


<b>Title</b>	Development of a toolbox for the reduction of hordeins in barley malt beers
<b>Author(s)</b>	Taylor, Joshua P.
<b>Publication date</b>	2016
<b>Original citation</b>	Taylor, J. P. 2016. Development of a toolbox for the reduction of hordeins in barley malt beers. PhD Thesis, University College Cork.
<b>Type of publication</b>	Doctoral thesis
<b>Rights</b>	<p>© 2016, Joshua P. Taylor.</p> <p><a href="http://creativecommons.org/licenses/by-nc-nd/3.0/">http://creativecommons.org/licenses/by-nc-nd/3.0/</a></p> 
<b>Item downloaded from</b>	<a href="http://hdl.handle.net/10468/3241">http://hdl.handle.net/10468/3241</a>

Downloaded on 2018-08-23T18:18:03Z



**UCC**

University College Cork, Ireland  
 Coláiste na hOllscoile Corcaigh

*Ollscoil na hÉireann*  
**THE NATIONAL UNIVERSITY OF IRELAND**  
*Coláiste na hOllscoile, Corcaigh*  
**UNIVERSITY COLLEGE CORK**  
*SCHOOL OF FOOD AND NUTRITIONAL SCIENCES*



**DEVELOPMENT OF A TOOLBOX FOR THE  
REDUCTION OF HORDEINS IN BARLEY MALT BEERS**

*Thesis presented by*

**Joshua P. Taylor**

BSc (Hons) Biotechnology

*Under the supervision of*

Prof. DSc. Dr. Elke K. Arendt

*For the degree of*

**Doctor of Philosophy**

(PhD in Food Science and Technology)

Head of School - Prof. Yrjö Roos

January 2016



# Table of Contents

<b>Table of Contents .....</b>	<b>iii</b>
<b>Abstract.....</b>	<b>1</b>
<b>Acknowledgements .....</b>	<b>3</b>
<b>Declaration.....</b>	<b>4</b>
<b>Chapter 1: Introduction .....</b>	<b>5</b>
Introduction .....	6
Literature cited.....	9
<b>Chapter 2: Literature review – Gluten-free beer .....</b>	<b>11</b>
Abstract.....	12
Introduction .....	13
Conclusion and future trends .....	38
Literature cited.....	41
<b>Chapter 3: A Fundamental Study on the Relationship between Barley Cultivar and Hordeins in Single Cultivar Beers.....</b>	<b>51</b>
Abstract.....	52
Introduction .....	53
Materials and methods.....	55
Results.....	57
Discussion.....	65

Conclusions .....	71
Literature cited.....	73
<b>Chapter 4: A study on Malt Modification, used as a Tool to Reduce Levels of Beer Hordeins .....</b>	<b>76</b>
Abstract.....	77
Introduction .....	78
Materials and methods .....	79
Results and discussion.....	83
Conclusion .....	94
Literature cited.....	95
<b>Chapter 5: Reduction of Hordein content in beer by applying Prolyl- endoprotease to the malting process.....</b>	<b>98</b>
Abstract.....	99
Introduction .....	100
Materials and methods .....	102
Results and discussion.....	105
Conclusion .....	119
Literature cited.....	120
<b>Chapter 6: Fundamental study on the impact of silica gel and tannic acid on hordein levels in beer .....</b>	<b>123</b>
Introduction .....	125
Materials and methods .....	127

Results and discussion.....	131
Conclusions .....	146
Literature cited.....	148
<b>Chapter 7: Fundamental Study on the Impact of Transglutaminase on Hordein Levels in Beer .....</b>	<b>152</b>
Introduction .....	154
Materials and methods .....	156
Results and discussion.....	161
Conclusion .....	177
Literature cited.....	179
<b>Chapter 8: Overall discussion and conclusion.....</b>	<b>184</b>
Overall discussion and conclusion .....	185
Literature cited.....	201

## Abstract

Gluten sensitive consumers and people suffering from coeliac disease account for up to 6% of the general population (Catassi et al., 2013). These consumers must avoid foods which contain gluten and related proteins found in wheat, rye or barley. Beer is produced from barley malt and therefore contains hordeins, (gluten like proteins). Beers labelled as gluten-free must contain below 10 mg/kg hordeins (10 mg/kg hordeins = 20 mg/kg gluten under current regulations) to be considered safe for gluten sensitive consumers. Currently there are a limited number of methods available for reducing beer hordeins, the studies outlined in this thesis provide a range of tools for the beverage industry to reduce the hordein content of beer

It is well known, that during malting and brewing hordeins are reduced, but they still remain in beer at levels above 10 mg/kg. During malting, hordeins are broken down to form new proteins in the growing plant. Model malting and brewing systems were developed and used to test, how the modification of the malting process could be used to reduce beer hordeins. It was shown, that by using a controlled malting and brewing regime, a range of barley cultivars produced beer with significant differences in levels of hordeins. Beer hordeins ranged from 10 mg/kg to 60 mg/kg. Another study revealed that when malting was prolonged, to maximise breakdown of proteins, beer hordeins can be reduced by up to 44%. The natural breakdown of hordein during malting enhanced in a further study, when a protease was added to support the hordein degradation during steeping and germination. The enzyme addition resulted in a 46% reduction in beer hordeins

when compared to the control. All of the malt treatments had little or no impact on malt quality.

The hordein levels can also be reduced during the beer stabilisation process. Levels of beer hordein were tested after stabilisation using two different concentrations of silica gel and tannic acid. Silica gel was very effective in reducing beer hordeins, 90% of beer hordeins were removed compared to the control beer. Beer hordeins could be reduced to below 10 mg/kg and the beer qualities such as foam, colour and flavour were not affected. Tannic acid also reduced beer hordein by up to 90%, but it reduced foam stability and affected beer flavours.

A further study described treatment of beer with microbial transglutaminase (mTG), to create bonds between hordein proteins, which increased particle size and allowed removal during filtration. The addition of the mTG led to a reduction of the beer hordein by up to 96% in beer, and the impact on the resulting beer quality was minimal.

These studies provide the industry with a toolbox of methods leading to the reduction of hordein in the final beer without negatively affecting beer quality.



## Acknowledgements

First of all thanks to Elke, for giving me the opportunity to study brewing and malting which allowed me to transform my hobby into my career. Thanks for all the support and ideas during the years as well as making sure there was always funding available. Thanks to Professor Jacob for his help, expertise and technical support analysing beer samples. Much appreciation also goes to Jim McNamara for keeping the brewery in working order as well as always helping out in everyway possible. Thanks to Donal, Tom and Jimmy too for all the help.

Thanks to Lorenzo who made working in the brewery very entertaining, aromatic and interesting. It was great to have you as a friend and colleague over the last few years. Thanks also to everyone else I worked with over the years in the lab in particular: Alice, Alina, Anika, Aylin, Birgit, Claudia, Christoph, Deborah, Eilish, Emanuele, Franzi, Ines, Kieran, Manuela, Marcus, Mareile, Markus, Max, Outi, Pedro, Sophie, Stefan, Steffie, Thibaut and Wil amongst many others. It was great to work with all of you and I appreciate all your help.

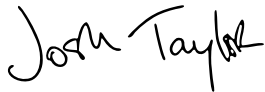
I'm always grateful to my parents Annie and Tats for all the help and support during my years in education, and my brother Reub for his critical analysis and amazing cakes.

Finally thanks to Noémie for all her support and hard work keeping me sane (ish) during my PhD and giving me a life outside of the lab

## Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

**Signature:**

A handwritten signature in black ink that reads "Josh Taylor". The signature is written in a cursive style with a large initial 'J' and a long, sweeping underline.

**Date:** 9/5/2016

# Chapter 1: Introduction

## **Introduction**

Beer has been an important part of the human diet for thousands of years and the production of beer is one of the earliest examples of biotechnology. The main ingredient in beer is malted barley and the malting of barley grain begins with a steep and then a controlled germination step. The early stages of growth in the grain kick-start the metabolic processes, which are required to turn the grain into a barley plant. Enzymes are placed into action and are actively synthesised, structures within the grain are broken down and new molecules are formed to help the barley embryo to develop. Then before all the starch reserves in the grain are used up, the germination is stopped by a well-controlled heating process (kilning). The grain is dried and cleaned and it is then ready to use for beer production. This malted grain still contains a large reserve of carbohydrate within the endosperm alongside non-starch polysaccharides and proteins. The starchy carbohydrate is the source of fermentable sugar needed for fermentation, and the enzymes which were synthesised during germination help to break starch into smaller fermentable sugars during brewing.

The proteins found within the grain are a combination of enzymes needed for embryo development and storage proteins. Up to half of all the proteins found in barley are storage proteins (Osman et al., 2002). The function of these proteins is to act as a reserve of peptides and amino acids, used by the barley embryo to grow. These storage proteins cause health problems for consumers sensitive to gluten.

Coeliac disease affects about 1% of the global population and non-coeliac gluten sensitivity affects an even larger percentage (Catassi et al., 2013; Tack, Verbeek, Schreurs, & Mulder, 2010). Gluten is a general term used for prolamin proteins

which are toxic for coeliacs. They are given different names depending on the grain they originate from (which is shown in brackets), coeliac toxic prolamins are found in rye (secalins), wheat (gliadins and glutelins), barley (hordeins) and other closely related cereals. These prolamins are not tolerated by consumers sensitive to gluten and the only current treatment is lifelong avoidance of gluten containing foods. Safe levels of gluten in foods are determined by regulations set out by the Codex Alimentarius Commission (Codex Alimentarius, 1979). The regulations state that, provided foods contain less than 20 mg/kg gluten, they can be labelled gluten-free. Levels of gluten must be determined using a suitable ELISA assay. The ELISA assay works by measuring soluble prolamins, a factor of two is then applied to calculate gluten (prolamin multiplied by two = gluten). This factor is suitable for some foods but not for others (Wieser & Koehler, 2009) and throughout this thesis results are presented as mg/kg hordein.

Beer is one of the many foods which contain gluten, normally in the form of hordeins. Although during the brewing process hordeins are considerably reduced (Celus, Brijs, & Delcour, 2006), they can persist at levels above 10 mg/kg, meaning barley based beers are not suitable for coeliacs to consume safely.

Levels of barley hordeins present in the grain vary depending on the environmental conditions during growth, and the barley cultivar (Shewry & Halford, 2002). During malting these hordeins are degraded and used up by the growing barley grain (Briggs & Hough, 1981). Although malt is the only source of hordeins in many beers there is little research on how malt production can affect beer hordeins.

There are published methods, which describe using enzymes to remove hordeins from beer (Guerdrum & Bamforth, 2012), but the use of enzymes during malting

has not been publicly reported. The only paper found in the literature focuses on the addition of cellulase, which was applied during germination to improve wort filtration and viscosity (Grujic, 1998).

Not all proteins which are found in beer affect gluten sensitive consumers. Examples of such proteins are LTP1 and Protein Z which stabilize foam (Leiper, Stewart, & McKeown, 2003) and therefore contribute to optimal beer quality (Bamforth, 1985). Other proteins in beer can form complexes with polyphenols over time, which are referred to as chill-haze. Beer stabilisation methods often remove proteins from beer to prevent chill-haze formation and therefore contribute to the increase of the beer shelf life (Siebert, Carrasco, & Lynn, 1996). The proteins involved in the formation of chill-haze are also the proteins, which cause health problems for gluten sensitive consumers (Dostalek, Hochel, Mendez, Hernando, & Gabrovska, 2006; Lewis & Bamforth, 2006; Van Landschoot, 2011). The nature of hordein proteins allow the selective removal from beer using a range of enzymes or stabilising aids

The objective of this thesis is to evaluate novel methods of hordein reduction in beer. The methods employed in this thesis to characterise malt, wort and beer quality are largely based on standard MEBAK methods of analysis. The research demonstrates there is a wide range of methods available with good potential to reduce beer hordeins. The methods developed in this thesis provide the brewing industry with a toolbox which allows them to produce gluten-free beer based on barley malt.

## Literature cited

- Bamforth, C. W. (1985). The Foaming Properties of Beer. *Journal of the Institute of Brewing*, 91(6), 370-383.
- Briggs, D. E., & Hough, J. S. (1981). Malting and brewing science (2nd ed., pp. 82, 83, 112). London, Chapman and Hall.
- Catassi, C., Bai, J. C., Bonaz, B., Bouma, G., Calabro, A., Carroccio, A., . . . Fasano, A. (2013). Non-Celiac Gluten Sensitivity: The New Frontier of Gluten Related Disorders. *Nutrients*, 5(10), 3839-3853. doi:10.3390/nu5103839
- Celus, I., Brijs, K., & Delcour, J. A. (2006). The effects of malting and mashing on barley protein extractability. *Journal of Cereal Science*, 44(2), 203-211. doi:10.1016/j.jcs.2006.06.003
- Codex Alimentarius (1979). Codex standard for foods for special dietary use for persons intolerant to gluten Codex Stan 118 - 1979
- Dostalek, P., Hochel, I., Mendez, E., Hernando, A., & Gabrovská, D. (2006). Immunochemical determination of gluten in malts and beers. *Food Additives and Contaminants*, 23(11), 1074-1078. doi:10.1080/02652030600740637
- Grujic, O. (1998). Application of a commercial enzyme preparation in the barley malting process. *Journal of the Institute of Brewing*, 104(5), 249-253.
- Guerdrum, L. J., & Bamforth, C. W. (2012). Prolamin Levels Through Brewing and the Impact of Prolyl Endoproteinase. *Journal of the American Society of Brewing Chemists*, 70(1), 35-38. doi:10.1094/Asbcj-2012-0130-01
- Leiper, K. A., Stewart, G. G., & McKeown, I. P. (2003). Beer polypeptides and silica gel - Part II. Polypeptides involved in foam formation. *Journal of the Institute of Brewing*, 109(1), 73-79.
- Lewis, M., & Bamforth, C. W. (2006). *Essays in brewing science*: Springer. New York, U.S.A.
- Osman, A. M., Coverdale, S. M., Cole, N., Hamilton, S. E., de Jersey, J., & Inkerman, P. A. (2002). Characterisation and assessment of the role of barley malt endoproteases during malting and mashing. *Journal of the Institute of Brewing*, 108(1), 62-67.
- Shewry, P. R., & Halford, N. G. (2002). Cereal seed storage proteins: structures, properties and role in grain utilization. *Journal of Experimental Botany*, 53(370), 947-958. doi:10.1093/jexbot/53.370.947
- Siebert, K. J., Carrasco, A., & Lynn, P. Y. (1996). Formation of protein-polyphenol haze in beverages. *Journal of Agricultural and Food Chemistry*, 44(8), 1997-2005. doi:10.1021/jf950716r

- Tack, G. J., Verbeek, W. H. M., Schreurs, M. W. J., & Mulder, C. J. J. (2010). The spectrum of celiac disease: epidemiology, clinical aspects and treatment. *Nature Reviews Gastroenterology & Hepatology*, 7(4), 204-213. doi:10.1038/nrgastro.2010.23
- Van Landschoot, A. (2011). Gluten-free barley malt beers. *Cerevisia*, 36(3), 93-97.
- Wieser, H., & Koehler, P. (2009). Is the calculation of the gluten content by multiplying the prolamin content by a factor of 2 valid? *European Food Research and Technology*, 229(1), 9-13. doi:10.1007/s00217-009-1020-5



## Chapter 2: Literature review – Gluten-free beer

*Anna-Sophie Hager, Josh P. Taylor, Deborah M. Waters and Elke K. Arendt*

Published as “Gluten-free beer - A review” in Trends in Food Science and Technology 36 (2014) 44 – 54

### **Author Contributions**

Dr Hager was the main author for the paper and compiled the completed final manuscript.

Joshua Taylor made significant contributions to conception of technical aspects of the review and helped writing sections as well as critically reviewing the final manuscript.

Dr Waters also made significant contributions to writing the paper as well as critically reviewing the final manuscript.

Prof Arendt helped with the conception of the paper as well as making contributions and suggestions throughout the writing process and critically reviewed the final manuscript and supervised the project.

## **Abstract**

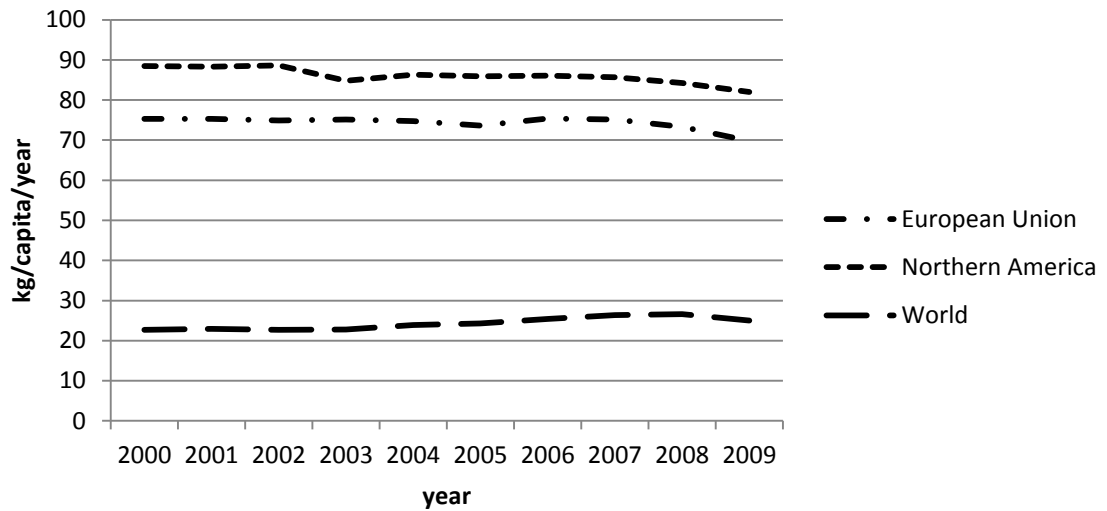
Beer is one of the most frequently consumed alcoholic beverages. However, the consumption of conventional barley beer is not safe for coeliac patients. The availability of tasty gluten-free beers significantly improves gluten-sensitive peoples' well-being. This review summarises legislation for the labelling of gluten-free products and compares state-of-the art techniques in gluten content determination. Several technical solutions exist for the reduction of hordein levels in barley based products, including precipitation and enzymatic hydrolysis. Furthermore, gluten-free beers can be produced using gluten-free cereals and pseudocereals. A third approach is the production of yeast fermented beverages based on fermentable sugars/syrups.

## **Introduction**

The gluten-free diet was introduced in the 1950's, originally as a standard therapy for coeliac disease patients (Dicke, Weijers, & Van De Kamer, 1953). It currently represents the sole treatment for this life-long autoimmune enteropathy. Damage done to the small intestine of genetically susceptible people is reversed when dietary gluten is excluded. Screening studies have revealed that coeliac disease affects about 1 - 2% of the general population in Western countries (Fasano et al., 2003; Lohi et al., 2007; Riestra, Fernandez, Rodrigo, Garcia, & Ocio, 2000; Schapira et al., 2003). A much higher percentage of the general population than this 1% consider themselves to be suffering from wheat sensitivity and exclude wheat from their diet (Catassi et al., 2013). Purchasers of gluten-free products are both diagnosed and undiagnosed individuals of above mentioned conditions as well as their relatives, and consumers who believe a gluten-free diet to be healthier or other lifestyle customers (Worosz & Wilson, 2012). Due to increased awareness and improved diagnoses, there are a growing number of individuals who desire a wider choice of better tasting gluten-free products and who are willing to pay a premium price. Hence, the production of high quality gluten-free products represents an important socio-economic issue and it is not surprising that the market has experienced significant growth over the past few years. In the years 2009-2011, sales of gluten-free foods have grown 50%, from US\$1.6 billion in 2009 to an estimated \$6.1 billion in 2011 (Spins, 2012).

Strict adherence to a gluten-free diet represents a difficult challenge for the consumer and their family and might seriously compromise the quality of life (Fera, Cascio, Angelini, Martini, & Guidetti, 2003; Ford, Howard, & Oyeboode, 2012;

Hauser, Gold, Stein, Caspary, & Stallmach, 2006; O'Leary et al., 2002). Poor availability of gluten-free products means that people end up losing the balance between health benefits and social sacrifices; often tolerating side effects such as stomach pain or diarrhoea in order to take part in popular activities like eating-out or drinking beer. For brewing, usually gluten-containing barley malts are used, with a growing proportion of beers also being produced from wheat malts. As such, beer is therefore unsuitable for consumption by coeliac disease patients. While one might argue that beer is not an essential part of human nutrition, it has to be acknowledged that an individual's diet encompasses more than just meeting the physiological need for nutrients. Beer is consumed all over the world and the average annual consumption of about 74 kg/capita in Europe and 86 kg/capita in Northern America (which includes Bermuda, Canada, Greenland, Saint Pierre and Miquelon and the United States of America) demonstrates the value of this beverage in many cultures (Fig. 2.1). Therefore, the availability of safe, healthy and tasty gluten-free beers would significantly improve peoples' well-being and perception of a normal social life.



**Figure 2.1.** Beer food supply quantity in kg/capita/year. Source: <http://faostat3.fao.org>; accessed on 5th of April 2013.

### Legal standing & labelling

For regulatory purposes “gluten” is defined as the protein fraction from wheat, rye, barley and oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant (Commision, 2009). From a scientific point of view, using the term “gluten” to describe storage proteins of rye, barley and oats is not completely correct as the coeliac-toxic fractions of these cereals are termed secalin, hordein and avenin, respectively.

To set gluten-free standards for international trade purposes, the Codex Alimentarius Commission concluded that gluten-free foods can not contain wheat, rye, barley, oats or their crossbred varieties, unless they have been specially processed to reduce the gluten level to below 20 mg/kg (Commision, 2009). The standard also states that oats can be tolerated by most but not all people who are intolerant to gluten. Therefore, the allowance of oats that are not contaminated

with wheat, rye or barley in foods covered by this standard may be determined at the national level. In the European Union foodstuffs for people intolerant to gluten, that contain a level of gluten not exceeding 100 mg/kg, may bear the term “very low gluten” (Codex Alimentarius, 1979). As stated in a final rule issued by the Food and Drug Administration (FDA), the definition of the term “gluten-free” for the labelling of foods in the U.S.A. is similar, i.e. any unavoidable presence of gluten in the food has to be below 20 mg/kg (Food and Drug Administration, 2013). A standardised method of analysis is needed to quantitatively determine gluten contents in food and beverages thus providing a basis for enforcing regulations (Thompson & Mendez, 2008).

#### **Assessment of gluten content**

Gluten peptides from wheat, rye or barley trigger the immune-mediated enteropathy of coeliac disease. Because they lack major cleaving points for gastrointestinal proteases they are highly resistant to breakdown and can reach the duodenum in an almost native state. Due to the great heterogeneity of peptides involved in the pathogenesis of coeliac disease, the characterisation of the complete repertoire of relevant epitopes has not been achieved yet (Camarca et al., 2009). The chemical diversity resulting from the different amino acid compositions makes the quantification of coeliac toxic peptide sequences a complex task. Specific detection can be achieved with immunoassays, based on specific interactions between the protein and its antibody. Several commercial test kits for quantification are available and the majority are based on ELISA (enzyme linked immunosorbent assay). The official standard method for gluten determination according to the Codex Alimentarius is an ELISA which uses the R5 antibody

(Kahlenberg et al., 2006). This antibody is capable of recognising several small repetitive coeliac toxic epitopes (QQPFP, LQPFP, QLPYP, QLPTP, QQSFP, QQTFP, PQQFP, QQPYP and PQQFP). Because the epitope QQPFP is present in wheat gliadin, barley hordein and rye secalin, R5 recognises all fractions of all three grains. A sandwich ELISA (RIDASCREEN gliadin kit) based on the monoclonal antibody R5 is available from R-Biopharm AG (Darmstadt, Germany). When choosing an assay for the determination of gluten in a fermented product such as beer, two aspects have to be taken into account; firstly, some test kits are suitable for wheat samples but are not able to accurately detect and quantify barley prolamins and; secondly, most assays cannot accurately quantify gluten that has been partially hydrolysed during production. The latter is due to the fact that certain ELISAs (sandwich method) require two antibody-binding sites (epitopes). When a protein has been partially broken down, the two epitopes can be lacking and thus gluten content is underestimated (Thompson & Mendez, 2008). Prolamins present in beer are partially hydrolysed into fragments with one or more epitopes. Consequently, these small fragments cannot be measured by the conventional sandwich R5 ELISA. Upon evaluation of above mentioned considerations, the RIDASCREEN gliadin *competitive* ELISA, also based on the R5 antibody, appears most suitable for the determination of gluten content in beer and has been independently validated and tested for testing hydrolysed prolamins (Haas-Lauterbach, Immer, Richter, & Koehler, 2012). The R5 ELISA method has been accepted by Codex alimentarius which regulates levels of allergens in food throughout Europe. All of the ELISA based methods use the assumption that prolamins multiplied by two = gluten because they detect the soluble fraction of gluten, this may overestimate gluten content in beer samples

because of the removal of insoluble proteins during the brewing process (Celus, Brijs, & Delcour, 2006; Wieser & Koehler, 2009).

Although the gluten-free industry relies on ELISA based kits for validation of the gluten-free status of beer, it has to be mentioned that to date there is no suitable single hordein standard for beer. Tanner, Colgrave, Blundell, Goswami, and Howitt (2013) showed that ELISA analysis calibrated with a single prolamin standard can lead to serious over or underestimation of the hordein content. It is difficult to identify appropriate controls because beers are often produced from a blend of barley varieties and additionally hordeins are modified during malting and brewing (i.e. hydrolysis, glycation, glycosylation, etc.) (Tanner, Blundell, Colgrave, & Howitt, 2013).

**Beers from traditional raw-materials processed to eliminate coeliac toxic proteins and peptides.**

Most beers brewed from barley or wheat based malt are generally considered unsuitable for individuals suffering from coeliac disease or gluten intolerance. However, the veracity of this conclusion has been questioned due to the modification and removal of proteins which occurs during traditional beer processing, as well as the fact that beers often contain significant quantities of gluten-free adjuncts, which serve to 'dilute' the initial raw material gluten content (Guerdrum & Bamforth, 2011). (Dostalek, Hochel, Mendez, Hernando, & Gabrovská, 2006) studied the gluten levels throughout the whole brewing process (Table 2.1).



**Table 2.1** Gluten levels throughout the brewing process

	Gluten (mg/kg) <sup>a</sup>	%	Gluten (mg/kg) <sup>b</sup>	%
<b>Malt</b>	18,780.0	100	13,664	100
<b>Sweet wort</b>	49.4	1.75	6864	50.2
<b>Wort</b>	48.0	1.70	5934	43.4
<b>Beer</b>	6.0	0.21	262	1.9
<b>Stabilised beer</b>	<3.0	<0.11	-	-

<sup>a</sup>(Dostalek et al., 2006) Samples were analysed using the RIDASCREEN Gliadin kit;

<sup>b</sup>(Guerdrum & Bamforth, 2012) Samples were analysed using the RIDASCREEN Gliadin *competitive* assay and prolamin levels were multiplied by 2.

During the mashing process when certain malt components are solubilised in water, most of the proteins are precipitated and only some are further hydrolysed into simple polypeptides. The majority of the precipitated protein remains in the spent grain after the lautering process and only a small proportion of gluten passes from malt to sweet wort (Celus, Brijs, & Delcour, 2006). Only negligible gluten depletion occurs during wort boiling. Throughout the primary and secondary fermentations, the pH decreases, causing precipitation of some polypeptides and their adsorption onto the yeast surface. As a result, only a very small percentage of the original gluten content remains in beer. Polyvinylpolypyrrolidone (PVPP) and silica gel are often used after the filtration process to stabilise beer by removing proteins and polyphenolic substances. This process also aids the elimination of coeliac toxic peptides (Dostalek et al., 2006). However, it has to be kept in mind that this study relied on an ELISA assay which is effective for intact but not hydrolysed proteins,

resulting in an underestimation of the gluten content after mashing and lautering. The significance of choosing the right assay method becomes clear when comparing results obtained by Dostalek et al. (2006) using the sandwich ELISA (RIDASCREEN Gliadin kit) to those of Guerdrum and Bamforth (2012) who used the RIDASCREEN Gliadin *competitive* assay (Table 2.1). Colgrave, Goswami, Howitt, and Tanner (2012) studied wort and beer using tandem mass spectrometry and showed that hordeins are indeed present in beer despite speculation of the contrary. In addition, multiple reaction monitoring mass spectrometry of non-barley based gluten-free beers targeting the major hordein protein families was performed and confirmed the absence of hordein in several gluten-free commercial beers (Colgrave, Goswami, Blundell, Howitt, & Tanner, 2014; Colgrave et al., 2012).

Using a sandwich ELISA, Tanner, Colgrave, et al. (2013) determined the hordein levels of 60 commercial beers. Results for four products labelled gluten-free were below the detection limit, but values up to 40,800 - 46,500 mg/kg were detected for three wheat beers. (Van Landschoot, 2011) analysed 58 commercial beers with the R5 antibody sandwich ELISA as well as the *competitive* ELISA. Using the sandwich ELISA, 83% of the beers were gluten-free. However, results suggested that not all of these are still considered gluten-free when analysed with the *competitive* ELISA. Guerdrum and Bamforth (2011) assessed a range of commercially available beers using the RIDASCREEN Gliadin *Competitive* ELISA kit. Products sold as "gluten-free" contained gliadin levels below the detection limit of 6 mg/kg gluten. With the exception of wheat beers, which tend to have significantly higher gluten contents (approx. 200 - 300 mg/kg), most of the analysed beers showed relatively low levels of gliadin. Several lager and ale samples were also

below 20 mg/kg. This leads to the question of how problematic these small amounts of gliadin-derived peptides in the beers actually are to coeliac disease patients. Even though several beers contained low gluten levels, it is unclear which quantities of these beers may be consumed by the coeliac consumer without resulting intestinal damage. During social gatherings, frequently more than one serving of beer is consumed which results in a gliadin accumulation effect (Guerdrum & Bamforth, 2011). At present, it is not clear what amounts of dietary gluten can be ingested by coeliac patients without damaging the mucosa of the small intestine, but (Catassi et al., 2007) suggested that gluten ingestion should be kept below 50 mg/day. From a labelling perspective, producers of beers made from barley, wheat or rye cannot label their product “gluten-free” unless they take measures to assure consistently low gluten levels. However, through optimised processing and the incorporation of processing aids, gluten-free status of beers produced from barley can be achieved.

### **Raw material selection**

Beer only contains about 0.2 - 0.6% protein or peptide material, originating mainly from malt (Picariello et al., 2011). Conventionally, malt is produced from the gluten containing grains barley or wheat. Comino et al. (2012) showed significant differences in coeliac immunotoxicity of barley varieties. Antibody guided searches have found wheat varieties which are naturally reduced in coeliac disease related gluten epitopes (Molberg et al., 2005; Spaenij-Dekking et al., 2005; van den Broeck et al., 2010). Thus, one possible method to produce low gluten beers is to select grains with fewer immunogenic epitopes for brewing grist production. Howitt (2014) patented a method for producing food or malt-based beverages with low

levels of hordeins suitable for coeliac patients. The invention is based on the observation that barley hordein production can be partly or fully abolished whilst still obtaining viable seeds which are able to germinate and produce barley plants in the field, despite the loss of the major storage form of nitrogen in the seed.

Dostalek et al. (2006) determined the gluten content of different commercial malt types and showed that the level of gluten varies significantly between the different samples. Values ranged from 19,000 mg/kg for Pilsner barley malt to 45,000 mg/kg for Carafa barley malt. Therefore, when gluten-free beer is produced from traditional raw materials through the elimination of toxic proteins and peptides, the right choice of malt facilitates this process.

### **Precipitation of hordeins**

Haze-active proteins in beer are largely derived from the proline-rich barley hordeins. Therefore, substances commonly used in brewing to remove these haze-active proteins have the potential to reduce gluten levels to below 20 mg/kg. These brewing aids include tannins and silica hydrogels. (Dostalek et al., 2006) used PVPP and silica gel for beer stabilisation and reported that the final product contained only 0.11% of the original gluten content. Silica gel binds to proline, which is present at high levels in hordein (Siebert & Lynn, 1997). Van Landschoot (2011) reported that by using tannins and enzymes even 100% barley malt beers can obtain gluten-free status. Hordeins and tannins form protein-polyphenol complexes held together by a combination of hydrogen and/or hydrophobic bonding (Siebert, 1999), which can then be removed by filtration.

### **Enzymatic Treatments**

Prolyl endoprotease (EC 3.4.21.26, also known as postproline endopeptidase or prolyl oligopeptidase) is an enzyme that specifically hydrolyses peptide linkages beside a proline residue. Therefore, it was hypothesised that it can be used to degrade the peptide sequences responsible for coeliac disease, as all of these toxic epitopes are proline-rich. Indeed, research published by (Van Landschoot, 2011) reported that 100% barley malt beers can be rendered gluten-free using prolyl endoprotease.

Guerdrum and Bamforth (2012) explored the impact of prolyl endoprotease derived from *Aspergillus niger* (AN-PEP) on the prolamin levels of beers produced from conventional malts. This enzyme, currently used in the brewing industry as a means of haze prevention, breaks down the proline-rich prolamin fraction of gluten. The authors concluded that this exogenous enzyme, when added during fermentation or to the finished product, renders beer essentially free of gluten, without negatively impacting foam stability. Tanner, Colgrave, and Howitt (2014) also confirmed the removal of gluten was successful by using mass spectrometry methods.

Pasternack, Marx, and Jordan (2008) patented a process for the production of prolamin reduced beverages, involving cross-linking enzymes and removal of the modified prolamin.

Apart from microbial enzymes, a range of endogenous seed proteases in cereals are known to destroy immunotoxic gluten epitopes. Germination provides the necessary hydrolytic enzymes to modify the grain and degrade storage proteins such as hordeins. Hartmann, Koehler, and Wieser (2006) demonstrated that proteases from germinated wheat, rye and barley rapidly cleave coeliac toxic

peptides into non-toxic fragments with less than nine amino acids. Knorr, Kerpes, et al. (2015); (Knorr, Wieser, & Koehler, 2015) produced an extract from barley malt that had increased peptidase activity and used it to produce gluten-free wort and beer. Stenman et al. (2009) showed that proteases from germinating wheat reduced the toxicity of pepsin and trypsin digested gliadin in vitro. However, in this study the immune response was only diminished and not eliminated because degradation of the toxic peptides was incomplete. Also glutamine-specific endoprotease two (EP-B2) from barley has shown promise for this purpose (Gass, Bethune, Siegel, Spencer, & Khosla, 2007). Luoto et al. (2012) studied whether malts from wheat, rye or barley differ in their auto-proteolytic potential regarding prolamin hydrolysis and showed that barley is more resistant to hydrolysis, probably due to steric hindrance by their more complex secondary structure. The same authors demonstrated that while the produced malt hydrolysates have substantially lower prolamin levels than the native malts, they are still too high to allow “very low in gluten” labelling (above 100 mg/kg gluten). Therefore, the authors further eliminated residual levels of toxic prolamin epitopes using prolyl endoprotease derived from *A. niger*.

Walter, Wieser, and Koehler (2014) demonstrated the versatility of AN-PEP to degrade gluten by treating wheat starch containing various levels of gluten. This AN-PEP treatment reduced the level of gluten from 2070 mg/kg to below 20 mg/kg. An alternative approach is the digestion of gluten peptides with bacterial derived peptidases during food or beverage processing (Caputo, Lepretti, Martucciello, & Esposito, 2010). The initial gluten contamination of 400 mg/kg in a gluten-free recipe was decreased to below 20 mg/kg by sourdough lactobacilli (Di Cagno et al.,

2008). Additionally, fermentation of dough by selected lactobacilli was proven as a potential tool to decrease the risks associated with rye contamination in gluten-free products (De Angelis et al., 2006). Rizzello et al. (2007) showed that fermentation with a mixture of sourdough lactobacilli together with fungal proteases decreased the concentration of gluten to below 10 mg/kg. It is important to note that the detoxifying effect of these microorganisms was observed in wheat and rye dough systems and results may differ when applying above mentioned techniques to barley wort systems. Although reported data suggests that fermentation with a mixture of selected lactic acid bacteria reduces toxicity, the flavour and aroma compounds produced by these microorganisms may have an effect on the taste of beer.

#### **Beers produced from alternative cereals or pseudocereal materials**

For naturally gluten-free beer, grains such as rice, corn, sorghum or millet are used as raw materials. These grains are only distantly related to wheat, rye and barley and therefore their consumption is safe for coeliac patients. Other starch-rich raw materials commonly used for food production are the so called “pseudocereals” quinoa, buckwheat and amaranth. They do not belong to the Poaceae (grass family), are therefore taxonomically unrelated to wheat and can hence be considered gluten-free. A detailed general review of these cereals and pseudocereals and their utilisation in the beverage industry can be found in a book by Arendt and Zannini (2013). The present review focuses on their potential for the production of gluten-free beers. Barley variety, the malting protocol and various brewing parameters such as temperature and pH of mashing, sparging, boiling, fermentation conditions, yeast strain used, pitching rate, temperature, pressure,

aeration, agitation and stirring as well as storage and ageing conditions influence the type and quality of beer (Sohrabvandi, Mortazavian, & Rezaei, 2012). Hence, it is not surprising that these parameters have to be adjusted when replacing barley with gluten-free raw materials. Gluten-free grains often require prolonged germination times compared to barley. However, excessive time under germination conditions can result in mould growth and a higher malting loss (Usansa et al., 2011). Frequently, a lack of suitability of gluten-free cereal malts for brewing is observed in comparison to barley malt, therefore the use of industrial enzyme preparations or gluten-free adjuncts, such as invert sugar syrup, agave syrup or maize grits, may be necessary (Kiss, Vecseri-Hegyessy, Kun-Farkas, & Hoschke, 2011). For yeast to produce carbon dioxide and ethanol from cereals, the starch must be saccharified, i.e. converted to simple sugars (glucose, maltose, and maltotriose), by the malt amylases, which are collectively referred to as the “malt diastatic system” (Delcour & Hoskeney, 2010). To render starch easily accessible to enzymes such as  $\alpha$ - and  $\beta$ -amylases, starch generally must first be gelatinised. The temperature at which gelatinisation commences varies depending on the starch properties of the raw material utilised. For barley, this temperature lies at around 63°C, while most gluten-free cereals gelatinise at significantly higher temperatures (Table 2.2).



**Table 2.2 Gelatinisation temperatures of milled wheat and gluten-free grains as determined by differential scanning calorimetry (Hager and Arendt, unpublished data)**

<b>Gelatinisation</b>	<b>Onset</b>	<b>Peak</b>	<b>End</b>
<b>T [°C]</b>			
<b>Wheat</b>	55 ± 1	61 ± 0	66 ± 0
<b>Rice</b>	61 ± 0	67 ± 0	72 ± 0
<b>Oat</b>	51 ± 0	56 ± 0	62 ± 1
<b>Quinoa</b>	52 ± 1	58 ± 0	64 ± 0
<b>Buckwheat</b>	59 ± 0	66 ± 0	72 ± 1
<b>Sorghum</b>	64 ± 0	69 ± 0	73 ± 0
<b>Maize</b>	64 ± 1	70 ± 0	75 ± 1
<b>Teff</b>	66 ± 1	71 ± 0	76 ± 1

In the case of most gluten-free malts, the gelatinisation point is above the optimal range of  $\beta$ -amylase (62-65°C), resulting in enzyme deactivation before starch saccharification occurs. Hence a temperature has to be chosen at which the majority of starch can gelatinise, yet amylolytic enzymes are not heat inactivated. Alternatively, exogenous industrial enzymes can be used to facilitate the process. This brings the advantage that the time point of enzyme addition can be chosen and the temperature regime can be adapted to the optimal temperatures of these enzymes.

During mash filtration/lautering the undissolved substances, referred to as “spent grain” are separated from the liquid “wort”. When mashing gluten-free malts, the

separation of supernatant and solids can pose difficulties (Ceppi & Brenna, 2010a) and often is addressed by use of a mash filter.

