. Additionally, the high enzyme complement in green malt could enable more efficient brewing with raw (unmalted) materials (e.g. raw barley) to create highly attenuated beers, without the use of exogenous enzymes. The resulting beers should be subjected to sensorial tasting.

- Against expectations, DMS levels in beers made of green malt were similar to the kilned malt controls. However, it would be of interest for the brewers to see if a different brewhouse design and lower fermentation temperatures can also cope with the high DMS potential.
- Green malt beers are reported (Chapter 3) to have a distinctive colour, arising from the prevalence of different pigments (polyphenols, riboflavin) relative to the melanoidins contributed by kilned malts. Therefore, the precise origins and qualities of the colour contributed by green malt should be further investigated.
- Based on the outcomes presented in this thesis, brewing with green malt has the potential to advance current knowledge of the impacts of raw materials on beer staling, particularly regarding the impacts of malt kilning on downstream flavour (in)stability. Thus, it is recommended to add worts and beers prepared from green malt to experiments investigating the flavour (in)stability of beers - even if the overall goal is not to develop a new product by using green malt. For example:
    - Worts and beers from untreated green malt had a very good oxidative stability. Interestingly, while worts of the re-steeped green malt brews performed equally as well, the finished fresh beers showed similar radical formation within the ESR study to the control beers. Thus, it would be interesting to establish whether precursors in green malt wort can be metabolised by yeast to form an antioxidative species. When re-steeping, these precursors might be removed or altered, and thus, significantly more radicals were formed than in untreated green malt beers. Furthermore, kilning or roasting was shown to impact the content of transition metal ions with prooxidative effects in the wort (Hoff et al., 2012, Jenkins et al., 2018, Pagenstecher et al., 2020, Poreda et al., 2015). Thus, it would be very interesting to further investigate the transition metal ion composition in wort and influence on oxidative stability when using green malt as the grist bill.
    - Untreated green malt beers developed significantly lower levels of staling marker aldehydes during forced ageing compared to the kilned malt control, despite the fact that free aldehyde levels were reduced to similar

levels during wort production and fermentation. This could become of great interest in the hunt for the origin of free and bound staling aldehydes.

Opportunities when using green malt in the industry:

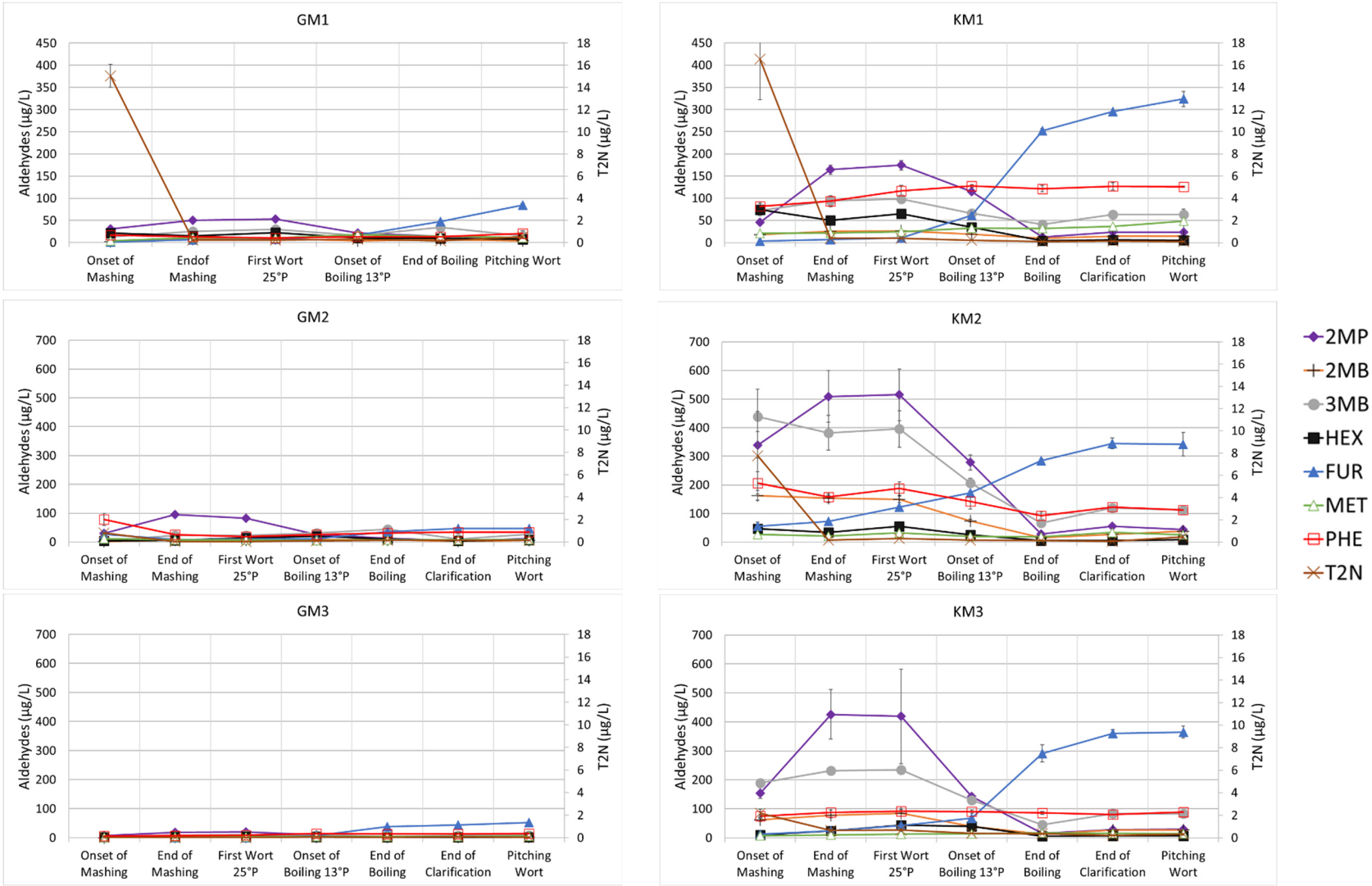
- Use as an enzyme-rich adjunct to digest unmalted adjunct materials.
- Marketing of an environmentally friendly beer, as consumer awareness and thus the demand for energy and water efficient products increases.
- Creating a new market for green malt beers (as opposed direct flavour match to existing kilned malt beers).
- Use with small quantities of roasted malts in the grist to adjust the colours and flavours and prepare different beer styles.
- Produce green malt extracts with greater storability

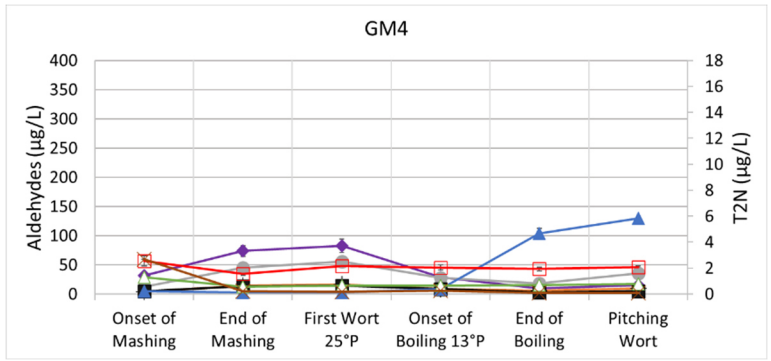
Appendix 1: Short heat treatment of green malt

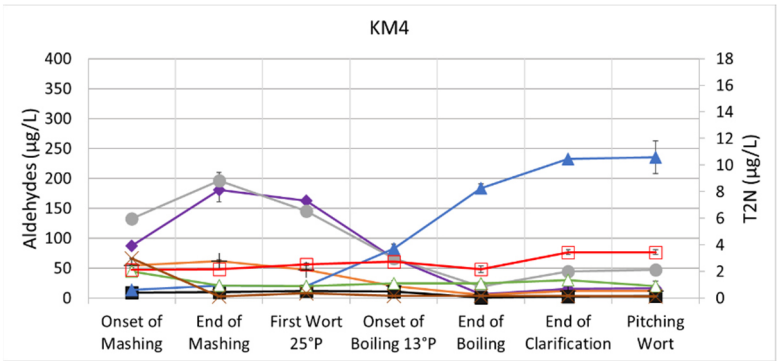
Lipoxygenase activity, α-amylase and β-amylase concentrations (n=18) modelled as a function of time (5-60 min) and temperature (65°C-90°C).

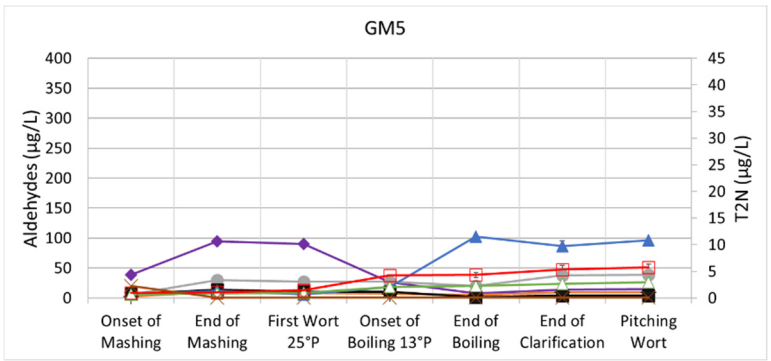
		Temp (°C)	Time (min)	LOX (U/g)	α-amylase (CU/g)	β-amylase (BU/g)
green malt batch 1	1	90	32.5	1.93	138.62	2.08
	2	90	60	0.00	120.37	0.62
	3	75	5	12.87	224.30	16.47
	4	77.5	32.5	4.42	216.20	12.55
	5	65	60	5.40	250.99	15.29
	6	65	60	5.02	228.50	14.56
green malt batch 2	7	82.5	16	8.35	131.91	6.06
	8	90	5	8.93	173.01	12.27
	9	65	32.5	8.11	220.11	14.32
	10	77.5	32.5	5.83	195.81	9.65
	11	90	60	0.00	95.74	0.40
	12	77.5	32.5	6.36	228.68	9.91
green malt batch 3	13	65	5	9.85	223.89	15.44
	14	75	60	2.93	194.46	12.28
	15	77.5	32.5	5.72	192.74	11.40
	16	65	5	7.46	229.56	16.51
	17	90	5	10.58	170.77	13.44
	18	82.5	49	0.50	125.98	2.60

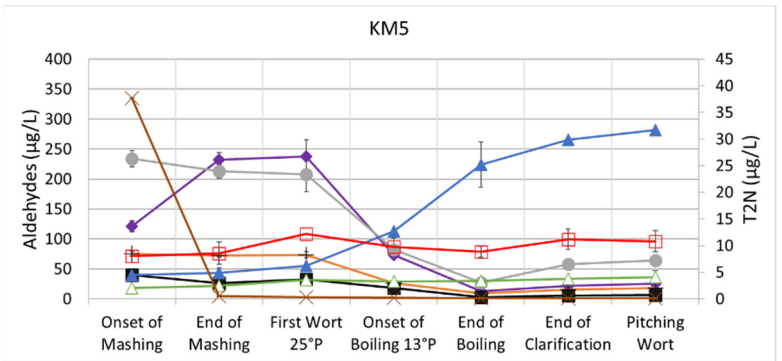
Appendix 2: Evolution of free staling aldehydes throughout the wort production process

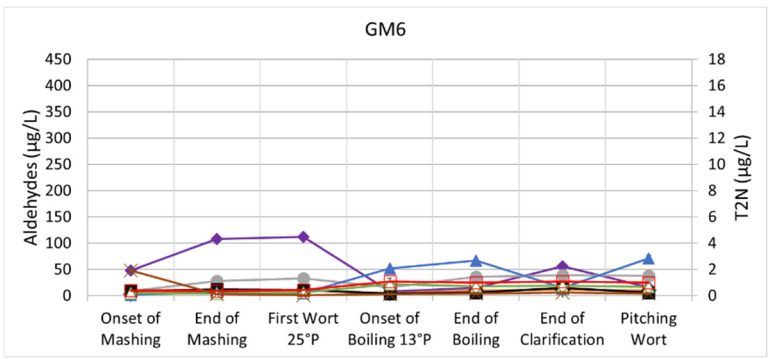


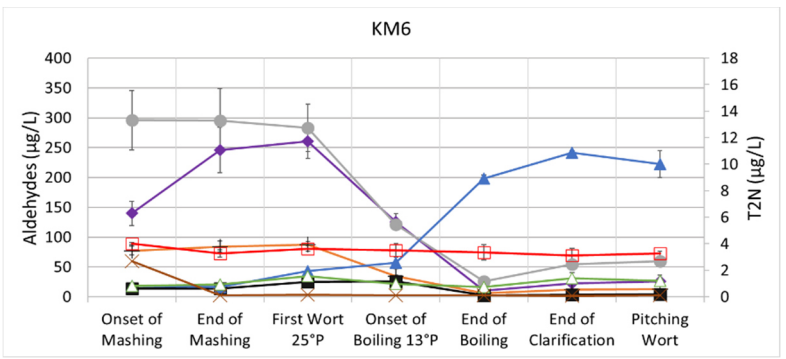

GM4
Aldehydes (µg/L)
T2N (µg/L)
Onset of Mashing
End of Mashing
First Wort 25°P
Onset of Boiling 13°P
End of Boiling
Pitching Wort

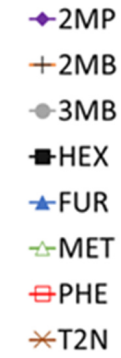

KM4
Aldehydes (µg/L)
T2N (µg/L)
Onset of Mashing
End of Mashing
First Wort 25°P
Onset of Boiling 13°P
End of Boiling
End of Clarification
Pitching Wort


GM5
Aldehydes (µg/L)
T2N (µg/L)
Onset of Mashing
End of Mashing
First Wort 25°P
Onset of Boiling 13°P
End of Boiling
End of Clarification
Pitching Wort


KM5
Aldehydes (µg/L)
T2N (µg/L)
Onset of Mashing
End of Mashing
First Wort 25°P
Onset of Boiling 13°P
End of Boiling
End of Clarification
Pitching Wort


GM6
Aldehydes (µg/L)
T2N (µg/L)
Onset of Mashing
End of Mashing
First Wort 25°P
Onset of Boiling 13°P
End of Boiling
End of Clarification
Pitching Wort


KM6
Aldehydes (µg/L)
T2N (µg/L)
Onset of Mashing
End of Mashing
First Wort 25°P
Onset of Boiling 13°P
End of Boiling
End of Clarification
Pitching Wort


2MP
2MB
3MB
HEX
FUR
MET
PHE
T2N

Appendix 3: Analysis of aged beers

FAN, total polyphenol (TP), flavanoid and proanthocyanidin concentration (mg/L) in fresh and aged (30°C) beers.

Beers	FAN		TP		Flavanoid		PA		Beers	FAN		TP		Flavanoid		PA	
	mean	±SD	mean	±SD	mean	±SD	mean	±SD		mean	±SD	mean	±SD	mean	±SD	mean	±SD
GM1	62.4	0.3	177.3	0.4	72.4	0.8	40.7	7.1	**KM1**	136.1	0.8	215.7	18.6	48.1	2.5	45.9	5.8
_30	61.4	0.5	179.2	5.3	76.5	3.5	30.4	4.9	**_30**	133.3	0.7	206.8	13.6	46.8	1.3	36.2	4.0
_60	58.5	0.8	179.6	6.9	75.5	5.1	37.4	7.0	**_60**	136.0	0.6	205.6	9.6	46.2	2.6	36.0	2.8
_90	64.2	0.4	183.5	3.4	73.0	5.2	38.3	5.9	**_90**	133.5	0.8	210.5	6.3	45.7	1.1	32.6	3.0
GM2	44.3	0.7	240.7	2.2	83.4	0.5	47.1	2.2	**KM2**	138.0	1.3	237.2	32.2	48.7	4.3	42.5	1.4
_30	44.5	0.3	244.4	5.6	73.4	3.9	37.8	3.2	**_30**	141.3	0.8	261.8	8.1	53.2	4.6	51.1	2.4
_60	45.1	0.5	245.2	7.1	74.3	6.9	41.0	6.7	**_60**	141.7	1.4	267.5	2.4	50.1	4.1	49.6	1.6
_90	49.5	0.5	231.4	3.8	75.0	2.4	48.8	4.6	**_90**	152.7	1.1	273.3	10.3	51.9	1.6	50.4	3.5
GM3	43.4	0.4	192.5	9.8	70.4	3.4	34.1	4.4	**KM3**	78.5	0.8	201.7	6.6	70.6	2.6	44.9	2.0
_30	49.2	0.3	188.4	3.7	70.4	1.6	34.4	2.7	**_30**	77.4	0.2	198.6	3.1	69.1	1.6	44.4	3.0
_60	49.9	0.3	184.7	3.3	67.6	2.7	35.0	0.3	**_60**	88.8	1.0	199.5	5.2	72.0	2.0	46.1	5.9
_90	47.3	0.0	185.5	2.7	69.2	3.0	32.3	2.1	**_90**	82.6	0.6	194.8	2.2	70.2	3.4	45.1	4.4
RGM4	82.7	0.4	242.3	5.2	59.8	0.8	43.7	2.7	**RKM4**	61.6	0.5	261.8	4.7	71.8	2.0	44.1	2.7
_30	80.7	0.8	249.1	3.5	59.5	1.8	39.6	2.3	**_30**	59.9	0.5	258.1	3.9	69.2	1.8	46.6	2.9
_60	85.0	0.6	248.7	1.7	56.9	4.2	43.7	5.0	**_60**	55.6	1.2	253.0	3.7	72.9	8.7	44.3	1.7
_90	89.1	0.3	245.8	5.2	57.9	2.6	44.8	1.9	**_90**	62.9	0.8	256.5	2.9	68.0	3.2	45.0	2.1
RGM5	100.3	0.9	211.2	9.4	69.8	1.4	41.8	2.3	**RKM5**	95.4	0.3	259.3	9.8	71.3	1.2	50.9	2.5
_30	99.4	1.1	214.0	5.1	66.7	2.3	41.9	4.3	**_30**	95.8	0.7	254.6	3.9	67.0	1.1	47.5	2.5
_60	100.4	0.8	216.5	3.2	66.9	1.3	45.7	1.4	**_60**	92.8	0.9	252.4	9.7	64.8	2.8	54.7	8.0
_90	96.7	0.8	217.1	5.2	66.4	4.1	42.4	3.2	**_90**	95.6	0.3	254.4	6.8	67.5	1.4	46.4	1.2
RGM6	52.9	1.0	221.4	4.1	74.3	3.4	37.7	1.0	**RKM6**	44.8	0.6	234.1	10.9	77.9	4.3	52.0	4.8
_30	49.4	0.7	211.8	1.4	74.6	0.9	31.3	0.6	**_30**	49.2	0.6	231.2	10.9	73.1	3.6	45.6	0.4
_60	56.1	0.6	212.4	4.0	73.2	1.9	36.1	2.0	**_60**	45.5	0.8	236.6	11.4	74.1	2.0	43.1	1.7
_90	50.4	1.1	210.1	3.5	73.4	1.0	39.1	1.1	**_90**	46.8	0.6	238.6	8.4	74.5	2.6	42.7	2.5

Selective quantification of isohumulones in fresh and aged (30°C) beers. Results expressed in mg/L.

Beers	t-ich	c-ich	t-ih	c-ih	t-iah	c-iah	total	Beers	t-ich	c-ich	t-ih	c-ih	t-iah	c-iah	total
GM1	1.4	3.9	1.2	4.7	0.4	1.2	12.8	**KM1**	1.9	4.6	2.2	6.8	0.7	1.7	17.8
_30	1.2	3.9	1.1	5.0	0.4	1.3	13.0	**_30**	1.6	4.5	1.7	6.3	0.6	1.6	16.3
_60	1.0	3.9	1.0	5.4	0.4	1.4	13.2	**_60**	1.5	4.7	1.6	6.5	0.5	1.7	16.5
_90	0.8	3.9	0.9	5.4	0.4	1.4	12.8	**_90**	1.3	4.7	1.4	6.6	0.5	1.8	16.3
GM2	2.5	6.2	3.4	9.7	1.0	2.4	25.3	**KM2**	2.4	5.2	2.5	7.5	0.7	1.8	20.0
_30	2.0	5.7	2.4	8.4	0.9	2.2	21.5	**_30**	1.9	5.0	2.0	7.1	0.6	1.8	18.3
_60	1.7	5.8	2.0	8.5	0.9	2.2	21.0	**_60**	1.6	5.1	1.9	7.1	0.5	1.8	18.1
_90	1.4	5.5	1.5	7.6	0.7	2.0	18.7	**_90**	1.2	5.1	1.6	6.9	0.4	1.8	17.2
GM3	1.8	4.8	1.7	5.7	0.6	1.5	16.1	**KM3**	1.3	3.5	1.0	3.8	0.4	1.0	10.9
_30	1.4	4.6	1.4	5.6	0.5	1.4	15.0	**_30**	1.3	4.1	1.2	5.2	0.4	1.3	13.7
_60	1.2	4.4	1.0	5.1	0.5	1.4	13.5	**_60**	1.1	4.1	1.0	5.2	0.4	1.3	13.1
_90	1.0	4.5	1.0	5.7	0.4	1.5	14.1	**_90**	1.0	4.1	0.9	5.0	0.3	1.3	12.6
RGM4	1.5	3.8	1.5	5.1	0.6	1.3	13.8	**RKM4**	1.8	4.2	1.6	5.5	0.6	1.4	15.0
_30	1.6	4.2	1.5	5.6	0.4	1.4	14.6	**_30**	1.6	5.0	1.9	7.3	0.6	1.9	18.2
_60	1.4	4.3	1.3	5.5	0.4	1.4	14.2	**_60**	1.4	4.8	1.5	6.6	0.6	1.7	16.6
_90	1.1	4.2	1.0	5.3	0.3	1.3	13.2	**_90**	1.3	4.9	1.4	7.1	0.5	1.9	17.1
RGM5	1.6	3.8	1.1	3.6	0.5	1.1	11.6	**RKM5**	1.7	4.0	1.2	4.3	0.5	1.4	13.1
_30	1.4	4.2	1.0	3.9	0.4	1.2	12.1	**_30**	1.6	4.7	1.3	4.8	0.5	1.5	14.4
_60	1.2	4.1	0.8	3.9	0.4	1.2	11.6	**_60**	1.3	4.7	1.1	5.1	0.5	1.6	14.5
_90	0.9	4.0	0.7	3.7	0.3	1.2	10.9	**_90**	1.2	4.6	0.9	4.9	0.4	1.5	13.6
RGM6	1.6	4.1	1.0	3.6	0.4	1.1	11.8	**RKM6**	1.6	4.2	1.1	4.2	0.5	1.3	13.0
_30	1.4	4.6	0.9	4.0	0.4	1.2	12.3	**_30**	1.3	4.4	0.9	4.2	0.5	1.3	12.6
_60	1.1	4.5	0.7	3.8	0.3	1.2	11.7	**_60**	1.1	4.6	0.9	4.8	0.5	1.5	13.5
_90	0.9	4.4	0.5	3.7	0.3	1.2	11.0	**_90**	1.0	4.5	0.7	4.4	0.5	1.4	12.5

Data represent the average of 3 replicate measurements; t- ich: *trans*-isocohumulone; c-ich: *cis*-isocohumulone; t-ih: *trans*-isohumulone; c-ih: *cis*-isohumulone; t-iah: *trans*-isoadhumulone; c-iah: *cis*-isoadhumulone

Aldehyde concentrations in fresh and aged beers; FT = flavour thresholds according to Meilgaard (Meilgaard, 1975a). Results in µg/L.

GM	2-MP	2-MB	3-MB	HEX	FUR	MET	PHE	T2N	Sum	KM	2-MP	2-MB	3-MB	HEX	FUR	MET	PHE	T2N	Sum
FT	1000	1250	600	350	150000	250	1600	0.11	-	FT	1000	1250	600	350	150000	250	1600	0.11	-
GM1	6.0	0.6	5.1	0.2	14.2	0.6	6.0	0.039	32.7	**KM1**	9.0	1.3	6.6	0.3	10.4	1.0	5.6	0.041	34.2
_30	54.1	0.8	6.2	0.4	156.6	0.8	6.9	0.043	225.9	**_30**	75.9	2.0	8.5	0.4	94.1	2.1	7.3	0.044	190.3
_60	50.8	1.5	5.5	0.2	127.7	0.9	7.3	0.042	194.0	**_60**	124.0	2.4	8.4	0.4	182.0	2.2	7.3	0.050	326.7
_90	37.7	2.1	7.7	0.2	99.4	1.3	9.0	0.045	157.6	**_90**	145.3	2.8	8.9	0.5	279.1	2.8	8.1	0.054	447.6
GM2	8.2	0.5	8.9	0.2	4.9	0.1	1.7	0.032	24.6	**KM2**	13.7	1.1	7.5	0.3	6.9	2.3	4.2	0.042	35.9
_30	67.2	0.8	10.4	0.4	101.1	0.3	2.7	0.055	183.0	**_30**	139.2	1.8	10.5	0.4	76.2	3.7	5.6	0.044	237.4
_60	102.1	1.1	9.9	0.4	183.4	0.8	4.0	0.065	301.7	**_60**	215.1	2.5	11.8	0.4	144.8	4.1	7.6	0.045	386.4
_90	123.8	1.3	7.6	0.4	267.9	0.6	2.9	0.052	404.5	**_90**	253.6	3.1	10.5	0.4	268.7	3.7	7.6	0.051	547.8
GM3	6.3	0.5	5.7	0.2	11.5	0.6	6.9	0.030	31.8	**KM3**	5.3	0.9	6.1	0.3	17.4	0.9	6.9	0.042	37.9
_30	42.0	0.7	7.4	0.4	102.3	0.6	5.4	0.041	158.8	**_30**	42.0	1.4	8.0	0.4	240.6	1.3	7.9	0.053	301.5
_60	55.1	1.3	7.0	0.3	143.9	0.6	6.1	0.045	214.4	**_60**	65.6	1.8	8.3	0.5	440.9	1.4	8.8	0.072	527.5
_90	65.5	2.2	7.3	0.3	202.5	0.9	9.1	0.055	287.9	**_90**	84.3	2.1	6.9	0.5	557.7	1.4	8.7	0.066	661.6
RGM4	5.1	1.0	5.0	0.3	7.2	1.1	8.0	0.031	27.8	**RKM4**	3.0	0.9	4.3	0.2	11.0	1.0	5.5	0.023	26.0
_30	39.5	1.3	6.8	0.4	100.9	1.3	7.0	0.028	157.1	**_30**	16.9	1.4	5.9	0.3	209.3	2.5	8.4	0.043	244.8
_60	53.2	1.4	6.6	0.5	158.2	1.4	7.0	0.044	228.2	**_60**	23.9	1.6	6.4	0.4	308.8	2.5	8.4	0.065	352.1
_90	63.3	1.8	6.6	0.6	224.6	2.1	8.3	0.051	307.3	**_90**	27.3	2.0	6.3	0.5	457.2	2.8	8.2	0.078	504.3
RGM5	3.4	0.7	4.1	0.3	8.3	1.5	9.1	0.024	27.4	**RKM5**	3.2	0.8	4.2	0.2	11.7	1.2	7.5	0.020	28.9
_30	34.7	1.2	6.5	0.5	150.7	2.1	8.8	0.041	204.5	**_30**	30.9	1.5	6.4	0.3	243.3	2.4	8.8	0.043	293.7
_60	52.6	1.2	5.2	0.5	296.8	2.0	8.4	0.047	366.7	**_60**	44.7	2.0	5.9	0.4	494.7	2.5	8.5	0.045	558.8
_90	55.6	1.4	4.9	0.5	379.1	2.1	8.1	0.051	451.7	**_90**	54.8	2.5	7.5	0.5	640.8	2.8	8.6	0.058	717.5
RGM6	3.0	0.5	3.4	0.2	7.9	0.4	1.6	0.029	17.1	**RKM6**	2.4	0.6	3.2	0.2	12.4	0.4	1.4	0.033	20.6
_30	27.3	0.9	5.6	0.4	188.9	0.5	2.2	0.046	225.8	**_30**	13.7	0.9	3.8	0.3	231.5	0.5	2.7	0.049	253.3
_60	37.2	1.0	4.6	0.5	380.6	0.5	2.1	0.057	426.6	**_60**	20.6	1.3	4.4	0.3	454.2	0.5	2.9	0.063	484.4
_90	41.2	1.3	4.3	0.5	526.8	0.5	1.8	0.057	576.4	**_90**	24.1	1.6	3.8	0.3	579.6	0.6	3.0	0.067	613.1

Data represent the average of 2-3 replicate measurements.