Probably the most commonly used gluten-free grain, industrially and for research purposes, is rice (*Oryza sativa*). Rice is a cheap nutrient source, it consists of about 80% starch and its proteins are not considered coeliac toxic. In spite of its high gelatinisation temperature, dehulled unmalted rice is often used as an adjunct in brewing, after a pre-cooking stage. However, there is little information available concerning malting and brewing with 100% rice. Usansa et al. (2011) optimised the malting conditions of black waxy (high amylopectin) and non-waxy rice (low amylopectin). The rice malts obtained had much lower extract contents, showed poorer  $\beta$ -amylase activities but higher amounts of limit-dextrinase and  $\alpha$ -amylase activities, than barley malt. Ceppi and Brenna (2010b) evaluated different rice varieties for their suitability to produce rice malt and showed that a good rice malt could be obtained, but it had a lower enzymatic activity than barley malt. Compared to barley malt, rice malts had a lower soluble protein percentage and a low Kolbach index, i.e. soluble/total protein ratio, which means that they were poorly modified during extraction. The same authors then prepared a beer-like beverage with rice malt as the only ingredient, apart from hops, yeast and water (Ceppi & Brenna, 2010a). They reported reduced brewhouse yields when compared to those obtained with barley malt, as incomplete saccharification caused a lower yield, which was further reduced by a more difficult filtration process. Upon fermentation with lager yeast, beers of acceptable final gravity (3.1 - 3.6°P) and alcohol content (3.6 - 4.5%) were obtained. A rice beer called "Zutho" is a traditional alcoholic beverage produced from sprouted rice in rural areas of India. It

is a whitish porridge-like slurry containing 5.0% (v/v) alcohol. It has a fruity aroma, sour taste and its aroma characteristics are similar to those of Japanese sake (Teramoto, Yoshida, & Ueda, 2002). Sorghum (*Sorghum bicolor*) and maize (*Zea mays*) are two closely related species. The latter is grown worldwide and ranks third only to wheat and rice in world grain consumption statistics (FAO, 2013). Even though maize supplies many micro- and macro-nutrients necessary for human metabolism, the amounts of some essential nutrients are inadequate (Nuss & Tanumihardjo, 2010). Although the proximate composition and nutritional value of sorghum is similar to that of maize, its proteins are less digestible (Wrigley, Corke, & Walker, 2004). Sorghum (*Sorghum bicolor*) is a cereal of remarkable genetic variability, with particularly waxy varieties being suitable for industrial brewing. Waxy varieties contain high levels of amylopectin and low amounts of amylose (up to 95% amylopectin of total starch) and, due to the former polymer's physicochemical properties, can gelatinise more rapidly. These sorghums are more susceptible to hydrolysis by amylolytic and proteolytic enzymes (Del Pozo-Insfran, Urias-Lugo, Hernandez-Brenes, & Saldivar, 2004; Goode, Halbert, & Arendt, 2003; Obeta, Okungbowa, & Ezeogu, 2000). The two most important differences between sorghum and barley are the significantly higher gelatinisation temperatures of the starch (Table 2.2) and the lower level of  $\beta$ -amylase activity in sorghum malt. Sorghum beer is produced in many African countries using *Saccharomyces cerevisiae* and *Lactobacillus* cultures (Lyumugabe, Gros, Nzungize, Bajyana, & Thonart, 2012). It is produced by souring (lactic acid bacteria fermentation), cooking, mashing, straining and fermenting (yeast fermentation). The souring process is carried out by inoculating a water suspension of ground malt with

*Lactobacillus leichmannii*. When the desired degree of acidification is reached, water and adjuncts are added. This sour is then cooked to gelatinise the starch of the unmalted grains. During the mashing process sorghum malt is added. After a straining step, yeast fermentation is carried out over a few days (Zweytick, Sauerzopf, & Berghofer, 2005). Compared to conventional beer, sorghum beer is a rather viscous beverage. The taste is slightly sweetish and due to the formation of lactic acid, it can be a little sour. Its colour can be yellowish, when sorghum malt and millet are used for brewing, or pinkish, when sorghum malt and maize are used. The depth of the colour depends on the pH of the product (Zweytick & Berghofer, 2009).

The fundamentally different cell wall compositions of gluten-free grains such as maize or sorghum may also pose problems during malting. Cell walls of barley are mainly composed of  $\beta$ -glucans, whereas in sorghum and maize the much more complex water-insoluble glucurono-arabinoxylans predominate. Glucurono-arabinoxylans are much more complex and highly substituted compared to arabinoxylans found in barley (Verbruggen, Beldman, & Voragen, 1995). The resistance of these cell walls to enzymatic attack during germination inhibit the access of amylolytic enzymes to the starch inside the cells during the brewing process (Taylor, Schober, & Bean, 2006) when supplementing the sorghum malt with cell wall degrading enzymes such as xylanases, arabinofuranosidases and glucuronidase, Verbruggen et al. (1995) observed that glucurono-arabinoxylans were partially solubilised during mashing but only partly degraded. Several researchers have observed that steeping gluten-free grains such as sorghum in dilute sodium hydroxide can give malts with improved diastatic power, FAN, protein

and carbohydrate mobilisation, as well as reduced malting loss (Ezeogu & Okolo, 1999; Okolo & Ezeogu, 1996a, 1996b; Rojas-Molina et al., 2007). A possible explanation for these findings is that alkali destroys the molecular structure of the non-starch polysaccharides found in these cell walls. In addition, alkaline steeping also prevents microbial spoilage which is a common problem when malting gluten-free cereals (De Meo et al., 2011). An optimization of conditions for mashing with unmalted sorghum and commercial enzymes was performed by (Goode et al., 2003). The optimization results suggested that the potential for brewing a high-quality beer from unmalted sorghum could be improved by adjusting the calcium content of the mash-in liquor to 200 mg/kg, adjusting the mash-in pH to 6.5, using a heat-stable  $\alpha$ -amylase, a neutral protease and a fungal  $\alpha$ -amylase.

Maize is nowadays well integrated into the brewing process of sorghum beer. Its suitability for brewing as a malted grain is poor; therefore it is mainly used as an adjunct. However, Zweytick and Berghofer (2009) produced maize malt on a pilot scale to brew bottom-fermented beer using 100% maize. The authors reported that the resulting beer was clear, light yellow in colour, with good foam stability, and boasting a taste comparable to that of conventional beer (Zweytick & Berghofer, 2009). The relatively low price of maize and rice, in comparison the other GF grains, makes them the most commonly used gluten-free raw materials in brewing and indeed other GF food applications.

Pearl millet and finger millet, like sorghum, have highly resistant endosperm cell walls and high gelatinisation temperatures (Zweytick & Berghofer, 2009). Pelembe, Dewar, and Taylor (2002) compared pearl millet to sorghum malt and reported similar levels of free amino nitrogen, diastatic power and comparable malting loss.

In contrast to sorghum brewing which is done in Africa on a large, commercial scale since the late 1980's (Ilori, Makinwa, & Irefin, 1996), millet malting and brewing is still at the experimental stage (Taylor et al., 2006). Eneje, Obiekezie, Aloh, and Agu (2001) compared infusion, double decoction and decantation methods for mashing of millet malt and found that the latter was most suitable as it produced the highest extract contents. However, using the decantation procedure, lower levels of free amino nitrogen were obtained and wort filtered more slowly. Nzelibe and Nwasike (1995) compared malting and brewing characteristics of two millet varieties (*Pennisetum typhoides* and *Digitaria exilis*) to those of sorghum and observed that the development of hydrolytic enzymes was significantly higher in the two millet varieties. All three malts produced worts suitable for conventional brewing. Although high levels of starch degrading enzymes are present in *D. exilis*, the authors concluded that due to high malting losses, the use of this grain is uneconomical. However, a blend of *D. exilis* with pearl millet or sorghum produces malt comparable to barley malt. Chiba et al. (2012) showed that proso millet and sorghum produce wide spectra of substrates (sugars and amino acids) when malted and mashed. Zarnkow, Kessler, Back, Arendt, and Gastl (2010) optimised the malting conditions of proso millet and Zarnkow, Kessler, et al. (2007) optimised the mashing procedure for 100% malted proso millet. In another publication by Zarnkow, Faltermaier, Back, Gastl, and Arendt (2010) a variety of top-fermenting yeasts were used to brew beer from proso millet malt.

Teff, a small seeded tropical grain (*Eragrostis tef*), can be considered a minor crop when compared to the former discussed millets. The small-seeded annual grass falls into the group of millet and originated in Ethiopia where it is used for the

production of several types of flat breads or a local beer called Shamit (Tatham et al., 1996). Gebremariam, Zarnkow, and Becker (2014) reviewed the potential of teff for malting and brewing processes and concluded that they have not been intensively investigated except for a study by Zarnkow et al. (2008), where four different teff varieties were used to obtain malts.

Amaranth is a foxtail plant which was a basic food in pre-Columbian times is currently an underutilised crop, mainly grown in the Andes. This pseudocereal has very small seeds, low amylase content and a high gelatinisation temperature. Beer from 100% amaranth malt was produced in a previous study, resulting in a slightly opaque and yellow product which was excessively bitter to taste. Additionally, beer foam stability was reported to be unsatisfactory (Zweytick & Berghofer, 2009).

Quinoa (*Chenopodium quinoa*) is a typical crop of the Andean region. It has been recognised as an extremely nutritious grain, due to the good quality and high quantity of its protein and essential fatty acids (Wrigley et al., 2004). To the authors' knowledge, only few publications exist on the utilisation of this grain for brewing purposes. Zarnkow, Geyer, et al. (2007) investigated the influence of degree of steeping as well as germination time and temperature on the quality of quinoa malt and developed an optimised malting procedure. Quinoa beer was produced by (Zweytick et al., 2005) and the authors reported a slightly opaque yellow product with acceptable foam and taste. A patent by Kamelgard (2012) describes a method for the production of a yeast fermented beverage based on malted quinoa, where quinoa is pre-conditioned to remove off-flavours. Quinoa has a high proportion of D-xylose, maltose and fructose, suggesting that it is suitable for the production of malt based beverages (Ogungbenle, 2003). However, the

suitability of quinoa for malting is limited by its very small grain size and the significantly lower enzyme activities compared to wheat or barley. Also radicle growth is rapid resulting in high malting losses.

Optimised malting and mashing conditions for 100% buckwheat malt have been described in a number of publications (Phiarais, Schehl, Oliveira, & Arendt, 2006; Wijngaard, Ulmer, & Arendt, 2005, 2006; Wijngaard, Ulmer, Neumann, & Arendt, 2005) that by using commercial enzymes, the production of wort from 100% buckwheat malt is feasible. These authors showed that the utilisation of commercial cellulase, amyloglycosidase and  $\alpha$ -amylase can sufficiently increase extract levels, fermentability, total fermentable extract, total soluble nitrogen, free amino nitrogen (FAN) and Kolbach index. In a further study, Phiarais et al. (2010) brewed top fermented beer from 100% buckwheat malt. They reported difficulties with lautering and filtration, but the resulting beer was comparable to wheat beer with regards to pH, FAN, fermentability and total alcohol. However, the extract of buckwheat wort was lower. Sensory analyses indicated that these buckwheat beers were acceptable regarding odour, purity of taste, mouthfeel, tingling and bitterness. A patent by Maccagnan, Pat, Collavo, Ragg, and Bellini (2004) describes the procedure for obtaining gluten-free beer with organoleptic properties similar to beer made from barley. A mixture composed of buckwheat (40 – 60%) and syrup obtained by the hydrolysis of gluten-free starch (20 – 60%) is used as starting material.

The status of oats (*Avena sativa*) in the gluten-free diet is controversial. Most but not all people with intolerance to gluten can include oats in their diet without adverse effect on their health (Commision, 2009). In former times, oats represented



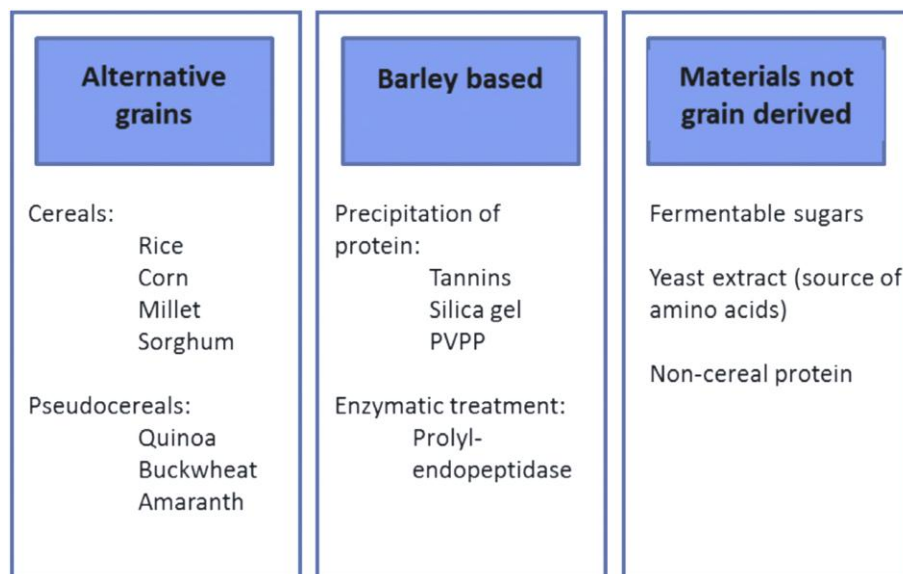
one of the most important cereals and was also used for brewing purposes. However, the utilisation of oat can lead to astringent and bitter tasting beers. Mutioz-Insa, Gastl, Zarnkow, and Becker (2011) studied the influence of germination time and temperature, as well as degree of steeping, on the quality of two oat cultivars in order to optimise the malting process. These authors concluded that oats are an alternative cereal with potential as a raw material for malting and brewing purposes. Hubner, O'Neil, Cashman, and Arendt (2010) studied the influence of germination time on protein breakdown of buckwheat and oat. They found that in oat malts, total nitrogen was not affected, however levels of soluble nitrogen increased with prolonged germination times. Protease activity in oat malts was strongly increased by choosing appropriate germination conditions and using capillary electrophoresis it could be shown that protein breakdown is more pronounced in samples germinated for longer times in both grains. The synthesis and changes of oat proteins during germination were reviewed by Klose and Arendt (2012). Hubner et al. (2010) studied the changes on the contents of some bioactive compounds in oats caused by varying germination conditions. Slight changes in the mineral content were observed, mainly caused by steeping. Degradation of phytate in oats was significantly enhanced by prolonging the germination period. It was possible to retain the amounts of soluble dietary fibre, when short germination periods were applied, although this may not be desired in beer it is interesting for other food applications. However, long germination periods caused an extensive breakdown of soluble dietary fibre, especially beta-glucan. The content of insoluble fibre was increased by applying long germination periods. Klose, Thiele, and Arendt (2010) investigated the changes of the protein profile of oats during brewing and

fermentation by means of two dimensional gel and capillary electrophoresis. Compared to barley beer, oat beers showed similar protein profiles. This is interesting as protein distribution is very different in barley and oats (i.e. mainly prolamins and glutelins in barley and mainly globulins in oats). Huebner, Schehl, Thiele, and Arendt (2009) investigated the impact of germination time and temperature during malting on the quality of oat malt. They found that activities of  $\alpha$ - and  $\beta$ -amylase and proteases were affected by germination time, whereas  $\beta$ -glucanase activity was not significantly influenced. Fermentability of Congress mash worts increased with prolonged germination times to maximum values and then declined. High viscosities and low extract contents of oat malt remained unaffected by the varied germination parameters. Optimal germination conditions were observed for germination times between 88 and 124 h at temperatures between 19 and 20°C or at 10°C, yielding malts with fermentability and soluble nitrogen in the range expected for barley malt but slightly less free amino nitrogen. Klose et al. (2011) brewed 100% oat malt beer. Oat wort was not able to reach the same final attenuation and alcohol values as wort produced from barley and the pH did not drop as low as in barley beers. The colour of the oat beer was slightly different from the barley control and foam stability was relatively poor. However, the resulting 100% oat malt beers were comparable to barley malt beers. In addition, flavour analyses of oat beer revealed some special characteristics such as a strong berry flavour and a better reaction towards staling. The authors found that due to a higher husk content, oat mash lautered faster than barley mashes.

**Beers derived from fermentable sugar and excluding grain-derived materials**

A third approach for the production of gluten-free beers is the use of materials that do not contain cereal proteins. Into this category fall Japanese products, which are based on fermented sugar syrups, with yeast extract as source of amino acids, using hop materials for flavouring, caramel for colour and protein from peas, soybeans or corn (Nakatani, 2007). The patent of Klisch (2009) describes production of gluten-free beer by dissolving an enzyme hydrolysed maltose syrup, from rice or sorghum or a combination thereof, in water to produce an aqueous solution. A yeast nutrient, protein coagulant and hops are added to form an aqueous brew which is then fermented by the addition of yeast to produce gluten-free beer. Additionally, Scott (2005) provides a method to produce a liquid base facilitating gluten-free beer brewing. This mixture may include filtered water and at least two sugar sources, such as honey and molasses. The liquid mixture may also include different hops varieties as bittering agents or may further include a protein coagulant, a yeast nutrient which is then fermented by yeast cells.

**Figure 2.2** Different approaches for the production of gluten-free beer



## **Conclusion and future trends**

Fig. 2.2 summarises the different strategies of producing gluten-free beers. For barley and wheat grist based beers, malting and brewing processes are well established. However, this is not the case for alternative cereal or pseudocereal substrates, which is a serious limiting factor in the production of gluten-free alternative beers. Scientific and industry interest in this growing area has peaked in the last decade, as supported by the relatively higher number of publications during that time. As mentioned, knowledge on the use of alternative cereals or pseudocereals for beer production is still limited. Apart from the fact that few publications exist on brewing with this raw materials, many of the gluten-free grains are generally poorly understood and hence fundamental studies still have to be carried out in order to optimise their use (e.g. optimal pH and temperatures of enzyme activities, potential haze forming compounds, flavour profile, foaming properties of proteins). Due to the diverse composition and physico-chemical properties of millet, rice, sorghum, maize or pseudocereals such as quinoa, teff or amaranth, their utilisation often results in products which differ significantly in taste and quality from beverages derived from their barley counterparts, which is negatively perceived by consumers and producers alike. Therefore, inventive food technology-based solutions are needed to counterbalance undesirable effects and result in a more pleasant beer.

One potential solution is to test several combinations of grain-based raw materials to get a final brewed product which closely resembles traditional beers. However, this over simplistic methodology often needs further scientific input to be successful. Another option is the use enzymes or processing aids to render barley or

wheat malt-based beers gluten-free (i.e. <20 mg/kg detected by ELISA). Additionally, the breeding of barley varieties which contain little or undetectable coeliac toxic epitopes for brewing purposes has potential for success in this field and warrants further research attention. Regarding taste and aroma, barley based gluten-free beers are certainly most similar to conventional beers, while beers made from alternative raw-materials often show distinct flavour profiles.

Another issue in producing gluten-free beers, whether based on barley, wheat or non-coeliac toxic grain raw materials, is to develop and improve standards for the determination of gluten levels in beer. Comparisons between ELISA and other methods such as mass spectrometry have raised concerns regarding accuracy and repeatability of the standard method currently suggested by the Codex Alimentarius.

The majority of gluten-free beers sold currently on the market are produced by small local specialised breweries. Due to increasing demand, it is likely that this area will also be investigated by multinational breweries who want to acquire their share of this profitable and growing market in the near future. Examples are gluten-free sorghum beer which is produced and sold in the U.S. (Redbridge, Anheuser-Busch InbeV) or barley based gluten-free beer (Estrella Damm Daura, S.A. Damm). The gluten-free market is one of the fastest growing food sectors and hence more gluten-free products including beers will become available within the next few years, providing a greater variety for coeliac patients and other customers. Furthermore, this expanding market creates an ideal niche for arable crop farmers to increase production of alternative cereals and stimulate supply chain

diversification. Supplying beer of high quality and safety to the customer while having an economically feasible process will remain a challenge for breweries.

## Literature cited

Food and Drug administration Final Rule 78 FR 47154 on food labelling; gluten-free labeling of foods of 5 August 2013, (2013).

Arendt, E. K., & Zannini, E. (2013). *Cereal grains for the food and beverage industries*. Cambridge, UK: Woodhead Publishing Limited.

Camarca, A., Anderson, R. P., Mamone, G., Fierro, O., Facchiano, A., Costantini, S., . . . Gianfrani, C. (2009). Intestinal T Cell Responses to Gluten Peptides Are Largely Heterogeneous: Implications for a Peptide-Based Therapy in Celiac Disease. *Journal of Immunology*, *182*(7), 4158-4166. doi:10.4049/jimmunol.0803181

Caputo, I., Lepretti, M., Martucciello, S., & Esposito, C. (2010). Enzymatic strategies to detoxify gluten: implications for celiac disease. *Enzyme Res*, *2010*, 174354. doi:10.4061/2010/174354

Catassi, C., Bai, J. C., Bonaz, B., Bouma, G., Calabro, A., Carroccio, A., . . . Fasano, A. (2013). Non-Celiac Gluten Sensitivity: The New Frontier of Gluten Related Disorders. *Nutrients*, *5*(10), 3839-3853. doi:Doi 10.3390/Nu5103839

Catassi, C., Fabiani, E., Iacono, G., D'Agate, C., Francavilla, R., Biagi, F., . . . Fasano, A. (2007). A prospective, double-blind, placebo-controlled trial to establish a safe gluten threshold for patients with celiac disease. *American Journal of Clinical Nutrition*, *85*(1), 160-166.

Celus, I., Brijs, K., & Delcour, J. A. (2006). The effects of malting and mashing on barley protein extractability. *Journal of Cereal Science*, *44*(2), 203-211. doi:10.1016/j.jcs.2006.06.003

Ceppi, E. L. M., & Brenna, O. V. (2010a). Brewing with Rice Malt - A Gluten-free Alternative. *Journal of the Institute of Brewing*, *116*(3), 275-279.

Ceppi, E. L. M., & Brenna, O. V. (2010b). Experimental Studies To Obtain Rice Malt. *Journal of Agricultural and Food Chemistry*, *58*(13), 7701-7707. doi:10.1021/jf904534q

Chiba, Y., Bryce, J. H., Goodfellow, V., MacKinlay, J., Agu, R. C., Brosnan, J. M., . . . Harrison, B. (2012). Effect of Germination Temperatures on Proteolysis of the Gluten-Free Grains Sorghum and Millet during Making and Mashing. *Journal of Agricultural and Food Chemistry*, *60*(14), 3745-3753. doi:10.1021/jf300965b

Colgrave, M. L., Goswami, H., Blundell, M., Howitt, C. A., & Tanner, G. J. (2014). Using mass spectrometry to detect hydrolysed gluten in beer that is

responsible for false negatives by ELISA. *Journal of Chromatography A*, 1370, 105-114. doi:10.1016/j.chroma.2014.10.033

Colgrave, M. L., Goswami, H., Howitt, C. A., & Tanner, G. J. (2012). What is in a Beer? Proteomic Characterization and Relative Quantification of Hordein (Gluten) in Beer. *Journal of Proteome Research*, 11(1), 386-396. doi:10.1021/pr2008434

Comino, I., Real, A., Gil-Humanes, J., Piston, F., de Lorenzo, L., Moreno, M. D., . . . Sousa, C. (2012). Significant differences in coeliac immunotoxicity of barley varieties. *Molecular Nutrition & Food Research*, 56(11), 1697-1707. doi:10.1002/mnfr.201200358

Codex Alimentarius (1979). Codex standard for foods for special dietary use for persons intolerant to gluten Codex Stan 118 - 1979.

De Angelis, M., Coda, R., Silano, M., Minervini, F., Rizzello, C. G., Di Cagno, R., . . . Gobbetti, M. (2006). Fermentation by selected sourdough lactic acid bacteria to decrease coeliac intolerance to rye flour. *Journal of Cereal Science*, 43(3), 301-314. doi:10.1016/j.jcs.2005.12.008

De Meo, B., Freeman, G., Marconi, O., Booer, C., Perretti, G., & Fantozzi, P. (2011). Behaviour of Malted Cereals and Pseudo-Cereals for Gluten-Free Beer Production. *Journal of the Institute of Brewing*, 117(4), 541-546.

Del Pozo-Insfran, D., Urias-Lugo, D., Hernandez-Brenes, C., & Saldivar, S. O. S. (2004). Effect of amyloglucosidase on wort composition and fermentable carbohydrate depletion in sorghum lager beers. *Journal of the Institute of Brewing*, 110(2), 124-132.

Delcour, J. A., & Hosney, R. C. (2010). *Principles of cereal science and technology*. AACC International Press.

Di Cagno, R., Rizzello, C. G., De Angelis, M., Cassone, A., Giuliani, G., Benedusi, A., . . . Gobbetti, M. (2008). Use of selected sourdough strains of *Lactobacillus* for removing gluten and enhancing the nutritional properties of gluten-free bread. *Journal of Food Protection*, 71(7), 1491-1495.

Dicke, W. K., Weijers, H. A., & Van De Kamer, J. H. (1953). Coeliac disease. II. The presence in wheat of a factor having a deleterious effect in cases of coeliac disease. *Acta paediatrica*, 42(1), 34-42.

Dostalek, P., Hochel, I., Mendez, E., Hernando, A., & Gabrovska, D. (2006). Immunochemical determination of gluten in malts and beers. *Food Additives and Contaminants*, 23(11), 1074-1078. doi:10.1080/02652030600740637



- Eneje, L. O., Obiekezie, S. O., Aloh, C. U., & Agu, R. C. (2001). Effect of milling and mashing procedures on millet (*Pennisetum maiwa*) malt wort properties. *Process Biochemistry*, *36*(8-9), 723-727. doi:Doi 10.1016/S0032-9592(00)00268-5
- Ezeogu, L., & Okolo, B. N. (1999). Relationship between some sorghum malt quality characteristics and nature of alkaline steep liquor. *Journal of the Institute of Brewing*, *105*(1), 49-54.
- Fasano, A., Bertl, I., Gerarduzzi, T., Not, T., Colletti, R. B., Drago, S., . . . Horvath, K. (2003). Prevalence of celiac disease in at-risk and not-at-risk groups in the United States - A large multicenter study. *Archives of Internal Medicine*, *163*(3), 286-292. doi:DOI 10.1001/archinte.163.3.286
- Fera, T., Cascio, B., Angelini, G., Martini, S., & Guidetti, C. S. (2003). Affective disorders and quality of life in adult coeliac disease patients on a gluten-free diet. *European Journal of Gastroenterology & Hepatology*, *15*(12), 1287-1292. doi:10.1097/01.meg.0000085512.01212.c5
- Food and Drug Administration, Final Rule 78 FR 47154 on food labelling; gluten-free labeling of foods ,of 5 August 2013, (2013).
- Ford, S., Howard, R., & Oyebode, J. (2012). Psychosocial aspects of coeliac disease: A cross-sectional survey of a UK population. *British Journal of Health Psychology*, *17*, 743-757. doi:10.1111/j.2044-8287.2012.02069.x
- Gass, J., Bethune, M. T., Siegel, M., Spencer, A., & Khosla, C. (2007). Combination enzyme therapy for gastric digestion of dietary gluten in patients with celiac sprue. *Gastroenterology*, *133*(2), 472-480. doi:10.1053/j.gastro.2007.05.028
- Gebremariam, M. M., Zarnkow, M., & Becker, T. (2014). Teff (*Eragrostis tef*) as a raw material for malting, brewing and manufacturing of gluten-free foods and beverages: a review. *Journal of Food Science and Technology-Mysore*, *51*(11), 2881-2895. doi:10.1007/s13197-012-0745-5
- Goode, D. L., Halbert, C., & Arendt, E. K. (2003). Optimization of mashing conditions when mashing with unmalted sorghum and commercial enzymes. *Journal of the American Society of Brewing Chemists*, *61*(2), 69-78.
- Guerdrum, L. J., & Bamforth, C. W. (2011). Levels of gliadin in commercial beers. *Food Chemistry*, *129*(4), 1783-1784. doi:DOI 10.1016/j.foodchem.2011.06.021
- Guerdrum, L. J., & Bamforth, C. W. (2012). Prolamin Levels Through Brewing and the Impact of Prolyl Endoproteinase. *Journal of the American Society of Brewing Chemists*, *70*(1), 35-38. doi:Doi 10.1094/Asbcj-2012-0130-01

- Haas-Lauterbach, S., Immer, U., Richter, M., & Koehler, P. (2012). Gluten Fragment Detection with a Competitive ELISA. *Journal of Aoac International*, *95*(2), 377-381. doi:10.5740/jaoacint.SGE\_Haas-Lauterbach
- Hartmann, G., Koehler, P., & Wieser, H. (2006). Rapid degradation of gliadin peptides toxic for coeliac disease patients by proteases from germinating cereals. *Journal of Cereal Science*, *44*(3), 368-371. doi:10.1016/j.jcs.2006.10.002
- Hauser, W., Gold, J., Stein, J., Caspary, W. F., & Stallmach, A. (2006). Health-related quality of life in adult coeliac disease in Germany: results of a national survey. *European Journal of Gastroenterology & Hepatology*, *18*(7), 747-754.
- Howitt, T. (2014). Barley with low levels of hordeins. Patent number:US 8642846 B2
- Hubner, F., O'Neil, T., Cashman, K. D., & Arendt, E. K. (2010). The influence of germination conditions on beta-glucan, dietary fibre and phytate during the germination of oats and barley. *European Food Research and Technology*, *231*(1), 27-35. doi:10.1007/s00217-010-1247-1
- Huebner, F., Schehl, B. D., Thiele, F., & Arendt, E. K. (2009). Investigation of the Malting Behavior of Oats for Brewing Purposes. *Journal of the American Society of Brewing Chemists*, *67*(4), 235-241. doi:10.1094/Asbcj-2009-0929-01
- Ilori, M. O., Makinwa, E. O., & Irefin, I. A. (1996). Indigenous technological capability development in the brewing industry in Nigeria: An engineering economic assessment and policy implications. *Food Reviews International*, *12*(4), 511-523.
- Kahlenberg, F., Sanchez, D., Lachmann, I., Tuckova, L., Tlaskalova, H., Mendez, E., & Mothes, T. (2006). Monoclonal antibody R5 for detection of putatively coeliac-toxic gliadin peptides. *European Food Research and Technology*, *222*(1-2), 78-82. doi:DOI 10.1007/s00217-005-0100-4
- Kamelgard. (2012) Quinoa-based beverages and method of creating quinoa-based beverages. Patent number: US 20120201924 A1.
- Kiss, Z., Vecseri-Hegybes, B., Kun-Farkas, G., & Hoschke, A. (2011). Optimization of Malting and Mashing Processes for the Production of Gluten-Free Beers. *Acta Alimentaria*, *40*, 67-78. doi:10.1556/AAlim.40.2011.Suppl.7
- Klisch, R. J. (2009) Gluten-free beer and method for making the same. Patent Number: US20090068309 A1.

- Klose, C., & Arendt, E. K. (2012). Proteins in Oats; their Synthesis and Changes during Germination: A Review. *Critical Reviews in Food Science and Nutrition*, 52(7), 629-639. doi:10.1080/10408398.2010.504902
- Klose, C., Mauch, A., Wunderlich, S., Thiele, F., Zarnkow, M., Jacob, F., & Arendt, E. K. (2011). Brewing with 100% Oat Malt. *Journal of the Institute of Brewing*, 117(3), 411-421.
- Klose, C., Thiele, F., & Arendt, E. K. (2010). Changes in the Protein Profile of Oats and Barley During Brewing and Fermentation. *Journal of the American Society of Brewing Chemists*, 68(2), 119-124. doi:10.1094/Asbcj-2010-0312-01
- Knorr, V., Kerpes, R., Wieser, H., Zarnkow, M., Becker, T., & Koehler, P. (2015). Production and application of barley malt extract with high peptidase activity for the degradation of gluten in wort. *European Food Research and Technology*, 1-13. doi:10.1007/s00217-015-2568-x
- Knorr, V., Wieser, H., & Koehler, P. (2015). Production of gluten-free beer by peptidase treatment. *European Food Research and Technology*, 1-12. doi:10.1007/s00217-015-2617-5
- Lohi, S., Mustalahti, K., Kaukinen, K., Laurila, K., Collin, P., Rissanen, H., . . . Maki, M. (2007). Increasing prevalence of coeliac disease over time. *Alimentary Pharmacology & Therapeutics*, 26(9), 1217-1225. doi:10.1111/j.1365-2036.2007.03502.x
- Luoto, S., Jiang, Z. Q., Brinck, O., Sontag-Strohm, T., Kanerva, P., Bruins, M., . . . Lojonen, J. (2012). Malt hydrolysates for gluten-free applications: Autolytic and proline endopeptidase assisted removal of prolamins from wheat, barley and rye. *Journal of Cereal Science*, 56(2), 504-509. doi:10.1016/j.jcs.2012.06.004
- Lyumugabe, F., Gros, J., Nzungize, J., Bajyana, E., & Thonart, P. (2012). Characteristics of African traditional beers brewed with sorghum malt: a review. *Biotechnologie Agronomie Societe Et Environnement*, 16(4), 509-530.
- Maccagnan, G., Pat, A., Collavo, F., Ragg, G. L., & Bellini, M. P. (2004) Gluten-free beer containing rice malt. Patent number: CA 2268506 A1.
- Molberg, O., Uhlen, A. K., Jensen, T., Flaete, N. S., Fleckenstein, B., Arentz-Hansen, H., . . . Sollid, L. M. (2005). Mapping of gluten T-cell epitopes in the bread wheat ancestors: Implications for Celiac disease. *Gastroenterology*, 128(2), 393-401. doi:10.1053/j.gastro.2004.11.003

- Mutioz-Insa, A., Gastl, M., Zarnkow, M., & Becker, T. (2011). Optimization of the malting process of oat (*Avena sativa* L.) as a raw material for fermented beverages. *Spanish Journal of Agricultural Research*, 9(2), 510-523.
- Nakatani, K. (2007). Beer in Japan - present and future trends. *Brewer and Distiller International*(3(5)), 54-57.
- Nuss, E. T., & Tanumihardjo, S. A. (2010). Maize: A Paramount Staple Crop in the Context of Global Nutrition. *Comprehensive Reviews in Food Science and Food Safety*, 9(4), 417-436. doi:10.1111/j.1541-4337.2010.00117.x
- Nzelibe, H. C., & Nwasike, C. C. (1995). The Brewing Potential of Acha (*Digitaria-Exilis*) Malt Compared with Pearl-Millet (*Pennisetum-Typhoides*) Malts and Sorghum (*Sorghum-Bicolor*) Malts. *Journal of the Institute of Brewing*, 101(5), 345-350.
- O'Leary, C., Wieneke, P., Buckley, S., O'Regan, P., Cronin, C. C., Quigley, E. M. M., & Shanahan, F. (2002). Celiac disease and irritable bowel-type symptoms. *American Journal of Gastroenterology*, 97(6), 1463-1467. doi:Pii S0002-9270(02)04046-7 Doi 10.1016/S0002-9270(02)04046-7
- Obeta, J. A. N., Okungbowa, J., & Ezeogu, L. I. (2000). Malting of sorghum: Further studies on factors influencing alpha-amylase activity. *Journal of the Institute of Brewing*, 106(5), 295-304.
- Ogungbenle, H. N. (2003). Nutritional evaluation and functional properties of quinoa (*Chenopodium quinoa*) flour. *International Journal of Food Sciences and Nutrition*, 54(2), 153-158. doi:10.1080/0963748031000084106
- Okolo, B. N., & Ezeogu, L. I. (1996a). Enhancement of amyolytic potential of sorghum malts by alkaline steep treatment. *Journal of the Institute of Brewing*, 102(2), 79-85.
- Okolo, B. N., & Ezeogu, L. I. (1996b). Promoting sorghum reserve protein mobilisation by steeping in alkaline liquor. *Journal of the Institute of Brewing*, 102(4), 277-284.
- Pasternack, R., Marx, S., & Jordan, D. (2008). Prolamin-Reduced Beverages and Methods for the Preparation Thereof. Patent number: WO 2006051093 A1
- Pelembe, L. A. M., Dewar, J., & Taylor, J. R. N. (2002). Effect of malting conditions on pearl millet malt quality. *Journal of the Institute of Brewing*, 108(1), 7-12.
- Phiarais, B. P. N., Mauch, A., Schehl, B. D., Zarnkow, M., Gastl, M., Herrmann, M., . . . Arendt, E. K. (2010). Processing of a Top Fermented Beer Brewed from 100% Buckwheat Malt with Sensory and Analytical Characterisation. *Journal of the Institute of Brewing*, 116(3), 265-274.

- Phiarais, B. P. N., Schehl, B. D., Oliveira, J. C., & Arendt, E. K. (2006). Use of response surface methodology to investigate the effectiveness of commercial enzymes on buckwheat malt for brewing purposes. *Journal of the Institute of Brewing*, 112(4), 324-332.
- Picariello, G., Bonomi, F., Iametti, S., Rasmussen, P., Pepe, C., Lilla, S., & Ferranti, P. (2011). Proteomic and peptidomic characterisation of beer: Immunological and technological implications. *Food Chemistry*, 124(4), 1718-1726. doi:10.1016/j.foodchem.2010.07.111
- Riestra, S., Fernandez, E., Rodrigo, L., Garcia, S., & Ocio, G. (2000). Prevalence of coeliac disease in the general population of northern Spain - Strategies of serologic screening. *Scandinavian Journal of Gastroenterology*, 35(4), 398-402.
- Rizzello, C. G., De Angelis, M., Di Cagno, R., Camarca, A., Silano, M., Losito, A., . . . Gobbetti, M. (2007). Highly efficient gluten degradation by lactobacilli and fungal proteases during food processing: New perspectives for celiac disease. *Applied and Environmental Microbiology*, 73(14), 4499-4507. doi:10.1128/Aem.00260-07
- Rojas-Molina, I., Gutierrez-Cortez, E., Palacios-Fonseca, A., Banos, L., Pons-Hernandez, J. L., Guzman-Maldonado, S. H., . . . Rodriguez, M. E. (2007). Study of structural and thermal changes in endosperm of quality protein maize during traditional nixtamalization process. *Cereal Chemistry*, 84(4), 304-312. doi:10.1094/Cchem-84-4-0304
- Schapira, M., Maisin, J. M., Ghilain, J. M., De Maeght, S., Deltenre, P., & Henrion, J. (2003). Epidemiology of coeliac disease. *Acta Gastro-Enterologica Belgica*, 66(3), 234-236.
- Scott, D. R. (2005) Liquid mixture for producing a substantially gluten-free beer in conformity with Jewish Orthodox law. Patent number: US 20050170042 A1.
- Siebert, K. J. (1999). Effects of protein-polyphenol interactions on beverage haze, Stabilization and analysis. *Journal of Agricultural and Food Chemistry*, 47(2), 353-362. doi:DOI 10.1021/jf980703o
- Siebert, K. J., & Lynn, P. Y. (1997). Mechanisms of beer colloidal stabilization. *Journal of the American Society of Brewing Chemists*, 55(2), 73-78.
- Sohrabvandi, S., Mortazavian, A. M., & Rezaei, K. (2012). Health-Related Aspects of Beer: A Review. *International Journal of Food Properties*, 15(1-2), 350-373. doi:10.1080/10942912.2010.487627

- Spaenij-Dekking, L., Kooy-Winkelaar, Y., Van Veelen, P., Drijfhout, J. W., Jonker, H., Van Soest, L. Koning, F. (2005). Natural variation in toxicity of wheat: Potential for selection of nontoxic varieties for celiac disease patients. *Gastroenterology*, *129*(3), 797-806. doi:10.1053/j.gastro.2005.06.017
- Spins. (2012). Gluten-free trends highlights. Gluten-free trend highlights. [http://www.expowest.com/ew12/CUSTOM/Uploads/Glutenfree\\_Kerry\\_Watson.pdf](http://www.expowest.com/ew12/CUSTOM/Uploads/Glutenfree_Kerry_Watson.pdf), Accessed 11th April
- Stenman, S. M., Venalainen, J. I., Lindfors, K., Auriola, S., Mauriala, T., Kaukovirta-Norja, A., Maki, M. (2009). Enzymatic detoxification of gluten by germinating wheat proteases: Implications for new treatment of celiac disease. *Annals of Medicine*, *41*(5), 390-400. doi:Pii 91027436910.1080/07853890902878138
- Tanner, G. J., Blundell, M. J., Colgrave, M. L., & Howitt, C. A. (2013). Quantification of Hordeins by ELISA: The Correct Standard Makes a Magnitude of Difference. *Plos One*, *8*(2). doi:ARTN e5645610.1371/journal.pone.0056456
- Tanner, G. J., Colgrave, M. L., Blundell, M. J., Goswami, H. P., & Howitt, C. A. (2013). Measuring Hordein (Gluten) in Beer - A Comparison of ELISA and Mass Spectrometry. *Plos One*, *8*(2). doi:ARTN e5645210.1371/journal.pone.0056452
- Tanner, G. J., Colgrave, M. L., & Howitt, C. A. (2014). Gluten, Celiac Disease, and Gluten Intolerance and the Impact of Gluten Minimization Treatments with Prolylendopeptidase on the Measurement of Gluten in Beer. *Journal of the American Society of Brewing Chemists*, *72*(1), 36-50. doi:10.1094/Asbcj-2014-0129-01
- Tatham, A. S., Fido, R. J., Moore, C. M., Kasarda, D. D., Kuzmicky, D. D., Keen, J. N., & Shewry, P. R. (1996). Characterisation of the major prolamins of tef (*Eragrostis tef*) and finger millet (*Eleusine coracana*). *Journal of Cereal Science*, *24*(1), 65-71. doi:DOI 10.1006/jcrs.1996.0038
- Taylor, J. R. N., Schober, T. J., & Bean, S. R. (2006). Novel food and non-food uses for sorghum and millets. *Journal of Cereal Science*, *44*(3), 252-271. doi:10.1016/j.jcs.2006.06.009
- Teramoto, Y., Yoshida, S., & Ueda, S. (2002). Characteristics of a rice beer (zutho) and a yeast isolated from the fermented product in Nagaland, India. *World Journal of Microbiology & Biotechnology*, *18*(9), 813-816. doi:Doi 10.1023/A:1021293804327
- Thompson, T., & Mendez, E. (2008). Commercial Assays to Assess Gluten Content of Gluten-Free Foods: Why They Are Not Created Equal. *Journal of the American Dietetic Association*, *108*(10), 1682-1687. doi:10.1016/j.jada.2008.07.012

- Usansa, U., Burberg, F., Geiger, E., Back, W., Wanapu, C., Arendt, E. K., . . . Zarnkow, M. (2011). Optimization of Malting Conditions for Two Black Rice Varieties, Black Non-Waxy Rice and Black Waxy Rice (*Oryza sativa* L. Indica). *Journal of the Institute of Brewing*, *117*(1), 39-46.
- van den Broeck, H., Chen, H. B., Lacaze, X., Dusautoir, J. C., Gilissen, L., Smulders, M., & van der Meer, I. (2010). In search of tetraploid wheat accessions reduced in celiac disease-related gluten epitopes. *Molecular Biosystems*, *6*(11), 2206-2213. doi:10.1039/c0mb00046a
- Van Landschoot, A. (2011). Gluten-free barley malt beers. *Cerevisia*, *36*(3), 93-97.
- Verbruggen, M. A., Beldman, G., & Voragen, A. G. J. (1995). The Selective Extraction of Glucuronoarabinoxylans from Sorghum Endosperm Cell-Walls Using Barium and Potassium Hydroxide Solutions. *Journal of Cereal Science*, *21*(3), 271-282. doi:DOI 10.1006/jcrs.1995.0030
- Walter, T., Wieser, H., & Koehler, P. (2014). Production of gluten-free wheat starch by peptidase treatment. *Journal of Cereal Science*, *60*(1), 202-209. doi:10.1016/j.jcs.2014.02.012
- Wieser, H., & Koehler, P. (2009). Is the calculation of the gluten content by multiplying the prolamin content by a factor of 2 valid? *European Food Research and Technology*, *229*(1), 9-13. doi:10.1007/s00217-009-1020-5
- Wijngaard, H. H., Ulmer, H. M., & Arendt, E. K. (2005). The effect of germination temperature on malt quality of buckwheat. *Journal of the American Society of Brewing Chemists*, *63*(1), 31-36. doi:10.1094/Asbcj-63-0031
- Wijngaard, H. H., Ulmer, H. M., & Arendt, E. K. (2006). The effect of germination time on the final malt quality of buckwheat. *Journal of the American Society of Brewing Chemists*, *64*(4), 214-221. doi:10.1094/Asbcj-64-0214
- Wijngaard, H. H., Ulmer, H. M., Neumann, M., & Arendt, E. K. (2005). The effect of steeping time on the final malt quality of buckwheat. *Journal of the Institute of Brewing*, *111*(3), 275-281.
- Worosz, M. R., & Wilson, N. L. W. (2012). A Cautionary Tale of Purity, Labeling and Product Literacy in the Gluten-Free Market. *Journal of Consumer Affairs*, *46*(2), 288-318. doi:10.1111/j.1745-6606.2012.01230.x
- Wrigley, C., Corke, H., & Walker, C. E. (2004). *Encyclopedia of grain science*. Elsevier Academic Press, Amsterdam, Netherlands.