The concentration of selected volatile compounds in finished beers (fresh and aged). Results in mg/L Data represent the average of 2-3 technical replicate (bottles) measurements. FT= Flavour threshold according to Meilgaard (Meilgaard, 1975a)

	EAc	IsoAA	IsoBA	Ebut	Ehex	Eoct	1Prop	IsoB	3M1B		EAc	IsoAA	IsoBA	Ebut	Ehex	Eoct	1Prop	IsoB	3M1B
FT	30	1.6	1.6	0.4	0.23	0.9	800	200	70		30	1.6	1.6	0.4	0.23	0.9	800	200	70
GM1	31.2	1.07	0.06	0.09	0.08	0.14	33.1	88.4	126.1	**KM1**	97.6	2.14	0.07	0.15	0.15	0.37	28.9	33.7	81.0
_30	30.3	0.96	0.05	0.07	0.08	0.13	32.2	86.2	123.2	**_30**	93.4	1.70	0.05	0.15	0.15	0.40	30.5	33.5	82.4
_60	30.9	0.84	0.05	0.08	0.07	0.12	33.4	87.2	123.4	**_60**	87.8	1.43	0.05	0.14	0.15	0.39	29.5	33.7	79.7
_90	31.7	0.73	0.05	0.02	0.07	0.10	33.9	89.7	122.8	**_90**	86.9	1.06	0.04	0.13	0.14	0.37	29.9	32.5	78.6
GM2	59.2	1.93	0.08	0.12	0.13	0.35	25.6	43.1	85.1	**KM2**	118.0	2.89	0.09	0.21	0.23	0.70	32.2	36.9	88.4
_30	58.5	2.02	0.09	0.14	0.14	0.39	25.3	43.0	86.1	**_30**	102.9	2.22	0.08	0.17	0.23	0.70	36.9	40.6	90.7
_60	53.2	1.23	0.06	0.10	0.09	0.24	25.6	40.7	81.8	**_60**	86.2	1.58	0.06	0.13	0.22	0.73	32.8	37.1	91.6
_90	52.0	1.41	0.07	0.12	0.12	0.33	25.0	41.4	84.4	**_90**	87.1	1.28	0.05	0.13	0.23	0.74	32.7	37.0	89.7
GM3	64.3	1.95	0.11	0.10	0.11	0.33	24.2	49.0	81.7	**KM3**	64.3	2.07	0.09	0.10	0.09	0.21	29.0	58.5	100.3
_30	60.6	1.60	0.10	0.10	0.10	0.29	25.3	48.6	82.6	**_30**	57.4	2.24	0.09	0.10	0.12	0.27	28.6	59.1	101.4
_60	61.0	1.49	0.10	0.10	0.10	0.31	25.0	48.2	80.2	**_60**	55.0	2.01	0.09	0.10	0.11	0.26	29.5	59.8	101.8
_90	50.4	1.37	0.09	0.09	0.11	0.28	23.2	47.9	83.0	**_90**	50.1	1.83	0.07	0.09	0.11	0.27	28.4	57.5	101.3
RGM4	86.3	3.50	0.11	0.13	0.15	0.42	30.7	46.4	99.1	**RKM4**	88.1	3.07	0.10	0.14	0.17	0.50	33.5	40.6	82.9
_30	89.1	3.02	0.09	0.12	0.13	0.37	32.1	47.5	100.4	**_30**	79.2	2.61	0.09	0.12	0.16	0.48	32.1	38.9	80.7
_60	88.8	2.84	0.09	0.13	0.13	0.37	32.5	48.0	99.8	**_60**	79.3	2.44	0.08	0.12	0.16	0.50	32.6	39.4	82.1
_90	97.3	2.28	0.08	0.04	0.11	0.31	31.5	47.7	99.4	**_90**	80.1	2.26	0.08	0.12	0.16	0.50	34.0	40.9	83.5
RGM5	60.8	3.12	0.11	0.12	0.15	0.42	34.6	57.7	108.6	**RKM5**	101.3	3.34	0.11	0.14	0.16	0.53	45.4	41.8	85.1
_30	75.6	2.63	0.11	0.12	0.12	0.32	37.5	61.4	111.0	**_30**	87.8	3.05	0.10	0.13	0.17	0.56	41.9	39.7	83.7
_60	69.7	2.80	0.11	0.14	0.15	0.42	39.0	62.5	111.9	**_60**	87.1	2.71	0.10	0.13	0.16	0.52	42.2	39.5	83.3
_90	59.0	2.41	0.09	0.12	0.13	0.35	36.1	60.4	113.9	**_90**	92.9	2.64	0.10	0.14	0.16	0.51	43.9	41.6	84.7
RGM6	72.5	3.44	0.16	0.14	0.16	0.48	39.9	60.2	103.4	**RKM6**	75.5	2.74	0.10	0.11	0.14	0.51	36.2	41.2	80.4
_30	70.4	3.03	0.14	0.14	0.15	0.43	39.8	58.5	100.2	**_30**	77.8	2.55	0.10	0.11	0.14	0.49	38.0	42.2	83.0
_60	72.1	2.57	0.13	0.14	0.13	0.40	42.4	60.2	100.1	**_60**	75.4	2.34	0.09	0.11	0.14	0.45	38.1	43.1	81.4
_90	74.7	2.51	0.13	0.14	0.14	0.37	41.6	60.4	100.5	**_90**	74.7	1.98	0.07	0.03	0.10	0.33	38.9	40.9	78.1

EAc.= ethyl acetate, IsoAA = isoamyl acetate, IsoBA = isobutyl acetate, Ebut = ethyl butyrate, Ehex = = ethyl hexanoate, Eoct = ethyl octanoate, 1Prop = 1-propanol, IsoB = isobutanol, 3M1B= 3-methyl-1-butanol

Appendix 4: Standard beer parameters

Standard analysis of beers. Data represent the average of all fresh and aged (30, 60 and 90 days, 30°C) beers. * = re-steeping trials

Brew Nr.		pH	Colour (EBC)	SG	density	Alc % (v/v)	PG	OG	P (%)	RDF (%)	p (g/100mL)
1	GM	4.1	4.8	1.0052	1.0034	4.1	5.2	49.0	12.1	73.2	12.7
	±SD	0.1	0.1	0.0000	0.0001	0.1	0.1	0.2	0.0	0.1	0.0
	KM	4.4	6.9	1.0084	1.0066	4.4	8.4	50.3	12.4	68.1	13.0
	±SD	0.0	0.2	0.0001	0.0001	0.0	0.1	0.5	0.1	0.0	0.1
2	GM	4.1	7.4	1.0077	1.0059	5.4	7.7	48.9	12.0	68.9	12.6
	±SD	0.0	1.3	0.0001	0.0001	0.0	0.1	0.1	0.0	0.1	0.0
	KM	4.5	11.0	1.0097	1.0079	5.6	9.7	52.5	12.9	66.7	13.5
	±SD	0.0	0.4	0.0001	0.0001	0.0	0.1	0.1	0.0	0.1	0.0
3	GM	4.1	5.0	1.0089	1.0070	5.0	8.8	47.3	11.7	66.4	12.2
	±SD	0.0	0.3	0.0001	0.0001	0.0	0.1	0.2	0.0	0.1	0.0
	KM	4.2	7.2	1.0065	1.0047	5.5	6.5	48.9	12.1	71.0	12.6
	±SD	0.0	0.3	0.0000	0.0000	0.0	0.0	0.1	0.0	0.0	0.0
4*	GM	4.3	6.1	1.0090	1.0072	5.8	9.0	53.6	13.2	68.2	13.8
	±SD	0.0	0.2	0.0004	0.0004	0.0	0.4	0.4	0.1	0.5	0.1
	KM	4.2	8.4	1.0085	1.0067	5.7	8.5	52.6	12.9	68.7	13.6
	±SD	0.0	0.3	0.0000	0.0000	0.0	0.0	0.0	0.0	0.0	0.0
5*	GM	4.2	5.5	1.0055	1.0037	5.9	5.5	50.6	12.5	73.0	13.1
	±SD	0.0	0.2	0.0000	0.0000	0.0	0.0	0.1	0.0	0.0	0.0
	KM	4.2	10.8	1.0085	1.0066	5.7	8.4	52.5	12.9	68.7	13.6
	±SD	0.0	0.4	0.0001	0.0000	0.0	0.0	0.1	0.0	0.0	0.0
6*	GM	4.2	6.0	1.0088	1.0070	5.6	8.8	51.9	12.8	68.0	13.4
	±SD	0.0	0.4	0.0001	0.0001	0.0	0.1	0.1	0.0	0.2	0.0
	KM	4.1	7.9	1.0087	1.0069	5.4	8.7	50.3	12.4	67.6	13.0
	±SD	0.0	0.6	0.0000	0.0000	0.0	0.0	0.1	0.0	0.0	0.0

7 References

Abrahamsson, V, Hoff, S, Nielsen, NJ, Lund, MN & Andersen, ML 2012. Determination of Sulfite in Beer Based on Fluorescent Derivatives and Liquid Chromatographic Separation. *Journal of the American Society of Brewing Chemists,* 70**,** 296-302. https://doi.org/10.1094/ASBCJ-2012-1012-01

Andersen, ML, Gundermann, M, Danielsen, BP & Lund, MN 2017. Kinetic Models for the Role of Protein Thiols during Oxidation in Beer. *Journal of Agricultural and Food Chemistry,* 65**,** 10820-10828. https://doi.org/10.1021/acs.jafc.7b05012

Andersen, ML, Outtrup, H & Skibsted, LH 2000. Potential Antioxidants in Beer Assessed by ESR Spin Trapping. *Journal of Agricultural and Food Chemistry,* 48**,** 3106-3111. https://doi.org/10.1021/jf000354+

Andersen, ML & Skibsted, LH 1998. Electron Spin Resonance Spin Trapping Identification of Radicals Formed during Aerobic Forced Aging of Beer. *Journal of Agricultural and Food Chemistry,* 46**,** 1272-1275. https://doi.org/10.1021/jf9708608

Andersen, ML & Skibsted, LH 2017. ESR Spectroscopy for the Study of Oxidative Processes in Food and Beverages. *In:* WEBB, G. A. (ed.) *Modern Magnetic Resonance.* Cham: Springer International Publishing, p. 1-14.

Anderson, RJ, Clapperton, JF, Crabb, D & Hudson, JR 1975. Dimethyl sulphide as a feature of lager flavour. *Journal of the Institute of Brewing,* 81**,** 208-213. https://doi.org/10.1002/j.2050-0416.1975.tb03679.x

Andrews, J 2004. A review of progress in mash separation technology. *Tech. Q. Master Brew. Assoc. Am.,* 41**,** 45-49.

Anness, BJ 1980. The reduction of dimethyl sulphoxide to dimethyl sulphide during fermentation. *Journal of the Institute of Brewing,* 86**,** 134-137. https://doi.org/10.1002/j.2050-0416.1980.tb03973.x

Anness, BJ 1981. The determination of dimethyl sulphoxide in aqueous solution. *Journal of the Science of Food and Agriculture,* 32**,** 353-358. https://doi.org/10.1002/jsfa.2740320407

Anness, BJ 1984. Lipids of barley, malt and adjuncts. *Journal of the Institute of Brewing,* 90**,** 315-318. https://doi.org/10.1002/j.2050-0416.1984.tb04282.x

Anness, BJ & Bamforth, CW 1982. Dimethyl sulphide - A review. *Journal of the Institute of Brewing,* 88**,** 244-252. https://doi.org/10.1002/j.2050-0416.1982.tb04101.x

Anness, BJ, Bamforth, CW & Wainwright, T 1979. The measurement of dimethyl sulphoxide in barley and malt and its reduction to dimethyl sulphide by yeast. *Journal of the Institute of Brewing,* 85**,** 346-349. https://doi.org/10.1002/j.2050-0416.1979.tb03940.x

Anness, BJ & Reed, RJR 1985. Lipids in the brewery - A material balance. *Journal of the Institute of Brewing,* 91**,** 82-87. https://doi.org/10.1002/j.2050-0416.1985.tb04310.x

Aquilani, B, Laureti, T, Poponi, S & Secondi, L 2015. Beer choice and consumption determinants when craft beers are tasted: An exploratory study of consumer preferences. *Food Quality and Preference,* 41**,** 214-224. https://doi.org/10.1016/j.foodqual.2014.12.005

Aron, PM & Shellhammer, TH 2017. Profiling of Hop-Derived Flavan-3-ols from Lager Beer in Relation to Hopping Technology. *Journal of the American Society of Brewing Chemists,* 75**,** 276-282. https://doi.org/10.1094/ASBCJ-2017-3149-01

Back, JF, Oakenfull, D & Smith, MB 1979. Increased thermal stability of proteins in the presence of sugars and polyols. *Biochemistry,* 18**,** 5191-5196. https://doi.org/10.1021/bi00590a025

Baert, J. 2015. *Unravelling the role of free and bound-state aldehydes in beer flavour instability.* PhD, KU Leuven.

Baert, JJ, De Clippeleer, J, Bustillo Trueba, P, Jaskula-Goiris, B, De Rouck, G, Aerts, G & De Cooman, L 2018. Exploring Aldehyde Release in Beer by 4-Vinylpyridine and the Effect of Cysteine Addition on the Beer's Pool of Bound Aldehydes. *Journal of the American Society of Brewing Chemists,* 76**,** 257-271. https://doi.org/10.1080/03610470.2018.1518639

Baert, JJ, De Clippeleer, J, De Cooman, L & Aerts, G 2015a. Exploring the Binding Behavior of Beer Staling Aldehydes in Model Systems. *Journal of the American Society of Brewing Chemists,* 73**,** 100-108. https://doi.org/10.1094/ASBCJ-2015-0109-01

Baert, JJ, De Clippeleer, J, Hughes, PS, De Cooman, L & Aerts, G 2012. On the origin of free and bound staling aldehydes in beer. *Journal of Agricultural and Food Chemistry,* 60**,** 11449-72. https://doi.org/10.1021/jf303670z

Baert, JJ, De Clippeleer, J, Jaskula-Goiris, B, Van Opstaele, F, De Rouck, G, Aerts, G & De Cooman, L 2015b. Further Elucidation of Beer Flavor Instability: The Potential Role of Cysteine-Bound Aldehydes. *Journal of the American Society of Brewing Chemists,* 73**,** 243-252. https://doi.org/10.1094/ASBCJ-2015-0531-01

Baldus, M, Kunz, T & Methner, F-J 2013. The analysis of dimethyl sulfoxide in malt, wort and beer using headspace gas chromatography combined with pulsed flame photometric detection (HS-GC-PFPD) - Methodology and applications. *BrewingScience,* 66**,** 154-161.

Bamforth, C 1999a. The science and understanding of the flavour stability of beer: A critical assessment. *Brauwelt International,* 2**,** 98-110.

Bamforth, C 2004. A critical control point analysis for flavor stability of beer. *Tech. Q. Master Brew. Assoc. Am.,* 41**,** 97-103.

Bamforth, CW 1999b. Beer Haze. *Journal of the American Society of Brewing Chemists,* 57**,** 81-90. https://doi.org/10.1094/ASBCJ-57-0081

Bamforth, CW 1999c. Enzymic and Non-Enzymic Oxidation in the Brewhouse: A Theoretical Consideration. *Journal of the Institute of Brewing,* 105**,** 237-242. https://doi.org/10.1002/j.2050-0416.1999.tb00025.x

Bamforth, CW 2009. Current perspectives on the role of enzymes in brewing. *Journal of Cereal Science,* 50**,** 353-357. https://doi.org/10.1016/j.jcs.2009.03.001

Bamforth, CW 2013. *Flavor: Practical Guides for Beer Quality,* St Paul MN, American Society of Brewing Chemists.

Bamforth, CW 2014. Dimethyl Sulfide – Significance, Origins, and Control. *Journal of the American Society of Brewing Chemists,* 72**,** 165-168. https://doi.org/10.1094/asbcj-2014-0610-01

Bamforth, CW & Lentini, A 2009. The flavor instability of beer. *Beer: A Quality Perspective.* p. 85-109.

Bamforth, CW & Martin, HL 1981. Beta-glucan and beta-glucan solubilase in malting and mashing. *Journal of the Institute of Brewing. Institute of Brewing (Great Britain),* 87**,** 365-371. https://doi.org/10.1002/j.2050-0416.1981.tb04052.x

Bamforth, CW & Martin, HL 1983. The degradation of β-glucan during malting and mashing: the role of β-glucanase. *Journal of the Institute of Brewing,* 89**,** 303-307. https://doi.org/10.1002/j.2050-0416.1983.tb04190.x

Bamforth, CW, Roza, JR & Kanauchi, M 2009. Storage of Malt, Thiol Oxidase, and Brewhouse Performance. *Journal of the American Society of Brewing Chemists,* 67**,** 89-94. https://doi.org/10.1094/ASBCJ-2009-0219-01

Bamforth, CW & Simpson, WJ 1995. Ionic equilibria in brewing. *Brew Guard,* 124**,** 18-24.

Barber, MG, Jackson, EA & Smith, DB 1994. Total and individual barley (1–3), (1–4)-β-D-glucanase activities in some green and kilned malts. *Journal of the Institute of Brewing,* 100**,** 91-97. https://doi.org/10.1002/j.2050-0416.1994.tb00812.x

Bate-Smith, EC 1973. Haemanalysis of tannins: The concept of relative astringency. *Phytochemistry,* 12**,** 907-912. https://doi.org/10.1016/0031-9422(73)80701-0/

Baxter, ED 1982. Lipoxidases in Malting and Mashing. *Journal of the Institute of Brewing,* 88**,** 390-396. https://doi.org/10.1002/j.2050-0416.1982.tb04130.x

Baxter, ED 1984. Recognition of two lipases from barley and green malt. *Journal of the Institute of Brewing,* 90**,** 277-281. https://doi.org/10.1002/j.2050-0416.1984.tb04273.x

Beal, AD & Mottram, DS 1994. Compounds contributing to the characteristic aroma of malted barley. *Journal of Agricultural and Food Chemistry,* 42**,** 2880-2884. https://doi.org/10.1021/jf00048a043

Belitz, HD 2009. *Food chemistry,* Berlin, Heidelberg, Springer Verlag.

Benthin, S & Villadsen, J 1995. Different inhibition of Lactobacillus delbrueckii subsp. bulgaricus by D- and L-lactic acid: effects on lag phase, growth rate and cell yield. *Journal of Applied Bacteriology,* 78**,** 647-654. https://doi.org/10.1111/j.1365-2672.1995.tb03111.x

Bettenhausen, HM, Barr, L, Broeckling, CD, Chaparro, JM, Holbrook, C, Sedin, D & Heuberger, AL 2018. Influence of malt source on beer chemistry, flavor, and flavor stability. *Food Research International,* 113**,** 487-504. https://doi.org/10.1016/j.foodres.2018.07.024

Bokulich, NA & Bamforth, CW 2013. The Microbiology of Malting and Brewing. *Microbiology and Molecular Biology Reviews,* 77**,** 157-172. https://doi.org/10.1128/mmbr.00060-12

Bonnely, S, Peyrat-Maillard, MN, Rondini, L, Masy, D & Berset, C 2000. Antioxidant activity of malt rootlet extracts. *Journal of Agricultural and Food Chemistry,* 48**,** 2785-92. https://doi.org/10.1021/jf990793c

Boudnitskaya, EV & Borisova, IG 1972. Investigation of lipoxygenase functions in chloroplasts and mitochondria from Pisum sativum seedlings. *FEBS Letters,* 24**,** 359-362.

Boulton, C & Quain, D 2006. The Biochemistry of Fermentation. *Brewing Yeast and Fermentation***,** 69-142. https://doi.org/10.1002/9780470999417.ch3

Braidot, E, Petrussa, E, Micolini, S, Tubaro, F, Vianello, A & Macrì, F 2004. Biochemical and immunochemical evidences for the presence of lipoxygenase in plant mitochondria. *Journal of Experimental Botany,* 55**,** 1655-1662. https://doi.org/10.1093/jxb/erh197

Brewers of Europe. 2018. *Exports boost Europe's beer production to eight-year high* [Online]. Available: https://brewersofeurope.org/site/media-centre/post.php?doc_id=970 [Accessed December 2019].

Briggs, DE 1978. Weeds, pests and diseases in the growing crop. *In:* BRIGGS, D. E. (ed.) *Barley.* Dordrecht, Netherlands: Springer, p. 339-368.

Briggs, DE 1986. Accelerating Malting: A Review of Some Lessons of the Past from the United Kingdom. *Journal of the American Society of Brewing Chemists,* 45**,** 1-6.

Briggs, DE 1998a. The biochemistry of malting. *Malts and Malting.* London, UK: Blackie Academic & Professional, p. 163, 192.

Briggs, DE 1998b. Grain Physiology. *Malts and Malting.* London, UK: Blackie Academic & Professional, p. 81.

Briggs, DE 1998c. Grains and Pulses. *Malts and Malting.* London, UK: Blackie Academic & Professional, p. 43-47.

Briggs, DE & McGuinness, G 1993. Microbes on barley grains. *Journal of the Institute of Brewing,* 99**,** 249-255. https://doi.org/10.1002/j.2050-0416.1993.tb01168.x

Briggs, DE, Stevens, R, Young, TW & Hough, JS 1981a. *Malting and Brewing Science: Malt and Sweet Wort*, Springer US.

Briggs, DE, Stevens, R, Young, TW & Hough, JS 1981b. *Malting and Brewing Science: Malt and Sweet Wort,* London, Chapman & Hall.

Briggs, DE & Woods, J 1993. Dormancy in malting barley: studies on drying, storage, biochemistry and physiology. *HGCA Project Report (United Kingdom)*.

Brookes, PA, Lovett, DA & Macwilliam, IC 1976. The steeping of barley. A review of the metabolic consequences of water uptake, and their practical implications. *Journal of the Institute of Brewing,* 82**,** 14-26. https://doi.org/10.1002/j.2050-0416.1976.tb03716.x

Brudzynski, A & Roginski, H 1969. Comparative studies of kilned and freeze-dried malts. *Journal of the Institute of Brewing,* 75**,** 472-476. https://doi.org/10.1002/j.2050-0416.1969.tb06385.x

Buchs, M & Sylke, S 2013. UK Households' Carbon Footprint: A Comparison of the Association between Household Characteristics and Emissions from Home Energy, Transport and Other Goods and Services. IZA Discussion Paper No. 7204.

Bustillo Trueba, P, De Clippeleer, J, Van der Eycken, E, Guevara Romero, JS, De Rouck, G, Aerts, G & De Cooman, L 2018. Influence of pH on the Stability of 2-Substituted 1,3-Thiazolidine-4-Carboxylic Acids in Model Solutions. *Journal of the American Society of Brewing Chemists,* 76**,** 272-280. https://doi.org/10.1080/03610470.2018.1546094

Bustillo Trueba, P, Jaskula-Goiris, B, De Clippeleer, J, Goiris, K, Praet, T, Sharma, UK, Van der Eycken, E, Sanders, MG, Vincken, JP, De Brabanter, J, De Rouck, G, Aerts, G & De Cooman, L 2019. Validation of an ultra-high-performance liquid chromatography-mass spectrometry method for the quantification of cysteinylated aldehydes and application to malt and beer samples. *Journal of Chromatography A,* 1604**,** 460467. https://doi.org/10.1016/j.chroma.2019.460467

Callemien, D & Collin, S 2007. Involvement of flavanoids in beer color instability during storage. *Journal of agricultural and food chemistry,* 55**,** 9066. https://doi.org/10.1021/jf0716230

Caplice, E & Fitzgerald, GF 1999. Food fermentations: role of microorganisms in food production and preservation. *International Journal of Food Microbiology,* 50**,** 131-49. https://doi.org/10.1016/S0168-1605(99)00082-3

CarbonTrust 2011. Industrial Energy Efficiency Accelerator - Guide to the maltings sector. *Malting Sector Guide,* CTG053.

Chandra, C, Buggey, L, Peters, S, Cann, C & Liegeois, C 2001. Factors affecting the development of antioxidant properties of malts during the malting and roasting process. *HGCA PROJECT REPORT*.

Chu, FL & Yaylayan, VA 2008. Model Studies on the Oxygen-Induced Formation of Benzaldehyde from Phenylacetaldehyde Using Pyrolysis GC-MS and FTIR. *Journal of Agricultural and Food Chemistry,* 56**,** 10697-10704. https://doi.org/10.1021/jf8022468

Coghe, S, Gheeraert, B, Michiels, A & Delvaux, FR 2006. Development of maillard reaction related characteristics during malt roasting. *Journal of the Institute of Brewing,* 112**,** 148-156. https://doi.org/10.1002/j.2050-0416.2006.tb00244.x

Coghe, S, Martens, E, D'Hollander, H, Dirinck, PJ & Delvaux, FR 2004. Sensory and Instrumental Flavour Analysis of Wort Brewed with Dark Specialty Malts. *Journal of the Institute of Brewing,* 110**,** 94-103. https://doi.org/10.1002/j.2050-0416.2004.tb00188.x

Cohen, D & Paleg, LG 1967. Physiological effects of gibberellic Acid. X. The release of gibberellin-like substances by germinating barley embryos. *Plant physiology,* 42**,** 1288-1296. https://doi.org/10.1104/pp.42.9.1288

Conway, J. 2019. *Beer production worldwide from 1998 to 2018* [Online]. Available: https://www.statista.com/statistics/270275/worldwide-beer-production/ [Accessed July 2020].

Cook, AH & Hudson, JR 1964. New Possibilities in Grists for Brewing. *Proceedings. Annual meeting - American Society of Brewing Chemists,* 22**,** 29-33. https://doi.org/10.1080/00960845.1964.12006733

Cortés, N, Kunz, T, Methner, F-J, Suárez, AF & Hughes, P 2010. Development and correlation between the organic radical concentration in different malt types and oxidative beer stability. *Journal of the American Society of Brewing Chemists,* 68**,** 107-113. https://doi.org/10.1094/ASBCJ-2010-0412-01

Curtis, NS 1966. Head and haze - A review. *Journal of the Institute of Brewing,* 72**,** 240-250. https://doi.org/10.1002/j.2050-0416.1966.tb02958.x

Dalgliesh, CE 1977. Flavour Stability. *Proc. Eur. Brew. Conv. Cong.,* 16**,** 623-659.

Davies, N 2010. Cutting the Carbon Footprint of Malting by 75%--Achievable or Just Hot Air? *Tech. Q. Master Brew. Assoc. Am.,* 47**,** 23-28. https://doi.org/10.1094/tq-47-4-0902-01

De-Jing, M, Jian, L, Wei, F, Jian-Jun, D, Jianhua, Z, Wei-Bao, K, Yan, L & Lian-Ju, S 2009. Optimization of extraction conditions for antioxidant phenolic compounds from malt rootlets using response surface methodology. *Journal of Food Biochemistry,* 33**,** 291-305. https://doi.org/10.1111/j.1745-4514.2009.00218.x

De Buck, A, Aerts, G, Bonte, S & Dupire, S. Rapid evaluation of the two lipoxygenase isoenzyme activities in malt. Proc. Eur. Brew. Conv. Cong., 1995 Brussels. IRL Press, Oxford, 209-216.

De Buck, A, Aerts, G, Bonte, S, Dupire, S & Van den Eynde, E. Relation between lipoxygenase extraction during brewing, reducing capacity of the wort and the organoleptical stability of beer. Proc. Eur. Brew. Conv. Cong., 1997 Maastricht. IRL Press, Oxford, 333-340.

De Clippeleer, J. 2013. *Flavour stability of pale lager beer. Chemical-analytical characterisation of critical factors related to wort production and hopping.* PhD, KU leuven.

De Clippeleer, J, De Rouck, G, De Cooman, L & Aerts, G 2010a. Influence of the hopping technology on the storage-induced appearance of staling aldehydes in beer. *Journal of*

the Institute of Brewing, 116**,** 381-398. https://doi.org/10.1002/j.2050-0416.2010.tb00789.x

De Clippeleer, J, Van Opstaele, F, De Cooman, L & Aerts, G. The contribution of aldehyed to the staling taste and odour of pale large beer. Proceedings of the Congress of the European Brewery Convention, 2011 Glasgow, UK.

De Clippeleer, J, Van Opstaele, F, Vercammen, J, Francis, GJ, De Cooman, L & Aerts, G 2010b. Real-time profiling of volatile malt aldehydes using selected ion flow tube mass spectrometry. *LC GC North America,* 28**,** 386-395.

De Cooman, L, Aerts, G, Overmeire, H & De Keukeleire, D 2000. Alterations of the Profiles of Iso-α-Acids During Beer Ageing, Marked Instability of Trans-Iso-α-Acids and Implications for Beer Bitterness Consistency in Relation to Tetrahydroiso-α-Acids. *Journal of the Institute of Brewing,* 106**,** 169-178. https://doi.org/10.1002/j.2050-0416.2000.tb00054.x

De Rouck, G, De Clippeleer, J, Poiz, S, De Cock, J, van Waesberghe, J, De Cooman, L & Aerts, G 2007. Prolonged flavour stability by production of beer with low residual FAN using active dry yeast. *Proceedings of the 31st Congress of the European Brewery Convention*.

De Rouck, G, Flores-González, AG, Clippeleer, J, De Cock, J, De Cooman, L & Aerts, G 2010. Sufficient formation and removal of dimethyl sulfide (DMS) without classic wort boiling. *BrewingScience,* 63**,** 31-40.

De Rouck, G, Jaskula-Goiris, B, De Causmaecker, B, Opstaele, F, Clippeleer, J, Cooman, L & Aerts, G 2013a. The impact of wort production on the flavour quality and stability of pale lager beer. *BrewingScience,* 66**,** 1-11.