- Zarnkow, M., Almaguer, C., Burberg, F., Back, W., Arendt, E. K., & Kreis, S. (2008). The use of response surface methodology to optimise malting conditions of teff (*Eragrostis tef* (Zucc.) Trotter) as a raw material for gluten free foods and beverages. *Brewing Science*, *61*, 94-104.
- Zarnkow, M., Faltermaier, A., Back, W., Gastl, M., & Arendt, E. K. (2010). Evaluation of different yeast strains on the quality of beer produced from malted proso millet (*Panicum miliaceum* L.). *European Food Research and Technology*, *231*(2), 287-295. doi:10.1007/s00217-010-1268-9
- Zarnkow, M., Geyer, T., Lindemann, B., Burberg, F., Back, W., & Arendt, E. K. (2007). The use of response surface methodology to optimise malting conditions of quinoa (*Chenopodium quinoa* L.) as a raw material for gluten-free foods. *Brewing Science*, *60*, 118-126.
- Zarnkow, M., Kessler, M., Back, W., Arendt, E. K., & Gastl, M. (2010). Optimisation of the Mashing Procedure for 100% Malted Proso Millet (*Panicum miliaceum* L.) as a Raw Material for Gluten-free Beverages and Beers. *Journal of the Institute of Brewing*, *116*(2), 141-150.
- Zarnkow, M., Kessler, M., Burberg, F., Back, W., Arendt, E. K., & Kreis, S. (2007). The use of response surface methodology to optimise malting conditions of proso millet (*Panicum miliaceum* L.) as a raw material for gluten-free foods. *Journal of the Institute of Brewing*, *113*(3), 280-292.
- Zweytick, G., & Berghofer, E. (2009). Production of gluten free beer. In E. Gallagher (Ed.), *Gluten-free food science and technology*. Oxford, UK Wiley-Blackwell.
- Zweytick, G., Sauerzopf, E., & Berghofer, E. (2005, 11-14 September). *Production of gluten-free beer*. Paper presented at the AACC annual meeting, Orlando, FL.



# **Chapter 3: A Fundamental Study on the Relationship between Barley Cultivar and Hordeins in Single Cultivar Beers**

*Joshua P. Taylor, Elke K. Arendt*

Accepted for publication in the Journal of Institute of Brewing - January 2016

## **Author Contributions**

Joshua Taylor was the main author for the paper, contributed to the conception and performed all the laboratory work and experiments.

Prof Arendt helped with the conception of the paper as well as making contributions and suggestions throughout the experimental process and supervised the project. Prof Arendt also critically reviewed the final manuscript

## **Abstract**

The hordein proteins found in beer are not suitable for gluten sensitive consumers. Hordeins are storage proteins found in barley and have limited solubility in water. It is not currently known if the nitrogen concentration of barley directly impacts on the hordeins present in beer. In this study a controlled malting was performed on eight barley cultivars and produced a single cultivar model beer from each. The single cultivar model beers were then examined for differences in content of hordeins. The quality of barley and malt was assessed and the parameters measured were compared to the beer hordeins using a Pearson correlation matrix. The results showed significant differences in content of beer hordeins, depending on the barley malt used. Correlations between results showed a positive relationship between malt nitrogen and a negative relationship to friability. The results suggest it may be possible to optimise choice of barley cultivar and malting conditions in order to produce beer low in hordeins.

## Introduction

Coeliac disease affects about 1% of the world population (Catassi, Gatti, & Fasano, 2014) and non-coeliac gluten sensitivity has been estimated to affect up to 6% of the population (Catassi et al., 2013). This portion of the population has contributed to the surge in the amount of consumers avoiding gluten in the past 10 years. The gluten-free market value has been growing steadily and since 2009 has increased in value from US\$ 1.4 Billion to US\$ 2.6 billion in 2014 (Euromonitor passport accessed 2-10-15, <http://www.euromonitor.com/passport>). This is not only due to the increase in the number of patients with medical conditions who are being advised to avoid gluten as a part of treatment. A large number of consumers are choosing to avoid food containing gluten due to perceived health benefits, despite there being no published evidence to support this idea (Worosz & Wilson, 2012).

The main protein fraction found in wheat, rye, barley and other members of the Triticeae tribe are the alcohol soluble prolamins. These prolamins, rich in proline and glutamine (giving the prol-amin title), are more commonly known as the gluten proteins found in wheat (gliadins and glutenlins), rye (secalins), barley (hordeins) and some people are sensitive to prolamins found in oats (avenins). Prolamins can account for up to 50% of the total grain protein (Tatham & Shewry, 2012).

Beer is traditionally produced from barley malt and as malt contains hordeins, gluten sensitive consumers do not drink traditional beers. Levels of hordeins and other prolamins found in beers vary considerably (Kanerva, Sontag-Strohm, & Lehtonen, 2005) depending on the ingredients used. The most up to date method for testing prolamins in beer is provided by Codex Alimentarius. These guidelines

recommend an R5 antibody based *competitive* ELISA assay to test levels of prolamins in beer (Codex Alimentarius, 1979).

Beers produced using barley malt as the main ingredient and marketed as gluten-free are widely available (Van Zandycke, 2013), these can be treated with enzymes to degrade or remove prolamins (Guerdrum & Bamforth, 2012; Taylor, Jacob, & Arendt, 2015) or use filtration processes that reduce prolamins (Taylor, Jacob & Arendt, 2015). Beer below 10 mg/kg prolamins (assuming prolamins multiplied by two = gluten) can be labelled as gluten-free (Codex Alimentarius, 1979).

Hordein proteins are reduced significantly during malting and brewing, for example hordeins are reduced by over 30% during malting (Briggs, 1998), during lautering hordeins are also removed (Celus, Brijs, & Delcour, 2006), and often commercial beers can be low in prolamins (Guerdrum & Bamforth, 2011).

How the barley cultivar impacts the content of hordeins in beers is not known. As there is significant degradation of hordeins during malting and during the brewing process (Celus et al., 2006), differences caused by the choice of barley cultivar may not be significant. On the other hand, as hordeins are storage proteins, which account for up to half of protein found in barley, protein nitrogen might be a good indicator of levels of beer hordeins.

Differences between hordeins in beers produced from single cultivar malts were examined, and using correlation analysis we assessed if there was a relationship to any of the quality parameters measured.

## **Materials and methods**

### **Barley**

Barley seed was obtained from breeders at sites in Carlow and Waterford in Ireland. Five different barley cultivars were from Seedtech, based in Waterford (Quench, Taberna, Propino, Overture and Mickle). Three barley cultivars from Glanbia, based in Carlow (Quench, Propino and Cropton) were also examined. The barley cultivars were chosen from the list of recommended spring barleys in Ireland for 2013. Two of the barley varieties evaluated were common to both sites.

### **Barley analyses**

Analyses of barley was carried out according to standard MEBAK (2011) (Mitteleuropäische Brautechnische Analysenkommision) methods. Germination (MEBAK 1.4.2.5), barley nitrogen (MEBAK 1.5.2.1) and thousand kernel weight (TKW) (MEBAK 1.3.2) tests were all carried out on the barley.

### **Malting**

Malting was performed according to a standard micromalting method (MEBAK 1.5.3). Each barley cultivar was steeped in a temperature controlled water bath at 14°C using a combination of wet steeps and air rests for three days until the moisture level increased to 45% w/w. Air rests were performed in a humidity and temperature controlled chamber. The steeped barley was then held at constant humidity (80%) and temperature in the same chamber for a further three days. The final kilning step of the malting process took place in a Joe White malting machine (Joe White, Australia) over 23 hours before cleaning and removal of the rootlets using a thresher (Wintersteiger LD180, Wintersteiger AG, Ried, Austria).

### **Malt analyses**

Malt analyses were also carried out according to MEBAK recommended methods. Malt nitrogen (MEBAK 1.5.2.1) and friability (MEBAK 3.1.3.6.1) were carried out on the whole grains. Wort produced from a congress mash was used as the basis for measuring extract (MEBAK 3.1.4.2.2), viscosity (MEBAK 3.1.4.4.1), soluble nitrogen (MEBAK 3.1.4.5.2.1), Kolbach index (MEBAK 3.1.4.5.3) and apparent limit of attenuation (fermentability) (MEBAK 3.1.4.10.1.2).

### **Alpha amylase activity**

Alpha amylase activity was determined by Megazyme Ceralpha  $\alpha$ -Amylase Assay Kit and expressed in ceralpha units/g (McCleary & Sheehan, 1987).

### **Proteolytic activity**

Endoproteolytic activity was determined using azocasein as a substrate following the method of Brijs, Trogh, Jones, & Delcour (2002). Increase in absorbance at 440nm per hour was reported.

### **Model beer production**

Single malt model beers were produced using a modified congress mash method (MEBAK 3.1.4.2). The wort (300 ml) produced from the congress mash was added to a one litre boiling flask and boiled with 0.25 g target hops (11.41%  $\alpha$ -acid, T90, Simply Hops, Kent. UK) for one hour. After boiling each flask was cooled to room temperature and any reduction in weight due to evaporation was replaced with water. Boiled wort was then filtered through a fluted paper filter to remove hops and any solids. The boiled and filtered wort (200 g, the remainder was discarded) was then added to 500 ml conical flask and shaken prior to addition of yeast (160 mg *S. Cerevisiae*, Fermentis Safale US-05,).

Fermentation took place at 15°C for 10 days followed by 10 days at 1°C without stirring. Fermentation broths were then filtered at 1°C using fluted paper filters to remove yeast prior to prolamin determination.

### **Prolamin determination**

Prolamin levels (hordeins) in each model beer were measured by the R5 *competitive* ELISA kit from R-Biopharm following manufacturer's instructions for beer samples (MEBAK method 2.6.5).

### **Statistics**

ANOVA with Tukey post-hoc analysis ( $\alpha= 0.05$ ) was used to determine significant differences between means and two way Pearson correlations were used to look at relationships between the quality parameters using SPSS (version 20, IBM, Armonk U.S.A).

All analyses were carried out in at least triplicate.

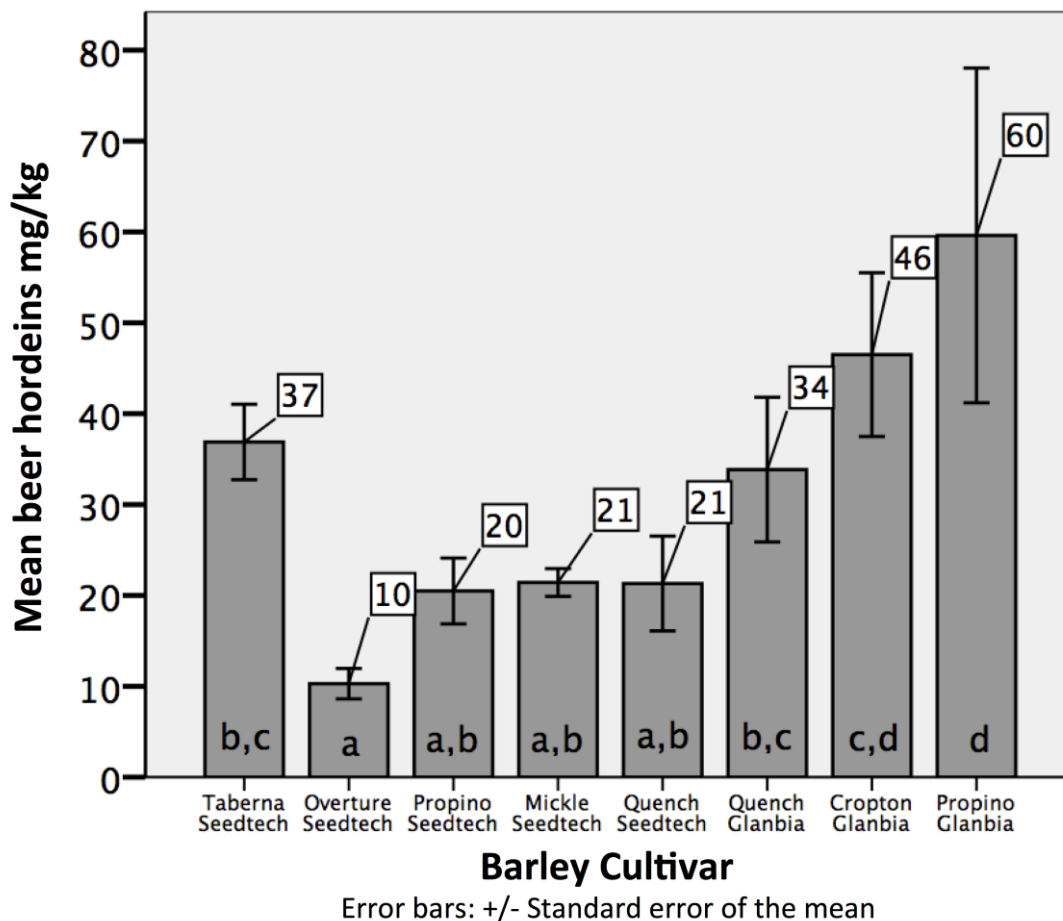
### **Results**

#### **Hordeins in model beers and correlations to malt quality**

The prolamins found in barley, malt and malt-based beer are hordeins. Levels of hordeins measured by ELISA in the single cultivar beers are shown in figure 3.1, levels range from 10 - 60 mg/kg hordeins. Overture malt (Seedtech) beer had the lowest level of hordeins and beer produced from Propino malt (Glanbia) had the highest level of hordeins for all beers tested.

The level of hordeins in most model beers were significantly different, depending on the barley cultivar used. Significant differences between means are shown in figure 3.1.

**Figure 3.1.** Levels of beer hordeins in single cultivar model beers in mg/kg. Different letters in bars indicates sig differences between them at alpha level of 0.05 (two-tailed) using a one way ANOVA model.



Hordeins from the model beers showed correlations with malt quality parameters. Significant negative correlations were shown between content of beer hordeins and friability ( $r = -0.690$ ,  $p < .01$ ), Kolbach Index ( $r = -0.465$ ,  $p < .05$ ) and limit of attenuation ( $r = -0.467$ ,  $p < .05$ ) when data were analysed (table 3.1). There were also positive correlations shown between hordeins in the beer and nitrogen of the malt ( $r = 0.592$ ,  $p < .01$ ), TKW ( $r = 0.610$ ,  $p < .01$ ), and barley nitrogen ( $r = 0.580$ ,  $p < .01$ ).

#### **Malt quality**



## **Germination**

According to standard methods of analysis (MEBAK) the barley must germinate to a level of at least 95% by the third day of growth to be considered standard malting quality. Table 3.2 shows the germination percentage of each barley.

Three of the barleys (Taberna, Propino and Quench, all from Seedtech) had levels of germination above 95%. The other barleys showed between 91% and 94% germination (Table 3.2). Although barley showing below 95% germination would not normally be considered for malting, the quality analysis and malting was performed on all samples.



### **Barley and malt nitrogen concentration**

Barley nitrogen results are shown in table 3.2. Taberna from Seedtech had the highest level of nitrogen at 2.02% (w/w (d.m.)). The range of values found in the other barleys was between 1.54% (w/w (d.m.)) for Overture from Seedtech up to 1.82% (w/w (d.m.)), measured in Quench from Seedtech and Cropton from Glanbia (table 3.2). There were positive correlations between barley nitrogen and soluble nitrogen ( $r = 0.790$ ,  $p < .01$ ), malt nitrogen ( $r = 0.943$ ,  $p < .01$ ) and beer hordeins. Significant negative correlations were also found between barley nitrogen and fermentability ( $r = -0.906$ ,  $p < .01$ ), extract ( $r = -0.768$ ,  $p < .01$ ), Kolbach index ( $r = -0.512$ ,  $p < .05$ ) and friability ( $r = -0.476$ ,  $p < 0.05$ ) (table 3.1).

Nitrogen concentration of the malt was very similar to that of the barley prior to malting (table 3.2). Nitrogen levels in malt were correlated negatively with extract ( $r = -0.860$ ,  $p < .01$ ), fermentability ( $r = -0.903$ ,  $p < .01$ ) and Kolbach index ( $r = -0.711$ ,  $p < .01$ ). Nitrogen levels in malt were positively correlated with barley nitrogen and both soluble nitrogen in the wort ( $r = 0.689$ ,  $p < .01$ ) and hordeins in the model beer.

### **Thousand kernel weight (TKW) and friability**

TKW gives a measure of the size of the barley kernels. Propino from Glanbia showed the highest values with 48.5 g. The lowest TKW was measured in Overture from Seedtech at 37 g. Friability measures how hard a malt kernel is. Higher percentage indicates it breaks more readily. Friability of the malted barley ranged from 61% for Propino (Glanbia) to 83% for Overture (Seedtech). Significant differences in the means are shown in table 3.

**Table 3.2.** Basic barley and malt quality analysis. Different superscript letters beside mean values indicates sig differences at alpha level of 0.05 (two-tailed). Values without a letter beside them were not tested for significance.

		Barley and Malt Quality									
		Germination		Barley Nitrogen		TKW		Malt Nitrogen		Friability	
		Mean %	Standard Error	Mean % (d.m.)	Standard Error	Mean (g)	Standard Error	Mean % (d.m.)	Standard Error	Mean %	Standard Error
Cultivar	Taberna	97	1	2.02 <sup>e</sup>	0.01	40.7 <sup>b</sup>	0.1	1.93 <sup>f</sup>	0	75 <sup>c,d</sup>	0.1
	Overture	91	3	1.54 <sup>a</sup>	0.01	39.1 <sup>a</sup>	0.14	1.44 <sup>a</sup>	0.01	83 <sup>e</sup>	0.2
	Propino	96	1	1.55 <sup>a</sup>	0	43.8 <sup>c</sup>	0.05	1.61 <sup>c</sup>	0.01	82 <sup>e</sup>	0.6
	Mickle	94	1	1.61 <sup>b</sup>	0.01	44.8 <sup>d</sup>	0.07	1.56 <sup>b</sup>	0.01	76 <sup>c,d</sup>	0.6
	Quench	97	1	1.82 <sup>d</sup>	0.01	44.0 <sup>c</sup>	0.07	1.73 <sup>e</sup>	0	75 <sup>c</sup>	0.4
	Quench	93	1	1.74 <sup>c</sup>	0.01	44.9 <sup>d</sup>	0.3	1.67 <sup>d</sup>	0.01	69 <sup>b</sup>	0.3
	Cropton	91	1	1.8 <sup>d</sup>	0.01	44.8 <sup>d</sup>	0.05	1.76 <sup>e</sup>	0	77 <sup>d</sup>	0.4
	Propino	92	1	1.82 <sup>d</sup>	0.01	48.5 <sup>e</sup>	0.15	1.73 <sup>e</sup>	0	61 <sup>a</sup>	0.1

TKW was found to correlate negatively with friability ( $r = -0.735$ ,  $p < .01$ ), proteolytic activity ( $r = -0.550$ ,  $p < .01$ ) and  $\alpha$ -amylase activity ( $r = -0.556$ ,  $p < .05$ ). Table 3.1 shows TKW demonstrating positive correlations with beer hordeins. Friability correlated negatively with viscosity ( $r = -0.800$ ,  $p < .01$ ) and positively with fermentability (table 3.1).

### Wort quality results

#### Viscosity

The malt from Propino and Quench barley grown by Glanbia produced wort with the highest viscosity (table 3.3). The lowest viscosity wort was produced from Propino cultivar barley malt grown by Seedtech. Viscosity measurements ranged between 1.55-1.62 mPa x S and correlated with friability (table 3.3).

**Table 3.3.** Wort quality results using varietal malts. Different superscript letters beside mean values indicates significant differences at alpha level of 0.05 (two-tailed). Values without a letter beside them were not tested for significance.

Cultivar		Wort quality									
		Viscosity		Apparent starting extract		Extract		Apparent limit of Attenuation		Soluble Nitrogen	
		Mean mPa·s	Standard Error	Mean extract (% w/w)	Standard Error	Mean app. extract (% w/w)	Standard Error	Mean %	Standard Error	Mean (d.m.)	Standard Error
Taberna	Seedtech	1.57 <sup>b,c</sup>	0	9.05	0.01	84.7 <sup>a</sup>	0.1	73.8 <sup>a</sup>	0.2	0.92 <sup>d</sup>	0
Overture	Seedtech	1.58 <sup>c</sup>	0	9.32	0.01	87.3 <sup>e</sup>	0.2	79.5 <sup>e</sup>	0.2	0.85 <sup>b</sup>	0.01
Propino	Seedtech	1.55 <sup>a</sup>	0	9.24	0.01	86.4 <sup>c,d</sup>	0.1	78 <sup>d,e</sup>	0.2	0.81 <sup>a</sup>	0
Mickle	Seedtech	1.57 <sup>b,c</sup>	0	9.22	0.00	86.2 <sup>c,d</sup>	0.1	79.2 <sup>e</sup>	0.6	0.81 <sup>a</sup>	0
Quench	Seedtech	1.58 <sup>c</sup>	0.01	9.21	0.01	86.3 <sup>c,d</sup>	0.1	75.7 <sup>b</sup>	0.3	0.94 <sup>e</sup>	0
Quench	Glanbia	1.62 <sup>d</sup>	0	9.18	0.01	86 <sup>b,c</sup>	0.1	76.7 <sup>b,c,d</sup>	0.3	0.88 <sup>c</sup>	0
Cropton	Glanbia	1.56 <sup>a,b</sup>	0	9.16	0.01	85.6 <sup>b</sup>	0.2	77.4 <sup>c,d</sup>	0.2	0.93 <sup>d,e</sup>	0
Propino	Glanbia	1.61 <sup>d</sup>	0	9.23	0.01	86.6 <sup>d</sup>	0.1	76.2 <sup>b,c</sup>	0.3	0.88 <sup>c</sup>	0

### Extract

Extract achieved from each of the malts was good for pale malt standards (table 3.3). Quench from Glanbia had the lowest extract at a level of 84% (w/w, d.m.). The highest level of extract achieved was 87% (w/w d.m.), generated by Overture from Seedtech. Extract levels positively correlated with limit of attenuation ( $r = 0.699$ ,  $p < .01$ ) and Kolbach Index ( $r = 0.731$ ,  $p < .01$ ) (table 3.1) and were negatively correlated with soluble nitrogen ( $r = -0.477$ ,  $p < 0.05$ ), barley nitrogen and malt nitrogen (table 3.1).

### Apparent limit of attenuation (fermentability)

Table 3.3 shows the fermentability of the worts, attenuation ranged from 74% (Taberna from Seedtech) up to 80% (Overture from Seedtech). Limit of attenuation results were correlated positively with friability, extract, Kolbach Index and negatively with malt nitrogen, soluble nitrogen, beer hordeins and barley nitrogen (table 3.1).

### Soluble nitrogen and Kolbach index

Mickle and Propino from Seedtech had the lowest levels of soluble nitrogen and Taberna from Seedtech had the highest. Soluble nitrogen measurements are shown in table 3.3 and results were between 0.81% (d.m.) and 0.92% (d.m.). There was a correlation between nitrogen levels of both barley and malt with soluble nitrogen. Kolbach index for all malts was > 41% and was negatively correlated with nitrogen levels and positively correlated with proteolytic activity ( $r = 0.710$ ,  $p < 0.01$ ) (table 3.1).

#### **Alpha amylase and proteolytic activity**

Activity of alpha amylase varied for each of the malts and ranged between 115 – 290 ceralpha units/g (table 3.4). Highest activity was measured in Taberna from Seedtech and the lowest was found in Quench from Glanbia. Measurements negatively correlated with the TKW of the grains (table 3.1).

Overture malt from Seedtech had the highest level of proteolytic activity at 0.333 (abs@440nm/hr) and Propino from Seedtech showed the lowest activity at 0.195 (abs@440nm/hr) (table 3.4). Proteolytic activity results positively correlated with Kolbach index and negatively correlated with TKW.

**Table 3.4.** Alpha amylase and proteolytic enzyme activity of individual malts. Different superscript letters beside mean values indicates sig differences at alpha level of 0.05 (two-tailed).

		Enzyme activity			
		Alpha amylase activity		Proteolytic activity	
		Mean Ceralpha Units/g	Standard Error	Mean abs at 440nm/hr	Standard Error
Cultivar	Taberna Seedtech	293.79 <sup>d</sup>	7.16	0.26 <sup>b,c</sup>	0.01
	Overture Seedtech	286.03 <sup>d</sup>	5.14	0.33 <sup>d</sup>	0
	Propino Seedtech	289.78 <sup>d</sup>	7.84	0.19 <sup>a</sup>	0.02
	Mickle Seedtech	201.62 <sup>a,b</sup>	6.07	0.21 <sup>a,b</sup>	0.01
	Quench Seedtech	203.25 <sup>b</sup>	2.38	0.28 <sup>c</sup>	0.01
	Quench Glanbia	177.45 <sup>a</sup>	1.64	0.24 <sup>a,b,c</sup>	0.01
	Cropton Glanbia	187.6 <sup>a,b</sup>	4.55	0.24 <sup>b,c</sup>	0.01
	Propino Glanbia	238.44 <sup>c</sup>	2.01	0.23 <sup>a,b,c</sup>	0

## Discussion

### Principle findings

The findings of this study are twofold. It was shown that the hordeins in beers produced from single cultivar barley malts are significantly different from each other. Secondly, comparing all barley malts and using correlation analysis common relationships were shown between friability and malt nitrogen with the content of hordeins in beers.

### Level of hordeins in single cultivar model beers

Between barley varieties it is known that there is variation in the composition of hordeins and these differences can be used to detect individual varieties from single barley kernels (Shewry, Pratt, & Mifflin, 1978). During malting and mashing the hordein proteins are significantly modified and this affects their solubility. Using a model beer system allowed us to measure the hordeins that make it into the final beer and not those removed during the mash filtration steps. We tested the level of hordeins in single barley cultivar beers and detected significant differences

between them, depending on the barley used. Overture barley malt produced a beer that was significantly lower in hordeins than four of the other cultivars (figure 3.1). On the other hand, Propino from Glanbia produced a beer, which was significantly higher in hordeins than almost all of the other beers.

Environmental conditions during barley growth can also have a significant effect on the hordein proteins in a developing seed. It has been shown that increasing amounts of nitrogen fertiliser causes an increase in yield and also corresponding increases in hordein protein in the grain (Shewry, Tatham, & Halford, 2001). The amount of nitrogen available to the developing plant is dependent on many factors, such as level of nitrogen in the soil, sulphur availability, rainfall and drought (Garstang & Spink, 2011; Shewry et al., 2001). Application of fertiliser has already been mentioned, but the timing of application also has an effect. If nitrogen is applied early in plant development it has a different effect to being applied in the late stages of development (Briggs, 1978).

This effect of environmental conditions was observed in our samples. Propino variety barley grown in Carlow and Propino variety grown in Waterford had very similar levels of extract (table 3.3) but significantly different levels of barley nitrogen, which then carried over into the malt and subsequently affected the hordeins in the model beers. Figure 3.1 shows the mean values for hordeins in all the model beers, Propino from Glanbia has the highest value at 60 mg/kg and Propino from Seedtech was much lower at 20 mg/kg hordeins. An independent t-test showed that the levels of hordein were significantly different between the two sites of growth for Propino ( $t(5) = 4.9, p = 0.005$ ) The barley and malt nitrogen levels were also different in both Quench variety examples (table 3.2). However,



the two beers made from Quench barley malt, from Seedtech and Glanbia, showed differences that were smaller, with 21 mg/kg and 34 mg/kg hordeins respectively. Independent t-tests on the beers made using Quench malt also showed significant differences in beer hordeins caused by environmental factors ( $t(6) = 2.6, p = 0.04$ ). As environmental conditions can influence the hordeins in barley and there are clear differences between the level of hordeins in the beers, shown in figure 3.1, we were interested in looking at any common quality parameters between them that related to the hordeins in beer.

### **Quality parameters – results and correlations**

#### **Barley**

Germination is the most important parameter when looking at malting barley, without germination there is no malt. Germination levels (table 3.2) were at, or just below standard levels for malting barley. The lowest was Overture from Seedtech at just under 91% but it still performed well in other quality analysis. Taberna and Quench varieties from Seedtech both had 97% germination rates, the maximum of all samples tested.

Malt quality parameters are often correlated with each other (Briggs, 1998), this is a consequence of all the processes that are kick started during the malting. Germination starts the growth of the plant and this activates enzymes, breaks down cell structures and degrades storage proteins. As this complex biological process continues it is not surprising that related correlations occur.

Nitrogen concentration in barley and malt is another important quality characteristic. Acceptable levels of nitrogen in malting quality barley are between 1.5% and 2.1% (d.m.), all of the barley cultivars were within this range (table 3.2).

The nitrogen concentration measured in the malt was a little lower than that measured in the barley (table 3.2). This is normally due to losses of rootlets. Propino from Seedtech was the exception, which had a small increase which could be due to increased respiratory losses (Briggs & Hough, 1981).

Using a two way Pearson correlation we found a significant positive correlation between barley and malt nitrogen with beer hordeins (table 3.1). Given that hordeins are storage proteins, a positive correlation between malt and barley nitrogen with beer hordeins makes some sense. Higher malt nitrogen levels result in higher levels of hordeins in the beers produced. Negative correlations found between beer hordeins, friability and Kolbach index are also logical. During modification of malt, storage proteins are degraded (Baxter, 1981). Both friability and Kolbach Index are measures of the modification of malt suggesting the higher the friability and Kolbach index, the higher the level of degradation of proteins in the malt.

Beer hordeins are also correlated positively with TKW. Malting is a very complex process and it is possible modification does not progress as completely in larger grains. TKW is negatively correlated with friability, which suggests a relationship with modification. The reduced level of modification may result in the positive correlation between TKW and beer hordeins.

Thousand kernel weight results were normal for air-dried barley, Overture and Taberna (Seedtech) barley is classified as light, Propino from Glanbia is heavy barley and the remaining are medium weight barleys. TKW was negatively correlated with friability and alpha amylase, as our sample grains got larger they were less modified and tended to have lower alpha amylase activity.

Viscosity of the wort produced was negatively correlated with friability and this is commonly found. As malt is modified, barley cell wall components such as  $\beta$ -glucan are broken down and this reduces the wort viscosity (Bathgate, 1983).

All the malt samples showed a normal level of extract and this was correlated positively with fermentability, which has been described before (Briggs, 1998). Fermentability depends on several factors including malt enzymes, FAN, vitamins and minerals present in the malt (Garstang & Spink, 2011). The positive relationship between fermentability and level of extract has been shown previously by Briggs (1998). The negative correlation between fermentability and nitrogen concentration shown in our results was also reported elsewhere (Briggs, 1998).

It might be expected that soluble nitrogen levels in wort were correlated to the content of hordeins in the beer, but our results did not show this. Soluble nitrogen was positively related to malt and barley nitrogen levels (table 3.1). Kolbach index of all samples was above 41% which would be considered high, and showed negative correlations to beer hordeins. The Kolbach index is another measure of modification for malt, the higher the Kolbach index the more modified the malt is. This corresponds to the theory that hordeins are degraded during malting. Kolbach index also displayed negative correlations with nitrogen level in the malt and barley, showing our samples with high nitrogen levels did not modify as highly as our lower nitrogen barleys.

The  $\alpha$ -amylase activity levels of our malts (table 3.4) were in normal ranges as found by other researchers (McCleary et al., 2002; Oliveira, Mauch, Jacob, Waters, & Arendt, 2012). Our results showed a negative correlation between TKW and  $\alpha$ -amylase activity (table 3.1). The relationship between TKW and activities has been

shown to vary between positive and negative depending on environmental conditions during the harvest year (Krupnova, 2010).

### **Implications**

Our results show that choice of barley has a significant effect on the content of beer hordeins. The relationship between beer hordeins and barley quality has not been directly studied before and the plausible relationship we found between malt nitrogen and beer hordeins is an interesting result. It may be useful for selecting a range of varietal malts with low nitrogen concentration and then screened using our model beer system to select those that produce beer very low in hordeins. Therefore, using very low nitrogen barley for malting may have a negative influence on the enzyme content and other nitrogen containing compounds in the malt. The site of growth has an impact on the hordein levels in beers, the Quench and Propino barley grown at Glanbia produced beer with significantly higher levels of hordein than same cultivars grown at Seedtech.

We also found a negative correlation between friability and beer hordeins, which suggests it may be possible to influence beer hordeins during malting. Optimising malting to reduce levels of beer hordeins is an interesting possibility. However, care must be taken ensure that over-modification of low nitrogen barley still produces malt, wort and beer of high quality.

These findings could help to develop a method of producing beer low in hordeins using standard ingredients. The model beer system could also be used to screen batches of commercially available malts for those which produce beers lowest in hordeins.

### **Strengths and limitations**

Using the same malting and brewing regime on all barley samples allowed us to compare differences between them under controlled malting, mashing and fermentation conditions. Controlling these processes and specifically looking at differences in beer hordeins allowed us to focus on quality factors which may have a significant influence on beer hordeins.

The method used for determination of hordeins in our beer samples is the current best practice (Codex Alimentarius, 1979) but researchers are still working on more accurate methods. Previous sandwich ELISA versions of the method did not detect hydrolysed prolamins (found in beer) and used a gliadin standard which overestimated hordeins (Tanner, Blundell, Colgrave, & Howitt, 2013). However, the current R5 *competitive* ELISA detects hydrolysed prolamins and uses a standard composed of equal parts hydrolysed hordeins, gliadins and secalins (Haas-Lauterbach, Immer, Richter, & Koehler, 2012). There are still difficulties testing levels of hordeins in malt (personal communication with P.Koehler) but the method has been independently verified and accepted for testing prolamins levels in beer. The ultimate test for the beers would be clinically controlled trials with gluten sensitive consumers but this was not possible for our study.

Although our study was small, the results were significant and showed the possibility of a relationship between malt friability and nitrogen with beer hordeins.

### **Conclusions**

The model beer system developed was effective at screening malts for those with potential for producing beer low in hordeins. The results showed large differences in beer hordeins depending on the malts used for brewing. The relationship between friability and malt protein nitrogen with beer hordeins had not been

shown previously and provides useful information about levels of hordeins in single cultivar malt beers.

## Literature cited

- Bathgate, G. N. (1983). The Relationship between Malt Friability and Wort Viscosity. *Journal of the Institute of Brewing*, 89(6), 416-419.
- Baxter, E. D. (1981). Hordein in Barley and Malt - a Review. *Journal of the Institute of Brewing*, 87(3), 173-176.
- Briggs, D. E. (1978). Agricultural practices and yield *Barley* (pp. 303). New York, U.S.A. Wiley.
- Briggs, D. E. (1998). Malting conditions and their influence on malting *Malts and malting* (1st ed ed., pp. 642). London, UK: Blackie Academic.
- Briggs, D. E., & Hough, J. S. (1981). The biochemistry of malting grain *Malting and Brewing Science Volume I* (2nd ed ed., pp. 83). London, UK ; Chapman and Hall.
- Brijs, K., Trogh, I., Jones, B. L., & Delcour, J. A. (2002). Proteolytic enzymes in germinating rye grains. *Cereal Chemistry*, 79(3), 423-428. doi:Doi 10.1094/Cchem.2002.79.3.423.
- Catassi, C., Bai, J. C., Bonaz, B., Bouma, G., Calabro, A., Carroccio, A., Fasano, A. (2013). Non-Celiac Gluten Sensitivity: The New Frontier of Gluten Related Disorders. *Nutrients*, 5(10), 3839-3853. doi:Doi 10.3390/Nu5103839.
- Catassi, C., Gatti, S., & Fasano, A. (2014). The New Epidemiology of Celiac Disease. *Journal of Pediatric Gastroenterology and Nutrition*, 59, S7-S9. doi:DOI 10.1097/01.mpg.0000450393.23156.59.
- Celus, I., Brijs, K., & Delcour, J. A. (2006). The effects of malting and mashing on barley protein extractability. *Journal of Cereal Science*, 44(2), 203-211. doi:10.1016/j.jcs.2006.06.003.
- Codex Alimentarius (1979). Codex standard for foods for special dietary use for persons intolerant to gluten Codex Stan 118 - 1979
- Garstang, J. R., & Spink, J. H. (2011). Cultural practices: Focus on major barley producing regions. In S. E. Ullrich (Ed.), *Barley, production, improvement, and uses* (pp. x, 637 p.). Chichester, UK: Wiley-Blackwell.
- Guerdrum, L. J., & Bamforth, C. W. (2011). Levels of gliadin in commercial beers. *Food Chemistry*, 129(4), 1783-1784. doi:DOI 10.1016/j.foodchem.2011.06.021.
- Guerdrum, L. J., & Bamforth, C. W. (2012). Prolamin Levels Through Brewing and the Impact of Prolyl Endoproteinase. *Journal of the American Society of Brewing Chemists*, 70(1), 35-38. doi:Doi 10.1094/Asbcj-2012-0130-01.

- Haas-Lauterbach, S., Immer, U., Richter, M., & Koehler, P. (2012). Gluten Fragment Detection with a Competitive ELISA. *Journal of Aoac International*, *95*(2), 377-381. doi:10.5740/jaoacint.SGE\_Haas-Lauterbach.
- Kanerva, P., Sontag-Strohm, T., & Lehtonen, P. (2005). Determination of prolamins in beers by ELISA and SDS-PAGE. *Journal of the Institute of Brewing*, *111*(1), 61-64.
- Krupnova, O. V. (2010). Relation between grain weight and falling number in soft spring wheat. *Russian Agricultural Sciences*, *36*(5), 321-323. doi:10.3103/S1068367410050010.
- McCleary, B. V., McNally, M., Monaghan, D., Mugford, D. C., Black, C., Broadbent, R., . . . Themeier, H. (2002). Measurement of alpha-amylase activity in white wheat flour, milled malt, and microbial enzyme preparations, using the ceralpha assay: Collaborative study. *Journal of Aoac International*, *85*(5), 1096-1102.
- McCleary, B. V., & Sheehan, H. (1987). Measurement of Cereal Alpha-Amylase - a New Assay Procedure. *Journal of Cereal Science*, *6*(3), 237-251.
- MEBAK. (2013). *Mitteleuropäische Brautechnische Analysenkommission (MEBAK)* Vol. Wort, Beer and Beer-based Beverages, Freising-Weihenstephan, Germany.
- Oliveira, P. M., Mauch, A., Jacob, F., Waters, D. M., & Arendt, E. K. (2012). Fundamental study on the influence of Fusarium infection on quality and ultrastructure of barley malt. *International Journal of Food Microbiology*, *156*(1), 32-43. doi:10.1016/j.ijfoodmicro.2012.02.019.
- Shewry, P. R., Pratt, H. M., & Mifflin, B. J. (1978). Varietal Identification of Barley by Analysis of Hordein Polypeptides. *Journal of the Science of Food and Agriculture*, *29*(7), 587-596. doi:DOI 10.1002/jsfa.2740290703.
- Shewry, P. R., Tatham, A. S., & Halford, N. G. (2001). Nutritional control of storage protein synthesis in developing grain of wheat and barley. *Plant Growth Regulation*, *34*(1), 105-111. doi:Doi 10.1023/A:1013382803849.
- Tanner, G. J., Blundell, M. J., Colgrave, M. L., & Howitt, C. A. (2013). Quantification of Hordeins by ELISA: The Correct Standard Makes a Magnitude of Difference. *Plos One*, *8*(2). doi:ARTN e5645610.1371/journal.pone.0056456.
- Tatham, A. S., & Shewry, P. R. (2012). The S-poor prolamins of wheat, barley and rye: Revisited. *Journal of Cereal Science*, *55*(2), 79-99. doi:10.1016/j.jcs.2011.10.013.
- Taylor, J. P., Jacob, F., & Arendt, E. K. (2015). Fundamental study on the impact of silica gel and tannic acid on hordein levels in beer. *Innovative Food Science &*



Taylor, J. P., Jacob, F., & Arendt, E. K. (2015). Fundamental Study on the Impact of Transglutaminase on Hordein Levels in Beer. *Journal of the American Society of Brewing Chemists*, 73(3), 253-260. doi:10.1094/Asbcj-2015-0527-01.