De Rouck, G, Jaskula, B, De Causmaecker, B, Malfliet, S, Van Opstaele, F, De Clippeleer, J, De Brabanter, J, De Cooman, L & Aerts, G 2013b. The influence of very thick and fast mashing conditions on wort composition. *Journal of the American Society of Brewing Chemists,* 71**,** 1-14. https://doi.org/10.1094/ASBCJ-2013-0113-01

De Schutter, D. 2008. *The influence of thermal load during wort boiling on the flavour stability of beer.* Leuven : K.U.Leuven. Faculteit Bio-ingenieurswetenschappen.

De Schutter, DP, Saison, D, Delvaux, F, Derdelinckx, G, Rock, JM, Neven, H & Delvaux, FR 2008. Release and evaporation of volatiles during boiling of unhopped wort. *Journal of Agricultural and Food Chemistry,* 56**,** 5172-80. https://doi.org/10.1021/jf800610x

Debourg, A, Laurent, M, Goossens, E, Borremans, E, Van De Winkel, L & Masschelein, CA 1994. Wort Aldehyde Reduction Potential in Free and Immobilized Yeast Systems. *Journal of the American Society of Brewing Chemists,* 52**,** 100-106. https://doi.org/10.1094/ASBCJ-52-0100

Dickenson, CJ 1979. The relationship of dimethyl sulfide levels in malt, wort and beer. *Journal of the Institute of Brewing,* 85**,** 235-239. https://doi.org/10.1002/j.2050-0416.1979.tb03914.x

Ditrych, M, Filipowska, W, De Rouck, G, Jaskula-Goiris, B, Aerts, G, Andersen, M & De Cooman, L 2019. Investigating the evolution of free staling aldehydes throughout the wort production process. *BrewingScience,* 72**,** 10-17. https://doi.org/10.23763/BrSc18-21ditrych

Djekic, I, Tomic, N, Bourdoux, S, Spilimbergo, S, Smigic, N, Udovicki, B, Hofland, G, Devlieghere, F & Rajkovic, A 2018. Comparison of three types of drying (supercritical CO2, air and

freeze) on the quality of dried apple – Quality index approach. *LWT - Food Science and Technology,* 94**,** 64-72. https://doi.org/10.1016/j.lwt.2018.04.029

Doderer, A, Kokkelink, I, van der Veen, S, Valk, BE, Schram, AW & Douma, AC 1992. Purification and characterization of two lipoxygenase isoenzymes from germinating barley. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology,* 1120**,** 97-104. https://doi.org/10.1016/0167-4838(92)90429-H

Dong, L, Piao, Y, Zhang, X, Zhao, C, Hou, Y & Shi, Z 2013. Analysis of volatile compounds from a malting process using headspace solid-phase micro-extraction and GC–MS. *Food Research International,* 51**,** 783-789. https://doi.org/10.1016/j.foodres.2013.01.052

Doug, S 2010. Emissions, energy, water and malt. The carbon conundrum in the maltings. *Brewer & Distiller International***,** 38-41.

Douglas, PE & Flannigan, B 1988. A Microbiological Evaluation of Barley Malt Production. *Journal of the Institute of Brewing,* 94**,** 85-88. https://doi.org/10.1002/j.2050-0416.1988.tb04562.x

Drost, BW, van den Berg, R, Freijee, FJM, van der Velde, EG & Hollemans, M 1990. Flavor stability. *Journal of the American Society of Brewing Chemists,* 48**,** 124 - 131. https://doi.org/10.1094/ASBCJ-48-0124

Duff, SR 1963. Use of green malt in brewing. *Journal of the Institute of Brewing,* 69**,** 249-252. https://doi.org/10.1002/j.2050-0416.1963.tb01925.x

Dufour, J-P, Leus, M, Baxter, AJ & Hayman, AR 1999. Characterization of the reaction of bisulfite with unsaturated aldehydes in a beer model system using nuclear magnetic resonance spectroscopy. *Journal of the American Society of Brewing Chemists,* 57**,** 138-144. https://doi.org/10.1094/ASBCJ-57-0138

Edward, CL, Baker, CW & Don, CZ 1981. Metabolism of Linoleic Acid by Barley Lipoxygenase and Hydroperoxide Isomerase. *Plant Physiology,* 68**,** 950-955.

EUREKA SWAN Project. 2006. *EUREKA SWAN - Reduction of water use in the EU Malting Industry* [Online]. Available: http://sciencesearch.defra.gov.uk/Default.aspx?Menu=Menu&Module=More&Location=None&Completed=0&ProjectID=12449 [Accessed].

Euromalt. 2017. *Malt Production Capacity from 2012 to 2017* [Online]. Available: http://www.euromalt.be/list_infos/euromalt%20statistics/1011306087/list1353668317.html [Accessed August 2020].

Evans, D, Wallace, W, Lance, R & McLeod, LC 1997. Measurement of beta-amylase in malting barley (Hordeum vulgare L.). Part 2. The effect of germination and kilning. *Journal of Cereal Science,* 26**,** 241-250. https://doi.org/10.1006/jcrs.1997.0120

Evans, DE, Li, C & Eglinton, JK 2008. Improved Prediction of Malt Fermentability by Measurement of the Diastatic Power Enzymes β-Amylase, α-Amylase, and Limit Dextrinase: I. Survey of the Levels of Diastatic Power Enzymes in Commercial Malts. *Journal of the American Society of Brewing Chemists,* 66**,** 223-232. https://doi.org/10.1094/ASBCJ-2008-0909-02

Evans, DE, Stenholm, K, Vilpola, A, Home, S & Hughes, G 1998. Pilot scale evaluation of mash filter performance of malts with variable quality. *Tech. Q. Master Brew. Assoc. Am.,* 35**,** 189-195.

Evers, T & Millar, S 2002. Cereal Grain Structure and Development: Some Implications for Quality. *Journal of Cereal Science,* 36**,** 261-284. https://doi.org/10.1006/jcrs.2002.0435

Fenzl, V & Schönberger, ØL 2018. Improved Method for Measuring Lipoxygenase Activity in Barley and Malt. *Journal of the American Society of Brewing Chemists,* 76**,** 24-28. https://doi.org/10.1080/03610470.2017.1402583

Ferrari-John, RS, Katrib, J, Zerva, E, Davies, N, Cook, DJ, Dodds, C & Kingman, S 2017. Electromagnetic Heating for Industrial Kilning of Malt: a Feasibility Study. *Food and Bioprocess Technology,* 10**,** 687-698. https://doi.org/10.1007/s11947-016-1849-0

Filipowska, W, Jaskula-Goiris, B, Ditrych, M, Schlich, J, De Rouck, G, Aerts, G & De Cooman, L 2020. Determination of optimal sample preparation for aldehyde extraction from pale malts and their quantification via headspace solid-phase microextraction followed by gas chromatography and mass spectrometry. *Journal of Chromatography A,* 1612**,** 460647. https://doi.org/10.1016/j.chroma.2019.460647

Fincher, GB 1975. Morphology and chemical composition of barley endosperm cell walls. Journal of the Institute of Brewing, 81: 116-122. https://doi.org/10.1002/j.2050-0416.1975.tb03672.x

Flannigan, B 1996. The microflora of barley and malt. *In:* PRIEST, F. G. & CAMPBELL, I. (eds.) *Brewing Microbiology.* Boston, MA: Springer US, p. 83-125.

Flannigan, B, Okagbue, RN, Khalid, R & Teoh, CK 1982. Mould flora of malt in production and storage. *Brewing & Distiller International,* 31.

Fornaroli, S, Petrussa, E, Braidot, E, Vianello, A & Macrì, F 1999. Purification of a plasma membrane-bound lipoxygenase from soybean cotyledons. *Plant Science,* 145**,** 1-10. https://doi.org/10.1016/S0168-9452(99)00066-7

Franke, W & Frehse, H 1953. Zur Autoxydation der ungesättigten Fettsäuren. VI. Über die Lipoxydase der Gramineen, im besonderen der Gerste. *Hoppe-Seyler´s Zeitschrift für physiologische Chemie.*

Fratianni, AJ 2001. Results of the effect of storage temperature on packaged beer flavor. *Tech. Q. Master Brew. Assoc. Am.,* 38**,** 159-161.

Furukawa Suárez, A, Kunz, T, Cortes Rodriguez, N, Mackinlay, J, Hughes, PS & Methner, F-J 2011. Impact of colour adjustment on flavour stability of pale lager beers with a range of distinct colouring agents. *Food Chemistry,* 125**,** 850-859. https://doi.org/10.1016/j.foodchem.2010.08.070

Garbe, LA, Barbosa de Almeida, R, Nagel, R, Wackerbauer, K & Tressl, R 2006. Dual positional and stereospecificity of lipoxygenase isoenzymes from germinating barley (green malt): biotransformation of free and esterified linoleic acid. *Journal of Agricultural and Food Chemistry,* 54**,** 946-55. https://doi.org/10.1021/jf051993t

Garshol, LM 2020. Malt. *Historical Brewing Techniques: The Lost Art of Farmhouse Brewing.* Boulder, Colorado, USA: Brewers Publications, p. 78.

Gastl, M, Spieleder, E, Hermann, M, Thiele, F, Burberg, F, Kogin, A, Ikeda, H, Back, W & Narziss, L 2006. The influence of malt quality and malting technology on the flavour stability of beer. *Monatsschrift für Brauwissenschaft,* 59**,** 163-175.

Ge, S-J & Lee, T-C 1997. Kinetic Significance of the Schiff Base Reversion in the Early-Stage Maillard Reaction of a Phenylalanine−Glucose Aqueous Model System. *Journal of Agricultural and Food Chemistry,* 45**,** 1619-1623. https://doi.org/10.1021/jf960458d

Greenwood, CT & MacGregor, AW 1965. The isolation of α-amylase from barley and malted barley, and a study of the properties and and action-patterns of the enzymes. *Journal*

of the Institute of Brewing, 71**,** 405-417. https://doi.org/10.1002/j.2050-0416.1965.tb06366.x

Griffin, OT, Collier, JA & Shields, PD 1968. The development of liquid malt. *Journal of the Institute of Brewing,* 74**,** 154-163. https://doi.org/10.1002/j.2050-0416.1968.tb03109.x

Guido, L, Boivin, P, Benismail, N, R. Gonçalves, C & A. Barros, A 2005. An early development of the nonenal potential in the malting process. *European Food Research and Technology,* 220**,** 200-206. https://doi.org/10.1007/s00217-004-1079-y

Guido, LF, Curto, AF, Boivin, P, Benismail, N, Goncalves, CR & Barros, AA 2007. Correlation of malt quality parameters and beer flavor stability: multivariate analysis. *Journal of Agricultural and Food Chemistry,* 55**,** 728-33. https://doi.org/10.1021/jf0623079

Guiga, W, Boivin, P, Ouarnier, N, Fournier, F & Fick, M 2008. Quantification of the inhibitory effect of steep effluents on barley germination. 43**,** 311-319.

Gunst, F & Verzele, M 1978. On the sunstruck flavour of beer. *Journal of the Institute of Brewing,* 84**,** 291-292. https://doi.org/10.1002/j.2050-0416.1978.tb03891.x

Hämäläinen, JJ & Reinikainen, P 2007. A simulation model for malt enzyme activities in Kilning. *Journal of the Institute of Brewing,* 113**,** 159-167. https://doi.org/10.1002/j.2050-0416.2007.tb00273.x

Hashimoto, N 1966. Studies on volatile carbonyl compounds in beer. *Report of the Research Laboratories of Kirin Brewery Co., Ltd,* 9.

Hashimoto, N & Eshima, T 1979. Oxidative degradation of isohumulones in relation to flavour stability of beer. *Journal of the Institute of Brewing,* 85**,** 136-140. https://doi.org/10.1002/j.2050-0416.1979.tb06843.x

Hashimoto, N & Kuroiwa, Y 1975. Proposed Pathways for the Formation of Volatile Aldehydes during Storage of Bottled Beer. *Proceedings. Annual meeting - American Society of Brewing Chemists,* 33**,** 104-111. https://doi.org/10.1080/00960845.1975.12007147

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

Henson, CA, Duke, SH & Vinje, MA 2014. A Comparison of Barley Malt Amylolytic Enzyme Thermostabilities and Wort Sugars Produced during Mashing. *Journal of the American Society of Brewing Chemists,* 72**,** 51-65. https://doi.org/10.1094/ASBCJ-2014-0130-01

Herrmann, M, Gastl, M, Thiele, F & Back, W 2007. Malt volatile compounds (Part I). *BrewingScience,* 60**,** 110-117.

Herrmann, M, Klotzbücher, B, Wurzbacher, M, Hanke, S, Kattein, U, Back, W, Becker, T & Krottenthaler, M 2010. A new validation of relevant substances for the evaluation of beer aging depending on the employed boiling system. *Journal of the Institute of Brewing,* 116**,** 41-48. https://doi.org/10.1002/j.2050-0416.2010.tb00396.x

Hertrich, J 2013a. Topics in Brewing: Malting. *Tech. Q. Master Brew. Assoc. Am.,* 50**,** 131-141.

Hertrich, J 2013b. Topics in Brewing: Malting Barley. *Tech. Q. Master Brew. Assoc. Am.,* 50**,** 29-41.

Hieronymus, S 2012. *For The Love of Hops: The Practical Guide to Aroma, Bitterness and the Culture of Hops,* Boulder, Colorado, Brewers Publications.

Hirota, N, Kaneko, T, Kuroda, H, Kaneda, H, Takashio, M, Ito, K & Takeda, K 2005. Characterization of lipoxygenase-1 null mutants in barley. *Theoretical and Applied Genetics,* 111**,** 1580-1584. https://doi.org/10.1007/s00122-005-0088-y

Hirota, N, Kuroda, H, Takoi, K, Kaneko, T, Kaneda, H, Yoshida, I, Takashio, M, Ito, K & Takeda, K 2006. Development of novel barley with improved beer foam and flavor stability - The impact of lipoxygenase-1-less barley in the brewing industry. *Tech. Q. Master Brew. Assoc. Am.,* 30**,** 131-135.

Hoff, S, Larsen, FH, Andersen, ML & Lund, MN 2013. Quantification of protein thiols using ThioGlo 1 fluorescent derivatives and HPLC separation. *Analyst,* 138**,** 2096-103. https://doi.org/10.1039/c3an36672c

Hoff, S, Lund, MN, Petersen, MA, Jespersen, BM & Andersen, ML 2012. Influence of Malt Roasting on the Oxidative Stability of Sweet Wort. *Journal of Agricultural and Food Chemistry,* 60**,** 5652-5659. https://doi.org/10.1021/jf300749r

Hoki, T, Kanatani, R, Saito, W, Iimure, T, Zhou, TS, Takoi, K, Tanigawa, A, Kihara, M & Ogushi, K 2018. Breeding of lipoxygenase-1-less malting barley variety 'Satuiku 2 go'. *Journal of the Institute of Brewing,* 124**,** 112-120. https://doi.org/10.1002/jib.483

Hoki, T, Saito, W, Hirota, N, Shirai, M, Takoi, K, Yoshida, S, Shimase, M, Saito, T, Takaoka, T, Makoto, K & Yamada, S 2013. Breeding of Lipoxygenase-1-less Malting Barley Variety CDC PolarStar and Effect of Lipoxygenase-1-less Trait on Beer Quality at Pilot and Commercial Scale Brewing. *BrewingScience,* 66**,** 37-45.