Van Zandycke, S. (2013, Nov/Dec). Gluten-reduced beers. *The New Brewer*, 79-84.

Worosz, M. R., & Wilson, N. L. W. (2012). A Cautionary Tale of Purity, Labeling and Product Literacy in the Gluten-Free Market. *Journal of Consumer Affairs*, 46(2), 288-318. doi:10.1111/j.1745-6606.2012.01230.

## **Chapter 4: A study on Malt Modification, used as a Tool to Reduce Levels of Beer Hordeins**

*Joshua P. Taylor, Fritz Jacob, Elke K. Arendt*

Submitted to the Journal of Institute of Brewing May 2016

### **Author Contributions**

Joshua Taylor was the main author for the paper, contributed to the conception and performed all the laboratory work and experiments.

Prof Jacob helped with technical aspects of the paper and reviewed the final manuscript.

Prof Arendt helped with the conception of the paper as well as making contributions and suggestions throughout the experimental process and supervised the project. Prof Arendt also critically reviewed drafts and the final manuscript

## **Abstract**

Storage proteins from barley, wheat and rye are toxic to gluten sensitive consumers. These consumers include those suffering from coeliac disease, which account for up to 1% of the global population, and Non-Coeliac Gluten Sensitivity (NCGS) that may affect even greater numbers of the population. Codex Alimentarius has published guidelines and limits of gluten in gluten-free foods, which are applied in Europe (Codex Alimentarius, 1979) and similar guidelines apply in the rest of the world.

The storage proteins present in barley are hordeins, these proteins are broken down and used by the plant as a source of amino acids during germination and growth of the barley embryo. The objective of this study was to extend the germination stage of the malting process and look at the effect on beer hordeins.

Standard MEBAK methods were used to develop an extended malting process and produce three different malts, germinated for either three days, five days or seven days. The quality of malt was assessed and model beers were produced from each malt to test the effect of modification on levels of beer hordeins.

Malt germinated for seven days produced beer with 44% less hordeins than beer made from malt germinated for three days. The malting loss was increased during the seven days of germination but otherwise all malts were of high quality. Results showed that malting conditions have a significant impact on beer hordeins.

## Introduction

Gluten sensitive consumers account for a significant portion of the population with estimates suggesting it could affect up to 5% of the global population (Elli et al., 2015). Beer is not recommended for consumption by anyone sensitive to gluten, as it is usually produced from barley malt. Barley contains proteins, which are toxic to gluten sensitive consumers. The storage proteins that cause the problems are hordeins.

Levels of gluten in food must be below a threshold of 20 mg/kg before they can be labelled gluten-free. Recommended ELISA methods for testing gluten in beer can detect soluble proteins which contain coeliac toxic epitopes common to wheat, barley and rye. The *competitive* ELISA test detects hydrolysed hordein fragments in barley malt beers and ELISA results are then multiplied by two to account for insoluble gluten proteins. This assumes that hordein x two = gluten which is not always accurate (Wieser & Koehler, 2009). In this paper results are reported as mg/kg hordeins as many insoluble proteins are removed during the brewing process (Celus, Brijs, & Delcour, 2006). To convert mg/kg hordeins into mg/kg gluten the CODEX regulations recommend applying a factor of two.

Hordeins are significantly degraded during germination and used by the developing grain (Shewry, Napier, & Tatham, 1995). A crucial step in malting is the controlled germination of barley, by extending this stage of the malting process it may be possible to maximize the breakdown of hordeins. During germination there is a breakdown of structural molecules, cell walls and various cell components (Oh & Briggs, 1989). These structural changes in the endosperm are known as modification when used in reference to malting.

Levels of beer hordeins vary (Guerdrum & Bamforth, 2011) and can be influenced by many factors (Hager, Taylor, Waters, & Arendt, 2014; Taylor, Jacob, & Arendt, 2015). It is not currently known if beer hordeins can be influenced by malt modification and the aim of this study was to look at this possibility.

The experimental approach used in this paper focused on a single barley cultivar and used MEBAK standard methods to test levels of hordeins in model beers and assess general malt and wort quality.

### **Materials and methods**

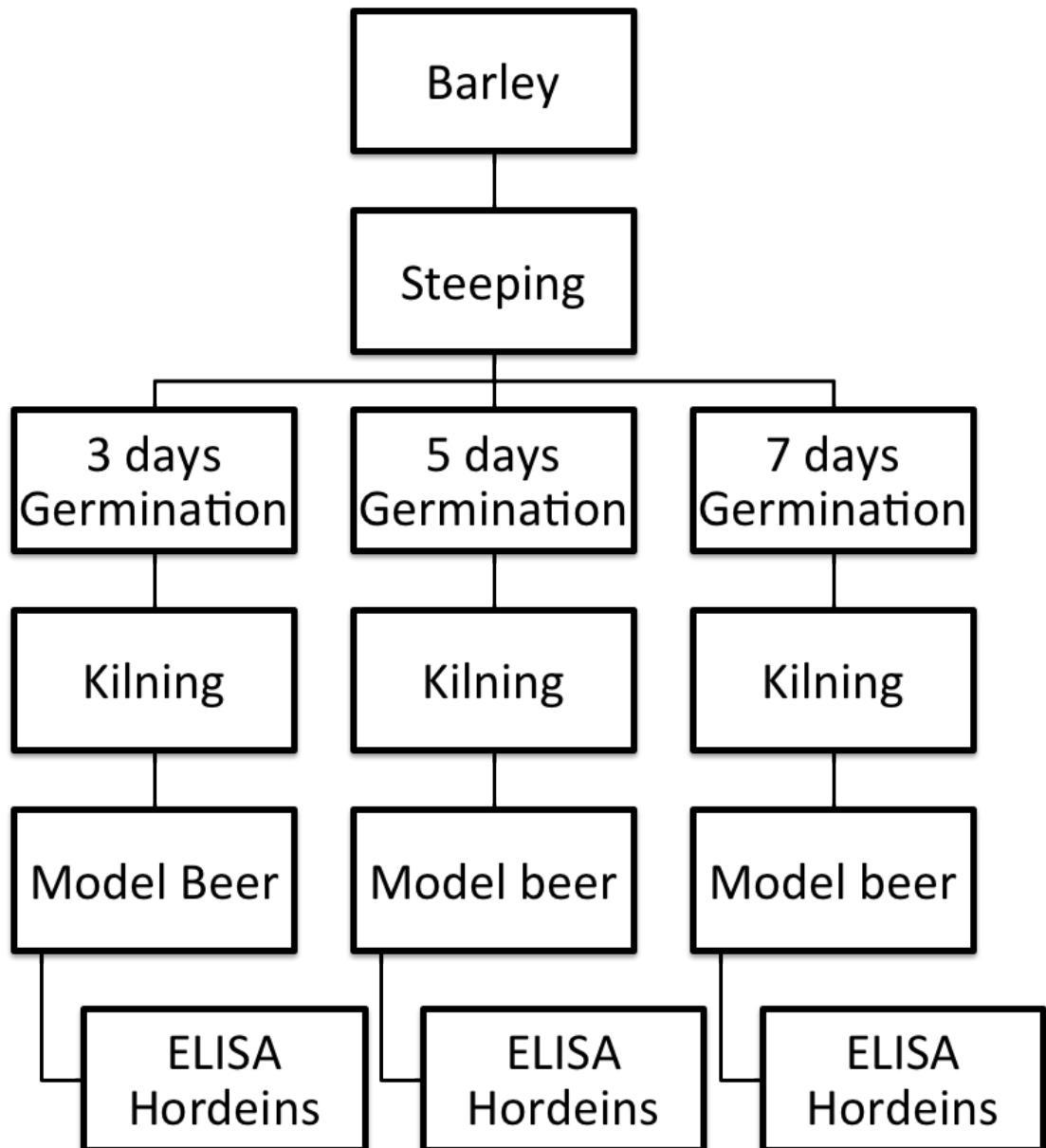
Spring malting barley (Beatrix cultivar) was sourced from Saaten Union, France because of its high malting quality. Standard quality tests were carried out to ensure the suitability of the barley for malting according to Mitteleuropäische Brautechnische Analysenkommission (MEBAK) guidelines. Moisture (MEBAK 1.5.1.1), germination (MEBAK 1.4.2.5), nitrogen (MEBAK 3.1.4.5.1.1) and thousand kernel weight (TKW) (MEBAK 1.3.2) were all tested.

### **Malting**

The barley was steeped in water at 14 °C according to MEBAK micromalting instructions (1.5.3). After a series of wet steeps and air rests for three days, the level of water inside the grain was increased to 45%. The air rests and germination were performed in a temperature-controlled chamber. Barley was turned daily to prevent rootlets from matting together. Three periods of germination were performed, three days, five days, and seven days (fig 4.1). All the grains were maintained at 45% moisture for the germination period. Kilning was performed according to MEBAK directions (1.5.3) and after kilning malt was

cleaned using a thresher (LD 180 Wintersteiger, Austria) prior to further analysis.

**Figure 4.1.** Differences between malting conditions of each experimental malt



### **Malt analysis**

TKW dry matter (d.m.) was measured for all three malts, which allowed the malting loss to be calculated as a percentage weight loss compared to the TKW of the barley.

Friability was determined using a friabilimeter (MEBAK 3.1.3.6.1) and nitrogen of each of the malts was measured using Kjeldahl methods (MEBAK 3.1.4.5.1.1).

### **Proteolytic activity**

Endo-proteolytic activity was tested in the malts, by extracting proteases and degrading azo-casein following the method of Brijs, Trogh, Jones, and Delcour (2002). Increase in absorbance at 440 nm/hr was reported.

### **Wort**

Congress mash analysis (MEBAK 3.1.4.2.1) was performed on each malt which allowed several wort characteristics to be tested. The ability of each malt to convert starch into sugars was tested using an iodine-based method (MEBAK 3.1.4.2.4). Viscosity of the wort was tested using a falling ball viscometer (MEBAK 3.1.4.1) and extract (% d.m.) of the wort was measured with an Anton-Paar density meter (DM4500 with Alcolyzer BEER ME module, Anton-Paar, Austria).

The nitrogen content of the wort (soluble nitrogen) was measured using a kjeldahl based method (MEBAK 3.1.4.5.2.1) and the Kolbach index was calculated according to MEBAK 3.1.4.5.3.

### **Hordein extraction and SDS-PAGE**

Finely ground malt (100 mg) or barley was weighed exactly into two ml microfuge tube. Hordein extraction buffer (40% propan-1-ol with 1% DTT) was

added (500 ul) and the tube was shaken at 50°C for 10 mins (Kanerva, Sontag-Strohm, Brinck, & Salovaara, 2011). Each sample was then centrifuged at 14,000 x g for 10 mins and the supernatant was removed and saved. This extraction was repeated twice more and each supernatant fraction was added to the initial tube of supernatant. The supernatant was then heated at 50°C until dry. The hordein pellet was then re-suspended on a one ul : one mg (d.m.) basis (based on weight of original extract material) in laemmli sample buffer (Laemmli, 1970) and 10 ul was loaded onto a 4 - 20% gel (Bio-Rad, Berkeley, U.S.A.) and run until the dye reached the end of the gel . Molecular weight marker (Precision Plus Protein Standards, All Blue, Bio-Rad, Berkeley, U.S.A.) was also run alongside the samples. The gel was then stained in coomassie blue and imaged on a flatbed scanner.

### **Model beer system**

Model beers were produced from each malt to test the content of beer hordeins. The model beers were made from wort produced using a congress mash method (MEBAK 3.1.4.2). The wort (250 ml) was boiled with hops (0.25 g Target 11.41%  $\alpha$ -acid, Simply Hops, Tonbridge, UK) for 60 mins and then cooled. Losses due to evaporation during boiling were replaced and 200 g of the wort was fermented with 200 mg yeast (Saflager S-23, Fermentis, Marcq-en-Barœul, France) for 15 days at 15°C. After the primary fermentation the beers were cooled to 1°C for 10 days before filtration through fluted paper filters at 1°C.

### **ELISA Analysis of Hordeins**



The filtered model beers were prepared and tested using a commercially available *competitive* ELISA kit (R7021 R-Biopharm, Darmstadt, Germany), according to MEBAK method 2.6.5 and manufacturers instructions.

### **Statistical analysis**

All analysis was performed in at least triplicate. Differences in mean were analysed using one-way ANOVA methods (SPSS, version 20, IBM, Armonk, U.S.A) and statistical significance was tested using Tukeys post hoc test ( $\alpha = 0.05$ ).

### **Results and discussion**

#### **Barley**

The TKW of barley gives an indication how big the grain is. The TKW of the Beatrix barley was 41.8g (d.m.)  $\pm$  0.2g, which is a large kernel size. The importance of germination is clear for malting barley, where failure to germinate means failure of the whole malting process. The germination rate was 98%, which is considered good for malting barley. Nitrogen levels in barley can influence levels of extract and enzymatic potential (Briggs, 1998). The level of nitrogen in the barley was 1.64% (d.m.) which is within the normal range (MEBAK, 2013). The moisture level was 13.1%, which is a suitable level for long term grain storage (MEBAK 1.5.1).

#### **Malt**

##### **Malting loss**

Malting loss is the decrease in weight (d.m.) that occurs over the entire malting process. The malting loss was lowest at 8% for day 3

three malt. Malting loss increased with germination time and day five had 10% losses. Day seven had the highest malting loss at 11% (table 4.1). The TKW of malt is reduced as the total weight losses due to respiration and rootlet losses are combined.

**Table 4.1** Mean values for malt and barley quality measurements determined using standard MEBAK methods. Different superscript letters beside mean values indicates sig differences at alpha level of 0.05 (two-tailed).

Sample	TKW g (d.m.)	SD	Malting loss % (d.m.)	SD	Friability %	SD
Day 3 Malt	38.32 <sup>a</sup>	0.21	8.28 <sup>a</sup>	0.50	78.31 <sup>a</sup>	0.89
Day 5 Malt	37.69 <sup>ab</sup>	0.29	9.80 <sup>ab</sup>	0.69	91.00 <sup>b</sup>	1.23
Day 7 Malt	37.17 <sup>b</sup>	0.47	11.03 <sup>b</sup>	1.13	96.87 <sup>c</sup>	0.32

### **Friability**

Friability also increased with extended germination time, day three malt had the lowest (78%), day five had 91% friability and this was increased to a maximum of 97% for day seven malt (table 4.1). As the barley embryo develops, structures within the endosperm are degraded, resulting in a modified, more friable endosperm (Aastrup & Erdal, 1980).

### **Nitrogen**

The majority of nitrogen contained in malt is due to the proteins, which are present. As hordeins are storage proteins, the total nitrogen content and soluble nitrogen content are of interest. The total nitrogen level of the malts ranged from 1.54% (d.m.) for day three to 1.48% (d.m.) for day seven malt (table 4.2), decreasing as germination proceeded. This decrease is caused by increased losses of nitrogen when longer rootlets containing protein are removed during the de-culming step (Briggs, 1998).

**Table 4.2.** Wort quality determined according to MEBAK methods. Means values displayed. Different superscript letters beside mean values indicates sig differences at alpha level of 0.05 (two-tailed).

Sample	Extract% (d.m.)	SD	Total nitrogen % (d.m.)	SD	Soluble nitrogen % (d.m.)	SD	Kolbach index (%)	SD
Day 3 Malt	83.66 <sup>a</sup>	0.82	1.54 <sup>a</sup>	0.03	0.83 <sup>a</sup>	0.01	54 <sup>a</sup>	0.03
Day 5 Malt	84.47 <sup>a</sup>	0.60	1.50 <sup>b</sup>	0.02	0.84 <sup>a</sup>	0.00	56 <sup>b</sup>	0.45
Day 7 Malt	82.32 <sup>a</sup>	0.91	1.48 <sup>b</sup>	0.01	0.84 <sup>a</sup>	0.01	57 <sup>b</sup>	1.04

The soluble nitrogen of all malts was very similar around 0.84% (table 4.2). Soluble nitrogen levels normally increase over the first days of germination, thanks to the actions of proteolytic enzymes. The levels can then remain almost constant after initial increases (Briggs, 1998). Our samples did not display an increase, soluble nitrogen content by day three was already high for malt (MEBAK 3.1.4.5.2.1) and remained the same until day seven (table 4.2). This suggests any increase in soluble nitrogen had already occurred within the first three days of germination.

The Kolbach index is the ratio of soluble protein to total protein. The Kolbach index was high for all the malts, indicating they were all well modified. It ranged from 54% for day three to 57% for day seven (table 4.2) and this would be expected to increase over time as it is another malt quality that increases with extended germination (Briggs, 1998). Care must be taken when using malt with a very high Kolbach index as they can negatively affect beer foam, which must be considered (Kunze, 2010).

### **Endo-Proteolytic activity**

Activities of many enzymes are increased during germination (Kuntz & Bamforth, 2007), as proteases are responsible for breaking down hordeins within the grain, the proteolytic activity in the malt is relevant when studying beer hordeins. Endo-proteolytic activity for day three malt was lowest at 0.317 (abs at 440 nm / hr), activity increased in day five to (0.368 abs@440nm/hr). Day seven malt displayed the maximum activity of 0.378 (abs at 440 nm / hr) (table 4.3). The extra enzyme activity found during germination is needed for the grain to continue developing (Jones, 2005) and during a long germination period likely has an impact on beer hordeins.

**Table 4.3.** Mean values for wort quality and proteolytic activity determined according to MEBAK methods. Different superscript letters beside mean values indicates sig differences at alpha level of 0.05 (two-tailed).

Sample	Viscosity (mPa x s)	SD	Fermentability (%)	SD	endo protease abs 440nm/hr	SD
Day 3 Malt	1.59 <sup>a</sup>	0.006	80.40 <sup>a</sup>	0.3	0.317 <sup>a</sup>	0.020
Day 5 Malt	1.54 <sup>b</sup>	0.003	82.00 <sup>b</sup>	0.1	0.368 <sup>b</sup>	0.020
Day 7 Malt	1.51 <sup>c</sup>	0.007	82.60 <sup>c</sup>	0.3	0.378 <sup>b</sup>	0.020

### Wort

Wort must provide certain qualities, which brewers expect and need to produce a good quality product. Saccharification is the breakdown of starch into fermentable sugars and should occur as quickly as possible during brewing. Saccharification was achieved for all malts within 10 minutes of reaching 70°C during mashing, times of less than 15 minutes are normal for pale malts (MEBAK 3.1.4.2.4).

Viscosity of wort helps to predict, if there will be problems in the course of wort processing. The wort viscosity was highest in wort from day three (1.59 mPa x s), 1.54 mPa x s for day five wort and lowest from day seven wort (1.51 mPa x s) (table 4.3). The viscosity of all worts tested was normal (MEBAK 3.1.4.4.1) and mash filtration time for each was also normal (MEBAK 3.1.4.2.5). As malt becomes more modified, high molecular weight substances such as arabinoxylans and  $\beta$ -glucan are broken down (Kuntz & Bamforth, 2007), causing a decrease in wort viscosity.

Wort should contain between 79 – 82% extract (d.m.) to be considered high quality. Day seven wort contained 82% extract (d.m.), day five contained 84% extract (d.m.) and day three provided 83% extract (d.m.) (table 4.2). The levels of extract were not significantly different, but the levels increased between day three and day five and then begin to drop again for day seven malt, as losses due to metabolism of the malt increased (Briggs & Hough, 1981).

The extract should also be fermentable, and fermentability of wort must be between 77 - 83% to be considered good quality (MEBAK 3.1.4.10.1.2). The fermentability of the wort was lowest from day three (80%), day five was 82% and most fermentable was from day seven (83%) (table 4.3). Fermentability of the wort has been reported to increase with level of modification (Edney et al., 2007). This could again be influenced by the increased breakdown of  $\beta$ -glucan during germination, which may increase access for enzymes to the endosperm. Also, complete hydrolysis of  $\beta$ -glucan yields glucose which adds to the fermentable sugars and increases fermentability (Molina-Cano et al., 2002). The overall quality of the wort from all malts was high and extending the period of

germination did not negatively affect the results of our tests. Further work would be needed to assess the impact of using highly modified malts on beer quality.

### **SDS-PAGE of total malt hordeins**

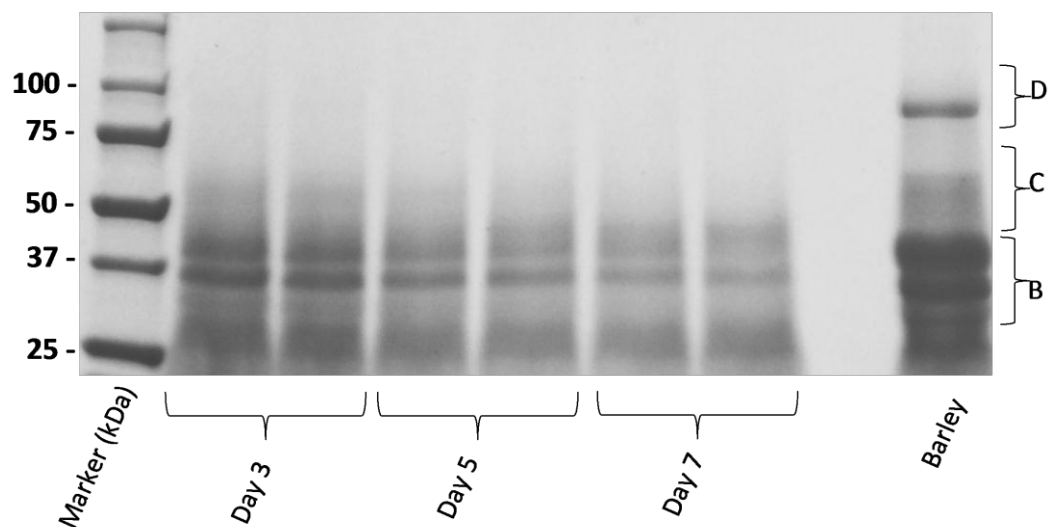
SDS-PAGE analysis of total hordeins extracted from malt is shown in fig 4.2. Hordeins from unmalted barley (lane 9) show a band approximate to the 100 kDa marker corresponding to D-hordeins. There are also major bands between 25 and 50 kDa, which correspond mainly to B-hordeins and potentially some C-hordeins (Field, Shewry, Miflin, & March, 1982). The B and C-hordeins are the major protein bands present in the extract, showing the highest amount of protein (fig 4.2). Day three hordein extract (lanes 3 and 4) shows bands which correspond to B and C-hordeins between 25 and 50 kDa. There is no evidence of D-hordeins in the extract and they are probably degraded within the first three days of germination (Weiss, Postel, & Gorg, 1992). The B and C –hordeins are clearly degraded when compared to the hordeins from unmalted barley. Day five hordein extract also shows B and C-hordeins, again without any D-hordeins. The bands from five day B and C-hordeins are not as strong as shown from day three hordein extract. This is probably due to the increased number of proteins broken down as the germination is prolonged, the developing embryo needs more proteins to be degraded to peptides and amino acids where they can then form the basis of new proteins for the next stages of development (Jones, 2005). Day seven hordein extract shows the greatest reduction in proteins present in the hordein extract. The B and C – hordeins are visible again around the 25 – 50 kDa marker, no other bands were present. The bands from the day



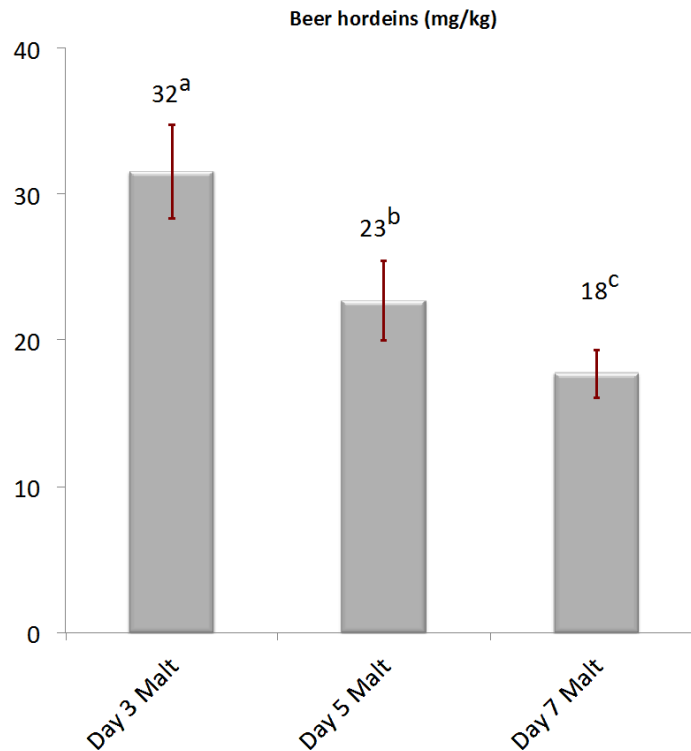
seven hordein extract were the weakest of all, which would be expected because the barley embryo was active for the longest period. The SDS-PAGE result shows that total hordeins in Beatrix cultivar were degraded to a greater extent with a longer germination period.

Although the amounts of hordeins that persist to the final beer are approximately only 0.2% of the level present in the initial malt (Dostalek, Hochel, Mendez, Hernando, & Gabrovská, 2006), the beers that were produced from these malts also had lower levels of hordeins, corresponding to longer germination times (fig 4.3). This shows that the malting regime has a significant impact on beer hordeins.

**Fig 4.2.** SDS-PAGE of hordeins extracted from each malt and un-malted barley. Day three, five and seven are the different period of germination for each malt. Duplicate extractions run next to each other on the same gel. The results are representative of results from all samples.



**Fig 4.3.** Level of hordeins in model beers produced from each malt. Error bars represent standard deviation, different letters beside values indicates a significant difference ( $\alpha = 0.05$ )



### **Model beer hordeins**

Extending the germination time of the malting process for Beatrix cultivar resulted in a significant decrease in the levels of beer hordeins (fig 4.3). Beer hordeins from small scale fermentations of each malt showed the day three beer contained the highest level of hordeins (32 mg/kg). This was reduced by 28% in day five beer and the least amount of hordeins was found in day seven beer which was reduced by 44% compared to hordeins in day three beer (shown in fig 4.3). Hordeins are degraded during barley germination (Baxter, Booer, & Wainwright, 1978), our results confirm that the changes that hordeins undergo during malting influences the content of beer hordeins in the barley

cultivar tested. The SDS-PAGE shows a reduction in total hordeins that are extracted from the malt germinated for seven days, compared to germination for three days. This is probably due to the increasing amount of degradation that occurs during malting. This increase in protein breakdown can alter protein solubility. If hordeins are modified sufficiently, the amount of hordeins that are soluble in wort may increase. It is possible this would have resulted in a net increase in beer hordeins if germination was extended longer, but this was not evident in our model beers. The levels of beer hordeins instead decreased during extended germination. This corresponds to proteins being consumed by the developing barley embryo and much of the remainder being broken down to a point at which they no longer contain epitopes for the ELISA antibody to react with. This complete degradation means that any hordein remnants, which no longer react with ELISA assay, should be degraded to the point which they are no longer toxic to gluten sensitive consumers (Wieser & Koehler, 2012).

## **Conclusion**

This study shows beer hordeins were reduced when highly modified malts were used. The barley cultivar used was commercially available and results are likely to be valid for other cultivars. There is also potential for further reductions of beer hordeins by treating malt with gibberellic acid to increase the production of malt enzymes. This work demonstrates that simple changes to the malting process could influence beer hordeins and these adjustments could be used as a tool to help produce beers very low in hordeins.

## Literature cited

- Aastrup, S., & Erdal, K. (1980). Quantitative-Determination of Endosperm Modification and Its Relationship to the Content of 1,3-1,4-Beta-Glucans during Malting of Barley. *Carlsberg Research Communications*, 45(5), 369-379. doi:Doi 10.1007/Bf02906161
- Baxter, E. D., Booer, C. D., & Wainwright, T. (1978). Degradation of Hordein by the Proteolytic Enzymes of Barley and Malt. *Journal of the Institute of Brewing*, 84(1), 30-33. doi:10.1002/j.2050-0416.1978.tb03834.x
- Briggs, D. E. (1998) *Malts and malting* (1st ed., pp. 69, 170, 628, 175). London, Blackie Academic.
- Briggs, D. E., & Hough, J. S. (1981). Malting and brewing science (2nd ed., pp. 82, 83, 112). London, Chapman and Hall.
- Brijs, K., Trogh, I., Jones, B. L., & Delcour, J. A. (2002). Proteolytic enzymes in germinating rye grains. *Cereal Chemistry*, 79(3), 423-428. doi:Doi 10.1094/Cchem.2002.79.3.423
- Celus, I., Brijs, K., & Delcour, J. A. (2006). The effects of malting and mashing on barley protein extractability. *Journal of Cereal Science*, 44(2), 203-211. doi:10.1016/j.jcs.2006.06.003
- Codex Alimentarius (1979). Codex standard for foods for special dietary use for persons intolerant to gluten *Codex Stan 118 - 1979*
- Dostalek, P., Hochel, I., Mendez, E., Hernando, A., & Gabrovska, D. (2006). Immunochemical determination of gluten in malts and beers. *Food Additives and Contaminants*, 23(11), 1074-1078. doi:Doi 10.1080/02652030600740637
- Edney, M. J., Eglinton, J. K., Collins, H. M., Barr, A. R., Legge, W. G., & Rossnagel, B. G. (2007). Importance of endosperm modification for malt Wort fermentability. *Journal of the Institute of Brewing*, 113(2), 228-238.
- Elli, L., Branchi, F., Tomba, C., Villalta, D., Norsa, L., Ferretti, F., . . . Bardella, M. T. (2015). Diagnosis of gluten related disorders: Celiac disease, wheat allergy and non-celiac gluten sensitivity. *World Journal of Gastroenterology*, 21(23), 7110-7119. doi:10.3748/wjg.v21.i23.7110
- Field, J. M., Shewry, P. R., Mifflin, B. J., & March, J. F. (1982). The Purification and Characterization of Homologous High Molecular-Weight Storage Proteins from Grain of Wheat, Rye and Barley. *Theoretical and Applied Genetics*, 62(4), 329-336.

- Guerdrum, L. J., & Bamforth, C. W. (2011). Levels of gliadin in commercial beers. *Food Chemistry*, 129(4), 1783-1784. doi:DOI 10.1016/j.foodchem.2011.06.021
- Hager, A. S., Taylor, J. P., Waters, D. M., & Arendt, E. K. (2014). Gluten free beer - A review. *Trends in Food Science & Technology*, 36(1), 44-54. doi:10.1016/j.tifs.2014.01.001
- Jones, B. L. (2005). Endoproteases of barley and malt. *Journal of Cereal Science*, 42(2), 139-156. doi:10.1016/j.jcs.2005.03.007
- Kanerva, P., Sontag-Strohm, T., Brinck, O., & Salovaara, H. (2011). Improved extraction of prolamins for gluten detection in processed foods. *Agricultural and Food Science*, 20(3), 206-216.
- Kuntz, R. J., & Bamforth, C. W. (2007). Time course for the development of enzymes in barley. *Journal of the Institute of Brewing*, 113(2), 196-205.
- Kunze, W. (2010). *Technology Brewing and Malting*, Malt production (pp. 199). VLB, Berlin, Germany.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227(5259), 680-685.
- MEBAK. (2013). *Mitteleuropäische Brautechnische Analysenkommission (MEBAK)* Vol. Wort, Beer and Beer-based Beverages, Freising-Weihenstephan, Germany.
- Molina-Cano, J. L., Romera, E., Aikasalo, R., Perez-Vendrell, A. M., Larsen, J., & Rubio, A. (2002). A reappraisal of the differences between Scandinavian and Spanish barleys: Effect of beta-glucan content and degradation on malt extract yield in the cv. Scarlett. *Journal of the Institute of Brewing*, 108(2), 221-226.
- Oh, S. Y., & Briggs, D. E. (1989). Modification in Malting Barley. *Journal of the Institute of Brewing*, 95(2), 83-88.
- Shewry, P. R., Napier, J. A., & Tatham, A. S. (1995). Seed Storage Proteins - Structures and Biosynthesis. *Plant Cell*, 7(7), 945-956. doi:DOI 10.1105/tpc.7.7.945
- Taylor, J. P., Jacob, F., & Arendt, E. K. (2015). Fundamental study on the impact of silica gel and tannic acid on hordein levels in beer. *Innovative Food Science & Emerging Technologies*, 31, 177-184. doi:10.1016/j.ifset.2015.07.007
- Weiss, W., Postel, W., & Gorg, A. (1992). Qualitative and Quantitative Changes in Barley Seed Protein-Patterns during the Malting Process Analyzed by

Sodium Dodecyl Sulfate-Polyacrylamide Gel-Electrophoresis with Respect to Malting Quality. *Electrophoresis*, 13(9-10), 787-797. doi:DOI 10.1002/elps.11501301172

Wieser, H., & Koehler, P. (2009). Is the calculation of the gluten content by multiplying the prolamin content by a factor of 2 valid? *European Food Research and Technology*, 229(1), 9-13. doi:10.1007/s00217-009-1020-5

Wieser, H., & Koehler, P. (2012). Detoxification of Gluten by Means of Enzymatic Treatment. *Journal of Aoac International*, 95(2), 356-363. doi:10.5740/jaoacint.SGE\_Wieser

# **Chapter 5: Reduction of Hordein content in beer by applying Prolyl-endoprotease to the malting process**

*Joshua P. Taylor, Fritz Jacob, Elke K. Arendt*

Submitted to the Journal American Society of Brewing Chemists (ASBC) - January  
2016

## **Author Contributions**

Joshua Taylor was the main author for the paper, contributed to the conception and performed all the laboratory work and experiments.

Prof Jacob helped with technical aspects of the paper and reviewed the final manuscript.

Prof Arendt helped with the conception of the paper as well as making contributions and suggestions throughout the experimental process and supervised the project. Prof Arendt also critically reviewed drafts and the final manuscript.



## **Abstract**

Barley malt contains hordein proteins, which gluten sensitive consumers cannot tolerate. Beer produced from barley malt also contains hordeins. *Aspergillus Niger* Prolyl Endoprotease (AN-PEP) is an enzyme that has been used very effectively to reduce beer hordeins during fermentation. The objective of this study was to apply AN-PEP during the steeping and germination of barley and evaluate the impact on malt quality characteristics and the hordein content of model beers.

Pilot scale malting trials were performed and the barley was germinated for either three days or five days with and without AN-PEP. Model beers were produced from malts and the levels of beer hordeins were tested using R5 antibody based *competitive* ELISA. The malt friability, extract, viscosity and several other quality parameters were measured using industry standard MEBAK methods.

Treatment of malt with AN-PEP for five days resulted in a 46% reduction in beer hordeins compared to beer produced from the five day control malt and the quality of the AN-PEP treated malt was comparable to untreated malt.

Applying enzymes to germinating grain is a novel way to influence the levels of hordeins in barley malt beers.

## Introduction

Coeliac disease affects about 1% of global population (Tack, Verbeek, Schreurs, & Mulder, 2010), it is caused by an immune reaction to cereal storage proteins (gluten proteins) found in wheat, rye, barley and closely related cereals (Sollid & Lundin, 2009). Several other medical conditions (Biesiekierski et al., 2011; Catassi et al., 2013), also benefit from avoiding gluten in the diet. The gluten proteins present in barley are hordeins, they contain a high proportion of proline, are largely insoluble in water and can be extracted in aqueous alcohol solutions (Shewry, Napier, & Tatham, 1995).

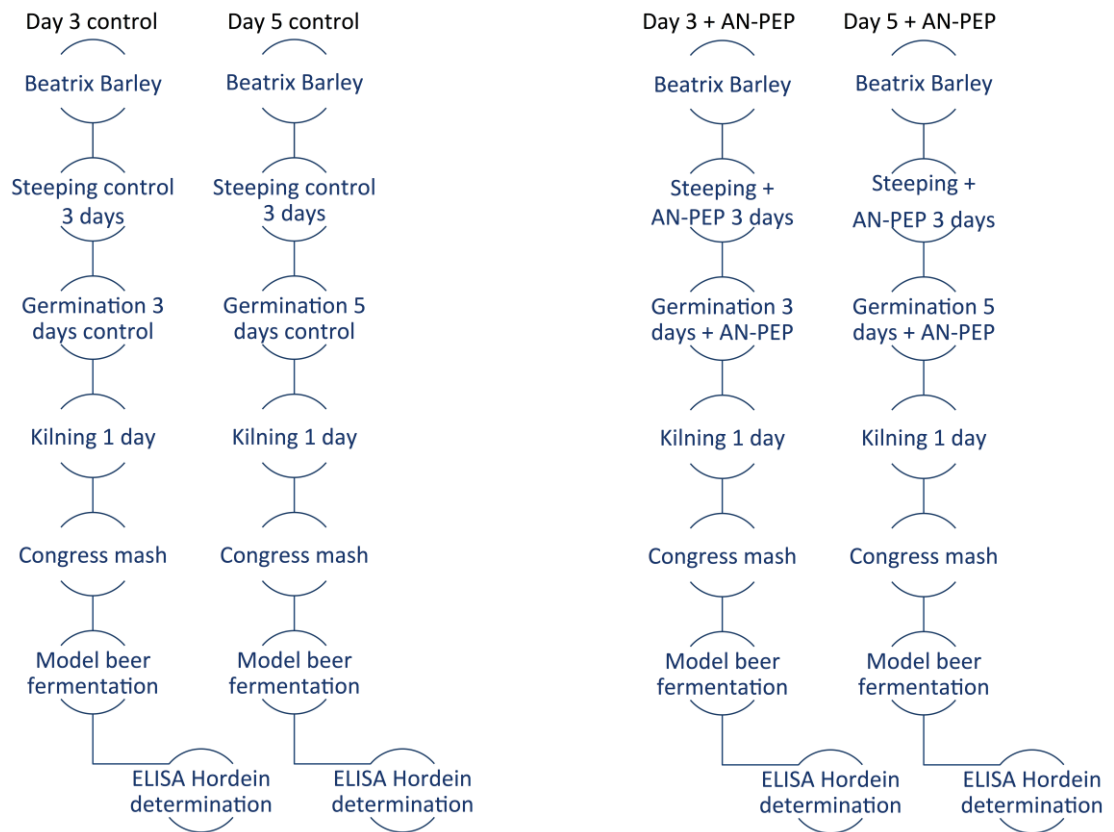
Proteolytic enzymes have been reported to degrade coeliac toxic proteins (Shan, Martin, Sollid, Gray, & Khosla, 2004) and have been considered as a way to treat coeliac patients by degrading ingested gluten in the stomach (Tack et al., 2013). *Aspergillus Niger* Prolyl-Endoprotease (AN-PEP) is a protease, that specifically targets proteins containing proline (Stepniak et al., 2006).

Researchers have also shown, that treatment of beer with AN-PEP enzymes during fermentation is very effective in reducing levels of hordein in beer (Guerdrum & Bamforth, 2012). AN-PEP has also been applied in the food industry to produce gluten-free wheat starch (Walter, Wieser, & Koehler, 2014). However, the use of enzymes during processing of malt is not well documented and there has been no published use of AN-PEP applied during the malting process.

In this study AN-PEP was applied during steeping and germination steps of malting. Model scale beers were then produced from each malt (fig 5.1).

The objective of this experiment was to determine if application of AN-PEP during steeping and germination of barley could reduce beer hordeins. The impacts on malt quality were also evaluated.

**Fig 5.1.** Outline of malting conditions comparing treatment with enzyme during steeping and germination and the untreated control.



## **Materials and methods**

### **Barley and malting**

Spring barley (Beatrix cultivar) was purchased from Saaten union (France). All standard methods used were performed according to Mittleuropäische Brautechnische Analysenkommission (MEBAK, 2013) directions. Germination (MEBAK 1.4.2.5) and thousand kernel weight (TKW) (MEBAK 1.3.2) of the barley were measured. The nitrogen content of the barley and malt was determined using kjeldahl method (MEBAK 1.5.2.1).

### **Malting**

Samples (500 g) of barley were malted according to a modified MEBAK (1.5.3) micromalting method. Steeping took place in perforated stainless steel boxes placed into plastic 2.5 L trays holding steep water for five hours at 14 ° C. This was followed by an air rest in a temperature controlled chamber (14 ° C) for 19 hours. The second step was three hours followed by 21 hour air rest to achieve a final moisture content of 45%. Germination took place in perforated stainless steel germination trays held at 14 ° C. Moisture levels were checked daily and maintained at 45% by spraying.

The enzyme treated samples were subject to the same conditions (Fig 5.1) with the addition of AN-PEP (1.25% v/v) to the steep water. In germination, enzyme treated samples were maintained at a moisture level of 45% by spraying water containing AN-PEP (1.25% v/v). The enzyme used was a commercially available product (Brewers Clarex, DSM, Netherlands), which is commonly used for preventing chill-haze in beer.

After three days and five days germination (Fig 5.1), control samples (three day control, five day control) and enzyme treated samples (three day + AN-PEP, five day + AN-PEP) were removed and kilned in a computer controlled malting machine (B3000 Joe White, Australia) following MEBAK methods. Malted samples were then cleaned using a thresher (WINTERSTEIGER LD180, AG, Austria) to remove rootlets.

### **Malt and wort analysis**

The TKW of the malt was tested (MEBAK 1.3.2), Congress mash was also performed (MEBAK 3.1.4.2) and the mash produced from this was used to perform a saccharification test (MEBAK 3.1.4.2.4), filtration (MEBAK 3.1.4.2.5), viscosity (MEBAK 3.1.4.4.1), extract (MEBAK 3.1.4.2.2), soluble nitrogen (MEBAK 3.1.4.5.2.1) and Kolbach index analysis (MEBAK 3.1.4.5.3) were also determined.

### **SDS-PAGE on hordeins**

Hordeins were extracted from 100 mg finely ground malt (0.2 mm in disc mill) using 500 ul 40% 1-propanol containing 1% w/v DTT according to Kanerva, Sontag-Strohm, Brinck, and Salovaara (2011). The sample was vortexed thoroughly before extraction at 50 °C with shaking for 20 mins. Samples were then centrifuged at 14,000 x g for 10 mins at room temp. The supernatant was transferred to another tube and the pellet was re-suspended in extraction buffer and the process was repeated a further two times. The supernatants were combined and evaporated to dryness at 50 °C. Evaporated samples were re-suspended on a one ul to one mg (d.m.) basis in SDS-PAGE sample buffer (Laemmli, 1970). The samples were vortexed and then heated to 95 °C and vortexed again to re-suspend completely. Samples were then centrifuged at

14,000 x g before loading onto a 4 - 20% gradient precast gel (Biorad, California, Berkeley, U.S.A.). Molecular weight marker (Precision Plus Protein Standards, All Blue, Bio-Rad, Berkeley, U.S.A.) was also run on the gel. The gel was then stained in Coomassie blue (Diezel, Kopperschlager, & Hofmann, 1972) prior to imaging.

### **Model Beer Production**

Model Beer was made using a congress wort based system for each of the malts. Congress wort was produced (MEBAK 3.1.4.2) and then 250 ml was boiled with target hops (11.41%  $\alpha$ -acid, T90, Simply Hops, UK) for one hour. Flasks were then cooled and any evaporation of water during boiling was replaced. The boiled, hopped wort (200 g) was then fermented with 200 mg dried yeast (Saflager S-23, Fermentis, France). Fermentation temperatures were 15 °C for 15 days followed by six days at 1 °C. Model beer was then filtered through fluted paper filters at 1 °C before determination of hordeins.

### **Hordein determination**

Beer hordeins were measured using a *competitive* ELISA kit (R7021, R-Biopharm AG, Germany). Hordeins from the beer were extracted in 60% ethanol containing 10% fish gelatin. The extracted hordeins were diluted and assayed using the *competitive* ELISA according to MEBAK method 2.6.5 and manufacturer's guidelines.

### **Statistical Analysis**

Data was analysed with SPSS (version 20, IBM, Armonk, U.S.A.) using one way ANOVA and Tukey HSD post-hoc test ( $\alpha = 0.05$ ) all analyses were performed at least three times.

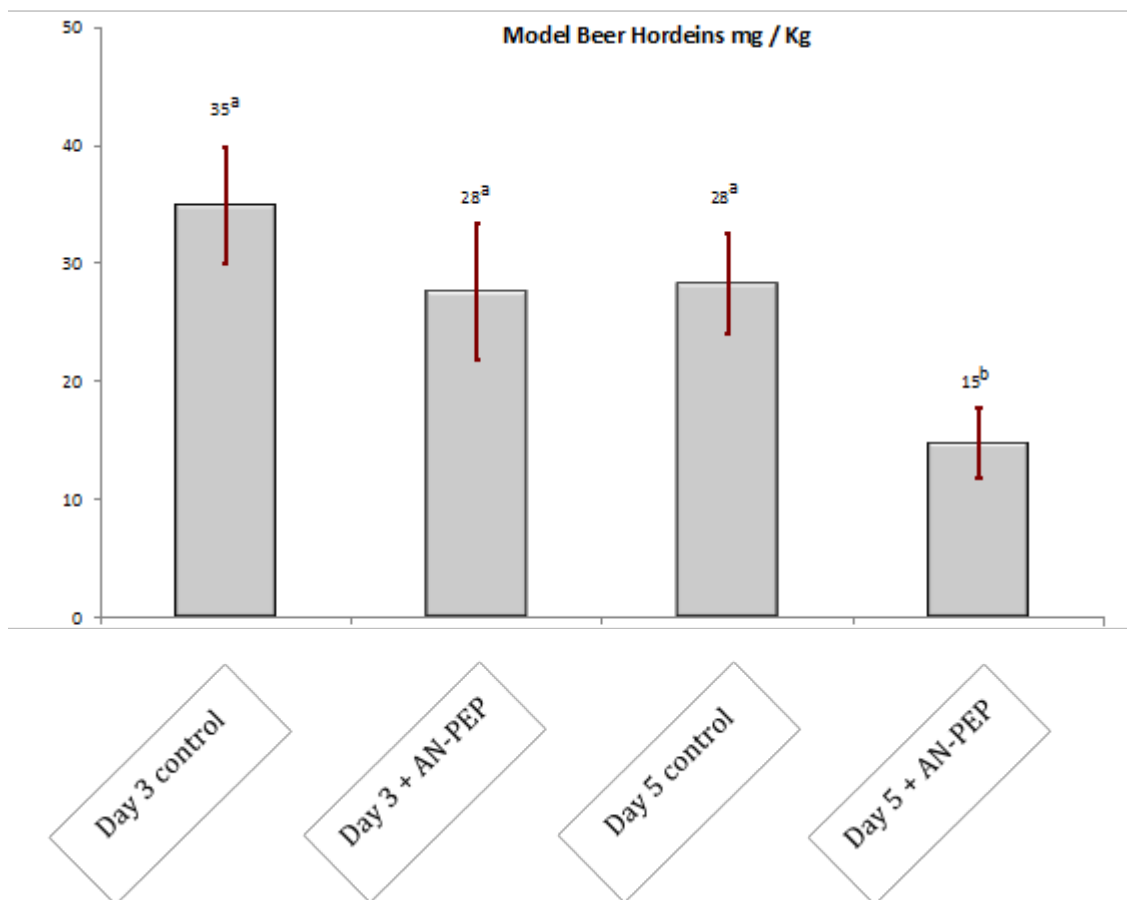
## Results and discussion

### Model beer – levels of hordein proteins

The small scale beers produced from each malt showed if a reduction in hordeins was possible by treating malt with AN-PEP. *Competitive* ELISA results showed, that three day control beer contained 35 mg/kg hordeins (fig 5.2), whereas the three day + AN-PEP beer contained 28 mg/kg hordeins. This difference was not large enough to be significant, but with extended treatment reductions could be greater.

Beer from five day control contained 28 mg/kg hordeins and five day + AN-PEP produced a beer with 15 mg/kg hordeins. The longer germination time combined with the extended contact with the AN-PEP enzyme results in a decrease in beer hordein levels. As the malting process proceeds, cell walls and  $\beta$ -glucan are broken down (Briggs, 1978; Edney et al., 2007) and this should allow greater access for the applied AN-PEP enzymes to penetrate into the barley kernel. This may let the AN-PEP breakdown hordeins deeper in the endosperm.

**Fig 5.2.** Mean values for beer hordeins determined in model beers using competitive ELISA assay. Different superscript letters beside values indicate significant differences at  $\alpha = 0.05$  (two-tailed) and error bars represent standard deviation.



Extending the germination time also caused a reduction in the level of beer hordeins. The control beer made from five day germinated malt had lower hordeins than the control beer made from three day germinated malt. This could be due to endogenous enzymes present in the germinating barley breaking down the storage proteins during germination (Bethune, Strop, Tang, Sollid, & Khosla, 2006).

The use of enzymes during the malting process has not been researched in great detail, one example being the use of a cellulase during steeping, which produced a wort with improved filtration rate and reduced viscosity (Grujic, 1998).



Overall the levels of beer hordeins were reduced significantly with AN-PEP treatment of malt. However, in order to be useful, AN-PEP treatment should not result in poor quality malt.

### **Barley Quality**

AN-PEP was applied to malting barley during the steeping and germination process to determine if hordein levels in beer could be reduced. The length of germination was either the standard MEBAK micromalting three day duration (3 day control) or extended to five day germination (5 day control) (fig 5.1). The concentration of AN-PEP used was selected after small scale germination trials. A range of AN-PEP concentrations were applied (0.125, 1.25 and 12.5% v/v AN-PEP). The highest concentration tested which did not affect germination was 1.25% v/v AN-PEP and this was chosen for application during malting (results not shown).

The barley used was of high quality and suitable for producing high quality malt according to all tests performed (table 5.1). Malting quality barley contains nitrogen at a level between 1.6 – 1.76% (d.m.) (MEBAK 3.1.4.5.1.1) and Beatrix barley contained 1.64% nitrogen (d.m.). A germination rate of 98% was achieved and the barley had a thousand kernel weight (d.m.) of 41.8 g. All of these parameters showed this had potential for high quality malt production according to MEBAK guidelines.

**Table 5.1** Mean values for malt quality parameter determined using MEBAK methods. Different superscript letters beside values indicate significant differences at  $\alpha = 0.05$  (two-tailed) standard deviation (SD) shown for each value.

Sample	TKW (g d.m.)	SD	Malting loss (% d.m.)	SD	Friability %	SD
3 day control	38.9 <sup>a</sup>	0.3	6.8 <sup>ab</sup>	0.7	75.0 <sup>a</sup>	1.0
3 day + AN-PEP	39.3 <sup>a</sup>	0.2	6.0 <sup>a</sup>	0.5	72.0 <sup>b</sup>	1.4
5 day	38.3 <sup>b</sup>	0.5	8.4 <sup>bc</sup>	1.1	90.3 <sup>c</sup>	0.8
5 day + AN-PEP	38.0 <sup>b</sup>	0.2	9.0 <sup>c</sup>	0.4	89.9 <sup>c</sup>	0.8

## Malt Quality

### Malting loss

Malting loss is of great economic interest to the maltster as a high malting loss increases costs. TKW is a measure of barley grain size, the TKW of 41.8 g (d.m.) indicated Beatrix grains were large according to MEBAK (1.3.2). During malting there are reductions in weight due to metabolism and loss of rootlets, these losses can be measured by calculating the TKW of the malt. The difference between the weight of barley and malt was calculated as a percentage malting loss.

Treating the malt with AN-PEP during steeping and germination did not have a large effect on the malting loss. There was a lower malting loss for three day control than for five day control due to increased losses of rootlets and the metabolism of the germinating grain (Briggs, 1998).

The three day control had a TKW similar to its enzyme treated counterpart (table 5.2). The difference between three day control and three day + AN-PEP was not significant when comparing TKW of the malts. Malting loss for three day control was 6.0% and loss for day three + AN-PEP was 6.8%, but the

differences were not significant ( $p > 0.05$ ). The five day control showed a higher malting loss than the three day control (8.4%) and the five day + AN-PEP had a malting loss of 9% (table 5.1). These differences between malt treated with AN-PEP and the control are not significantly different.

Malting loss is known to increase with extended germination (Briggs, 1998), the AN-PEP enzyme treatment of barley did not significantly affect the malting loss (table 5.1).

**Table 5.2** Wort quality determined using MEBAK methods. Mean values shown with standard deviations. Different superscript letters beside values indicate significant differences at  $\alpha = 0.05$  (two tailed).

Sample	Extract		Total nitrogen %		Soluble nitrogen %		Kolbach index	
	d.m.	SD	d.m.	SD	d.m.	SD	index	SD
3 day control	82.8 <sup>ab</sup>	0.45	1.49 <sup>a</sup>	0.00	0.79 <sup>a</sup>	0.00	53.05 <sup>a</sup>	0.38
3 day + AN-PEP	82.2 <sup>a</sup>	0.63	1.49 <sup>a</sup>	0.01	0.79 <sup>a</sup>	0.01	53.22 <sup>a</sup>	0.25
5 day	83.5 <sup>b</sup>	0.48	1.47 <sup>a</sup>	0.03	0.87 <sup>b</sup>	0.02	59.11 <sup>b</sup>	0.15
5 day + AN-PEP	83.1 <sup>ab</sup>	0.64	1.48 <sup>a</sup>	0.01	0.88 <sup>b</sup>	0.00	59.35 <sup>b</sup>	0.40

## **Friability**

The friability of malt demonstrates how hard the grain is, it was used to assess modification levels in the malt. Germination causes cell walls and structural proteins to be broken down making the malt 'friable' and easy to break. The three day control was 75% friable but the three day + AN-PEP was only 72% friable. This difference was significant (table 5.1) and could be due to AN-PEP interfering with the germination process. During germination proteins are broken down into amino acids and taken up by the developing embryo (Briggs & Hough, 1981). Treatment with AN-PEP may inhibit the natural process of enzyme development, perhaps by degrading parts of enzymes containing proline which are needed for the development.

The five day control malt had a friability of 90%, the longer germination period resulted in more complete breakdown of the endosperm structure. The five day + AN-PEP also had a friability of 90%. During the extended germination any differences in friability caused by applying AN-PEP are not significant (table 5.1). The limited impact of treating germinating barley with AN-PEP is not obvious after five days of germination. It is possible the earlier stages of development and growth are more sensitive to interference from AN-PEP but the grain can recover in the subsequent days.

## **Malt Nitrogen**

Malt nitrogen levels are indicators of overall protein content in malt. High levels can cause problems with haze, processing and reduced extract levels. Upper and lower limits for nitrogen levels in malt are between 1.2% and 2.2% (d.m.) (MEBAK 3.1.4.5.1.1). Nitrogen levels in the three day control were 1.49% (d.m.)

and the three day + AN-PEP levels were the same (table 5.2). The nitrogen level in the five day control was 1.47% (d.m.) and five day + AN-PEP was again very similar at 1.48% (d.m.).

The largest change in total nitrogen content of grain is due to proteins lost when rootlets are removed. Proteases cause solubility of proteins to change, but not the nitrogen content. Overall neither the period of germination nor the application of AN-PEP resulted in a change in malt nitrogen.

### **Soluble Nitrogen**

During germination there is normally an increase in soluble nitrogen as proteases breakdown proteins in the grain (Briggs & Hough, 1981). The three day control and three day + AN-PEP both had levels of soluble nitrogen at 0.79% (table 5.2). The five day control had soluble nitrogen levels of 0.87% (d.m.) and five day + AN-PEP had 0.88% (d.m.).

The increased level of soluble nitrogen over the period of germination is well reported and is due to the overall endosperm degradation that occurs during malting. This causes a degradation of storage proteins, an increase in water-soluble nitrogen containing compounds as well as the formation of new proteins for the developing embryo (Briggs, 1998; Briggs & Hough, 1981).

Addition of an exogenous protease might be expected to increase the levels of soluble nitrogen, but application of AN-PEP did not have an impact on soluble nitrogen. This could be due to AN-PEP acting on proteins in the germinating malt that are present in the soluble protein fraction found in wort. It could also be a result of the complex interactions that occur during germination, limiting access to proteins.  $\beta$ -glucanases, cellulases, arabinoxylanases and several

different types of protease (Jones, 2005; Kanauchi & Bamforth, 2008; Taiz & Honigman, 1976) are released by the developing grain to breakdown structures as and when the embryo needs them which demonstrate the complexity involved. The developing embryo needs to remove these structures to get access to the endosperm, it is likely there are also obstacles for the applied AN-PEP.

### **Wort quality**

#### **Extract**

Extract is the percentage of sugars which can be extracted from the malt during mashing. Extract achieved during the congress mash procedure is primarily dependent on  $\alpha$  and  $\beta$ -amylase mediated breakdown of starch present in the malt. This allows a prediction of how well malt will perform during the beer production process. The term used for complete starch breakdown into smaller sugars is saccharification. The saccharification time of all the malts was normal at less than 10 minutes after reaching 70 °C during mashing (MEBAK 3.1.4.2.4). The three day control produced clear wort with a nice aroma. Extract for good quality malt is considered to be between 79 – 82% extract (MEBAK 3.1.4.2.2). The three day control obtained 82.8% extract (d.m.) likewise, three day + AN-PEP appearance was very similar and it achieved 82.2% extract (table 5.2). The five day control also produced clear wort with a pleasant aroma and had a good extract level of 83.5% (table 5.2). Similarly five day + AN-PEP had 83.1% extract, clear wort and good aroma. The extract levels of both controls were similar and of high quality. The three day + AN-PEP extract level was a little less than three day control but the difference was significant. It is likely this is due to

AN-PEP inhibiting a part of the germination process. Because of the intrinsic need for germination to occur to produce malt, many qualities are affected. But as with the case of friability results (table 5.1), after five days of germination the five day control and five day + AN-PEP do not show any significant difference in extract, and the germinating recovers to full potential after five days.

### Fermentability

Extract produced during mashing should be highly fermentable by yeast in order to produce high quality beer. Fermentability of three day control was 79.6% (d.m.), very similar to three day + AN-PEP (79.4% (d.m.)) shown in table 5.3.

**Table 5.3** Mean values for wort quality determined using MEBAK methods and malt proteolytic activity with standard deviations. Different superscript letters beside values indicate significant differences at  $\alpha = 0.05$  (two-tailed).

Sample	Viscosity (mPa x s)	SD	Fermentability (%)	SD	endo- proteolytic activity	SD
3 day control	1.65 <sup>a</sup>	0.01	79.6 <sup>a</sup>	0.22	0.35 <sup>a</sup>	0.02
3 day + AN-PEP	1.69 <sup>a</sup>	0.07	79.4 <sup>a</sup>	0.14	0.34 <sup>a</sup>	0.02
5 day	1.56 <sup>b</sup>	0.01	81.4 <sup>b</sup>	0.24	0.40 <sup>b</sup>	0.02
5 day + AN-PEP	1.57 <sup>b</sup>	0.01	81.5 <sup>b</sup>	0.46	0.40 <sup>b</sup>	0.02

The five day control wort was more fermentable (81.4%) than the day three control wort, caused by the increased level of malt modification which has been shown to have an effect on the fermentability (Edney et al., 2007). The fermentability of five day + AN-PEP wort was 81.5% which is no different to the control and higher than both day three control and day three + AN-PEP.

Fermentability is influenced by several factors, one of the main factors being  $\beta$ -glucan breakdown. The incomplete  $\beta$ -glucan breakdown can restrict movements of enzymes (Eastwood & Morris, 1992). Hydrolysis of  $\beta$ -glucan results in the release of glucose which improves fermentability (Edney et al., 2007). The fermentability of worts was not affected by AN-PEP in our experiments.

### **Wort viscosity**

Wort viscosity is a reliable test for filtration issues during brewing. Highly viscous worts are slow to lauter and cause problems during processing. High levels of viscosity in wort can be caused by insufficient modification, where problem causing polysaccharides such as  $\beta$ -glucan remain un-degraded.

The viscosity of three day control wort (1.65 mPa x s) was higher than expected for a congress wort (MEBAK 3.1.4.4.1). The average viscosity of the three day + AN-PEP wort was higher again at 1.69 mPa x s (table 5.3).

This increase in viscosity for three day + AN-PEP wort is once again probably due to the impact AN-PEP had on the early stages of germination. All malt properties are linked to the germination of the grain, this is likely why extract, viscosity and friability are all impacted by the effects of AN-PEP treatment

Well modified malt produces wort with lower viscosity, caused by the degradation of high molecular weight substances such as  $\beta$ -glucan, arabinoxylan that occurs with a more complete modification (Briggs & Hough, 1981). The day five control had a lower viscosity (1.57 mPa x s) than wort from day three control (table 5.2) and five day + AN-PEP had viscosity almost the same (1.57



mPa x s). Any differences in viscosity caused by AN-PEP are not significant after five days of germination.

### **Endo-proteolytic activity**

As AN-PEP is an endo-protease, it is possible the endoproteolytic activity in the malt would be increased after treatment. An azo-casein based endo-protease assay was used to check the activity levels (Brijs, Trogh, Jones, & Delcour, 2002). The three day control had an activity of (0.34 abs@440nm/hour), this was similar to the three day + AN-PEP at (0.35 abs@440nm/hour) (table 5.3). The five day control had a slightly higher activity of (0.40 abs@440nm/hour) and was the same in five day + AN-PEP (table 5.3). Endo proteolytic activity of the malt was not changed when the enzyme was applied, this could be because after treatment with AN-PEP the germinating grain was kilned. Kilning subjects the grain to high temperatures for 23 hours (MEBAK 1.5.3) which would have inactivated the applied AN-PEP.

The malt and wort quality parameters tested were not hugely affected by AN-PEP treatment. The three day + AN-PEP had a lower friability, extract and viscosity which are all likely related to some interference from the AN-PEP during the natural germination process. The developing grain manages to overcome these problems and the five day + AN-PEP is of the same quality as the five day control but produces a model beer with 46% less hordeins.

### **SDS-PAGE on hordein extract of barley and malt**

Hordeins are classified based on protein solubility studies performed by Osborne in the 1930's. These fractions correspond to groups of proteins with separate functions in the grain. In barley seeds the complete hordein fraction

can be extracted using an alcohol based buffer with a reducing agent (Kanerva et al., 2011). This technique was used to extract hordeins from malt. Malt proteins undergo huge degradation during the germination process, proteins are broken down into peptides and amino acids. These breakdown products are then taken up by the embryo and used to create new proteins needed for development (Briggs, 1998).

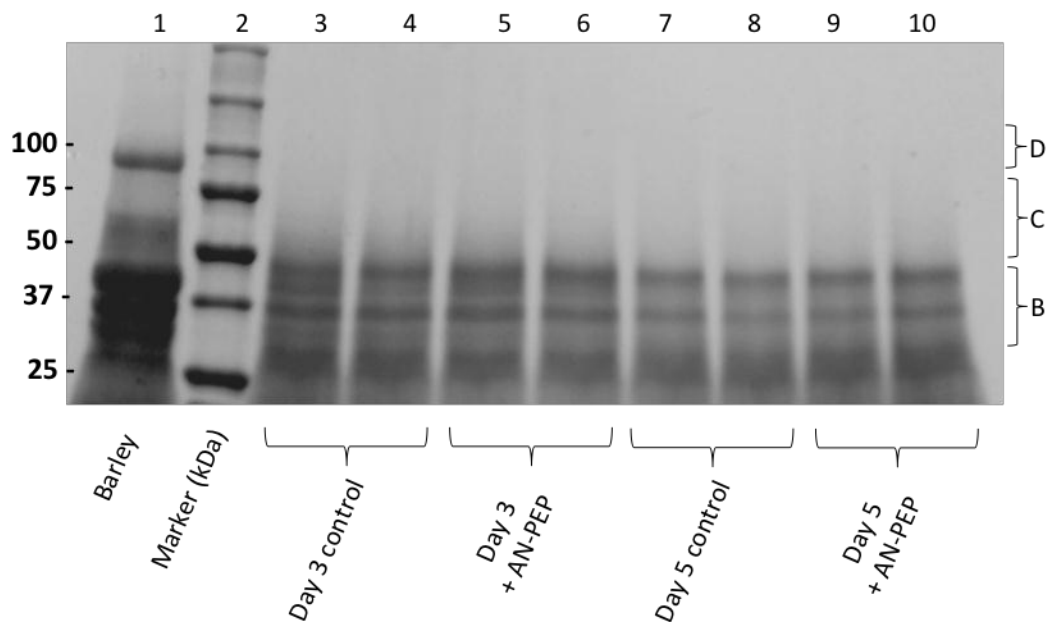
The SDS-PAGE analysis shows staining of hordein proteins (fig 5.3) from barley in lane one compared to hordeins extracted from each of the malts (lanes 3-10). The hordeins extracted from barley clearly show a band of D-hordeins around the 100 kDa marker. The B and C-hordeins are the major bands between 25-50 kDa with intense protein staining.

The hordein proteins extracted from three day control are shown in lanes 3 and 4. The D-hordeins have been completely degraded during the malting process. The B and C-hordeins are visible between 25-50 kDa, but due to degradation they are much weaker than those present in the barley extract. The extracted proteins from three day + AN-PEP (lanes 5 + 6) are virtually identical to the hordein proteins extracted from three day control. The difference shown by *competitive* ELISA results between three day control and three day + AN-PEP are not visible in the SDS-PAGE gel.

The proteins extracted from five day malt control shown on SDS-PAGE are very similar to three day control and three day + AN-PEP. There are bands corresponding to B and C-hordeins and D-hordeins are degraded (Figure 5.3).

The proteins extracted from five day + AN-PEP are again the same as the five

**Figure 5.3.** SDS-PAGE analysis of hordeins extracted from malt and barley. Separate extractions run next to each as duplicates. Results are representative of all samples tested.



day control, with no large differences compared to either of the three day samples.

The primary focus of the work was to determine if AN-PEP applied during malting could reduce beer hordeins, but any reduction in total hordeins from the malt would also be interesting. However, the SDS-PAGE does not show degradation of total hordeins by AN-PEP. This may be due to the complex reactions that occur during germination that may restrict enzyme access

(Eastwood & Morris, 1992; Edney et al., 2007). The lack of effect with AN-PEP on total malt hordein could be because the enzyme can only act on a small amount of free proteins.

Only a small fraction (0.2%) of hordeins present in the malt makes it into the final beer (Dostalek, Hochel, Mendez, Hernando, & Gabrovska, 2006). The differences in the hordein fraction, that make it into the beer are not evident on SDS-PAGE as they are only a very small portion of total malt hordein.

Although the limited solubility of hordeins suggests they will not enter into wort at all during brewing, the protein degradation during malting and mashing breaks down hordeins to a point where some of them become soluble in water (Celus, Brijs, & Delcour, 2006). These water soluble hordein peptides and smaller proteins can still contain epitopes toxic for gluten sensitive consumers and can be found in beer. The *competitive* ELISA method detects these hydrolysed fragments of hordeins that make it into the beer.

### **Implications**

This work shows in principle that the application of AN-PEP during steeping and germination can reduce beer hordeins. Increasing the variety of potential methods for reducing beer hordein content will benefit the consumer by offering more choice with regard to gluten-free beer. It is also possible, that other enzymes could be applied during malting to create functional malts, unique specialty malts or perhaps facilitate malting of alternative grains.

### **Strength and limitations**

Enzymes are rarely applied during malting and there have been no publications on use of AN-PEP during malting. In order to allow proper comparison the entire

malting process was controlled and a model scale brewing process was used to show differences due to the AN-PEP application. Standard methods of analysis were used allowing easy comparison of results.

In this work the Beatrix cultivar was examined after AN-PEP application, the results are significantly different and are likely applicable to other barley cultivars.

### **Conclusion**

Applying AN-PEP during pilot scale malting trials significantly reduced beer hordeins. A commercial malting barley cultivar was used and the quality of the malt remained comparable to the untreated control. The enzyme treatment did not require specialised equipment. By experimenting with other cultivars and using higher enzyme concentrations greater reductions may be possible. Using this method the level of hordeins in the beer made from five day + AN-PEP malt were reduced by almost half compared to beer made from five day control malt.

## Literature cited

- Bethune, M. T., Strop, P., Tang, Y. Y., Sollid, L. M., & Khosla, C. (2006). Heterologous expression, purification, refolding, and structural-functional characterization of EP-B2, a self-activating barley cysteine endoprotease. *Chemistry & Biology*, 13(6), 637-647. doi:10.1016/j.chembiol.2006.04.008
- Biesiekierski, J. R., Newnham, E. D., Irving, P. M., Barrett, J. S., Haines, M., Doecke, J. D., . . . Gibson, P. R. (2011). Gluten Causes Gastrointestinal Symptoms in Subjects Without Celiac Disease: A Double-Blind Randomized Placebo-Controlled Trial. *American Journal of Gastroenterology*, 106(3), 508-514. doi:10.1038/ajg.2010.487
- Briggs, D.E. (1978). *Barley*. London, Chapman and Hall.
- Briggs, D. E. (1998) *Malts and Malting* (1st ed., pp. 628, 175). London, Blackie Academic.
- Briggs, D. E., & Hough, J. S. (1981). *Malting and brewing science* (2nd ed., pp. 82, 83, 112). London, Chapman and Hall.
- Brijs, K., Trogh, I., Jones, B. L., & Delcour, J. A. (2002). Proteolytic enzymes in germinating rye grains. *Cereal Chemistry*, 79(3), 423-428. doi:10.1094/Cchem.2002.79.3.423
- Catassi, C., Bai, J. C., Bonaz, B., Bouma, G., Calabro, A., Carroccio, A., . . . Fasano, A. (2013). Non-Celiac Gluten Sensitivity: The New Frontier of Gluten Related Disorders. *Nutrients*, 5(10), 3839-3853. doi:10.3390/Nu5103839
- Celus, I., Brijs, K., & Delcour, J. A. (2006). The effects of malting and mashing on barley protein extractability. *Journal of Cereal Science*, 44(2), 203-211. doi:10.1016/j.jcs.2006.06.003
- Diezel, W., Kopperschlager, G., & Hofmann, E. (1972). An improved procedure for protein staining in polyacrylamide gels with a new type of Coomassie Brilliant Blue. *Anal Biochem*, 48(2), 617-620.
- Dostalek, P., Hochel, I., Mendez, E., Hernando, A., & Gabrovská, D. (2006). Immunochemical determination of gluten in malts and beers. *Food Additives and Contaminants*, 23(11), 1074-1078. doi:10.1080/02652030600740637
- Eastwood, M. A., & Morris, E. R. (1992). Physical-Properties of Dietary Fiber That Influence Physiological-Function - a Model for Polymers Along the Gastrointestinal-Tract. *American Journal of Clinical Nutrition*, 55(2), 436-442.

- Edney, M. J., Eglinton, J. K., Collins, H. M., Barr, A. R., Legge, W. G., & Rossnagel, B. G. (2007). Importance of endosperm modification for malt Wort fermentability. *Journal of the Institute of Brewing*, *113*(2), 228-238.
- Grujic, O. (1998). Application of a commercial enzyme preparation in the barley malting process. *Journal of the Institute of Brewing*, *104*(5), 249-253.
- Guerdrum, L. J., & Bamforth, C. W. (2012). Prolamin Levels Through Brewing and the Impact of Prolyl Endoprotease. *Journal of the American Society of Brewing Chemists*, *70*(1), 35-38. doi:Doi 10.1094/Asbcj-2012-0130-01
- Jones, B. L. (2005). Endoproteases of barley and malt. *Journal of Cereal Science*, *42*(2), 139-156. doi:10.1016/j.jcs.2005.03.007
- Kanauchi, M., & Bamforth, C. W. (2008). The Relevance of Different Enzymes for the Hydrolysis of beta-glucans in Malting and Mashing. *Journal of the Institute of Brewing*, *114*(3), 224-229.
- Kanerva, P., Sontag-Strohm, T., Brinck, O., & Salovaara, H. (2011). Improved extraction of prolamins for gluten detection in processed foods. *Agricultural and Food Science*, *20*(3), 206-216.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*(5259), 680-685.
- MEBAK. (2013). *Mitteleuropäische Brautechnische Analysenkommission (MEBAK) Vol. Wort, Beer and Beer-based Beverages*, Freising-Weihenstephan, Germany.
- Shan, L., Martin, T., Sollid, L. M., Gray, G. M., & Khosla, C. (2004). Comparative biochemical analysis of three bacterial prolyl endopeptidases: implications for coeliac sprue. *Biochemical Journal*, *383*, 311-318. doi:10.1042/Bj20040907
- Shewry, P. R., Napier, J. A., & Tatham, A. S. (1995). Seed Storage Proteins - Structures and Biosynthesis. *Plant Cell*, *7*(7), 945-956. doi:DOI 10.1105/tpc.7.7.945
- Sollid, L. M., & Lundin, K. E. A. (2009). Diagnosis and treatment of celiac disease. *Mucosal Immunology*, *2*(1), 3-7. doi:10.1038/mi.2008.74
- Stepniak, D., Spaenij-Dekking, L., Mitea, C., Moester, M., de Ru, A., Baak-Pablo, R., . . . Koning, F. (2006). Highly efficient gluten degradation with a newly identified prolyl endoprotease: implications for celiac disease. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *291*(4), G621-G629. doi:10.1152/ajpgi.00034.2006
- Tack, G. J., Van de Water, J. M. W., Bruins, M. J., Kooy-Winkelaar, E. M. C., van Bergen, J., Bonnet, P., . . . Koning, F. (2013). Consumption of gluten with

gluten-degrading enzyme by celiac patients: A pilot-study. *World Journal of Gastroenterology*, 19(35), 5837-5847. doi:10.3748/wjg.v19.i35.5837

Tack, G. J., Verbeek, W. H. M., Schreurs, M. W. J., & Mulder, C. J. J. (2010). The spectrum of celiac disease: epidemiology, clinical aspects and treatment. *Nature Reviews Gastroenterology & Hepatology*, 7(4), 204-213. doi:10.1038/nrgastro.2010.23

Taiz, L., & Honigman, W. A. (1976). Production of Cell-Wall Hydrolyzing Enzymes by Barley Aleurone Layers in Response to Gibberellic-Acid. *Plant Physiology*, 58(3), 380-386. doi:DOI 10.1104/pp.58.3.380

Walter, T., Wieser, H., & Koehler, P. (2014). Production of gluten-free wheat starch by peptidase treatment. *Journal of Cereal Science*, 60(1), 202-209. doi:10.1016/j.jcs.2014.02.012



# **Chapter 6: Fundamental study on the impact of silica gel and tannic acid on hordein levels in beer**

*Joshua P. Taylor, Fritz Jacob, Elke K. Arendt*

Published in the Journal: Innovative Food Science and Emerging Technologies –  
October 2015

## **Author Contributions**

Joshua Taylor was the main author for the paper, contributed to the conception and performed laboratory work and experiments.

Prof Jacob performed the technical analysis of the foam and beer flavours and reviewed the final manuscript.

Prof Arendt helped with the conception of the paper as well as making contributions and suggestions throughout the experimental process and supervised the project. Prof Arendt also critically reviewed drafts and the final manuscript

**Abstract:**

Demand for gluten-free foods has been increasing and although gluten-free beers are available, the range of styles is limited. In this study beer made from barley malt was treated with either silica gel or tannic acid and compared to unstabilised beer.

Hordein levels in the beers were analysed using Western blot and *competitive* ELISA. Beer quality parameters such as foam, colour and various flavours were also determined. There was no significant impact on beer quality when using silica gel to stabilise the beer and hordein levels were significantly reduced, the highest dose reducing the beer below 4 mg/kg.

Stabilisation with tannic acid reduced the hordein content significantly, the lowest dose reduced hordein to below 21 mg/kg without significant impact on beer quality. Although beer stabilised with the highest dose of tannic acid had a large reduction in hordein content (< 6 mg/kg), the quality of the beer, as indicated by colour, foam and flavour, was seriously affected.

## **Introduction**

In recent years gluten-free foods have seen an enormous surge in popularity, with 60% growth worldwide between the years 2008 – 2013. In 2013 alone, sales of gluten-free food accounted for over \$US two billion worldwide (source: Euromonitor Passport accessed 20-8-2014). These gluten sensitive consumers range from those suffering with medically diagnosed gluten sensitive conditions, to self-diagnosed individuals and those who believe the gluten-free diet is healthier.

Patients who are obliged to follow a gluten-free diet can suffer from a number of diagnosable conditions. Coeliac disease (CD) affects up to 1% of the population worldwide (Tack, Verbeek, Schreurs, & Mulder, 2010) and the only effective treatment is strict adherence to a gluten-free diet (Van De Kamer & Weijers, 1955). The disease is caused by an inappropriate immune response to ingested gluten proteins (Sollid & Jabri, 2013). This immune response results in damage to the intestine and can ultimately stop adsorption of essential nutrients, causing malnutrition and even cancer in untreated patients (Meresse, Ripoche, Heyman, & Cerf-Bensussan, 2009).

Recently there has been a lot of research done on the pathogenesis and epidemiology of non-coeliac gluten sensitivity (NCGS). This term is used for patients who do not suffer from the villus atrophy of CD or the abnormal levels of IgE antibodies associated with WA, but do have symptoms which are reduced when they adhere to a gluten-free diet (Sapone et al., 2012). The frequency of NCGS is still unclear due to varying definitions for the disease and possible cross-overs with other diseases like Irritable Bowel Syndrome (IBS) (Biesiekierski et al., 2011) but frequencies of NCGS of up to 6% are being reported (Catassi et al., 2013).

All of these conditions are aggravated by dietary gluten. Gluten is a general term for alcohol soluble prolamin storage proteins found in wheat, barley, rye and oats (toxicity of oat prolamins to gluten sensitive consumers is less common). Gluten proteins from wheat are also composed of glutenin which are not soluble in alcohol. Prolamin proteins in wheat are gliadins, in barley they are hordeins and in rye and oats they are secalins and avenins respectively. These prolamin proteins can be found in foods prepared using the aforementioned grains.

As with other allergens the Codex Alimentarius Commission has determined maximum safe levels of gluten allowed in gluten-free products (WHO/FAO, 1979). The Codex has determined that 20 mg/kg gluten is the maximum level permitted in gluten-free products. The level of prolamins are determined by ELISA analysis, and compared to a prolamin standard (Thompson & Mendez, 2008).

Gluten-free beers are now widely available in many countries and are produced by a variety of methods. The most common method is to use ingredients that do not contain gluten, alternative cereals like sorghum, buckwheat, maize and rice are used directly in the brewing process often with additions of thermostable amylolytic enzymes (Goode, Halbert, & Arendt, 2003; Hager, Taylor, Waters, & Arendt, 2014; Wijngaard & Arendt, 2006).

Another effective method for production of gluten-free beer is by application of enzymes. These enzymes can work either by detoxifying gluten proteins by protease action (Lopez & Edens, 2005), or on the other hand enzymes can be used to create covalent bonds between gluten proteins allowing removal by filtration (Wieser & Koehler, 2012). These methods allow use of traditional ingredients to produce beers which are low enough in prolamins to be labelled gluten-free.

A third option, which was used in this study, is to use standard stabilisation process methods to reduce hordein levels in beers. Stabilisation works by removing either haze-active proteins, polyphenols or both (Siebert, Carrasco, & Lynn, 1996). Without stabilisation, over time these haze-active precursors interact and form colloidal haze. By reducing either haze-active proteins or polyphenols stability is improved (Bamforth, 1999). Haze-active proteins tend to be very rich in the amino acid proline, much like hordein proteins. Removal of these proline rich proteins is an effective stabilisation method.

This study was focused on stabilising agents which target these haze-active proteins. Silica gel and tannic acid were chosen, they are both in common use within the brewing industry and previous research suggested their efficacy (Dostalek, Hochel, Mendez, Hernando, & Gabrovska, 2006; Lewis & Bamforth, 2006; Van Landschoot, 2011). Gluten content of unstabilised beers were compared with beer stabilised with different concentrations of each stabilising agent.

## **Materials and methods**

### **Wort production**

Beer was produced using the 10 hL pilot scale brewing facility in University College Cork. Propino ale malt was purchased from the Malting Company of Ireland Ltd, Cork. The extract content of the malt, dry matter (d.m.), was 82.2% (w/w). Total nitrogen content of the malt was 1.56% (d.m.) and soluble nitrogen was 0.62% (d.m.) which provided a soluble nitrogen ratio of 40%.

Malt (133.5 kg) was mixed with 400 L water and mashed at 50°C for 20 minutes, 62°C for 40 minutes and 72°C for 30 minutes. Lautering was performed for 90 minutes and 880 L wort was collected prior to boiling. Hop pellets (T-90) were

added at 10 min after start of boiling (400 g Hallertauer Tradition, 7.4%  $\alpha$ -acid, 510 g Spalter Select, 5.6%  $\alpha$ -acid Hopsteiner, Mainburg, Germany) and at the end of boiling (623 g Spalter Select from Hopsteiner and 267 g Cascade , 7.6%  $\alpha$ -acid, Simply Hops, Kent, U.K.). The boiled wort was rested 20 min in the whirlpool prior to cooling and aeration. A volume of 880 L of wort with an initial extract of 9.81% (w/w) was achieved.