Holtman, WL, Vredenbregt-Heistek, JC, Schmitt, NF & Feussner, I 1997. Lipoxygenase-2 oxygenates storage lipids in embryos of germinating barley. *Eur J Biochem,* 248**,** 452-8. https://doi.org/10.1111/j.1432-1033.1997.00452.x

Huang, S, Yu, J, Yin, H, Lu, J, Dong, J, Li, X, Hu, S & Liu, J 2016. Optimization of kilning progress for equilibrating multiple parameters that strictly affect malt flavour and sensory evaluation. *Journal of the Institute of Brewing,* 122**,** 706-713. https://doi.org/10.1002/jib.369

Huang, X, Han, Y & Yu, S 2004. The effect of steeping conditions on Arapiles malting. *Monatsschrift fur Brauwissenschaft,* 57**,** 13-15.

Hudson, JR & Birtwistle, SE 1966. Wort-boiling in relation to beer quality. *Journal of the Institute of Brewing,* 72**,** 46-50. https://doi.org/10.1002/j.2050-0416.1966.tb02933.x

Hughes, P 2000. The Significance of Iso-α-Acids for Beer Quality Cambridge Prize Paper. *Journal of the Institute of Brewing,* 106**,** 271-276. https://doi.org/10.1002/j.2050-0416.2000.tb00066.x

Hugues, M, Boivin, P, Gauillard, F, Nicolas, J, Thiry, J & Richardforget, F 1994. Two lipoxygenases from germinated barley - heat and kilning stability. *Journal Of Food Science,* 59**,** 885-889. https://doi.org/10.1111/j.1365-2621.1994.tb08150.x.

Huvaere, K & Andersen, ML 2008. Beer and ESR Spin Trapping. *In:* PREEDY, V. R. (ed.) *Beer in Health and Disease Prevention.* Burlington: Academic Press, p.

Hysert, DW & Morrison, NM 1976. Sulfate Metabolism during Fermentation. *Journal of the American Society of Brewing Chemists,* 34**,** 25-31. https://doi.org/10.1080/03610470.1976.12006180

Ilett, DR & Simpson, WJ 1995. Loss of sulphur dioxide during storage of bottled and canned beers. *Food Research International,* 28**,** 393-396. https://doi.org/https://doi.org/10.1016/0963-9969(95)00012-B

Jamieson, AM & Van Gheluwe, JEA 1970. Identifcation of a compound responsible for cardboard flavor in beer. *Proceedings. Annual meeting - American Society of Brewing Chemists,* 28**,** 192-197. https://doi.org/10.1080/00960845.1970.12006980

Jaskula-Goiris, B, De Causmaecker, B, De Rouck, G, Aerts, G & De Cooman, L. Influence of malt specification on beer quality and flavour stability. Proceedings of the XV Chair De Clerck, 2015 Belgium, Gent.

Jaskula-Goiris, B, De Causmaecker, B, De Rouck, G, Aerts, G, Paternoster, A, Braet, J & De Cooman, L 2019. Influence of transport and storage conditions on beer quality and flavour stability. *Journal of the Institute of Brewing,* 125**,** 60-68. https://doi.org/10.1002/jib.535

Jaskula-Goiris, B, De Causmaecker, B, De Rouck, G, De Cooman, L & Aerts, G 2011. Detailed multivariate modeling of beer staling in commercial pale lagers. *BrewingScience,* 64**,** 119-139.

Jaskula, B, Syryn, E, Goiris, K, De Rouck, G, Van Opstaele, F, De Clippeleer, J, Aerts, G & De Cooman, L 2007. Hopping Technology in Relation to Beer Bitterness Consistency and Flavor Stability. *Journal of the American Society of Brewing Chemists,* 65**,** 38-46. https://doi.org/10.1094/ASBCJ-2007-0112-03

Jenkins, D, James, S, Dehrmann, F, Smart, K & Cook, D 2018. Impacts of Copper, Iron, and Manganese Metal Ions on the EPR Assessment of Beer Oxidative Stability. *Journal of the American Society of Brewing Chemists,* 76**,** 50-57. https://doi.org/10.1080/03610470.2017.1402585

Jin, Y-L, Speers, RA, Paulson, AT & Stewart, RJ 2004. Effect of β-Glucans and Process Conditions on the Membrane Filtration Performance of Beer. *Journal of the American Society of Brewing Chemists,* 62**,** 117-124. https://doi.org/10.1094/ASBCJ-62-0117

Johnston, JHS 1954. Physical factors affecting the kilning of barley and malt. *Journal of the Institute of Brewing,* 60**,** 318-340. https://doi.org/10.1002/j.2050-0416.1954.tb02759.x

Jones, BL 2005. The endogenous endoprotease inhibitors of barley and malt and their roles in malting and brewing. *Journal of Cereal Science,* 42**,** 271-280. https://doi.org/https://doi.org/10.1016/j.jcs.2005.06.002

Jones, D, Lelyveld, TP, Mavrofidis, SD, Kingman, S & Miles, N 2002. Microwave heating applications in enviromnental engineering - a review. *Resources, Conservation and Recycling,* 34**,** 75-90. https://doi.org/10.1016/S0921-3449(01)00088-X

Jones, M & Pierce, JS 1964. Absorption of amino acids from wort by yeasts. *Journal of the Institute of Brewing,* 70**,** 307-315. https://doi.org/10.1002/j.2050-0416.1964.tb01996.x

Justé, A, Malfliet, S, Lenaerts, M, De Cooman, L, Aerts, G, Willems, K & Lievens, B 2011. Microflora during malting of Barley: Overview and Impact on malt quality. *BrewingScience,* 64**,** 22-31.

Kaneda, H, Kimura, T, Kano, Y, Koshino, S, Osawa, T & Kawakishi, S 1991. Role of fermentation conditions on flavor stability of beer. *Journal of Fermentation and Bioengineering,* 72**,** 26-30. https://doi.org/10.1016/0922-338X(91)90141-3

Kaneda, H, Osawa, T, Kawakishi, S, Munekata, M & Koshino, S 1994. Contribution of carbonyl-bisulfite adducts to beer stability. *Journal of Agricultural and Food Chemistry,* 42**,** 2428-2432. https://doi.org/10.1021/jf00047a012

Kaneda, H, Takashio, M, Tamaki, T & Osawa, T 1997. Influence of pH on flavour staling during beer storage. *Journal of the Institute of Brewing,* 103**,** 21-23. https://doi.org/10.1002/j.2050-0416.1997.tb00932.x

Kaukovirta-Norja, A, Laakso, S, Reinikainen, P & Olkku, J 1993. Lipolytic and oxidative changes of barley lipids during malting and mashing L. *Journal of the Institute of Brewing,* 99**,** 395-403. https://doi.org/10.1002/j.2050-0416.1993.tb01179.x

Kaukovirta-Norja, A, Laakso, S, Reinikainen, P & Olkku, J 1998. The effect of kilning on the capability of malt to oxidise lipids. *Journal of the Institute of Brewing,* 104**,** 327-332. https://doi.org/10.1002/j.2050-0416.1998.tb01004.x

Kavanagh, TE, Derbyshire, RC, Hildebrand, RP, Clarke, BJ & Meeker, FJ 1976. Dimethyl sulphide formation in malt—Effect of malting conditions. *Journal of the Institute of Brewing,* 82**,** 270-272. https://doi.org/10.1002/j.2050-0416.1976.tb03768.x

Kavanagh, TE, Steward, SR, Hildebrand, RP, Clarke, BJ & Meeker, FJ 1975. Trace volatile constituents of beer dimethyl sulphide formation in malt. *Journal of the Institute of Brewing,* 81**,** 322-327. https://doi.org/10.1002/j.2050-0416.1975.tb03700.x

Kleban, J & Nickerson, I 2012. To brew, or not to brew - That is the question: An analysis of competitive forces in the craft brew industry. *International Academy for Case Studies,* 18**,** 51-58.

Knudsen, S, Rils, P, Skadhauge, B, Bech, LM & Olsen, O. 2011. *Energy saving brewing method*. Patent number: US15413094.

Kobayashi, N, Kaneda, H, Kano, Y & Koshino, S 1993. The production of linoleic and linolenic acid hydroperoxides during mashing. *Journal of Fermentation and Bioengineering,* 76**,** 371-375. https://doi.org/10.1016/0922-338X(93)90024-3

Kobayashi, N, Kaneda, H, Kano, Y & Koshino, S 1994. Behavior of Lipid Hydroperoxides During Mashing. *Journal of the American Society of Brewing Chemists,* 52**,** 141-145. https://doi.org/10.1094/ASBCJ-52-0141

Kobayashi, N, Kaneda, H, Kuroda, H, Kobayashi, M, Kurihara, T, Watari, J & Shinotsuka, K 2000a. Simultaneous Determination of Mono-, Di-, and Trihydroxyoctadecenoic Acids in Beer and Wort. *Journal of the Institute of Brewing,* 106**,** 107-110. https://doi.org/10.1002/j.2050-0416.2000.tb00046.x

Kobayashi, N, Kaneda, H, Kuroda, H, Watari, J, Kurihara, T & Shinotsuka, K 2000b. Behavior of mono-, di-, and trihydroxyoctadecenoic acids during mashing and methods of controlling their production. *Journal of Bioscience and Bioengineering,* 90**,** 69-73. https://doi.org/10.1016/S1389-1723(00)80036-5

Kunz, T, Müller, C, Mato-Gonzales, D & Methner, FJ 2012a. The influence of unmalted barley on the oxidative stability of wort and beer. *Journal of the Institute of Brewing,* 118**,** 32-39. https://doi.org/10.1002/jib.6

Kunz, T, Müller, C, Methner, F-J, Kunz, T & Müller, C 2012b. EAP determination and Beverage Antioxidant IndeX (BAX) - advantageous tools for evaluation of the oxidative flavour stability of beverages. *BrewingScience,* 65**,** 12-20.

Kunz, T, Strähmel, A, Cortés, N, Kroh, LW & Methner, F-J 2013. Influence of Intermediate Maillard Reaction Products with Enediol Structure on the Oxidative Stability of Beverages. *Journal of the American Society of Brewing Chemists,* 71**,** 114-123. https://doi.org/10.1094/ASBCJ-2013-0429-01

Kunze, W 2014. *Technology; Brewing and Malting,* Berlin, Germany, VLB Berlin.

Kuroda, H, Furusho, S, Maeba, H & Takashio, M 2003. Characterization of factors involved in the production of 2(E)-nonenal during mashing. *Bioscience, Biotechnology, and Biochemistry,* 67**,** 691-7. https://doi.org/10.1271/bbb.67.691

Kuroda, H, Kobayashi, N, Kaneda, H, Watari, J & Takashio, M 2002. Characterization of Factors That Transform Linoleic Acid into Di- and Trihydroxyoctadecenoic Acids in Mash. *Journal of Bioscience and Bioengineering,* 93**,** 73-77. https://doi.org/10.1263/jbb.93.73

Leclercq, G 2020. Einsatz von Grünmalz - der Enzymkick. *Brauwelt,* 19**,** 498-500.

Lehnhardt, F, Gastl, M & Becker, T 2019. Forced into aging: Analytical prediction of the flavor-stability of lager beer. A review. *Critical Reviews in Food Science and Nutrition,* 59**,** 2642-2653. https://doi.org/10.1080/10408398.2018.1462761

Leiper, KA, Stewart, GG, McKeown, IP, Nock, T & Thompson, MJ 2005. Optimising beer stabilisation by the selective removal of tannoids and sensitive proteins. *Journal of the Institute of Brewing,* 111**,** 118-127. https://doi.org/10.1002/j.2050-0416.2005.tb00657.x

Lermusieau, G, Noël, S, Liégeois, C & Collin, S 1999. Nonoxidative mechanism for development of trans-2-nonenal in beer. *Journal of the American Society of Brewing Chemists,* 57**,** 29-33. https://doi.org/10.1094/ASBCJ-57-0029

Li, M 2009. Determination of Furfural in Beer by High- Performance Liquid Chromatography with Solid-Phase Extraction. *Journal of the Institute of Brewing,* 115**,** 226-231. https://doi.org/10.1002/j.2050-0416.2009.tb00373.x

Lidel, DR 1999. CRC handbook of chemistry and physics [electronic resource]. *Handbook of chemistry and physics.* Boca Raton, Florida, USA: CRC Press.

Liégeois, C, Meurens, N, Badot, C & Collin, S 2002. Release of deuterated (E)-2-nonenal during beer aging from labeled precursors synthesized before boiling. *Journal of agricultural and food chemistry,* 50**,** 7634-7638. https://doi.org/10.1021/jf020617v

Lund, MN, Kramer, AC & Andersen, ML 2015. Antioxidative Mechanisms of Sulfite and Protein-Derived Thiols during Early Stages of Metal Induced Oxidative Reactions in Beer. *Journal of Agricultural and Food Chemistry,* 63**,** 8254-61. https://doi.org/10.1021/acs.jafc.5b02617

MacWilliam, IC 1972. Effect of kilning on malt starch and on the dextrin content of resulting worts and beers. *Journal of the Institute of Brewing,* 78**,** 76-81. https://doi.org/10.1002/j.2050-0416.1972.tb03433.x

MacWilliam, IC, Hudson, JR & Whitear, AL 1963. Wort from green malt and unmalted cereals. *Journal of the Institute of Brewing,* 69**,** 303-308. https://doi.org/10.1002/j.2050-0416.1963.tb01930.x

Madigan, D, Perez, A & Clements, M 1998. Furanic aldehyde analysis by HPLC as a method to determine heat-induced flavor damage to beer. *Journal of the American Society of Brewing Chemists,* 56**,** 146-151. https://doi.org/10.1094/ASBCJ-56-0146

Malfliet, S, Van Opstaele, F, de Clippeleer, J, Syryn, E, Goiris, K, de Cooman, L & Aerts, G 2008. Flavour instability of pale lager beers: determination of analytical markers in relation to sensory ageing. *Journal of the Institute of Brewing,* 114**,** 180-192. https://doi.org/10.1002/j.2050-0416.2008.tb00324.x

Mallett, J 2014. From barley to malt. *Malt : a practical guide from field to brewhouse.* Boulder, Colorado, USA: Brewers Publications, p.

Manger, H-J 2017. *Maschinen, Apparate und Anlagen der Gärungs- und Getränkeindustrie: 2 Mälzerei,* Berlin, Germany, VLB Berlin.

Masaaki Yano, MMTYYO & Motoo, O 2019. Influence of Wort Boiling and Wort Clarification Conditions on Cardboard Flavor in Beer. *Tech. Q. Master Brew. Assoc. Am.,* 41**,** 317-320.

Maule, DR 1966. The fate of humulone during wort boiling and cooling. *Journal of the Institute of Brewing,* 72**,** 285-290. https://doi.org/10.1002/j.2050-0416.1966.tb02967.x

Mauthner, F, Hubmann, M, Christoph, B & Fink, C 2014. Manufacture of malt and beer with low temperature solar process heat. *Energy Procedia,* 48**,** 1188-1193. https://doi.org/10.1016/j.egypro.2014.02.134

McMurrough, I, Kelly, R, Byrne, J & O'Brien, M 1992. Effect of the Removal of Sensitive Proteins and Proanthocyanidins on the Colloidal Stability of Lager Beer. *Journal of the American Society of Brewing Chemists,* 50**,** 67-76. https://doi.org/10.1094/ASBCJ-50-0067

McMurrough, I, Madigan, D, Kelly, RJ & Smyth, MR 1996. The role of flavanoid polyphenols in beer stability. *Journal of the American Society of Brewing Chemists,* 54**,** 141-148. https://doi.org/10.1094/ASBCJ-54-0141

Meilgaard, MC 1975a. Flavor chemistry of beer. II. Flavor and threshold of 239 aroma volatiles. *Tech. Q. Master Brew. Assoc. Am.,* 12**,** 151-168.

Meilgaard, MC 1975b. Flavour chemistry of beer. Part 1: Flavour interaction between principal volatiles. *Tech. Q. Master Brew. Assoc. Am.,* 12**,** 107-117.

Meilgaard, MC, Dalgliesh, CE & Clapperton, JF 1979. Beer Flavour Terminology. *Journal of the Institute of Brewing,* 85**,** 38-42. https://doi.org/10.1002/j.2050-0416.1979.tb06826.x

Meng, D-J, Lu, J, Fan, W, Dong, J-J, Zhang, J, Kong, W-B, Lin, Y & Shan, L-J 2009. Optimization of extraction conditions for antioxidant phenolic compounds from malt rootlets using response surface methodology. *Journal of Food Biochemistry,* 33**,** 291-305. https://doi.org/10.1111/j.1745-4514.2009.00218.x

Menger, H-J 2006. Advantages of fine wet milling with a rotor/stator system and lautering with a thin-layer chamber mash filter. *Tech. Q. Master Brew. Assoc. Am.,* 43**,** 52-57.

Meura. *Hydromill* [Online]. Available: https://www.meura.com/products/hydromill.html [Accessed July 2020].

Michel, RH, McGovern, PE & Badler, VR 1993. The First Wine & Beer. *Analytical Chemistry,* 65**,** 408A-413A. https://doi.org/10.1021/ac00056a734

Moir, M 1992. The 1990 laurence bishop silver medal lecture*the desideratum for flavour control. *Journal of the Institute of Brewing,* 98**,** 215-220. https://doi.org/10.1002/j.2050-0416.1992.tb01107.x

Möller-Hergt, G, Roderfeld, HJ & Waterkamp, HJ. Changes of hydroxy acids and other ageing relevant compounds in wort and beer depending on storage of malt and barley. Monograph 31 of the European Brewery Convention Symposium 'Flavour and Flavour Stability', 2001 Nancy, France. Fachverlag Hans Carl, Nürnberg, Germany.

Müller, C, Kleinwächter, M, Selmar, D & Methner, FJ 2013. The influence of elevated steeping temperatures on the resulting malt homogeneity and malt quality. *BrewingScience,* 66**,** 114-122.

Müller, C, Kleinwaechter, M, Selmar, D & Methner, F-J 2014. The influence of the withering temperature on the resulting proteolytic and cytolytic modification of pale malt. *BrewingScience,* 67**,** 88-95.

Muntons. 2019. *Sustainability at Muntons* [Online]. Available: https://www.psmuntons.com/wp-content/uploads/2019/04/190424-Sustainability-Statement-2019.pdf [Accessed September 2019].

Narziss, L 1976. Polyphenolgehalt und Polymerisationsindex von Gersten und Kleinmalzen. *Monatsschrift für Brauwissenschaft,* 29**,** 9-19.

Narziss, L 1986. Technological factors of flavour stability. *Journal of the Institute of Brewing,* 92**,** 346-353. https://doi.org/10.1002/j.2050-0416.1986.tb04421.x

Narziss, L, Miedaner, H & Eichhorn, P 1999. Untersuchungen zur geschmacksstabilität des Bieres (teil 2). *Monatsschrift fur Brauwissenschaft,* 52**,** 80-85.

Narziss, L & Rusitka, P 1977. Variation of enzymic activities during drying and curing of malt. *Monatsschrift für Brauwissenschaft,* 20**,** 1-12.

Neven, H, Delvaux, F & Derdelinckx, G 1997. Flavor evolution of top fermented beers. *Tech. Q. Master Brew. Assoc. Am.,* 34**,** 115-118.

Noël, S, Liegeois, C, Lermusieau, G, Bodart, E, Badot, C & Collin, S 1999a. Release of deuterated nonenal during beer aging from labeled precursors synthesized in the boiling kettle. *Journal of Agricultural and Food Chemistry,* 47**,** 4323-6. https://doi.org/10.1021/jf990211r

Noël, S, Metais, N, Bonte, S, Bodart, E, Peladan, F, Dupire, S & Collin, S 1999b. The use of Oxygen 18 in appraising the impact of oxidation process during beer storage. *Journal of the Institute of Brewing,* 105**,** 269-274. https://doi.org/10.1002/j.2050-0416.1999.tb00519.x

O'Neil, MJ, Heckelman, PE, Koch, CB, J., RK, Kenny, CM & D'Arecca, MR 2006. *The Merck Index. An Encyclopedia of Chemicals, Drugs and Biologicals,* Whitehouse Station, NJ, Merck Research Laboratories.

O'Rourke, T 2002. The role of oxygen in brewing. *The Brewer International***,** 45-47.

O'Rourke, T 2015. Enzymes in Brewing. *Brewer and Distiller International,* October**,** 28-34.

O'Sullivan, TF, Walsh, Y, O'Mahony, A, Fitzgerald, GF & Sinderen, D 1999. A Comparative Study of Malthouse and Brewhouse Microflora. *Journal of the Institute of Brewing,* 105**,** 55-61. https://doi.org/10.1002/j.2050-0416.1999.tb00006.x

Özcan, MM, Aljuhaimi, F & Uslu, N 2018. Effect of malt process steps on bioactive properties and fatty acid composition of barley, green malt and malt grains. *Journal of food science and technology,* 55**,** 226-232. https://doi.org/10.1007/s13197-017-2920-1

Pagenstecher, M, Maia, C & Andersen, ML 2020. Retention of iron and copper during mashing of roasted malts. *Journal of the American Society of Brewing Chemists,* Published online. https://doi.org/10.1080/03610470.2020.1795609

Palmer, GH 1982. A reassessment of the pattern of endosperm hydrolysis (modification) in germinated barley. *Journal of the Institute of Brewing,* 88**,** 145-153. https://doi.org/10.1002/j.2050-0416.1982.tb04087.x

Palmer, GH 1995. Structure of ancient cereal grains. *Journal of the Institute of Brewing,* 101**,** 103-112. https://doi.org/10.1002/j.2050-0416.1995.tb00854.x

Palmer, GH 1998. Ultrastructure of the cell walls of the transport pathway for gibberellic acid in barley aleurone layer. *Journal of the Institute of Brewing,* 104**,** 137-142. https://doi.org/10.1002/j.2050-0416.1998.tb00987.x

Palmer, GH & Bathgate, GN 1976. Malting and Brewing. *Advances in Cereal Science and Technology,* 1**,** 237.

Pankoke, I. Securing beer and beverage quality during long distance distribution process–Using a special sensor system. European Brewing Congress, 2015 Porto.

Paternoster, A, Jaskula-Goiris, B, De Causmaecker, B, Vanlanduit, S, Springael, J, Braet, J, De Rouck, G & De Cooman, L 2019. The interaction effect between vibrations and temperature simulating truck transport on the flavor stability of beer. *Journal of the Science of Food and Agriculture,* 99**,** 2165-2174. https://doi.org/10.1002/jsfa.9409

Peppard, TL & Halsey, SA 1981. Malt flavour - transformation of carbonyl compounds by yeast during fermentation. *Journal of the Institute of Brewing,* 87**,** 386-390. https://doi.org/10.1002/j.2050-0416.1981.tb04055.x

Perpète, P & Collin, S 2000. How to improve the enzymatic worty flavour reduction in a cold contact fermentation. *Food Chemistry,* 70**,** 457-462. https://doi.org/10.1016/S0308-8146(00)00111-4

Peterreins, F & Van Waesberghe, J. 2003. *Production of wort/malt extract or wort granulates/malt extract granulates through stabilized green malt*. Patent number.

Petters, HI, Flannigan, B & Austin, B 1988. Quantitative and qualitative studies of the microflora of barley malt production. *Journal of Applied Bacteriology,* 65**,** 279-297. https://doi.org/10.1111/j.1365-2672.1988.tb01895.x

Peyrat-Maillard, MN, Bonnely, S, Rondini, L & Berset, C 2001. Effect of Vitamin E and Vitamin C on the Antioxidant Activity of Malt Rootlets Extracts. *LWT - Food Science and Technology,* 34**,** 176-182. https://doi.org/10.1006/fstl.2001.0752

Pimenta, MJ, Kaneta, T, Larondelle, Y, Dohmae, N & Kamiya, Y 1998. S-adenosyl-L-methionine:L-methionine S-methyltransferase from germinating barley. Purification and localization. *Plant physiology,* 118**,** 431-438. https://doi.org/10.1104/pp.118.2.431

Pires, EJ, Teixeira, JA, Brányik, T & Vicente, AA 2014. Yeast: the soul of beer's aroma--a review of flavour-active esters and higher alcohols produced by the brewing yeast. *Applied Microbiology and Biotechnology,* 98**,** 1937-49. https://doi.org/10.1007/s00253-013-5470-0

Pohl, P 2008. Determination and fractionation of metals in beer: A review. *Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment,* 25**,** 693-703. https://doi.org/10.1080/02652030701772323

Pohl, P & Prusisz, B 2010. Chemical fractionation of Cu, Fe and Mn in canned Polish beers. *Journal of Food Composition and Analysis,* 23**,** 86-94. https://doi.org/10.1016/j.jfca.2009.08.002

Poreda, A, Bijak, M, Zdaniewicz, M, Jakubowski, M & Makarewicz, M 2015. Effect of wheat malt on the concentration of metal ions in wort and brewhouse by-products. *Journal of the Institute of Brewing,* 121**,** 224-230. https://doi.org/10.1002/jib.226

Porter, JR & Bamforth, CW 2016. Manganese in brewing raw materials, disposition during the brewing process, and impact on the flavor instability of beer. *Journal of the American Society of Brewing Chemists,* 74**,** 87-90. https://doi.org/10.1094/ASBCJ-2016-2638-01

Prasad, A, Sedlářová, M, Kale, RS & Pospíšil, P 2017. Lipoxygenase in singlet oxygen generation as a response to wounding: in vivo imaging in Arabidopsis thaliana. *Scientific Reports,* 7**,** 9831. https://doi.org/10.1038/s41598-017-09758-1

Preece, IA 1948. Some aspects of stability of brewery malt α-amylase. *Journal of the Institute of Brewing,* 54**,** 141-147. https://doi.org/10.1002/j.2050-0416.1948.tb01367.x

Rakete, S, Klaus, A & Glomb, MA 2014. Investigations on the Maillard reaction of dextrins during aging of Pilsner type beer. *Journal of Agricultural and Food Chemistry,* 62**,** 9876-84. https://doi.org/10.1021/jf503038c

Ratti, C 2001. Hot air and freeze-drying of high-value foods: a review. *Journal of Food Engineering,* 49**,** 311-319. https://doi.org/10.1016/S0260-8774(00)00228-4

Rennie, H & Ball, K 1979. The influence of malt storage on wort separation. *Journal of the Institute of Brewing,* 85**,** 247-249. https://doi.org/10.1002/j.2050-0416.1979.tb03917.x

Rizzi, GP 2008. The Strecker Degradation of Amino Acids: Newer Avenues for Flavor Formation. *Food Reviews International,* 24**,** 416-435. https://doi.org/10.1080/87559120802306058

Rosahl, S 1996. Lipoxygenases in plants--their role in development and stress response. *Z Naturforsch C,* 51**,** 123-38. https://doi.org/10.1515/znc-1996-3-401

Ross, RP, Morgan, S & Hill, C 2002. Preservation and fermentation: past, present and future. *International Journal of Food Microbiology,* 79**,** 3-16. https://doi.org/10.1016/S0168-1605(02)00174-5

Saison, D, De Schutter, DP, Overlaet-Michiels, W, Delvaux, F & Delvaux, FR 2009a. Effect of Fermentation Conditions on Staling Indicators in Beer. *Journal of the American Society of Brewing Chemists,* 67**,** 222-228. https://doi.org/10.1094/ASBCJ-2009-0720-02

Saison, D, De Schutter, DP, Uyttenhove, B, Delvaux, F & Delvaux, FR 2009b. Contribution of staling compounds to the aged flavour of lager beer by studying their flavour thresholds. *Food Chemistry,* 114**,** 1206-1215. https://doi.org/10.1016/j.foodchem.2008.10.078

Saison, D, De Schutter, DP, Vanbeneden, N, Daenen, L, Delvaux, F & Delvaux, FR 2010a. Decrease of Aged Beer Aroma by the Reducing Activity of Brewing Yeast. *Journal of Agricultural and Food Chemistry,* 58**,** 3107-3115. https://doi.org/10.1021/jf9037387

Saison, D, Vanbeneden, N, De Schutter, DP, Daenen, L, Mertens, T, Delvaux, F & Delvaux, FR 2010b. Characterisation of the flavour and the chemical composition of lager beer after ageing in varying conditions. *BrewingScience,* 63**,** 41--53.