### **Fermentation and filtration**

Fermentation took place at 12°C for 15 days before maturation at 1°C. After maturation the beer was filtered using kieselguhr (FP-2 Celatom, EP minerals, Nevada, U.S.A.). The filtered beer was then added to kegs containing silica gel (Daraclar 920 from Grace) or tannic acid (Biotannin CS from Kerry). The recommended dose and 10 times the recommended dose of each was used. Silica gel was added at a rate of 50 g/hL and 500 g/hL. Tannic acid was added at 2 g/hL and 20 g/hL.

The beers were then held at 1°C for 15 mins before filtration through 1.5  $\mu$ m candle filter (ULTIPOR N66 1.5  $\mu$ m, Pall Corporation, New York, U.S.A.). A control unstabilised beer was filtered in the same manner. Each beer treatment was produced in duplicate and the beers were held in cold storage 1°C prior to bottling and pasteurisation (14 PU).

### **Western Blotting**

Beer samples from each treatment were separated using SDS-PAGE prior to western blotting. SDS-PAGE was carried out according to a modified Laemmli (1970) procedure using 4-20% precast TGX gradient gel (BioRad, Berkeley, California, U.S.A.). Beer samples were de-gassed and mixed (75ul) with SDS sample buffer

(25ul) resulting in final concentrations of 62.5mM Tris-HCl at pH 6.8, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 2% (w/v) SDS and 100 mM DTT. Each sample was heated to 100°C for five minutes before centrifugation (20,000 x g) for 30 mins. Samples (30ul) were then loaded onto the SDS-PAGE gel and it was run at 100V until the dye front reached the bottom of the gel.

The proteins were then transferred to a 0.45 µm nitrocellulose membrane (GE healthcare, UK) at 58V and 4°C for one hour as previously described (Kanerva, Sontag-Strohm, & Lehtonen, 2005). After transfer the membrane was rinsed in TBST (Tris Buffered Saline with Tween) (20 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) before blocking membrane overnight at 4°C in 5% BSA (Bovine Serum Albumin) in TBST. The membrane was then rinsed again in TBST before incubating two hours, shaking with 1:2000 anti-gliadin antibody conjugated to peroxidase (Sigma, Missouri, U.S.A.) diluted in 5% BSA in TBST. The membrane was then rinsed in TBST before performing three x five min washes in TBST prior to application of peroxidase substrate (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific, Illinois, U.S.A). The membrane was then exposed to X-Ray film (Kodak Omat LS) and developed in a dark room. Band signal intensity of the films was analysed using Licor's Image Studio Lite software.

### **Hordein determination**

The level of hordein in each treated beer was determined using a RIDASCREEN Gliadin *competitive* ELISA assay from R-Biopharm (Darmstadt, Germany). The samples were prepared and analysed according to MEBAK method 2.6.5. Prolamins were extracted from one ml beer by adding 9 ml 60% (v/v) ethanol containing 10% (w/v) of fish gelatine (Sigma G7765). Samples were then vortexed and shaken for 10

min prior to centrifugation. The supernatant was diluted and used for hordein determination according to the instructions of the manufacturer. The results from the assay were calculated based on a prolamin standard curve. The prolamin standard is comprised of equal parts gliadin, hordein and secalin (Haas-Lauterbach, Immer, Richter, & Koehler, 2012). The results here are presented as mg/kg hordein, and are not converted into gluten equivalents.

### **Beer analyses**

All standard analyses were carried out according to recognised methods published by Mitteleuropäische Brautechnische Analysenkommision (MEBAK, 2011).

Anton Paar density meter (Alcolyzer DMA 4500M with a Beer ME module, Anton Paar, Graz, Austria) was used to determine the extract and alcohol of the wort and beer.

Foam stability of the beers (MEBAK 2.18.4), shown in fig 6.3 panel a, was assessed and measured as a half-life time in seconds, using the Steinfurth Foam Stability Tester (Steinfurth Mess-Systeme GmbH, Essen, Germany).

### **Beer Flavour**

Common beer flavours were analysed using several methods, higher alcohols and esters were determined by gas chromatography (GC) using the headspace method (MEBAK 2.21.1). Fatty acids and remaining esters were measured using distillation methods (2.21.4 and 2.23.6) prior to quantification using GC. Diacetyl and other vicinal diketones were also measured using headspace technique and GC analysis (2.21.5.1, 2.21.5.4). Dimethyl sulphide (DMS) was measured using the headspace method and special GC equipment with a sulphur detector (2.23.1.1)

### **Colour**



Spectrophotometric colour of beer samples (fig 6.3, panel b) was measured using EBC (European Brewing Convention) colour units. Samples were filtered through 0.45  $\mu\text{m}$  membrane prior to analysis at 430 nm. This measurement was then multiplied by a factor of 25 in order to calculate the colour of the beer samples in EBC units (MEBAK 2.12.2).

### **Statistical analysis**

All determinations were carried out in triplicates and statistical analysis of data was performed using SPSS (version 20, IBM, Armonk, U.S.A.) using one way ANOVA and Dunnett's T3 post hoc test ( $\alpha = 0.05$ ) for unequal variance.

### **Results and discussion**

A large volume of beer was produced, and then stabilised with either tannic acid or silica gel and bottled. Hordein level of each beer was measured and beer quality was assessed. This allowed for a general quality appraisal of each beer treatment whilst maintaining a focus on hordein reduction. All results from stabilised beers were compared to unstabilised control beer.

The stabilisers used in this study work by selectively removing haze-active (proline rich) proteins. These are the proteins involved in colloidal haze formation (Siebert & Lynn, 1997). When levels of haze-active proteins are reduced, the formation of haze compounds is also reduced giving the product has a longer stable shelf life. The haze-active proline rich proteins are also responsible for the majority of gluten found in beer (Lewis & Bamforth, 2006).

Silica gel is effective as a beer stabiliser thanks to its highly porous structure and very large surface area containing a network of pores penetrating each particle. The surface of the silica gel is covered in silanol ( $\text{SiOH}$ ) groups which form interactions

with proline residues in haze-active proteins (Siebert & Lynn, 1997). Tannic acid on the other hand is a mixture of hydrolysable tannins, extracted from plants. Biotannin CS is derived from *Rhus semialata* and is comprised mainly of gallotannins (personal communication) and this is the case for most commercial products (Mueller-Harvey, 2001; Shahidi, 1997).

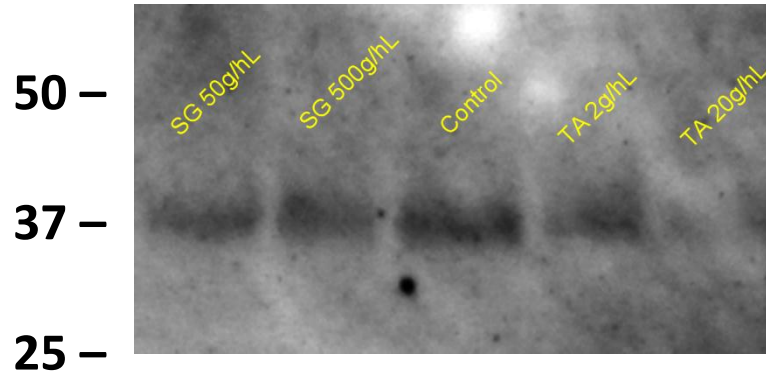
Stabilisation of beer using tannic acid results from reactions with sensitive proteins by several mechanisms. It has a large complex structure with many OH groups and aromatic rings which facilitates hydrogen bond formation between the tannic acid and sensitive proteins (Asano, Shinagawa, & Hashimoto, 1982; Mussche & de Pauw, 1999; Siebert, Troukhanova, & Lynn, 1996; Vanburen & Robinson, 1969).

Several quality parameters were assessed in this study to determine if there was any effect due to addition of these stabilisers.

### **Western blotting**

SDS-PAGE followed by western blotting transfer allowed the use of prolamin specific antibodies to show the low levels of detectable hordeins in beer samples. The band of prolamins which interacted with the antibody can be seen in fig 6.1 at 37 kDa for all beer samples. Band intensity was compared using densitometry software (Licor image studio lite). The stabilised beer samples in lane one (silica gel 50 g/hL) and lane two (silica gel 500 g/hL) show less intense bands than the unstabilised beer (lane three) with 60% of the band intensity compared to the control beer. The beer stabilised with tannic acid also showed less intensity with 40% and 4% of the control signal for 2 g/hL and 20 g/hL respectively. This reduction in signal would be expected if hordeins had been reduced in the beer samples.

**Figure 6.1.** Results from Western blot using samples of each beer. Commercially available anti-gliadin antibodies were used to detect hordeins. Image analysis was performed using Licor image studio lite. Unstabilised control beer band was used as the reference with the strongest band intensity at 37kDa. The intensity of the bands from silica gel 50g/hL and 500g/hL have 60% of the band intensity of the control beer sample. Beer stabilized with 2g/hL and 20g/hL tannic acid had 40% and 4% (respectively) of the intensity of the control in the western blot.



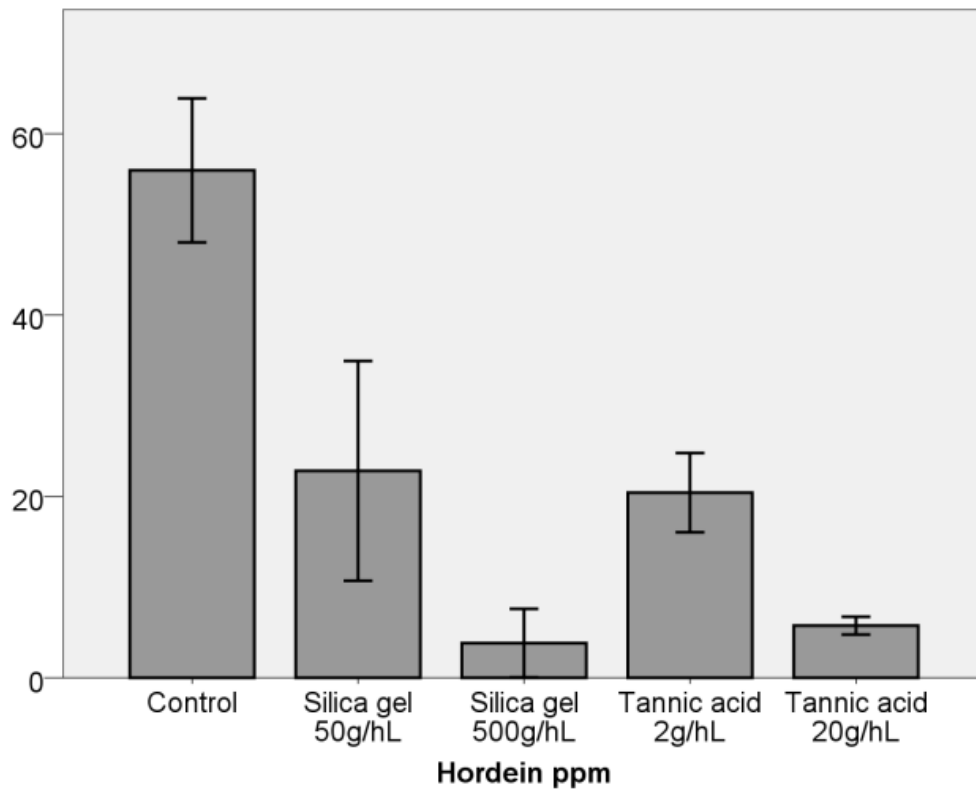
### Hordein determination

In order to quantify the hordein content of the different beer treatments in this study, a *competitive* ELISA assay was used. This test is the recommended method for measuring prolamin levels in food according to (EC) Commission Regulation No 41/2009 and Codex Stan 118 – 1979 (Codex Alimentarius, 1979). The Codex states that prolamin content of gluten is generally taken as 50% which implies a factor of two must be applied to convert into gluten equivalents. However, there is debate to the accuracy of this in relation to beer samples along with any food produced from barley or rye (Wieser & Koehler, 2009). In this study hordein results are reported without applying any factor to convert into gluten.

The hordein content of the unstabilised control beer was 56 mg/kg (fig 6.2). This result is in line with results found by Guerdrum and Bamforth (2012) for commercially available beers. Beer stabilised with 50 g/hL silica gel had lower hordein content than the control sample at 22.8 mg/kg (59% reduction). By applying ten times the recommended dose of silica gel (500 g/hL), hordein levels

were reduced significantly to 3.9 mg/kg which is a 90% reduction and is low enough to be labelled gluten-free.

**Figure 6.2.** Results from hordein determination using *competitive* ELISA. Beers were stabilised with either silica gel or tannic acid at different concentrations and compared to an unstabilised control. Error bars show 95% confidence interval.



Reduction of gluten level in beer using silica gel has been shown previously. Dostalek et al. (2006) reduced gluten content of beer by application of silica gel. This study has shown greater reductions are possible and levels can be reduced further by increasing concentration.

Stabilisation of beer samples using tannic acid at 2 g/hL also reduced hordein content to 20.4 mg/kg hordein which is significantly lower than the control but remained above the gluten-free labelling threshold. Again, when samples were

dosed with ten times the standard amount of tannic acid normally used for stabilisation (20 g / hl), the hordein levels dropped significantly to just 5.8 mg/kg.

Although the highest dose of tannic acid was very effective at reducing hordein levels, it also significantly reduced the foam stability and the flavours in the beer. Many of the flavours analysed were reduced substantially. Beer colour was also reduced considerably when stabilising with a high dose of tannic acid. The large number of unwanted side-effects which are found when overdosing with tannic acid may be reduced with optimised dosing or application at earlier steps of the brewing process.

Application of tannins to reduce gluten content of beer has been researched previously. Van Landschoot (2011) combined use of tannins with enzymes in order to reduce gluten content of beer. This study shows that application of tannic acid alone can reduce hordein to levels considered gluten-free. This is probably due to the fact that it preferentially binds to proteins rich in proline (Hagerman & Butler, 1981).

Our results also show by increasing the dosage of tannic acid, hordein content of our beer decreases correspondingly. Confidence intervals for our hordein determinations are wide due to the number of replicates, but reductions are still statistically significant.

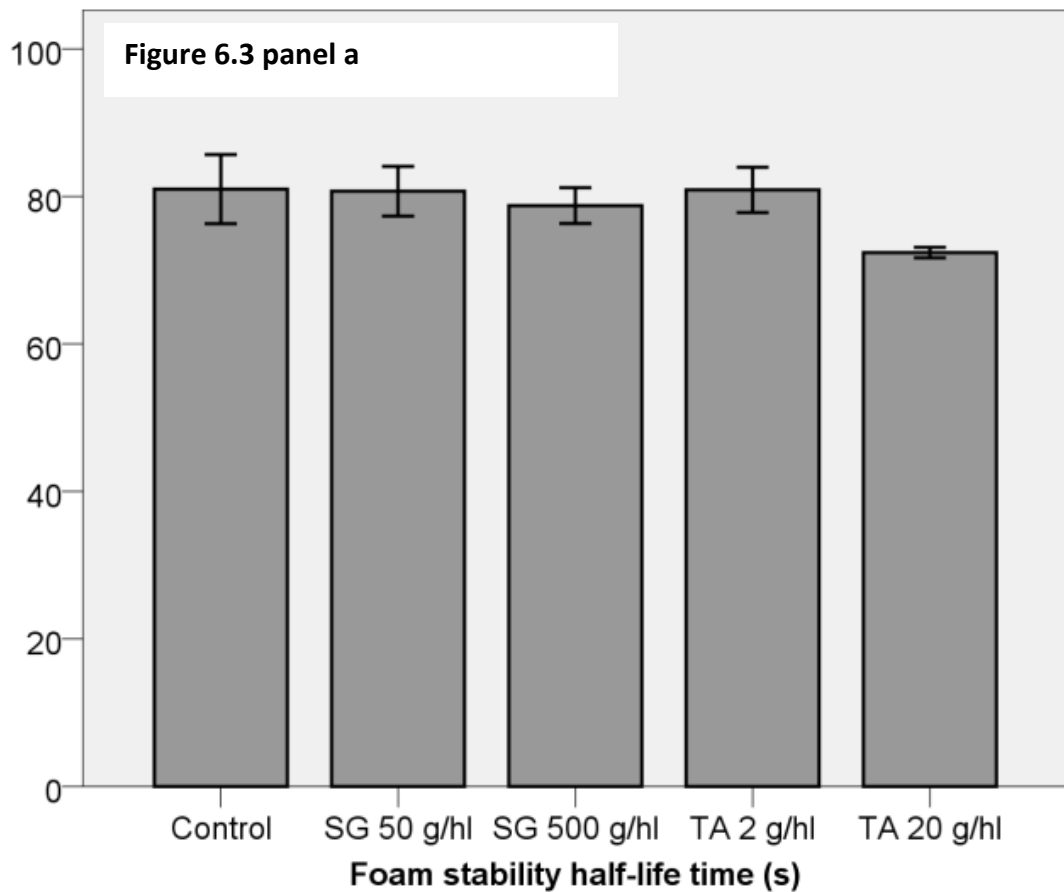
### **Foam Stability**

Initially beer quality is judged by appearance and foam stability is one of the first things that the consumer encounters. A beer without foam is not very attractive for most people (Evans & Sheehan, 2002), the stability of the foam was tested to see if there was an effect from any of the stabilising agents (fig 6.3, panel a). Foam

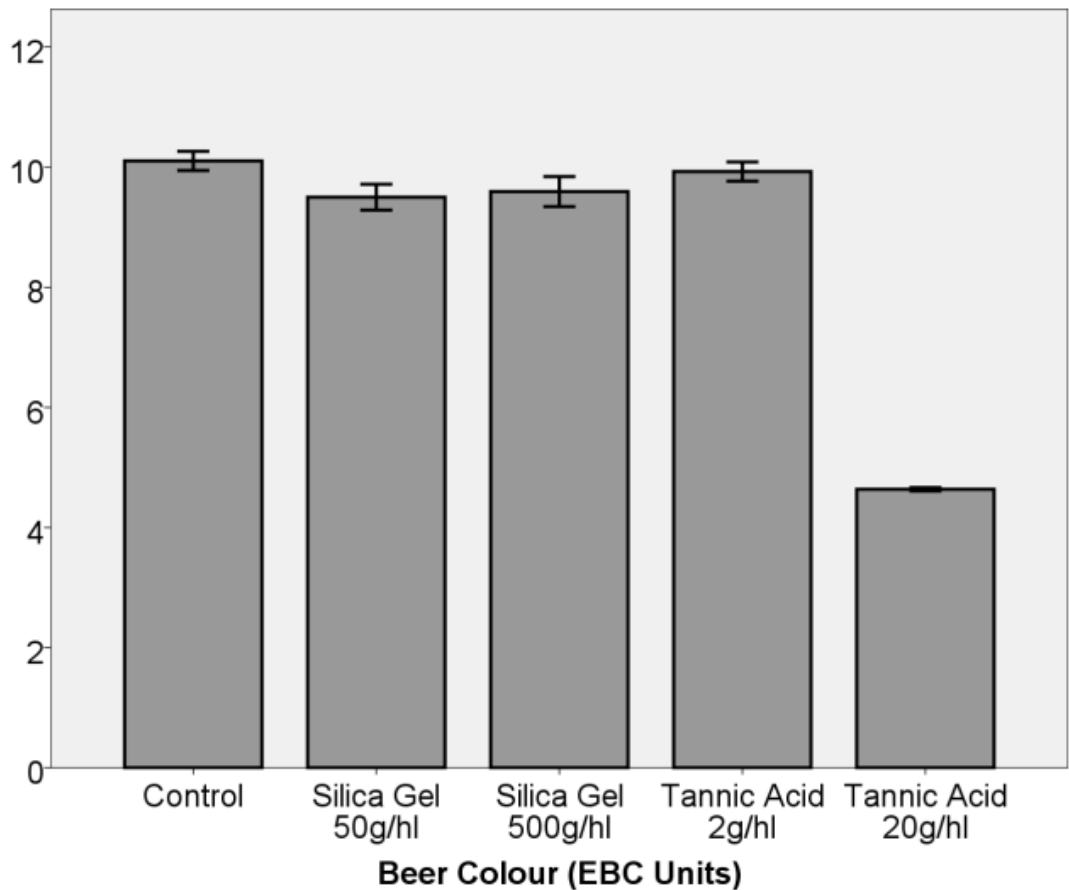
stability was measured based on the time taken for half of the total foam to collapse (half-life time). The foam half-life time of the unstabilised control beer was 81.0 seconds. Beer stabilised with silica gel (50 g/hL and 500 g / hL) had a half-life of 80.7 and 78.8 seconds respectively, which was not significantly different to the foam stability of the unstabilised control.

**Figure 6.3. Panel a:** Mean values of foam stability half-life in seconds for each beer stabilisation treatment. Stability determined using Steinfurth Foam Stability Tester. Error bars represent 95% CI.

**Panel b:** Mean values of EBC colour unit measurement for each of the beer stabilisation treatment. Error bars represent 95% CI



**Figure 6.3 panel b**



The foam stability of the beer treated with tannic acid at the recommended dose (2 g / hl) had a foam half-life of 80.9 seconds, the same foam stability as the control sample. The foam half-life was reduced to 72.4 seconds when 20 g/hL was used, causing a significant reduction in foam stability.

The foam which is formed when beer is poured depends on interactions of protein present in the beer and alpha acids from hops. Silica gel has been shown to be very effective at removing haze-active proteins (Leiper, Stewart, & McKeown, 2003) and these results also reflect those findings.

Tannic acid has also been shown to interact more generally with proteins by hydrogen bonding and also hydrophilic interactions (He, Shi, & Yao, 2006). As tannic acid is capable of numerous different type of chemical reaction, using an overdose

(20 g/hl) would make more hydroxyl groups, hydrophobic regions or charged areas of tannic acid molecules available for further chemical interactions.

### **Beer flavour**

Flavour is a combination of taste and smell and over years of research into beer, several critical compounds and their flavours have been identified (Meilgaard, 1975). These compounds can be identified and quantified using various gas chromatography techniques which have been standardised for beer samples (MEBAK, 2011).

When compounds are present above a certain threshold they are considered off-flavours and this can prove to be unacceptable by the consumer (Hughes & Baxter, 2001). The following results are divided into groups based on chemical structure of the flavour compounds. In this study 21 flavour compounds in each beer were analysed in order to show differences due to their relative stabilisation method.

### **Esters**

Esters contribute floral and fruity aromas to beer, these are desired in certain types of beer but can just as often be considered off-flavours. Esters considered crucial for beer quality include ethyl acetate (fruity/solvent), Isoamyl acetate (banana/apple) and ethyl hexanoate (apple, fruit-like), the levels of these esters in the unstabilised control beer in this study were below published sensory thresholds of 30 mg/L, 1.0 mg/L, 0.21 mg/L respectively (table 6.1). Further esters analysed (ethylbutyrate, isobutyl acetate, ethyl 2-phenylacetate, ethyl octanoate and ethyl decanoate) were also below sensory thresholds according to literature, depicted in table 6.1.



In beer treated with silica gel, levels of ethyl acetate, Isoamyl acetate and ethyl hexanoate were all within the same range as the control. Further esters analysed (ethylbutyrate, isobutyl acetate, ethyl 2-phenylacetate, ethyl octanoate and ethyl decanoate) also showed no significant differences (table 6.1).

Beer treated with tannic acid contained ethylbutyrate, isobutyl acetate, isoamyl acetate, ethyl 2-phenylacetate and ethyl acetate at levels not significantly different to the unstabilised beer.

When beer was dosed with 20 g/hL tannic acid there were reductions in levels of many esters by 1/3, shown in table 6.1. Ethyl hexanoate was reduced by 47.5%, ethyl octanoate was lower than the control by 60% and ethyl decanoate was less by 88% when beer was stabilised with 20 g/hL tannic acid (table 6.1).

Silica gel did not have an effect on esters but tannic acid did cause significant reductions. These reduced levels could be due to the excess of tannic acid being available for further reactions other than the desired proteo-tannic complex.

Table 6.1. Ester descriptives		Mean (mg / l)	95% Confidence Interval for mean lower and upper bound (mg / l)	Aroma	Sensory Threshold (mg / l)
Ethylbutyrate	Control	0.06	0.05 , 0.06	Fruity, jonquil	0.4 <sup>a</sup>
	SG 50 g/hl	0.06	0.05 , 0.06		
	SG 500 g/hl	0.06	0.04 , 0.07		
	TA 2 g/hl	0.06	0.05 , 0.06		
	TA 20 g/hl	0.04	0.02 , 0.06		
Isobutyl Acetate	Control	0.03	0.03 , 0.03	Fruity, floral	1.6 <sup>a</sup>
	SG 50 g/hl	0.03	0.03 , 0.03		
	SG 500 g/hl	0.03	0.03 , 0.03		
	TA 2 g/hl	0.03	0.03 , 0.03		
	TA 20 g/hl	0.02	0.01 , 0.03		
Isoamyl acetate	Control	0.80	0.78 , 0.82	Banana	1.0 <sup>b</sup>
	SG 50 g/hl	0.78	0.69 , 0.87		
	SG 500 g/hl	0.79	0.69 , 0.88		
	TA 2 g/hl	0.75	0.65 , 0.85		
	TA 20 g/hl	0.53	0.29 , 0.76		
Ethyl 2-phenylacetate	Control	0.20	0.19 , 0.21	Roses, honey, apple	3.8 <sup>b</sup>
	SG 50 g/hl	0.22	0.17 , 0.26		
	SG 500 g/hl	0.21	0.20 , 0.22		
	TA 2 g/hl	0.21	0.19 , 0.22		
	TA 20 g/hl	0.15	0.11 , 0.18		
Ethyl Acetate	Control	12.91	11.19 , 14.63	Solvent; fruity; sweet	30 <sup>a</sup>
	SG 50 g/hl	12.82	10.77 , 14.86		
	SG 500 g/hl	12.79	10.58 , 14.99		
	TA 2 g/hl	12.61	10.53 , 14.69		
	TA 20 g/hl	9.06	4.44 , 13.69		
Ethyl Hexanoate	Control	0.10	0.10 , 0.10	Fruity	0.21 <sup>a</sup>
	SG 50 g/hl	0.11	0.10 , 0.11		
	SG 500 g/hl	0.10	0.08 , 0.12		
	TA 2 g/hl	0.09	0.07 , 0.11		
	TA 20 g/hl	0.05	0.03 , 0.08		
Ethyl Octanoate	Control	0.22	0.18 , 0.25	Apples, sweet, fruity	0.9 <sup>b</sup>
	SG 50 g/hl	0.19	0.17 , 0.22		
	SG 500 g/hl	0.17	0.13 , 0.20		
	TA 2 g/hl	0.19	0.17 , 0.21		
	TA 20 g/hl	0.09	0.03 , 0.14		
Ethyl Decanoate	Control	0.04	0.03 , 0.06	Caprylic; fruity	1.5 <sup>a</sup>
	SG 50 g/hl	0.04	0.03 , 0.05		
	SG 500 g/hl	0.04	0.03 , 0.05		
	TA 2 g/hl	0.04	0.03 , 0.05		
	TA 20 g/hl	0.01	0.00 , 0.01		
a Meilgaard 1975 b Bamforth 2006					

## **Fatty acids and fusel alcohols**

Organic acids in general cause sour off flavours (Boulton & Quain, 2001) with individual fatty acids having unique unwanted flavours often referred to as 'goat-like'. Fatty acids are easily oxidised yielding carbonyl compounds which contribute to aged beer flavours (Charles W Bamforth, 2004; Esslinger, 2009).

Fusel alcohols are responsible for the warming character in some beers, they can also cause solvent-like and perfumed aromas in beers (Boulton & Quain, 2001; Hughes & Baxter, 2001). Levels of n-propanol, 2-phenylethanol, isobutanol and amyl alcohols were measured in the stabilised and non-stabilised beer (table 6.2).

Levels of caproic acid (cheesy / vegetable oil), caprylic acid (dairy / goaty), capric acid (dry, woody) and isovaleric acid (sweaty, cheese like) in the control beer were below sensory levels, shown in table 6.2.

Beer dosed with either 50 g/hL or 500 g/hL silica gel had no large differences in levels of fatty acids or fusel alcohols compared to the control, shown in table 6.2.

Table 6.2 shows samples treated with 2 g/hL of tannic acid also contained similar amounts of fatty acids and fusel alcohols to the control while beer treated with 20 g/hL tannic acid reduced levels of fatty acids and fusel alcohols by approximately 1/3 (table 6.2). Again this is likely due to unexpected interactions with excess tannic acid.

Table 6.2: Fatty Acid descriptives		Mean (mg / l)	95% Confidence Interval for Mean lower and upper bound	Aroma	Sensory Threshold (mg / l)
Caproic acid	Control	2.27	2.10 , 2.43	Vegetable oil, cheesy, fatty	8 <sup>c</sup>
	SG 50 g/hl	2.29	2.16 , 2.42		
	SG 500 g/hl	2.30	2.17 , 2.43		
	TA 2 g/hl	2.21	2.19 , 2.22		
	TA 20 g/hl	1.58	1.15 , 2.01		
Caprylic acid	Control	6.19	5.60 , 6.77	Dairy, goaty	15 <sup>b</sup>
	SG 50 g/hl	6.30	5.58 , 7.02		
	SG 500 g/hl	6.29	5.98 , 6.59		
	TA 2 g/hl	6.14	5.84 , 6.44		
	TA 20 g/hl	4.89	4.06 , 5.73		
Capric acid	Control	1.37	1.06 , 1.67	Dry, woody	10 <sup>c</sup>
	SG 50 g/hl	1.33	1.08 , 1.58		
	SG 500 g/hl	1.27	0.89 , 1.64		
	TA 2 g/hl	1.28	0.85 , 1.71		
	TA 20 g/hl	0.89	0.41 , 1.37		
Isovaleric acid	Control	0.86	0.69 , 1.03	Sweaty, cheesy, old-hop-like	1.5 <sup>c</sup>
	SG 50 g/hl	0.87	0.85 , 0.89		
	SG 500 g/hl	0.87	0.84 , 0.90		
	TA 2 g/hl	0.83	0.77 , 0.89		
	TA 20 g/hl	0.52	0.35 , 0.69		
b Bamforth 2006 c Esslinger 2009					

### Ketones, Linear aldehyde and sulphur compounds

Further compounds which affect beer flavour are the ketones, such as diacetyl, acetoin (buttery), and 2, 3-pentanedione (honey). Dimethyl sulphide (DMS) at levels above 0.03 mg/L can cause a cooked vegetable or cabbage like off flavour caused by this sulphur containing compound (Meilgaard, 1975). Acetaldehyde has a green apple type aroma at levels above 10 mg/L in beer and is often associated with

fermentation (Boulton & Quain, 2001; Meilgaard, 1975). The levels of off-flavours tested in the unstabilised beer were again all below sensory thresholds (table 6.4).

The beer stabilised with silica gel (50 g/hL or 500 g/hL) had levels of ketones, aldehydes and DMS slightly lower, but not significantly different to the control (table 6.3).

Tannic acid used at 2 g/hL also had little effect on levels of any of the ketones, DMS or acetaldehyde. However, when tannic acid was used at 20 g/hL, acetoin and 2, 3-pentanedione and were significantly reduced by 30% and 50% respectively. Diacetyl and DMS were also reduced (table 6.3). These results are in-line with the rest of the flavour analysis, when there is an excess of tannic acid used for stabilisation it causes significant reductions in several beer flavour compounds.

<b>Table 6.3:</b> Fusel Alcohol Descriptives		Mean (mg / l)	95% Confidence Interval for Mean lower and upper bound	Aroma	Sensory Threshold (mg / l)
n-Propanol	Control	10.64	9.18 , 12.11	Alcoholic	600 <sup>b</sup>
	SG 50 g/hl	10.51	8.69 , 12.33		
	SG 500 g/hl	10.38	8.33 , 12.43		
	TA 2 g/hl	10.28	8.62 , 11.93		
	TA 20 g/hl	7.37	3.56 , 11.18		
Iso Butanol	Control	7.74	5.75 , 9.73	Alcoholic	100 <sup>b</sup>
	SG 50 g/hl	7.57	5.30 , 9.83		
	SG 500 g/hl	7.57	5.39 , 9.75		
	TA 2 g/hl	7.42	5.35 , 9.49		
	TA 20 g/hl	5.37	1.38 , 9.36		
Amyl Alcohols (2-,3-methylbutanol)	Control	43.29	39.20 , 47.37	Alcohol, Vinous	50 <sup>b</sup>
	SG 50 g/hl	42.92	37.77 , 48.07		
	SG 500 g/hl	42.61	36.90 , 48.33		
	TA 2 g/hl	41.69	37.17 , 46.21		
	TA 20 g/hl	30.87	18.33 , 43.40		
2-Phenylethanol	Control	20.67	15.36 , 25.99	Floral; Roses; Perfume	40-100 <sup>b</sup>
	SG 50 g/hl	20.53	15.11 , 25.96		
	SG 500 g/hl	21.41	17.65 , 25.16		
	TA 2 g/hl	20.07	15.95 , 24.19		
	TA 20 g/hl	15.11	13.77 , 16.45		
b Bamforth 2006					

<b>Table 6.4:</b> Ketones, DMS, Aldehyde Descriptives		Mean (mg / l)	95% Confidence Interval for mean, and lower upper bound	Aroma	Sensory threshold (mg / l)
Acetaldehyde	Control	5.67	4.13 , 7.21	Green apples; fruit	10 <sup>c</sup>
	SG 50 g/hl	5.43	3.44 , 7.42		
	SG 500 g/hl	4.91	3.49 , 6.33		
	TA 2 g/hl	5.62	3.54 , 7.70		
	TA 20 g/hl	3.96	1.37 , 6.54		
Total diacetyl	Control	0.05	0.04 , 0.06	Buttery; butterscotch	0.1-0.14; 0.15 <sup>a</sup> <sup>d</sup> ,
	SG 50 g/hl	0.04	0.03 , 0.06		
	SG 500 g/hl	0.04	0.03 , 0.06		
	TA 2 g/hl	0.05	0.03 , 0.06		
	TA 20 g/hl	0.03	0.01 , 0.04		
total 2,3-Pentanedione	Control	0.02	0.02 , 0.02	Honey	0.9 <sup>b</sup>
	SG 50 g/hl	0.02	0.02 , 0.02		
	SG 500 g/hl	0.02	0.02 , 0.02		
	TA 2 g/hl	0.02	0.02 , 0.02		
	TA 20 g/hl	0.01	0.01 , 0.01		
Acetoin	Control	2.50	2.27 , 2.73	Buttery; dairy	50 <sup>a</sup>
	SG 50 g/hl	2.40	2.17 , 2.63		
	SG 500 g/hl	2.35	2.14 , 2.56		
	TA 2 g/hl	2.35	2.26 , 2.44		
	TA 20 g/hl	1.75	1.29 , 2.21		
Dimethyl sulfide (DMS Free)	Control	0.009	0.005 , 0.012	Sweetcorn; cooked vegetables	0.03 - 0.045 <sup>a</sup>
	SG 50 g/hl	0.011	0.011 , 0.012		
	SG 500 g/hl	0.010	0.010 , 0.011		
	TA 2 g/hl	0.008	0.005 , 0.010		
	TA 20 g/hl	0.007	0.007 , 0.008		
a Meilgaard 1975 b Bamforth 2006 c Esslinger 2009 d Hardwick 1995					

## **Colour**

Colour of beer is another crucial aspect of beer quality and any changes due to stabilisation were important to measure. Melanoidins are largely responsible for the colour in beer. They are non-enzymic maillard products formed during the malting process and during wort boiling (Nursten, 2005). Beer haze can also effect colour measurements made using a spectrophotometer.

The unstabilised beer had a colour measurement of 10.10 EBC units, within the reference value for pale beer. The beer stabilised with the recommended dose of silica gel had a slightly but significantly lower colour measurement of 9.50 EBC units (fig 6.3, panel b). The slight reduction in colour could be due to interactions of melanoidin molecules with the silanol groups of the silica gel. When the beer was treated with ten times the recommended dose of silica gel (500 g/hl) the colour was also slightly lower than the control with 9.59 EBC units.

Tannic acid treatment at the recommended level similarly did not have a large effect on colour with a measured value of 9.93 EBC units. But when dosed with 20 g/hL, colour is significantly reduced to 4.63 EBC units (fig 6.3, panel b). The complex structure of melanoidins may also result in many interaction sites for the excess tannic acid present in the beer treated with the highest dose.

## **Conclusions**

Silica gel was most effective at reducing hordein content at high doses with very little effect on the quality of the beer. Tannic acid was also very effective at reducing hordein content but high doses had serious impacts on beer quality. Foam stability, colour and flavour were all negatively affected. This research shows existing stabilisation methods are very effective at reducing hordein content.



Increasing dosage of silica gel or tannic acid resulted in a corresponding decrease in hordein content.

Innovative use of beer stabilisation methods successfully reduced hordein levels in beer significantly. Using ten times the recommended dose of either silica gel or tannic acid for stabilisation allowed very low hordein beer to be produced from a standard brewing process using 100% barley malt. According to current regulations the beer samples treated with high levels of silica gel or tannic acid could be labelled as gluten-free.

### **Acknowledgements**

Funding for this research was provided by the Irish National Development Plan 2007-2013 and administered through the Irish Department of Agriculture Food and the Marine with the Food Institutional Research Measure program. The authors would also like to acknowledge Iven Kelleher for his technical assistance with brewing.

## Literature cited

- Asano, K., Shinagawa, K., & Hashimoto, N. (1982). Characterization of haze-forming proteins of beer and their roles in chill haze formation. *Journal of the American Society of Brewing Chemists*, 40(4), 147-154. doi: 10.1094/ASBCJ-40-0147
- Bamforth, C. W. (1999). Beer haze. *Journal of the American Society of Brewing Chemists*, 57(3), 81-90. doi: 10.1094/ASBCJ-57-0081
- Bamforth, C. W. (2004). The Basics of Malting and Brewing: Product Safety and Wholesomeness *Beer: health and nutrition* (pp. 82). Oxford: Blackwell Science Ltd.
- Biesiekierski, J. R., Newnham, E. D., Irving, P. M., Barrett, J. S., Haines, M., Doecke, J. D., Gibson, P. R. (2011). Gluten Causes Gastrointestinal Symptoms in Subjects Without Celiac Disease: A Double-Blind Randomized Placebo-Controlled Trial. *American Journal of Gastroenterology*, 106(3), 508-514. doi: 10.1038/Ajg.2010.487
- Boulton, C., & Quain, D. (2001). Biochemistry of fermentation *Brewing yeast and fermentation* (pp. 116 - 126). Oxford: Blackwell Science
- Catassi, C., Bai, J. C., Bonaz, B., Bouma, G., Calabro, A., Carroccio, A., . . . Fasano, A. (2013). Non-Celiac Gluten Sensitivity: The New Frontier of Gluten Related Disorders. *Nutrients*, 5(10), 3839-3853. doi: 10.3390/Nu5103839
- Dostalek, P., Hochel, I., Mendez, E., Hernando, A., & Gabrovska, D. (2006). Immunochemical determination of gluten in malts and beers. *Food Additives and Contaminants*, 23(11), 1074-1078. doi: 10.1080/02652030600740637
- Esslinger, H. M. (2009). Stability of Beer *Handbook of brewing: processes, technology, markets* (pp. 399). Weinheim: John Wiley & Sons.
- Evans, D. E., & Sheehan, M. C. (2002). Don't be fobbed off: The substance of beer foam - A review. *Journal of the American Society of Brewing Chemists*, 60(2), 47-57. doi: 10.1094/ASBCJ-60-0047
- Goode, D. L., Halbert, C., & Arendt, E. K. (2003). Optimization of mashing conditions when mashing with unmalted sorghum and commercial enzymes. *Journal of the American Society of Brewing Chemists*, 61(2), 69-78. doi: 10.1094/ASBCJ-61-0069
- Guerdrum, L. J., & Bamforth, C. W. (2012). Prolamin Levels Through Brewing and the Impact of Prolyl Endoproteinase. *Journal of the American Society of Brewing Chemists*, 70(1), 35-38. doi: 10.1094/Asbcj-2012-0130-01

- Haas-Lauterbach, S., Immer, U., Richter, M., & Koehler, P. (2012). Gluten Fragment Detection with a Competitive ELISA. *J AOAC Int*, *95*(2), 377-381. doi: DOI 10.5740/jaoacint.SGE\_Haas-Lauterbach
- Hager, A. S., Taylor, J. P., Waters, D. M., & Arendt, E. K. (2014). Gluten free beer - A review. *Trends in Food Science & Technology*, *36*(1), 44-54. doi: 10.1016/j.tifs.2014.01.001
- Hagerman, A. E., & Butler, L. G. (1981). The specificity of proanthocyanidin-protein interactions. *Journal of Biological Chemistry*, *256*(9), 4494-4497.
- He, Q., Shi, B., & Yao, K. (2006). Interactions of galotannins with proteins, amino acids, phospholipids and sugars. *Food Chemistry*, *95*(2), 250-254. doi: 10.1016/j.foodchem.2004.11.055
- Hughes, P. S., & Baxter, E. D. (2001). Flavour Determinants of Beer Quality *Beer: quality, safety and nutritional aspects* (pp. 40-49). Cambridge: Royal Society of Chemistry.
- Kanerva, P., Sontag-Strohm, T., & Lehtonen, P. (2005). Determination of prolamins in beers by ELISA and SDS-PAGE. *Journal of the Institute of Brewing*, *111*(1), 61-64.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*(5259), 680-685.
- Leiper, K. A., Stewart, G. G., & McKeown, I. P. (2003). Beer polypeptides and silica gel - Part I. Polypeptides involved in haze formation. *Journal of the Institute of Brewing*, *109*(1), 57-72. doi: 10.1002/j.2050-0416.2003.tb00594.x
- Lewis, M., & Bamforth, C. W. (2006). Haze *Essays in brewing science* (pp. 55-56).: Springer. New York, U.S.A.
- Lopez, M., & Edens, L. (2005). Effective Prevention of Chill-Haze in Beer Using an Acid Proline-Specific Endoprotease from *Aspergillus niger*. *Journal of Agricultural and Food Chemistry*, *53*(20), 7944-7949. doi: 10.1021/jf0506535
- MEBAK. (2013). *Mitteleuropäische Brautechnische Analysenkommission (MEBAK)* Vol. Wort, Beer and Beer-based Beverages, Freising-Weihenstephan, Germany.
- Meilgaard, M. (1975). Flavor chemistry of beer. I. Flavor interaction between principal volatiles. *Technical Quarterly Master Brewers Association of the Americas*.
- Meresse, B., Ripoche, J., Heyman, M., & Cerf-Bensussan, N. (2009). Celiac disease: from oral tolerance to intestinal inflammation, autoimmunity and

lymphomagenesis. *Mucosal Immunology*, 2(1), 8-23. doi: 10.1038/mi.2008.75

Mueller-Harvey, I. (2001). Analysis of hydrolysable tannins. *Animal Feed Science and Technology*, 91(1-2), 3-20. doi: 10.1016/S0377-8401(01)00227-9

Mussche, R. A., & de Pauw, C. (1999). Total stabilisation of beer in a single operation. *Journal of the Institute of Brewing*, 105(6), 386-391. doi: 10.1002/j.2050-0416.1999.tb00030.x

Nursten, H. E. (2005). Introduction *The Maillard reaction: chemistry, biochemistry, and implications* (pp. 2-3). Cambridge: Royal Society of Chemistry.