Salama, A-RA, El-Sahn, MA, Mesallam, AS & Shehata, AME-T 1997. The chemical composition, the nutritive value and the functional properties of malt sprout and its components (acrospires, rootlets and husks). *Journal of the Science of Food and Agriculture,* 75**,** 50-56. https://doi.org/10.1002/(SICI)1097-0010(199709)75:1<50::AID-JSFA833>3.0.CO;2-0

Santos, J, Carneiro, J, Guido, L, Almeida, P, Rodrigues, J & Barros, AA 2003. Determination of E-2-nonenal by high-performance liquid chromatography with UV detection - Assay for the evaluation of beer ageing. *Journal of Chromatography A,* 985**,** 395-402. https://doi.org/10.1016/S0021-9673(02)01396-1

Schroeder, SW & MacGregor, AW 1998. Synthesis of Limit Dextrinase in Germinated Barley Kernels and Aleurone Tissues. *Journal of the American Society of Brewing Chemists,* 56**,** 32-37. https://doi.org/10.1094/ASBCJ-56-0032

Schwarz, P, Stanley, P & Solberg, S 2002a. Activity of Lipase during Mashing. *Journal of the American Society of Brewing Chemists,* 60**,** 107-109. https://doi.org/10.1094/ASBCJ-60-0107

Schwarz, PB, Jones, BL & Steffenson, BJ 2002b. Enzymes associated with Fusarium infection of barley. *Journal of the American Society of Brewing Chemists,* 60**,** 130-134. https://doi.org/10.1094/ASBCJ-60-0130

Schwarz, PB & Pyler, RE 1984. Lipoxygenase and Hydroperoxide Isomerase Activity of Malting Barley. *Journal of the American Society of Brewing Chemists,* 42**,** 47-53. https://doi.org/10.1094/asbcj-42-0047

Scott, PM 1996. Mycotoxins transmitted into beer from contaminated grains during brewing. *The Journal of AOAC International,* 79**,** 875-82.

Sheneman, JM & Hollenbeck, CM 1960. Microbial Patterns in Malt. Lactic Acid Bacteria. *Proceedings. Annual meeting - American Society of Brewing Chemists,* 18**,** 22-27. https://doi.org/10.1080/00960845.1960.12006892

Shimizu, C, Nakamura, Y, Miyai, K, Araki, S, Takashio, M & Shinotsuka, K 2001. Factors affecting 5-hydroxymethyl furfural formation and stale flavor formation in beer. *Journal of the American Society of Brewing Chemists,* 59**,** 51-58. https://doi.org/10.1094/ASBCJ-59-0051

Siebert, KJ & Lynn, PY 1998. Comparison of polyphenol interactions with polyvinylpolypyrrolidone and haze-active protein. *Journal of the American Society of Brewing Chemists,* 56**,** 24-31. https://doi.org/10.1094/ASBCJ-56-0024

Siebert, KJ & Lynn, PY 2008. On the mechanisms of adsorbent interactions with haze-active proteins and polyphenols. *Journal of the American Society of Brewing Chemists,* 66**,** 48-54. https://doi.org/10.1094/ASBCJ-2007-1116-02

Sissons, M, Taylor, M & Proudlove, M 1995. Barley Malt Limit Dextrinase: Its Extraction, Heat Stability, and Activity During Malting and Mashing. *Journal of the American Society of Brewing Chemists,* 53**,** 104-110. https://doi.org/10.1094/ASBCJ-53-0104

Skadhauge, B, Knudsen, S, Lok, F & Olsen, O. Barley for production of flavour-stable beer. Proc. Eur. Brew. Conv. Cong., 2005 Prague. Fachverlag HansCarl, Nürnberg, 1-13.

Smith, RJ, Davidson, D & Wilson, RJH 1998. Natural Foam Stabilizing and Bittering Compounds Derived from Hops. *Journal of the American Society of Brewing Chemists,* 56**,** 52-57. https://doi.org/10.1094/ASBCJ-56-0052

Sopanen, T & Laurière, C 1989. Release and Activity of Bound beta-Amylase in a Germinating Barley Grain. *Plant Physiology,* 89**,** 244-9. https://doi.org/10.1104/pp.89.1.244

Speers, RA, Jin, Y-L, Paulson, AT & Stewart, RJ 2003. Effects of β-Glucan, Shearing and Environmental Factors on the Turbidity of Wort and Beer. *Journal of the Institute of Brewing,* 109**,** 236-244. https://doi.org/10.1002/j.2050-0416.2003.tb00164.x

Steiner, E, Becker, T & Gastl, M 2010. Turbidity and Haze Formation in Beer - Insights and Overview. *Journal of the Institute of Brewing,* 116**,** 360-368. https://doi.org/10.1002/j.2050-0416.2010.tb00787.x

Thalacker, R & Böβendörfer, G 2005. Thiobarbituric acid index (TBI). *Brauwelt International,* 1**,** 35-39.

Trinci, APJ 1972. Culture turbidity as a measure of mould growth. *Transactions of the British Mycological Society,* 58**,** 467-IN14. https://doi.org/10.1016/S0007-1536(72)80095-0

Trummer, J, Poreda, A & Berski, W 2019. The effect of malting on green lentils and their suitability for wort production. *Przemysł Fermentacyjny i Owocowo-Warzywny,* 63**,** 14-18. https://doi.org/10.15199/64.2019.10.1

Turner, HM, Elmore, L, Walling, J, Lachowiec, J, Mangel, D, Fischer, A & Sherman, J 2019. Effect of Steeping Regime on Barley Malt Quality and Its Impacts on Breeding Program Selection. *Journal of the American Society of Brewing Chemists,* 77**,** 267-281. https://doi.org/10.1080/03610470.2019.1629794

Uchida, M, Suga, S & Ono, M 1996. Improvement for Oxidative Flavor Stability of Beer—Rapid Prediction Method for Beer Flavor Stability by Electron Spin Resonance Spectroscopy. *Journal of the American Society of Brewing Chemists,* 54**,** 205-211. https://doi.org/10.1094/ASBCJ-54-0205

Vahamidis, P, Stefopoulou, A, Kotoulas, V, Lyra, D, Dercas, N & Economou, G 2017. Yield, grain size, protein content and water use efficiency of null-LOX malt barley in a semiarid Mediterranean agroecosystem. *Field Crops Research,* 206**,** 115-127. https://doi.org/10.1016/j.fcr.2017.02.022

Van Mechelen, JR, Schuurink, RC, Smits, M, Graner, A, Douma, AC, Sedee, NJ, Schmitt, NF & Valk, BE 1999. Molecular characterization of two lipoxygenases from barley. *Plant Molecular Biology,* 39**,** 1283-98. https://doi.org/10.1023/A:1006118003998

Van Waesberghe, J, Aerts, G & De Cooman, L. Flavour stability starts with malt and compensations in the brewhouse for LOX and aldehydes in malt. EBC symposium on Flavour and Flavour Stability, 2001 Nancy, France. Fachverlag Hans Carl, Nürnberg, Germany, 1-16.

Van Waesberghe, JWM 1991. Practical investigations on the possible impact of mash separation time on beer flavor and its flavor stability. Influence of the husk fraction. *Tech. Q. Master Brew. Assoc. Am.,* 28**,** 33-37.

Vanderhaegen, B, Neven, H, Coghe, S, Verstrepen, KJ, Verachtert, H & Derdelinckx, G 2003. Evolution of Chemical and Sensory Properties during Aging of Top-Fermented Beer. *Journal of Agricultural and Food Chemistry,* 51**,** 6782-6790. https://doi.org/10.1021/jf034631z

Vanderhaegen, B, Neven, H, Verachtert, H & Derdelinckx, G 2006. The chemistry of beer aging - A critical review. *Food Chemistry,* 95**,** 357-381. https://doi.org/10.1016/j.foodchem.2005.01.006

Vaughan, A, O'Sullivan, T & Sinderen, D 2005. Enhancing the Microbiological Stability of Malt and Beer — A Review. *Journal of the Institute of Brewing,* 111**,** 355-371. https://doi.org/10.1002/j.2050-0416.2005.tb00221.x

Verstrepen, KJ, Iserentant, D, Malcorps, P, Derdelinckx, G, Van Dijck, P, Winderickx, J, Pretorius, IS, Thevelein, JM & Delvaux, FR 2004. Glucose and sucrose: hazardous fast-food for industrial yeast? *Trends in Biotechnology,* 22**,** 531-7. https://doi.org/10.1016/j.tibtech.2004.08.001

Vesely, P, Lusk, L, Basarova, G, Seabrooks, J & Ryder, D 2003. Analysis of Aldehydes in Beer Using Solid-Phase Microextraction with On-Fiber Derivatization and Gas Chromatography/Mass Spectrometry. *Journal of Agricultural and Food Chemistry,* 51**,** 6941-6944. https://doi.org/10.1021/jf034410t

Vriesekoop, F, Krahl, M, Hucker, B & Menz, G 2012. 125th Anniversary Review: Bacteria in brewing: The good, the bad and the ugly. *Journal of the Institute of Brewing,* 118**,** 335-345. https://doi.org/10.1002/jib.49

Wackerbauer, K & Meyna, S. The occurrence of free and triglyceride-bonded hydroxy fatty acids in barley and malt. EBC Symposium 'Flavour and Flavour Stability, 2001 Nancy, France. Fachverlag Hans Carl, Nürnberg, contribution 6, pp.16.

Wackerbauer, K & Meyna, S 2002a. Freie und triglycerid-gebundene Hydroxyfettsaeuren in Gerste und Malz I. Einfluss von Sorte, Provenienz und Erntejahr. *Monatsschift für Brauwissenschaft,* 55**,** 52-57.

Wackerbauer, K & Meyna, S 2002b. Freie und triglycerid-gebundene Hydroxyfettsaeuren in Gerste und Malz II. Die Entwicklung waehrend des Maelzungsprozesses. *Monatsschift für Brauwissenschaft,* 55**,** 110-114.

Wackerbauer, K, Meyna, S & Marre, S 2003. Hydroxy fatty acids as indicators for ageing and the influence of oxygen in the brewhouse on the flavour stability of beer. *Monatsschift für Brauwissenschaft,* 56**,** 174-178.

Wettstein, D, Jende-Strid, B, Ahrenst-Larsen, B & Erdal, K 1980. Proanthocyanidin-free barley prevents the formation of beer haze. *Tech. Q. Master Brew. Assoc. Am.,* 17**,** 16-23.

White, C & Zainasheff, J 2010. How to choose the right yeast. *Yeast: The Practical Guide to Beer Fermentation.* Brewers Publications, p.

White, FH & Parsons, R. The development of dimethyl sulphide in malting brewing and fermentation. Proc. Eur. Brew. Conv. Cong., 1975 Nice, France.

White, FH & Wainwright, T 1976a. Isolation and partial characterization of the dimethyl sulphide precursor in green malt and its effect on beer dimethyl sulphide levels. *Journal of the Institute of Brewing,* 82**,** 292-296. https://doi.org/10.1002/j.2050-0416.1976.tb03777.x

White, FH & Wainwright, T 1976b. The measurement of dimethyl sulphide precursor in malts, adjuncts, worts and beer. *Journal of the Institute of Brewing,* 82**,** 46-48. https://doi.org/10.1002/j.2050-0416.1976.tb03721.x

White, FH & Wainwright, T 1977. The presence of two dimethyl sulphide precursors in malt, their control by malt kilning conditions, and their effect on beer DMS levels. *Journal of the Institute of Brewing,* 83**,** 224-230. https://doi.org/10.1002/j.2050-0416.1977.tb03799.x

Whitehurst, RJ & Oort, Mv 2010. *Enzymes in food technology,* Oxford, Wiley-Blackwell.

Wietstock, P & Methner, F-J 2013. Formation of Aldehydes by Direct Oxidative Degradation of Amino Acids via Hydroxyl and Ethoxy Radical Attack in Buffered Model Solutions. *BrewingScience,* 66**,** 104-113.

Wietstock, PC, Kunz, T & Methner, FJ 2016. Relevance of Oxygen for the Formation of Strecker Aldehydes during Beer Production and Storage. *Journal of Agricultural and Food Chemistry,* 64**,** 8035-8044. https://doi.org/10.1021/acs.jafc.6b03502

Wietstock, PC & Shellhammer, TH 2011. Chelating Properties and Hydroxyl-Scavenging Activities of Hop α- and Iso-α-Acids. *Journal of the American Society of Brewing Chemists,* 69**,** 133-138. https://doi.org/10.1094/ASBCJ-2011-0718-01

Woonton, BW, Jacobsen, JV, Sherkat, F & Stuart, IM 2005. Changes in Germination and Malting Quality During Storage of Barley. *Journal of the Institute of Brewing,* 111**,** 33-41. https://doi.org/10.1002/j.2050-0416.2005.tb00646.x

Yabuuchi, S 1976. Occurrence of a New Lipoxygenase Isoenzyme in Germinating Barley Embryos. *Agricultural and Biological Chemistry,* 40**,** 1987-1992. https://doi.org/10.1080/00021369.1976.10862346

Yahya, H, Linforth, RST & Cook, DJ 2014. Flavour generation during commercial barley and malt roasting operations: A time course study. *Food Chemistry,* 145**,** 378-387. https://doi.org/10.1016/j.foodchem.2013.08.046

Yang, B, Schwarz, P & Horsley, R 1998. Factors involved in the formation of two precursors of dimethylsulfide during malting. *Journal Of The American Society Of Brewing Chemists,* 56**,** 85-92. https://doi.org/10.1094/ASBCJ-56-0085

Yang, G & Schwarz, P 1995. Activity of lipoxygenase isoenzymes during malting and mashing *Journal of the American Society of Brewing Chemists,* 53**,** 45-49. https://doi.org/10.1094/ASBCJ-53-0045

Yang, G, Schwarz, P & Vick, BA 1993. Purification and characterization of lipoxygenase isoenzymes in germinating barley. *Cereal Chemistry,* 70**,** 589-595.

Yaylayan, VA 2003. Recent Advances in the Chemistry of Strecker Degradation and Amadori Rearrangement: Implications to Aroma and Color Formation. *Food Science and Technology Research,* 9**,** 1-6. https://doi.org/10.3136/fstr.9.1

Ye, H, Harasymow, S, Zhang, X-Q, Paynter, B, Wu, D, Jones, M, Shu, X & Li, C 2014. Sequence variation and haplotypes of lipoxygenase gene LOX-1 in the Australian barley varieties. *BMC Genetics,* 15**,** 36-36. https://doi.org/10.1186/1471-2156-15-36

Yu, J, Huang, S, Dong, J, Fan, W, Huang, S, Liu, J, Chang, Z, Tian, Y, Hao, J & Hu, SM 2014. The influence of LOX-less barley malt on the flavour stability of wort and beer. *Journal of the Institute of Brewing,* 120**,** 93-98. https://doi.org/10.1002/jib.122

Zufall, C, Racioppi, G, Gasparri, M & Franquiz, J 2005. Flavour stability and ageing characteristics of light stable beer. *Proceedings of the 30th Congress of the European Brewery Convention.* Nürnberg, Germany.