Sapone, A., Bai, J. C., Ciacci, C., Dolinsek, J., Green, P. H. R., Hadjivassiliou, M., . . . Fasano, A. (2012). Spectrum of gluten-related disorders: consensus on new nomenclature and classification. *Bmc Medicine*, 10. doi: 10.1186/1741-7015-10-13

Shahidi, F. (1997). Methods for Determination of Condensed and Hydrolyzable Tannins *Antinutrients and phytochemicals in food* (pp. 212). Washington, DC: American Chemical Society.

Siebert, K. J., Carrasco, A., & Lynn, P. Y. (1996). Formation of protein-polyphenol haze in beverages. *Journal of Agricultural and Food Chemistry*, 44(8), 1997-2005. doi: 10.1021/Jf950716r

Siebert, K. J., & Lynn, P. Y. (1997). Mechanisms of beer colloidal stabilization. *Journal of the American Society of Brewing Chemists*, 55(2), 73-78. doi: 10.1094/ASBCJ-55-0073

Siebert, K. J., Troukhanova, N. V., & Lynn, P. Y. (1996). Nature of polyphenol-protein interactions. *Journal of Agricultural and Food Chemistry*, 44(1), 80-85. doi: 10.1021/Jf9502459

Sollid, L. M., & Jabri, B. (2013). Triggers and drivers of autoimmunity: lessons from coeliac disease. *Nature Reviews Immunology*, 13(4), 294-302. doi: 10.1038/Nri3407

Tack, G. J., Verbeek, W. H., Schreurs, M. W., & Mulder, C. J. (2010). The spectrum of celiac disease: epidemiology, clinical aspects and treatment. [Review]. *Nature reviews. Gastroenterology & hepatology*, 7(4), 204-213. doi: 10.1038/nrgastro.2010.23

Thompson, T., & Mendez, E. (2008). Commercial Assays to Assess Gluten Content of Gluten-Free Foods: Why They Are Not Created Equal. *Journal of the American Dietetic Association*, 108(10), 1682-1687. doi: 10.1016/j.jada.2008.07.012

- Van De Kamer, J. H., & Weijers, H. A. (1955). Coeliac disease. V. Some experiments on the cause of the harmful effect of wheat gliadin. *Acta paediatrica*, 44(5), 465-469. doi: 10.1111/j.1651-2227.1955.tb04269.x
- Van Landschoot, A. (2011). Gluten-free barley malt beers. *Cerevisia*, 36(3), 93-97. doi: 10.1016/j.cervis.2011.09.001
- Vanburen, J. P., & Robinson, W. B. (1969). Formation of Complexes between Protein and Tannic Acid. *Journal of Agricultural and Food Chemistry*, 17(4), 772-777. doi: 10.1021/Jf60164a003
- Codex Alimentarius (1979). Codex standard for foods for special dietary use for persons intolerant to gluten *Codex Stan 118 - 1979*
- Wieser, H., & Koehler, P. (2009). Is the calculation of the gluten content by multiplying the prolamin content by a factor of 2 valid? *European Food Research and Technology*, 229(1),9-13. doi: DOI 10.1007/s00217-009-1020-5
- Wieser, H., & Koehler, P. (2012). Detoxification of gluten by means of enzymatic treatment. *Journal of AOAC International*, 95(2), 356-363. doi: 10.5740/jaoacint.SGE\_Wieser
- Wijngaard, H. H., & Arendt, E. K. (2006). Optimisation of a Mashing Program for 100% Malted Buckwheat. *Journal of the Institute of Brewing*, 112(1), 57-65. doi: 10.1002/j.2050-0416.2006.tb00708.x

# **Chapter 7: Fundamental Study on the Impact of Transglutaminase on Hordein Levels in Beer**

*Joshua P. Taylor, Fritz Jacob, Elke K. Arendt*

Published in the journal of American Society of Brewing Chemists – September  
2015

## **Author Contributions**

Joshua Taylor was the main author for the paper, contributed to the conception and performed all the laboratory work and experiments.

Prof Jacob performed the technical analysis of the beer flavours and foam and reviewed the final manuscript.

Prof Arendt helped with the conception of the paper as well as making contributions and suggestions throughout the experimental process and supervised the project. Prof Arendt also critically reviewed drafts and the final manuscript.

**Abstract:**

Coeliac disease is widespread across the world with up to one in a hundred people diagnosed with the disease. Most beers are brewed using barley malt and are hence considered unsuitable for individuals suffering from coeliac disease or gluten intolerance. In this study lager beer was produced and treated with different concentrations of microbial transglutaminase (mTG). Quality aspects of each treatment, such as foam and flavour characteristics, were analysed and showed no significant differences. However, colour was significantly affected by mTG treatment. Dynamic light scattering (DLS) methods were used to analyse particle size of samples, which were found to increase significantly when treated with mTG. Western blotting was performed using anti-gliadin antibodies and showed gluten type proteins to be reduced in samples treated with the highest levels of mTG. The gluten content of the untreated beer (hordein mg/kg x two) was then quantitatively measured using a *competitive* ELISA assay giving a result of 88 mg/kg, application of mTG (9.2 g/hL, 92.5 g/hL or 231 g/hL), resulted in a significant reduction in gluten content (45 mg/kg, 12 mg/kg and 5 mg/kg respectively). The beers containing 12 mg/kg and 5 mg/kg gluten can be labelled gluten-free by Codex standards.

## **Introduction**

Coeliac disease is an autoimmune mediated enteropathy caused by an immune reaction to dietary gluten (Sollid & Jabri, 2013). It was first recognised as a disease of the intestine in the 2nd century AD by Aretaeus of Cappadocia (Aretaeus & Adams, 1856) and it may affect up to 1% of the global population, although these estimates of prevalence vary greatly between populations (Catassi, Gatti, & Fasano, 2014). The causative proteins were however not recognised until the 1950's by Dicke (Dicke, Weijers, & Van De Kamer, 1953). This discovery led to the introduction of the gluten-free diet, and this remains the only effective treatment for the disease (Meresse, Ripoche, Heyman, & Cerf-Bensussan, 2009; Van De Kamer & Weijers, 1955). There are also other non-coeliac types of gluten intolerance and wheat allergy which preclude gluten from the diet (Bizzaro, Tozzoli, Villalta, Fabris, & Tonutti, 2012; Brown, 2012). Gluten is a general term used for coeliac toxic storage proteins (prolamins) from wheat, barley, rye and possibly oats (Codex Alimentarius, 1979).

Safe levels of gluten in food are determined by the Codex Alimentarius, set up in 1963 by the World Health Organisation (WHO) and Food and Agriculture Organisation of the United Nations (FAO) in order to set out international food standards and guidelines. In reference to foods which may contain gluten it states that, if a food does not exceed 20 mg/kg gluten in total, it is considered a gluten-free food (Codex Alimentarius, 1979). The Codex recommends an Enzyme-linked Immunoassay (ELISA) using the R5 Mendez Method for gluten determination. This method detects soluble prolamins and multiplies the result by a factor of two to



account for insoluble gluten proteins. Beer produced from barley containing below 10 mg/kg hordein can therefore be labelled gluten-free.

Although prolamin levels are reduced significantly throughout the brewing process (Guerdrum & Bamforth, 2012), beer is not safe for coeliac consumers to consume. This is because it is traditionally produced using barley and wheat.

There are several existing methods for producing gluten-free beer. The starting material for brewing can be replaced, either completely or partly with a gluten-free alternative such as rice, buckwheat, maize or sorghum (Schehl, Mauch, & Arendt, 2009; Hager, Taylor, Waters, & Arendt, 2014; Phiarais et al., 2010). Alternatively barley malt can also be used, by addition of proline specific enzymes, gluten proteins can be degraded. These enzymes can be added during fermentation and specifically degrade gluten (Guerdrum & Bamforth, 2012).

A wide range of safe food choices are important for coeliac consumers to enjoy normal social activities, without the worry of exposure to allergens. Increasing the range of gluten-free foods for coeliacs to consume and use of appropriate labelling increases the quality of life for patients and reduces social exclusion (Clare Mills, 2007).

The objective of this study is to examine the effect of treating beer with microbial transglutaminase (mTG), in particular the effect on hordein levels and product quality. Hordein is the prolamin storage protein that occurs in barley (Shewry & Tatham, 1990). A patent for gluten-free beer production using mTG was applied for in 2006 (Pasternack, Marx, & Jordan, 2006) but there has been no fundamental research published on the topic to date.

Transglutaminase (TG) enzymes (EC 2.3.2.13) were initially discovered in animal liver and are now known to be involved in many protein cross linking reactions (Pisano, Finlayson, & Peyton, 1968). They are found in different taxonomic kingdoms, from animals to microorganisms. In mammals they are associated with numerous biological functions, ranging from G protein signalling and blood clotting to several disease states including neurodegenerative diseases and tissue fibrosis (Chen & Mehta, 1999; Griffin, Casadio, & Bergamini, 2002). TG2 is another example of a TG enzyme, it is endogenous to humans and is activated in the body by tissue damage as a repair system (Fesus & Piacentini, 2002). It is involved in the pathogenicity of coeliac disease and like other mammalian types of TG, and is calcium dependent (Griffin et al., 2002).

In contrast to TG, mTG is calcium independent, it has a lower molecular weight and does not catalyse deamidation reactions (Gianfrani et al., 2007), it has similar substrate specificity to TG and forms covalent crosslinks between glutamine rich proteins such as gluteins. Commercial applications for mTG are wide ranging, it is well known for producing restructured meat products, able to bind small pieces of meat together (Kuraishi, Sakamoto, & Soeda, 1996).

## **Materials and methods**

### **Wort production**

Beer was produced using the 10 hL pilot scale brewing facility in University College Cork. Propino ale malt was purchased from the Malting Company of Ireland Ltd, Cork. The extract content of the malt, dry matter (d.m.), was 82.2% (w/w). Total nitrogen content of the malt was 1.56% (d.m.) and soluble nitrogen was 0.62% (d.m.) which provided a soluble nitrogen ratio of 40%.

Malt (133.5 kg) was mixed with 400 l water and mashed at 50 °C for 20 minutes, 62 °C for 40 minutes and 72 °C for 30 minutes. Lautering was performed for 90 minutes and 880 l wort was collected prior to boiling. Hop pellets (T-90) were added at 10 min after start of boiling (400 g Hallertauer Tradition, 7.4%  $\alpha$ -acid, 510 g Spalter Select, 5.6%  $\alpha$ -acid Hopsteiner, Mainburg, Germany) and at the end of boiling (623 g Spalter Select from Hopsteiner and 267 g Cascade , 7.6%  $\alpha$ -acid, Simply Hops, Kent, U.K.). The boiled wort was rested 20 min in the whirlpool prior to cooling and aeration. A volume of 880 l of wort with an initial apparent extract of 9.81% (w/w) was achieved.

#### **Fermentation and filtration**

Fermentation took place at 12°C for 15 days before maturation at 1°C. After maturation the beer contained 2.05% (w/w) apparent extract. The beer was filtered using kieselguhr (FP-2 Celatom, EP minerals, Nevada, U.S.A.). The filtered beer was then added to kegs containing mTG from Ajinomoto Foods Europe S.A.S. (ACTIVA®WM, specific activity 81 – 135 AU/g), at concentrations of 9.23 g/hL, 92.3 g/hL and 231 g/hL. A control keg with no enzyme addition was also filled with beer. Each of the treatments and the control, along with all subsequent analyses were performed in at least duplicates. The beers were then held at 1°C for 20 h before filtration through 1.5  $\mu$ m candle filter (ULTIPOR N66 1.5um, Pall Corporation, U.S.A.).The beers were held in cold storage 1°C for 67 days before bottling and pasteurization using 14 pasteurising units (PU).

#### **Standard beer analyses**

All standard analyses were carried out according to methods published by Mitteleuropäische Brautechnische Analysenkommision (MEBAK, 2013).

Anton Paar density meter (Alcolyzer DMA 4500M with a Beer ME module, Anton Paar, Graz, Austria) was used to determine the extract and alcohol of the wort and beer.

Foam stability of the beers (MEBAK 2.18.4), shown in Fig. 7.1, was assessed and measured as a half-life time in seconds, using the Steinfurth Foam Stability Tester (Steinfurth Mess-Systeme GmbH, Essen, Germany).

### **Beer Flavour**

Common beer flavours were analysed using several methods, higher alcohols and esters were determined by gas chromatography (GC) using the headspace method (MEBAK 2.21.1). Fatty acids and remaining esters were measured using distillation methods (MEBAK 2.21.4 and 2.23.6) prior to quantification using GC. Diacetyl and other vicinal diketones were also measured using headspace technique and GC analysis (MEBAK 2.21.5.1, 2.21.5.4). Dimethyl sulphide was determined using the headspace method and special GC equipment with a sulphur detector (MEBAK 2.23.1.1)

### **Colour**

Spectrophotometric colour of beer samples (Fig. 7.2) was measured (MEBAK 2.12.2). Samples were filtered through 0.45 µm membrane prior to analysis at 430 nm.

### **Particle size**

Mean particle size was determined using Zetasizer Nano ZS (Malvern Instruments Ltd, UK). Particle size was determined using dynamic light scattering (DLS) based on Brownian motion of particles. The fluctuations in scattering intensity over time were used to calculate the hydrodynamic radius of particles in the sample, using

the Stokes-Einstein equation (Malvern, 2013). Beer samples were diluted 1:10 with water prior to analysis. The dispersant was water with a refractive index (RI) of 1.330. The RI of the analyte was set at 1.45 and absorbance at 0.001. The temperature used was 25°C and sample was analysed in a micro cuvette (40ul). Measurement duration, position and attenuator settings were determined automatically using Zetasizer Nano-ZS software (v 6.20).

### **Particle sedimentation**

Particle sedimentation was measured using an analytical centrifuge (Lumisizer, L.U.M. GmbH, Berlin Germany), undiluted beer samples were centrifuged at 4000 rpm at room temperature for one hour. Levels of light transmission along the length of the cuvette were recorded every 30 seconds for the duration of centrifugation.

### **SDS-PAGE and Western blotting**

SDS-PAGE analysis was performed on samples of each beer according to Laemmli (Laemmli, 1970). Lyophilised sample (20 mg) was suspended in 75 µl distilled water. SDS Sample buffer (25 µl) was added resulting in final concentrations of 62.5mM Tris-Cl at pH 6.8, 2% (w/v) SDS, 100 mM DTT, 10% (v/v) glycerol and 0.1% (w/v) bromophenol blue. Molecular weight marker (SigmaMarker S8445) was loaded alongside samples (10 µl, containing 60 µg protein according to Bradford) in the wells of a 15% polyacrylamide gel. Gels were imaged on a flatbed scanner.

SDS-PAGE was carried out as above for the immunoblotting, except that degassed beer samples (75 ul) were mixed with SDS buffer (25ul) directly, without concentration prior to separation. Samples (20ul) were loaded into the gel and electrophoresis was carried out until the dye front reached the end of the gel.

Proteins were then transferred from the gel using a standard immunoblotting method to a 0.45µm nitrocellulose membrane (Bio-Rad, Berkeley, U.S.A.) using transfer buffer containing 25 mM Tris, 192 mM glycine and 20% v/v methanol. The transfer voltage used was 58 V for one hour as previously described (Kanerva, Sontag-Strohm, & Lehtonen, 2005). The membrane was rinsed briefly in PBST (NaCl 137 mM, KCl 2.7 mM, Na<sub>2</sub>HPO<sub>4</sub> 10 mM and 0.1% v/v Tween 20). The membrane was then blocked in 5% (w/v) bovine serum albumin (BSA) in PBST shaking at 4°C overnight.

After blocking, the membrane was incubated in 5% (w/v) BSA in PBST containing one µl / ml anti-gliadin antibody conjugated to peroxidase (Sigma A1052) for two hours, shaking at room temperature. The membrane was then rinsed in PBST followed by three x five minute washes, before peroxidase substrate was applied (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific, Waltham, U.S.A.). The nitrocellulose membrane was then exposed to X-ray film (Amersham Hyperfilm, GE healthcare, Buckinghamshire, United Kingdom) and developed in a dark room.

### **Gluten determination**

The level of gluten in each treated beer was determined using a RIDASCREEN Gliadin *competitive* ELISA assay from R-Biopharm (Darmstadt, Germany). The samples were prepared and analysed according to MEBAK method 2.6.5. Prolamins were extracted from one ml beer by adding nine ml 60% (v/v) ethanol containing 10% (w/v) of fish gelatine (Sigma G7765). Samples were then vortexed for 10 min before mixing. Samples were centrifuged and the supernatant was diluted and used for gluten determination according to the instructions of the manufacturer. The

results from the assay were calculated based on a gliadin standard curve and multiplied by two to give total gluten of the sample.

Statistical analysis of data was performed using SPSS (version 20, IBM, Armonk, U.S.A.) using one way ANOVA and Dunnett's T3 post hoc test for unequal variance.

### **Results and discussion**

The main focus of this study was to examine the effect of treating beer with mTG, and determine if mTG was effective at reducing gluten content whilst maintaining product quality. Beer was produced on a large scale before filling into kegs which facilitated application of three different concentrations of mTG (9.23 g/hL, 92.3 g/hL and 231 g/hL) alongside untreated control samples. Beers were then filtered through a 1.5 µm candle filter prior to analysis. Parameters such as foam stability and colour, which could be affected by addition of a cross linking enzyme, were tested. Common off-flavours in beer were measured, and the proteins present in beer were studied using particle size analysis and SDS-PAGE techniques. The most important quality of gluten-free beer to be tested was the gluten content and this was determined using the recommended *competitive* ELISA method.

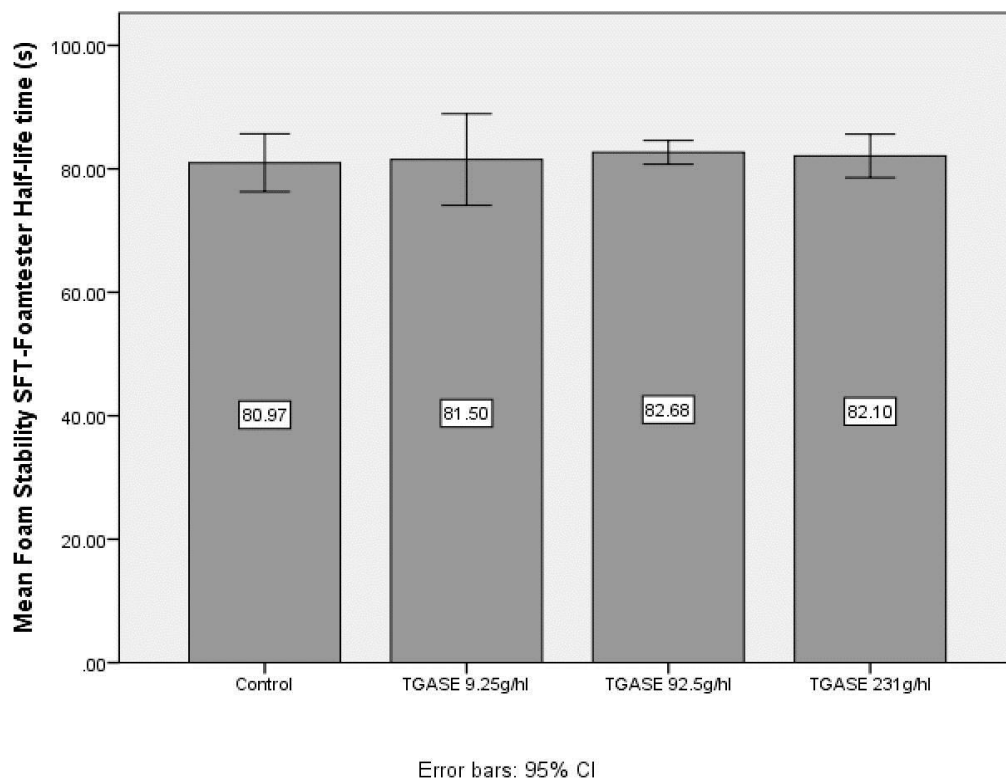
#### **Foam stability**

Foam is one of the first indicators of beer quality the consumer encounters and stability is affected by levels of proteins present in the beer. Interactions between these proteins and iso- $\alpha$ -acids from the hops along with many other factors such as level of carbonation and method of dispense all contribute to foam characteristics (Evans & Sheehan, 2002).

As mTG enables crosslinking of proteins, primarily between glutamine and lysine residues, this could influence properties of proteins involved in foam stability. The

half-life of the foam stability of the control sample was 81 seconds, shown in figure 7.1. Treatment with mTG at 9.25 g/hL had a foam half-life of 81.5 seconds, not significantly different to the control. Increasing the dosage of mTG to 92.5 g/hL or 231 g/hL also had no effect on foam stability with results of 82.7 and 82.1 seconds respectively. Samples treated with mTG, even at very high dosage, were not different in respect to the foam stability when compared to the control (Fig. 7.1). Removal of gluten proteins is not detrimental to foam stability (Bamforth, 2004) and in this study, proteins involved in foam were not significantly affected.

**Figure 7.1.** Foam stability half-life (in seconds) measured using SFT-Foamtester. Bars represent the different enzyme treatments and the control. Results show there were no differences between samples treated with mTG and the control sample



## Beer flavour

Beer quality is assessed by the consumer based many sensory characteristics, flavour being one of the most important. Flavour is a combination of taste and



smell, in beer it is primarily made up of four basic flavours, sweet, sour, salty and bitter. Flavour characteristics are a crucial factor for the consumer when they taste a beer (Hughes & Baxter, 2001). There are certain chemical compounds in beer which, when above a certain threshold, can cause off flavours (Meilgaard, 1975). Many of these can be quantified and give a measure of the flavour quality of the beer, 21 of these compounds were measured for each beer and compared in this study.

### **Esters**

Esters can contribute unwanted flavours in beer such as isoamyl acetate (banana-like) and ethyl hexanoate (apple, fruit-like), the levels of these esters in the control beer produced in this study was 0.80 mg/l and 0.10 mg/l respectively. This is below the sensory threshold, shown in table 7.1. Further esters analysed (ethylbutyrate, isobutyl acetate, ethyl 2-phenylacetate, ethyl acetate, ethyl octanoate and ethyl decanoate) were also below sensory thresholds (0.06 mg/l, 0.03 mg/l, 0.20 mg/l, 12.91 mg/l, 0.22 mg/l and 0.04 mg/l respectively) according to published literature, depicted in table 7.1. There was no significant difference between levels of esters in any of the mTG treated beers and the control.

Table 7.1.	Beer flavour/ aroma compounds (mg/L)	Control	mTG 9.25g/hL	mTG 92.5g/hL	mTG 231g/hL	Aroma	Sensory Threshold mg/L
Esters	Ethylbutyrate	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	Fruity, jonquil	0.4 <sup>d</sup>
	Isobutyl Acetate	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	Fruity floral	1.6 <sup>d</sup>
	Isoamyl acetate	0.80 ± 0.03	0.82 ± 0.03	0.80 ± 0.03	0.80 ± 0.03	Banana	1.0 <sup>c</sup>
	Ethyl 2-phenylacetate	0.20 ± 0.01	0.22 ± 0.01	0.23 ± 0.02	0.21 ± 0.01	Roses, honey, apple	3.8 <sup>c</sup>
	Ethyl Acetate	12.91 ± 0.62	13.33 ± 1.62	13.11 ± 1.62	12.89 ± 1.62	Solvent; fruity; sweet	30 <sup>d</sup>
	Ethyl Hexanoate	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	Fruity	0.21 <sup>d</sup>
	Ethyl Octanoate	0.22 ± 0.04	0.22 ± 0.04	0.21 ± 0.04	0.21 ± 0.04	Apples, sweet, fruity	0.9 <sup>c</sup>
	Ethyl Decanoate	0.04 ± 0.02	0.04 ± 0.02	0.05 ± 0.02	0.04 ± 0.02	Caprylic; fruity	1.5 <sup>d</sup>
Fatty Acids	Caproic acid	2.27 ± 0.15	2.38 ± 0.15	2.36 ± 0.15	2.34 ± 0.15	Vegetable oil; cheesy; fatty	8 <sup>e</sup>
	Caprylic acid	6.19 ± 0.82	6.80 ± 0.82	6.75 ± 0.82	6.89 ± 0.82	Dairy; goaty	15 <sup>c</sup>
	Capric acid	1.37 ± 0.26	1.46 ± 0.26	1.49 ± 1.49	1.45 ± 0.26	Dry; woody	10 <sup>e</sup>
	Isovaleric acid	0.86 ± 0.13	0.89 ± 0.13	0.88 ± 0.13	0.90 ± 0.13	Sweaty; cheese; old-hop-like	1.5 <sup>e</sup>

Fusel Alcohols	n-Propanol	10.64 ± 1.41	10.79 ± 1.41	10.81 ± 1.41	10.64 ± 1.41	Alcoholic	600 <sup>c</sup>
	2-Phenylethanol	20.67 ± 5.19	21.62 ± 5.19	22.59 ± 5.19	22.43 ± 5.19	Floral; roses; perfume	40-100 <sup>c</sup>
	Iso Butanol	7.74 ± 1.83	7.91 ± 1.83	7.84 ± 1.83	7.61 ± 1.83	Alcoholic	100 <sup>c</sup>
	Amyl Alcohols (2-,3-methylbutanol)	43.29 ± 3.45	43.56 ± 3.46	43.88 ± 3.46	43.75 ± 3.46	Alcohol, vinous	50 <sup>c</sup>
Ketones	Total diacetyl	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	Buttery; butterscotch	0.1-0.4 <sup>a</sup> , 0.15 <sup>d</sup>
	total 2,3-Pentanedione	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	Honey	0.9 <sup>c</sup>
	Acetoin (intermediate compound)	2.50 ± 0.23	2.50 ± 0.23	2.45 ± 0.23	2.50 ± 0.23	Buttery; dairy	50 <sup>d</sup>
Poly- sulphide	Dimethyl sulphide (DMS Free)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	Sweet corn; cooked vegetable	0.03-0.045 <sup>d</sup>
Linear Aldehyde	Acetaldehyde	5.67 ± 1.77	5.89 ± 1.75	5.84 ± 5.84	5.67 ± 1.75	Green apples; fruit	10 <sup>e</sup>

a Hardwick (1995)

b Saison, De Schutter, Uyttenhove, Delvaux, and Delvaux (2009)

c Bamforth (2006)

d Meilgaard (1975)

e Taylor and Organ (2009)

Table adapted from Oliveira, Mauch, Jacob, and Arendt (2012)

### **Fatty acids**

Fatty acids at levels above sensory thresholds can cause off flavours ranging from soapy and sweaty (caproic, caprylic and capric acid) to cheesy (iso-valeric acid) (Bamforth, 2004; Taylor & Organ, 2009). Levels in the control beer were 2.27 mg/l, 6.19 mg/l, 1.37 mg/l and 0.86 mg/l respectively. These levels are below sensory thresholds shown in table 7.1, all of the beers treated with mTG (9.25 g/hL, 92.5 g/hL and 231 g/hL) were also below sensory thresholds and had no significant difference in levels of fatty acids compared to the control.

### **Fusel alcohols**

Solvent-like, perfumed and vinous are flavours associated with high levels of fusel alcohols in beers (Hughes & Baxter, 2001). Levels of n-propanol, 2-phenylethanol, isobutanol and 2-, 3-methylbutanol were measured in the control beer (table 7.1). Results were below sensory limits (10.64 mg/l, 20.67 mg/l, 7.74 mg/l and 43.29 mg/l respectively) and again there was no difference between the control and beers treated with mTG.

### **Ketones**

Other common unwanted flavours in beer are often caused by high levels of compounds such as diacetyl, acetoin (buttery), and 2, 3-Pentanedione (honey). These compounds cause aromas that are not desired in most types of beer. They were detected at levels of 0.05 mg/l, 0.02 mg/l and 2.50 mg/l respectively. There was no significant difference between the control and beers treated with mTG and all were below sensory thresholds (table 7.1).

### **Linear aldehyde and sulphur compounds**

Dimethyl sulphide (DMS) at levels above 0.03 mg/l can cause a cooked vegetable or cabbage like off flavour. The control sample had a level of 0.01 mg/l, well below the threshold. Acetaldehyde has a green apple type aroma at levels above 10 mg/l in beer. The amount in the control beer was 5.67 mg/l, below the sensory limit. The levels of DMS and acetaldehyde in mTG treated beers were not significantly different to the control.

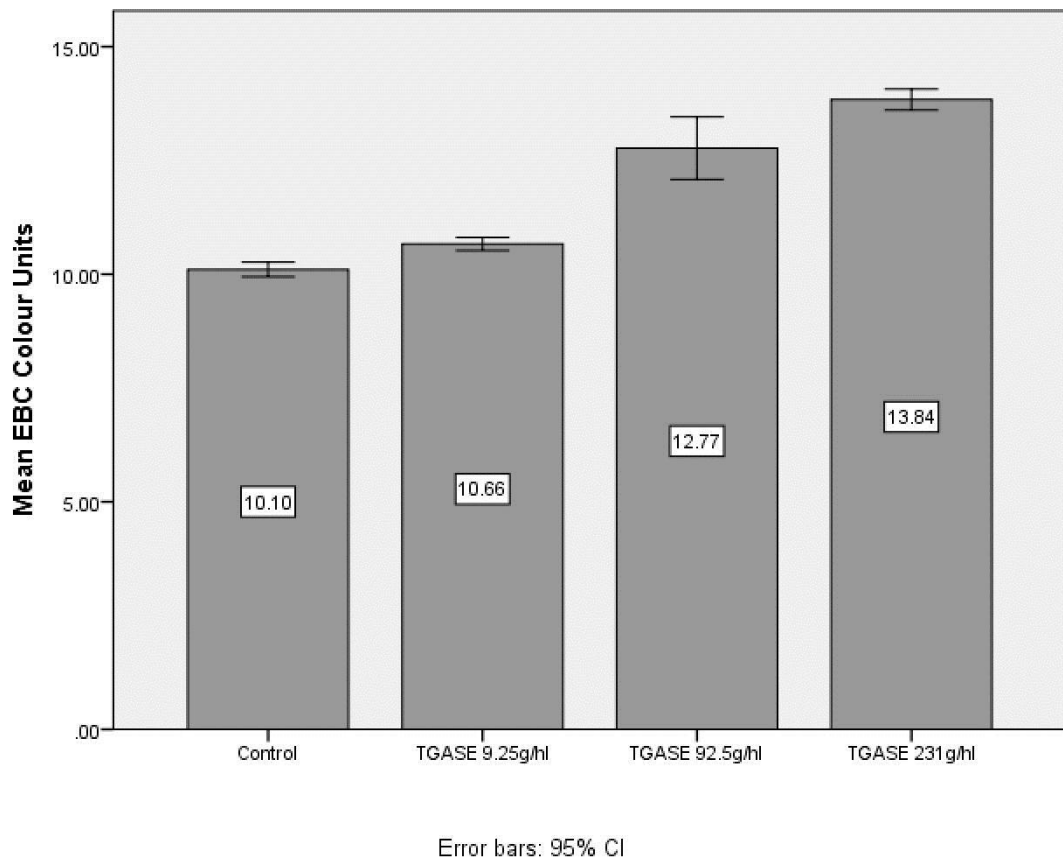
The levels of off-flavours tested in the beers were all below sensory thresholds (Table 7.1) and no differences between treatments were found ( $\alpha = 0.01$ ) showing application of mTG did not cause changes to flavours analysed.

### **Colour**

Beer colour is a very important aspect of beer quality, it is determined primarily from the colour of the malts used to produce the wort and further maillard reactions that occur during boiling. Therefore it was important to know if any reactions catalysed by enzyme additions had an effect on beer colour. Beer colour was compared between the different treatments and the control sample. Figure 7.2 shows the measured colour was 10 EBC colour units for the control sample. The colour of the beer treated with 9.25 g/hL mTG increased to 10.7 EBC units and the colour increased again in the beer treated with 92.5 g/hL, to 12.8 EBC units. This increasing colour trend continued in the highest dosage of mTG (231 g/hL) with a measured value of 13.8 EBC units. The colour of the samples increased significantly with the increasing dose of mTG, possibly due to crosslinking of proteins which could cause turbidity resulting from the presence of colloidal particles, any haze present may also cause an increase in the colour measurements. The samples were

filtered (0.45 µm) but this would still allow larger protein particles to remain in solution.

**Figure 7.2.** The colour of each beer treatment was measured (MEBAK 2.12.2). The colour increased corresponding to an increased dose of enzyme, the lowest colour measured was that of the control.



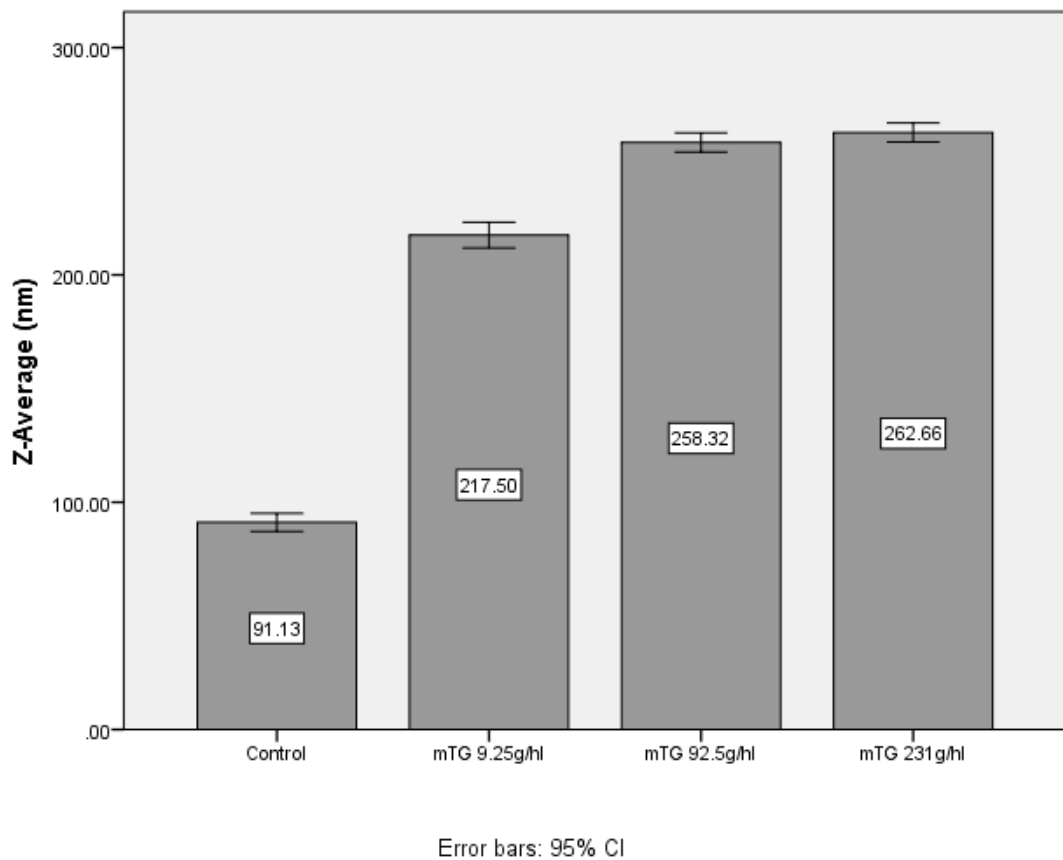
### Particle size

Particle analysis has previously been used to study colloidal stability in beer. Titze, Christian, Jacob, Parlar, and Ilberg (2010) predicted formation of haze based on the amount of charged particles in a sample. Particle size analysis using the DLS method is already used in many industries. In the pharmaceutical industry it is used to ensure a uniform size distribution throughout drug formulations (Shekunov, Chattopadhyay, Tong, & Chow, 2007). In the food industry it has been used in dairy

research to determine size differences between fat globules (Menard et al., 2010). In the brewing industry DLS has been used to study gushing potential in beer. Deckers et al. (2011) showed that only gushing samples had particles around 100 nm, and these particles were never detected in non-gushing samples. Differences in particle size due to the cross linking activity of mTG were determined in this study using DLS.

Particle size data was collected and analysed using the Zetasizer Nano-ZS. The Zetasizer Nano - ZS software (v 6.20) interpreted the data and determined an average z-value (particle size) from five measurements. The results from the particle size analysis showed that the mean particle size of the sample treated with mTG increased significantly when compared to the control (Figure 7.3). Average particle size of the control was 91 nm while the sample treated with the lowest concentration of mTG (9.25 g/hL) had a significantly higher average particle size at 217 nm. This particle size increase continued with the 92.5 g/hL and 231 g/hL additions of mTG which had average sizes of 258 nm and 262 nm respectively. When compared to the control, increasing dosage of mTG caused a significant increase in the particle size detected. These results fit with the theory that mTG is forming crosslinks between gluten proteins in beer.

**Figure 7.3.** Average particle size of each beer treatment measured with Zetasizer nano. Particle size increased significantly compared to the control which did not have mTG added.



### Particle sedimentation

Analytical centrifuges are used in food research to allow accelerated testing of creaming or sedimentation potential of micro-food dispersions. (D. Lerche, 2002; Dietmar Lerche, Sobisch, & Detloff, 2006). In this study, stability of the control sample and the sample containing the largest particles (treated with the highest level mTG) were tested with the Lumisizer. Both treated and untreated samples showed a constant integral transmission of 90% along the length of the cuvette during the centrifugation (data not shown) which indicated stability of both samples. The larger particles (262 nm) detected in samples treated with mTG (231 g/hL) remained in suspension and did not sediment during centrifugation at 4000 rpm.

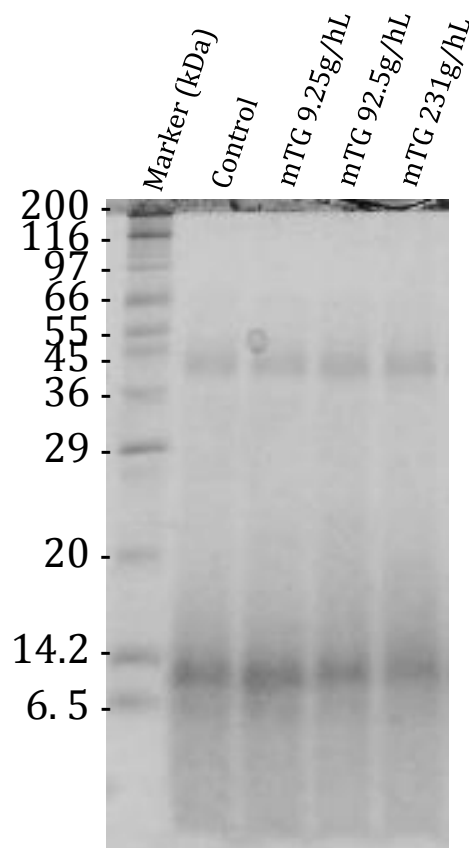


Changes in protein solubility are known to cause differences in scattering measurements (Steiner, Becker, & Gastl, 2010) and very small particles ( $< 0.1 \mu\text{m}$ ) can be detected by light scattering methods, although they are not visible to the naked eye (Bamforth, 1999). Protein crosslinking due to the action of mTG affects the molecular structure of proteins, creating large protein polymers (Bauer, Koehler, Wieser, & Schieberle, 2003). These larger particles may be detected using the DLS measurements, remaining in solution during sedimentation tests.

### SDS-PAGE and Western blotting

SDS-PAGE allows visualization of soluble proteins on a polyacrylamide gel. The particle size data from this study shows an increasing particle size corresponding

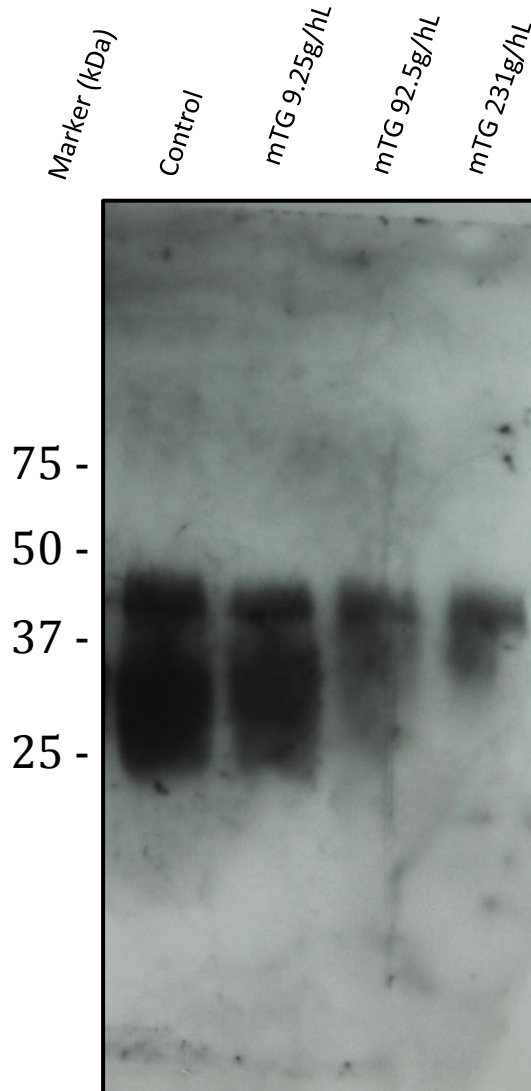
**Figure 7.4.** SDS-PAGE analysis of lyophilised beer samples (containing 2% SDS and 100mM DTT) on a 15% polyacrylamide gel shows bands at 40 kDa and 10 kDa.



with increasing dosage of mTG, and the stability of the mTG treated sample showed the particles did not sediment during centrifugation. In order to determine the molecular weight of the proteins in each beer treatment, samples were analysed using SDS-PAGE. Figure 7.4 shows all samples analysed using SDS-PAGE. The major bands in the samples were approximately 40 kDa and 10 kDa. The 40 kDa protein was likely protein Z and the 10 kDa band was probably lipid transfer protein one (LTP1), both of which are enriched throughout the brewing process due to resistance to degradation during both malting and brewing (Hejgaard, 1977; Leiper, Stewart, & McKeown, 2003). There were no visible differences in molecular weights of proteins between the samples. The larger particle sizes detected with the zetasizer were not visible on the SDS-PAGE gel, possibly due to the insoluble nature of the proteins cross-linked with mTG (Singh & MacRitchie, 2001).

Western blotting is a technique for detecting specific proteins using specially labelled antibodies. Samples are first separated by SDS-PAGE before the proteins are transferred to a membrane. This membrane is then incubated with the antibody which binds to a specific protein and emits a light signal when given an appropriate substrate, which can in turn be imaged.

**Figure 7.5.** Western blot analysis of neat beer samples, separated on a 15% polyacrylamide gel. Gluten proteins detected using peroxidase conjugated anti-gliadin antibody.



The western blot results (Figure 7.5) showed strong band intensity from control beer sample, indicating the presence of gluten proteins. The molecular weight of the signal, ranged from 50 kDa to 25 kDa and this could be due to several gluten proteins being detected by the antibody thus giving a strong signal. The intensity of the signal from the region around 37 – 50 kDa remains similar for all samples, but the sample treated with the two highest doses of mTG has a less intense band in

the region between 25 – 37 kDa, showing perhaps a stronger reaction between lower molecular weight hordeins such as B-hordeins and mTG. This might remove more B- hordein in the filtration step of brewing, resulting in a less intense western blot signal.

Overall the sample treated with mTG at a level 9.23 g/hL also had a strong signal, but slightly decreased compared to the control. Beer treated with 92.3 g/hL mTG had a weaker signal than the control and the lowest mTG concentration (9.23 g/hL). The sample treated with the highest concentrations of mTG (231 g/hL) had the weakest band intensity from the anti-gliadin antibodies. The higher the level of mTG probably resulted in less gluten in the beer samples to react with the anti-gliadin antibodies.

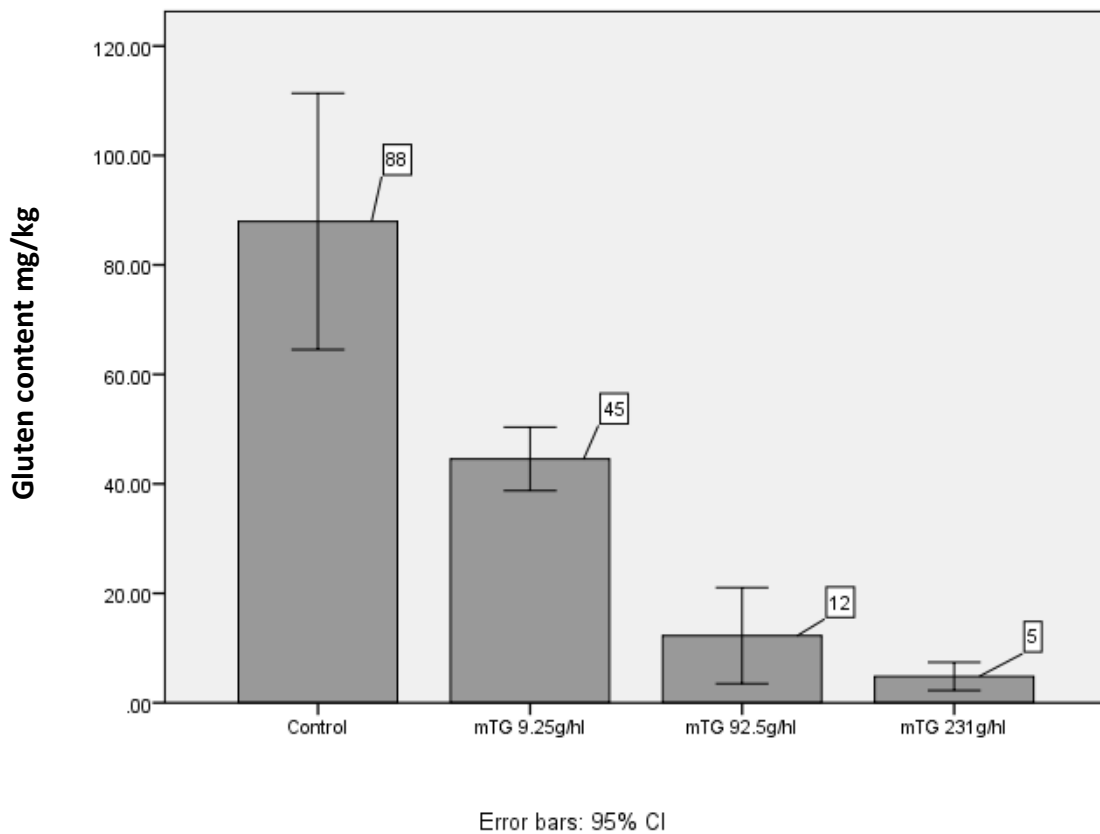
### **Gluten determination**

Analysed gluten levels in beer can vary widely depending on the type of beer and also the assay used to determine gluten content. If a large fraction of the grain bill is made up from gluten-free grain or sugar additions, the gluten content is inevitable lower than when 100% barley malt is used. Guerdrum and Bamforth (2011) measured gluten levels using the *competitive* gliadin ELISA in 25 beers and found seven of the beers, to be below the 20 mg/kg gluten threshold, although they were not labelled as gluten-free.

The levels of gluten in samples from this study were also determined using the *competitive gliadin* ELISA which is the current standard method accepted by the food industry (Gessendorfer, Koehler, & Wieser, 2009; Mena, Lombardia, Hernando, Mendez, & Albar, 2012). The gluten content of the control sample was 88 mg/kg (Fig. 6), this is in line with gluten determination of 100% barley malt beers

tested by other researchers (Guerdrum & Bamforth, 2012). The gluten levels of the mTG treated samples were all significantly lower ( $\alpha = 0.05$ ) than the control. The lowest concentration of mTG (9.25 g/hL) reduced the gluten level by approximately half to 45 mg/kg; 92.5 g / hL reduced the gluten content further to 12 mg/kg gluten, an 86% reduction. The highest concentration of mTG used, (231 g/hL) reduced gluten levels to 5 mg/kg gluten, a reduction of 96% when compared to the control sample. These results correspond to the western blot results where lower levels of gluten proteins were detected in beer treated with increasing dose of mTG.

**Figure 7.6.** Bars represent gluten content of each beer treatment as determined by RIDASCREEN Gliadin *competitive* ELISA. Control sample had the highest level of gluten with the level of gluten decreasing significantly with increasing dosage of mTG



There has also been research showing the possibility of epitope disguise when measuring gluten, low gluten results from *gliadin sandwich* ELISA did not correspond to Mass Spectrometry gluten results in a selection of beers tested (Tanner, Colgrave, Blundell, Goswami, & Howitt, 2013). Two different gluten specific antibodies were used in this study to show the reduction of gluten in our samples. Both the western blot analysis and *competitive* ELISA analysis support the theory that gluten proteins are reduced when beer is treated with mTG. The western blot analysis used a commercially available polyclonal anti-gliadin antibody and the *competitive* ELISA uses a monoclonal antibody that recognises peptides common to wheat rye and barley (Kahlenberg et al., 2006). Each antibody recognises different gluten epitopes which reduces the possibility both epitopes will be disguised (Kanerva, Sontag-Strohm, Brinck, & Salovaara, 2011).

These results confirm mTG can be used to substantially reduce gluten levels detected in beer as the related patent describes. Pasternack et al., (2006) outline production of gluten-free beer and beverages, whereby a beverage containing gluten is treated with mTG and the subsequent crosslinking reduces solubility of gluten proteins and allows removal by filtration (Wieser & Koehler, 2012). This study shows by application of mTG at 92.5 g/hL reduced the gluten level in the sample to 12 mg/kg gluten which allows gluten-free labelling (Codex Alimentarius, 1979). The highest dosage of mTG reduced levels of gluten to 5 mg/kg, below the 20 mg/kg threshold.

## Conclusion

Consumers suffering from coeliac disease and other forms of gluten intolerance must adhere to a strict gluten-free diet, this excludes beer and other malt based beverages. An increasing number of technologies to reduce or detoxify gluten are expanding the range of gluten-free food options for consumers. A single application of mTG to filtered beer reduced gluten levels significantly and the low temperature was suitable for a standard beer maturation process. Increasing dosage resulted in lower gluten content with the highest dose being lowest in gluten. The approximate cost of mTG acquired from Irish suppliers at the time the study was conducted was \$ 40 / Kg. Application of mTG is an effective method for reducing detectable gluten levels in beer, likely due to crosslinking of gluten proteins, which alters their solubility and allows removal by filtration (Wieser & Koehler, 2012). Research and other subsequent patents (Gianfrani, Rossi, & Siciliano, 2008; Gianfrani et al., 2007) show toxicity of wheat flour can be reduced in model systems by treatment with mTG. This reduction in toxicity occurs without removal of glutens, this may also be the case with mTG treated beers. The ultimate test of toxicity would be to conduct clinical studies but this was outside of the scope of this study.

The removal of the coeliac toxic proteins does not have an effect on the foam or the flavours analysed. The colour increased when the beer was treated with mTG enzyme and highest dosage used was above the colour range for a pale beer (MEBAK, 2013). Enzymatic treatments to reduce gluten, such as treatment with proline specific enzymes (Guerdrum & Bamforth, 2012), are an increasingly common way to use traditional beer ingredients to produce a product suitable for people sensitive to gluten. This study shows how treatment of beer with mTG could

be a viable option for reducing levels of coeliac toxic proteins whilst maintaining product quality.



## Literature cited

- Appendix III, (2008). Aretaeus, & Adams, F. (1856). *Causes and Symptoms of Chronic Diseases* (Vol. II). London, UK: The Sydenham Society.
- B. Schehl, A. Mauch, & Arendt, E. (2009). *Malting of sorghum and buckwheat for brewing purposes - A gluten free alternative to barley?* MN, USA AACC International.
- Bamforth, C. W. (1999). Beer haze. *Journal of the American Society of Brewing Chemists*, 57(3), 81-90.
- Bamforth, C. W. (2004). *The Basics of Malting and Brewing: Product Safety and Wholesomeness*. Oxford, UK: Blackwell Science.
- Bamforth, C. W. (2004). The relative significance of physics and chemistry for beer foam excellence: Theory and practice. *Journal of the Institute of Brewing*, 110(4), 259-266.
- Bamforth, C. W. (2006). *Scientific Principles of Malting and Brewing*: American Society of Brewing Chemists. MN, U.S.A.
- Bauer, N., Koehler, P., Wieser, H., & Schieberle, P. (2003). Studies on effects of microbial transglutaminase on gluten proteins of wheat. I. Biochemical analysis. *Cereal Chemistry*, 80(6), 781-786. doi:10.1094/Cchem.2003.80.6.781
- Bizzaro, N., Tozzoli, R., Villalta, D., Fabris, M., & Tonutti, E. (2012). Cutting-edge issues in celiac disease and in gluten intolerance. *Clinical reviews in allergy & immunology*, 42(3), 279-287. doi:10.1007/s12016-010-8223-1
- Brown, A. C. (2012). Gluten sensitivity: problems of an emerging condition separate from celiac disease. *Expert review of gastroenterology & hepatology*, 6(1), 43-55. doi:10.1586/egh.11.79
- Catassi, C., Gatti, S., & Fasano, A. (2014). The New Epidemiology of Celiac Disease. *Journal of Pediatric Gastroenterology and Nutrition*, 59, S7-S9. doi:10.1097/01.mpg.0000450393.23156.59
- Chen, J. S., & Mehta, K. (1999). Tissue transglutaminase: an enzyme with a split personality. *The international journal of biochemistry & cell biology*, 31(8), 817-836.
- Clare Mills, H. J. W., Karin Hoffmann-Sommergruber. (2007). *Managing Allergens in Food*. Cambridge, UK: CRC Press.

- Deckers, S. M., Lorgouilloux, Y., Gebruers, K., Baggerman, G., Verachtert, H., Neven, H., Martens, J. (2011). Dynamic Light Scattering (DLS) as a Tool to Detect CO<sub>2</sub>-Hydrophobin Structures and Study the Primary Gushing Potential of Beer. *Journal of the American Society of Brewing Chemists*, 69(3), 144-149. doi:Doi 10.1094/Asbcj-2011-0524-01
- Dicke, W. K., Weijers, H. A., & Van De Kamer, J. H. (1953). Coeliac disease. II. The presence in wheat of a factor having a deleterious effect in cases of coeliac disease. *Acta paediatrica*, 42(1), 34-42.
- Evans, D. E., & Sheehan, M. C. (2002). Don't be fobbed off: The substance of beer foam - A review. *Journal of the American Society of Brewing Chemists*, 60(2), 47-57.
- Fesus, L., & Piacentini, M. (2002). Transglutaminase 2: an enigmatic enzyme with diverse functions. *Trends in biochemical sciences*, 27(10), 534-539.
- Gessendorfer, B., Koehler, P., & Wieser, H. (2009). Preparation and characterization of enzymatically hydrolyzed prolamins from wheat, rye, and barley as references for the immunochemical quantitation of partially hydrolyzed gluten. *Analytical and Bioanalytical Chemistry*, 395(6), 1721-1728. doi:DOI 10.1007/s00216-009-3080-6
- Gianfrani, C., Rossi, M., & Siciliano, R. A. (2008). Treatment of cereal flour and semolina for consumption by celiac patients: Google Patents.
- Gianfrani, C., Siciliano, R. A., Facchiano, A. M., Camarca, A., Mazzeo, M. F., Costantini, S., . . . Rossi, M. (2007). Transamidation of wheat flour inhibits the response to gliadin of intestinal T cells in celiac disease. *Gastroenterology*, 133(3), 780-789. doi:10.1053/j.gastro.2007.06.023
- Griffin, M., Casadio, R., & Bergamini, C. M. (2002). Transglutaminases: nature's biological glues. *The Biochemical journal*, 368 (Pt 2), 377-396. doi:10.1042/BJ20021234
- Guerdrum, L. J., & Bamforth, C. W. (2011). Levels of gliadin in commercial beers. *Food Chemistry*, 129(4), 1783-1784. doi:DOI 10.1016/j.foodchem.2011.06.021
- Guerdrum, L. J., & Bamforth, C. W. (2012). Prolamin Levels Through Brewing and the Impact of Prolyl Endoproteinase. *Journal of the American Society of Brewing Chemists*, 70(1), 35-38. doi:Doi 10.1094/Asbcj-2012-0130-01
- Hager, A. S., Taylor, J. P., Waters, D. M., & Arendt, E. K. (2014). Gluten free beer - A review. *Trends in Food Science & Technology*, 36(1), 44-54. doi:DOI 10.1016/j.tifs.2014.01.001

- Hardwick, W. A. (1995). *Handbook of brewing*. NY, USA: M. Dekker.
- Hejgaard, J. (1977). Origin of a Dominant Beer Protein Immunochemical Identity with a Beta-Amylase-Associated Protein from Barley. *Journal of the Institute of Brewing*, 83(2), 94-96.
- Hughes, P., & Baxter, E. D. (2001). *Flavour Determinants of Beer Quality* (Vol. 84). Cambridge, UK: Royal Society of Chemistry.
- Kahlenberg, F., Sanchez, D., Lachmann, I., Tuckova, L., Tlaskalova, H., Mendez, E., & Mothes, T. (2006). Monoclonal antibody R5 for detection of putatively coeliac-toxic gliadin peptides. *European Food Research and Technology*, 222(1-2), 78-82. doi:DOI 10.1007/s00217-005-0100-4
- Kanerva, P., Sontag-Strohm, T., Brinck, O., & Salovaara, H. (2011). Improved extraction of prolamins for gluten detection in processed foods. *Agricultural and Food Science*, 20(3), 206-216.
- Kanerva, P., Sontag-Strohm, T., & Lehtonen, P. (2005). Determination of prolamins in beers by ELISA and SDS-PAGE. *Journal of the Institute of Brewing*, 111(1), 61-64.
- Kuraishi, C., Sakamoto, J., & Soeda, T. (1996). *The Usefulness of Transglutaminase for Food Processing* (Vol. 637). USA: American Chemical Society.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227(5259), 680-685.
- Leiper, K. A., Stewart, G. G., & McKeown, I. P. (2003). Beer polypeptides and silica gel - Part I. Polypeptides involved in haze formation. *Journal of the Institute of Brewing*, 109(1), 57-72.
- Lerche, D. (2002). Dispersion stability and particle characterization by sedimentation kinetics in a centrifugal field. *Journal of Dispersion Science and Technology*, 23(5), 699-709. doi:Doi 10.1081/Dis-120015373
- Lerche, D., Sobisch, T., & Detloff, T. (2006). *Determination of stability, consolidation and particle size distribution of liquid or semi-liquid food products by multisample analytical centrifugation*. Paper presented at the ISFRS 2006, Proceedings 4th international symposium on food rheology and structure, Zürich, Switzerland.
- Malvern. (2013). *Zetasizer Nano User Manual* (MAN0485 ed.). Malvern, U.K.: Malvern Instruments Ltd.

- MEBAK. (2013). *Mitteleuropäische Brautechnische Analysenkommission (MEBAK)* Vol. Wort, Beer and Beer-based Beverages, Freising-Weihenstephan, Germany.
- Meilgaard, M. (1975). Flavor chemistry of beer. I. Flavor interaction between principal volatiles. *Technical Quarterly Master Brewers Association of the Americas*.
- Mena, M. C., Lombardia, M., Hernando, A., Mendez, E., & Albar, J. P. (2012). Comprehensive analysis of gluten in processed foods using a new extraction method and a competitive ELISA based on the R5 antibody. *Talanta*, *91*, 33-40. doi:DOI 10.1016/j.talanta.2011.12.073
- Menard, O., Ahmad, S., Rousseau, F., Briard-Bion, V., Gaucheron, F., & Lopez, C. (2010). Buffalo vs. cow milk fat globules: Size distribution, zeta-potential, compositions in total fatty acids and in polar lipids from the milk fat globule membrane. *Food Chemistry*, *120*(2), 544-551. doi:DOI 10.1016/j.foodchem.2009.10.053
- Meresse, B., Ripoche, J., Heyman, M., & Cerf-Bensussan, N. (2009). Celiac disease: from oral tolerance to intestinal inflammation, autoimmunity and lymphomagenesis. *Mucosal Immunology*, *2*(1), 8-23. doi:10.1038/mi.2008.75
- Oliveira, P., Mauch, A., Jacob, F., & Arendt, E. K. (2012). Impact of Fusarium culmorum-infected Barley Malt Grains on Brewing and Beer Quality. *Journal of the American Society of Brewing Chemists*, *70*(3), 186-194. doi:Doi 10.1094/Asbcj-2012-0713-01
- Pasternack, R., Marx, S., & Jordan, D. (2006). Prolamin-reduced beverages and methods for the preparation thereof: Google Patents.
- Phiarais, B. P. N., Mauch, A., Schehl, B. D., Zarnkow, M., Gastl, M., Herrmann, M., Arendt, E. K. (2010). Processing of a Top Fermented Beer Brewed from 100% Buckwheat Malt with Sensory and Analytical Characterisation. *Journal of the Institute of Brewing*, *116*(3), 265-274.
- Pisano, J. J., Finlayson, J. S., & Peyton, M. P. (1968). [Cross-link in fibrin polymerized by factor 13: epsilon-(gamma-glutamyl)lysine]. *Science*, *160*(3830), 892-893.
- Saison, D., De Schutter, D. P., Uyttenhove, B., Delvaux, F., & Delvaux, F. R. (2009). Contribution of staling compounds to the aged flavour of lager beer by studying their flavour thresholds. *Food Chemistry*, *114*(4), 1206-1215. doi:DOI 10.1016/j.foodchem.2008.10.078
- Shekunov, B. Y., Chattopadhyay, P., Tong, H. H. Y., & Chow, A. H. L. (2007). Particle size analysis in pharmaceuticals: Principles, methods and applications.

*Pharmaceutical Research*, 24(2), 203-227. doi:DOI 10.1007/s11095-006-9146-7

Shewry, P. R., & Tatham, A. S. (1990). The Prolamin Storage Proteins of Cereal Seeds - Structure and Evolution. *The Biochemical journal*, 267(1), 1-12.

Singh, H., & MacRitchie, F. (2001). Application of polymer science to properties of gluten. *Journal of Cereal Science*, 33(3), 231-243. doi:DOI 10.1006/jcrs.2000.0360

Sollid, L. M., & Jabri, B. (2013). Triggers and drivers of autoimmunity: lessons from coeliac disease. *Nature Reviews Immunology*, 13(4), 294-302. doi:Doi 10.1038/Nri3407

Steiner, E., Becker, T., & Gastl, M. (2010). Turbidity and Haze Formation in Beer- Insights and Overview. *Journal of the Institute of Brewing*, 116(4), 360-368.

Tanner, G. J., Colgrave, M. L., Blundell, M. J., Goswami, H. P., & Howitt, C. A. (2013). Measuring Hordein (Gluten) in Beer - A Comparison of ELISA and Mass Spectrometry. *Plos One*, 8(2). doi:ARTN e56452 DOI 10.1371/journal.pone.0056452

Taylor, B., & Organ, G. (2009). *Sensory Evaluation*. Weinheim, Germany: Wiley VCH.

Titze, J., Christian, M., Jacob, F., Parlar, H., & Ilberg, V. (2010). The Possibilities of Particle Analysis Demonstrated by the Measurement of the Colloidal Stability of Filtered Beer. *Journal of the Institute of Brewing*, 116(4), 405-412.

Van De Kamer, J. H., & Weijers, H. A. (1955). Coeliac disease. V. Some experiments on the cause of the harmful effect of wheat gliadin. *Acta paediatrica*, 44(5), 465-469.

Wieser, H., & Koehler, P. (2012). Detoxification of Gluten by Means of Enzymatic Treatment. *Journal of the Association of Official Analytical Chemists*, 95(2), 356-363. doi:DOI 10.5740/jaoacint.SGE\_Wieser

## **Chapter 8: Overall discussion and conclusion**

## Overall discussion and conclusion

The market for gluten-free foods has been predicted to continue growing until 2020 (Euromonitor accessed 11-1-16). There has been an increase in the number and types of gluten-free beers associated with this growth. Gluten-free beer can be produced using several methods, as shown in chapter 2. They can be produced using gluten-free grains, which can often result in beers that have unusual flavours or appearance. They can be produced from sugar based syrups and can also be manufactured from barley malt based ingredients, provided it is processed in a way, that the final beer contains less than 20 mg/kg gluten (10 mg/kg hordein in barley malt beers).

Hordein levels in beers can vary considerably (Guerdrum & Bamforth, 2011) and are often at low levels in beers, that contain a high percentage of gluten-free adjuncts such as rice or maize (chapter two). There are differences in levels of beer hordeins between barley cultivars (chapter 3), extent of malt modification can have an effect (chapter four) and beer stabilisation technique can also impact on beer hordein content (chapter six). A commonly used technique for creating gluten-free beer is the application of a commercial *Aspergillus niger* prolyl-endoprotease (AN-PEP) preparation (Brewers Clarex) used to degrade chill haze proteins in beer during fermentation (Guerdrum & Bamforth, 2011). This enzyme allows barley malt to be used as an ingredient, during fermentation the AN-PEP is added, which then breaks down hordein peptides in the beer. A commercial example of beer produced using this enzyme is the range of gluten free beers from Omission brewery, available in the USA (Van Zandycke, 2013).

Alternative methods to using AN-PEP in beer for reducing beer hordeins are not well documented. These studies set out to find new techniques for reducing beer hordeins and optimise existing methods, while keeping a focus on beer quality. Expanding the variety of methods, which are known to reduce beer hordeins should increase the variety of gluten-free beers available to gluten sensitive consumers. Very little work has been published on how the malting process can impact on beer hordeins. Chapter three outlines the importance of choosing the barley cultivar carefully and chapter four then focuses on beer hordein reduction by modifying the malting conditions. Chapter five explores the use of enzymes during the malting process.

Chapters six and seven focus on ways to reduce hordeins in the final beer, taking advantage of stabilising aids and food grade enzymes that target chill haze proteins in beer. The techniques outlined here can be combined with existing knowledge, forming a toolbox of methods that significantly reduce hordein levels in beer.

The primary source of hordeins in beer is the raw material chosen to produce it. The hordeins proteins present in a barley cultivar are variable, depending on hordein genes passed on from parent cultivars as well as the conditions the barley plant was grown under (Shewry & Halford, 2002; Shewry, Tatham, & Halford, 2001). It makes sense that differences between various barley cultivars will have an impact on the hordein levels found in beer. Using established brewing techniques a system was developed to produce model beers, which allowed the control of every step of the brewing process, from malting to mashing and fermentation.

During malting, proteins are degraded as part of the germination process, and during mashing protease enzymes can also degrade proteins. Control over these



steps of the brewing process showed the differences in levels of beer hordein between each barley cultivar. The experiment also used a Pearson correlation matrix to compare all the barley qualities tested, including extract, friability, viscosity and soluble nitrogen.

Results from chapter three revealed that beer hordeins, were related to barley nitrogen levels prior to malting. In addition to the genetic influence, levels of barley nitrogen are influenced by many environmental factors including rainfall, drought, amount of fertiliser application and more (Shewry & Halford, 2002; Ullrich, 2011).

Beer hordeins also correlated with other interesting quality criteria, such as friability, which measures how modified the malt is. Structural changes occur in the malt during modification, which are caused by the breakdown of cell walls and various other cell components, including protein. The structural breakdown that occurs in the malt, negatively correlated with levels of beer hordeins. Overall, Chapter three shows that the choice of barley cultivar used to produce beer has a significant effect on the final level of hordeins. Positive correlations were found between the beer hordeins, and barley nitrogen whereas a negative correlation between the friability and beer hordein content could be observed. Barley nitrogen is a measure of protein present in the barley so a positive correlation with beer hordein was expected. Friability increases with grain modification and degradation of storage proteins also increases, providing amino acids for the developing barley embryo. Negative correlations between friability and beer hordeins would also be expected.

The malt quality results correlated well with previously published results (Briggs, 1998), as modification increased, qualities such as viscosity were reduced (due to  $\beta$ -

glucan and cell wall degradation). Kolbach index was negatively correlated with beer hordeins, as modification increases more proteins are degraded and resulting beer contains less hordeins.

Differences between barley hordeins due to cultivar have been shown previously (Shewry, Faulks, Parmar, & Miflin, 1980). They also showed that the differences between cultivars were still evident in the malted grain. Results from chapter three demonstrate, that the differences between single cultivar model beers can persist into the final beer. The differences in beer hordeins between cultivars are likely due to differences in genetics of the barley cultivar, as well as environmental conditions during the development of the barley.

Chapter four focused on how malting conditions can be altered to reduce beer hordeins. A single cultivar of barley (Beatrix) was used to determine the effect of malt modification on the level of hordeins in beer. During the germination stage of malting, there is a significant reduction in the amount of hordein present in the barley grain and up to 30% can be degraded (Briggs & Hough, 1981), as several proteases are active during grain development. Results in chapter 4, using SDS-PAGE gels, showed extending the period of germination decreased the amount of hordeins present in the malt.

The separation of hordeins, extracted from unmalted Beatrix barley, showed D-hordeins were present approximate to the 100 kDa marker, with B and C-hordeins shown between 25 – 50 kDa. After three days of germination, D-hordeins are completely degraded. The B and C-hordeins extracted from day three malt are also obviously degraded compared to the hordeins from unmalted barley. Day five malt shows further hordein degradation, but the greatest reduction in malt hordeins was

shown in extracts from day seven malt. This reduction of hordeins in malt is due to hordeins being degraded and used for the growing barley grain. As germination proceeds for longer periods, more hordeins are degraded.

Only a small percentage of the hordeins present in malt are found in the final beer, so reductions in beer hordeins had to be tested by producing model beers and testing the levels of hordeins in each one.

A reduction of 44% in beer hordeins was possible by extending the germination stage of malting by four days. This showed in principle that malting conditions can have a significant effect on the level of beer hordeins. It is well known that the longer a grain is germinated the less hordeins are present in the malt. This experiment shows, that the degradation of protein that occurs during malt modification also affects hordein levels in beer. The more highly modified a malt is, the less hordeins are present in the beer.

Endo-protease activity in the malts was assessed and was found to increase during the period of germination, the highest activity was found for the seven day germination process. This increase during germination has also been found by other researchers (Kuntz & Bamforth, 2007) and as protease enzymes breakdown hordeins, the result was expected. The general quality of malt and wort was also evaluated. Friability increased during the period of germination, ranging from 78% for three days of germination up to 97% for seven days of germination. Levels of extract were also good for all for all malt samples, fermentability increased during the course of germination, wort viscosity decreased and Kolbach index increased. However, malting loss was increased over the period of germination and day seven had losses of 11% in weight due to metabolism and losses of rootlets, which is very

high for a normal malting process, but is to be expected when germination is prolonged. The quality of malt and wort produced was as expected for highly modified malt and similar to other published results (Briggs, 1998).

Chapter five focused on novel ways to reduce hordein levels in beer during the malting process, by applying exogenous enzymes directly to the steeping and germination step and therefore increasing protein breakdown. There is already extensive protein breakdown occurring during the germination stage of malting. Extending this germination stage as seen in chapter 4, beer hordeins were reduced. The application of AN-PEP had the potential to reduce hordein levels even more. Guerdrum and Bamforth (2012) showed how AN-PEP can degrade beer hordeins during fermentation. AN-PEP specifically degrades proteins containing high amounts of proline, it has been used to produce gluten-free wheat starch (Walter, Wieser, & Koehler, 2014). This degradation of gluten-like proteins showed potential for reducing beer hordeins during the malting process.

Enzymes have previously been successfully used by other researchers during the malting process (Grujic, 1998) to decrease wort viscosity, but this is the first report of a protease used in the malting process.

The application of AN-PEP during steeping and germination, outlined in chapter 5, significantly reduced the levels of beer hordeins. The quality characteristics of the malt treated with AN-PEP for three days showed slight differences compared to the control. Viscosity was higher and friability was lower than in the control malt, these small differences in the early stages of germination were caused by application of the enzyme. However, after five days of germination there were no differences in quality of the AN-PEP treated malt compared to the untreated malt. The impact

enzyme treatment had on the developing grain was not observed after five days, and the germinating grain managed to recover to its full potential.

The malt treated with AN-PEP was used to produce model beers and hordein content was compared to beers made from untreated control malt. Day three + AN-PEP beer hordeins were not significantly reduced compared to the day three control beer. However, after five days, differences in hordein content were significant. The greatest reduction was evident in beer produced from malt treated for five days + AN-PEP. There was a 46% reduction in beer hordeins compared to the five day control beer. This chapter demonstrates the principle that applying AN-PEP during steeping and germination can result in a significant reduction of beer hordeins. The AN-PEP selectively degrades proline rich proteins such as hordein and application during steeping and germination helped to degrade hordeins, which reduced the levels found in the beer.

The AN-PEP enzyme is sold as a commercial product for reducing chill haze in beer, it is effective since it degrades the beer proteins involved in chill haze. Chill haze is formed as a complex between proline rich proteins and polyphenols found in beer. This complex cannot form if the proteins involved are not present. The proline rich proteins involved in chill haze formation are the hordein proteins that are dangerous for gluten sensitive consumers, so methods to reduce chill-haze in beer have the potential to reduce beer hordeins (Dostalek, Hochel, Mendez, Hernando, & Gabrovskaa, 2006; Lewis & Bamforth, 2006).

There are well established methods available to reduce levels of proteins involved in chill haze from beer (Kunze, 2010). The methods usually focus on removing either the protein or the polyphenol part of the chill haze complex (Siebert, Carrasco, &

Lynn, 1996). Chapter six focuses on two beer stabilisers, that are commonly used to reduce the protein part of the chill-haze complex. In order to test the impact of stabilisation on beer hordeins, beer was produced at pilot scale level and silica gel was applied at the recommended dose of 50 g/hl and also at 500 g/hl. The stabilised beer was compared to unstabilised beer in terms of quality and hordein content. The impact on beer quality was minimal, the flavours analysed were not significantly different from the control beer and the foam stability was also not affected. Applying 50 g/hl silica gel resulted in a 59% reduction in beer hordeins. This hordein reduction increased to 90% when 500 g/hl silica gel was applied. Hordein levels were reduced substantially and the beer quality was still in the acceptable range.

Silica gel successfully reduced beer hordeins due to its very large surface area covered in silanol (OH) groups (Siebert & Lynn, 1997). This binds to proline rich proteins and the silica gel is then removed by filtration. This selective removal of hordeins results in a large decrease in hordein levels with little effect on beer quality.

Stabilisation with tannic acid was also tested, at levels of 2 g/hl and 20 g/hl. Applying 2 g/hl of tannic acid resulted in a 64% reduction in beer hordeins without major impacts on beer quality. However, when 20 g/hl was applied the beer hordein was reduced by 90%, but unfortunately the high dose of tannic acid caused foam stability to be reduced significantly and there were also negative effects on flavour and colour.

Application of tannic acid at low levels reduced levels of beer hordein without negative effects on beer quality, this is due to reactions with proline rich proteins

(Asano, Shinagawa, & Hashimoto, 1982; Siebert, Troukhanova, & Lynn, 1996) however when tannic acid was applied at high levels, there is an excess of tannic acid and it is available for many more interactions within the beer (He, Shi, & Yao, 2006). This increased number of reactions causes removal of more proteins and other compounds from the beer, which reduces foam stability, reduces colour and many beer flavours are affected.

The beer stabilised with 2 g/hl tannic acid was just above the 10 mg/kg threshold of hordeins for gluten-free labelling, and the highest level of tannic acid produced beer with just 2.9 mg/kg hordein, but it affected beer quality negatively.

Tannic acid has been used previously to reduce beer hordeins, Van Landschoot (2011) showed that by applying of tannic acid it was possible to reduce beer hordeins very effectively. The author (Van Landschoot, 2011) used several applications of tannic acid during brewing and prolyl-endoprotease was added during fermentation. The focus for chapter six in this thesis outlined a single application of tannic acid at two different levels to evaluate the dose response. Results show that a single application of tannic acid at filtration can give large reductions in levels of beer hordein. There was also evaluation of the impact on beer quality which was not looked at previously. The results showed, when used at high doses, tannic acid was very effective at reducing beer hordein but had a negative effect on quality of beer.

Silica gel was more suited to reducing beer hordeins than tannic acid, after stabilisation using 500 g/hl silica gel, the beers were also below 10 mg/kg hordeins, and the beer was still of acceptable quality. By targeting the removal of chill-haze

proteins in the final beer it was possible to reduce beer hordeins and produce beer that could be labelled gluten-free.

Silica gel has previously been shown to be effective at reducing beer hordein by other researchers (Dostalek et al., 2006). However, the previous study used a single concentration of silica gel and did not study the side effects on beer quality in detail. The experiments outlined in this thesis show a dose related reduction in beer hordein and show that beer quality is not affected by treatment with silica gel.

Beer hordeins remaining in filtered beer were also the target of chapter 7. Microbial Transglutaminase (mTG) is a protein crosslinking enzyme used commonly in food production and although there are many examples of using mTG in food processing (Chiya, Jiro, & Takahiko, 1996), there are no published examples of its use in beer. The application of transglutaminase has been patented, but no scientific papers were available to support the patent. The enzyme creates covalent bonds between the glutamine residues in proteins, joining them together. This increases the physical size, which makes it possible to remove protein from beer during filtration. mTG was applied at three different concentrations and this resulted in a significant reduction in beer hordeins. The lowest concentration applied was 9.25 g/hl and this reduced hordein by 49%. This reduction went up to 86% when 92.5 g/hl was applied, which brought the beer below the 10 mg/kg hordein threshold to just 6 mg/kg hordeins. The maximum concentration applied reduced the level of beer hordeins down to just 2.5 mg/kg, which is a 94% reduction compared to the control. The quality of the beer was not substantially affected, but colour increased and particle size also increased as mTG facilitated crosslinks between proteins.



Hordein proteins are rich in proline and glutamine, silica gel and AN-PEP are successful in reducing beer hordein because of their affinity for proline. In contrast, mTG is effective at creating crosslinks between hordeins due to its affinity for glutamine amino acid residues. Proteins rich in glutamine, such as hordeins, bind together forming large protein complexes and the very large proteins in the beer were removed using filtration through 1.5  $\mu\text{m}$  filter. The beers treated with mTG were significantly lower in hordeins. The high level of glutamine found in hordein makes mTG very effective at binding them together. The enzyme treatment was applied at 1°C for 20 hours and when combined with normal beer filtration, it was very effective at reducing beer hordeins. There was no significant effect on foam stability or beer flavour, although there was an increase in beer colour.

The range of methods effective at reducing beer hordein suggests good potential for further research. In addition to technological methods for reducing beer hordeins, there is also a possibility of selecting varieties of barley that have low-hordein beer potential. This could be combined with controlling the nitrogen sources during growth of barley, which may have interesting results with regards to beer hordeins.

The optimisation of the malting process to reduce levels of beer hordein also has a great scope, by varying time, temperature and pH it may be possible to optimise even greater reductions in beer hordein during malting. Another possible way of increasing levels of enzymatic activity in malt would be to apply gibberellic acid during the germination (Briggs, 1998). This could boost production of proteases resulting in greater reductions of beer hordeins in a shorter time.

The range of options when it comes to applying enzymes during malting is of great interest also, the effect of applying AN-PEP was limited and perhaps by combining cellulase or a glucanase enzyme with AN-PEP, an increased effect may be possible.

The model beers produced from experimental malts contained reduced levels of hordeins, but they were still above the gluten-free threshold.

There has been little work prior to this on the effect of barley cultivar on levels of beer hordeins, a low gluten barley is being developed (Tanner, Blundell, Colgrave, & Howitt, 2015) but using commercial malting barley cultivars and demonstrating differences between levels of beer hordeins has not been shown before. Malting is well known to reduce hordein present in the grain, the impact of malting on beer hordeins has not been researched before, but results in chapter four showed significant decreases caused by extending the germination period of malting.

Enzymes have been used during malting before and AN-PEP reduced beer hordeins significantly when applied to germinating grain. This demonstrates the principle of applying enzymes during malting to reduce beer hordeins.

Beer stabilisers have already been shown to be effective at reducing beer hordeins. This study provides exact details of silica gel dosage and the corresponding reduction in beer hordein, while maintaining focus on beer quality, which was not reported by other researchers. The results from chapter six showed by increased dosage of silica gel, the beer hordein showed a corresponding decrease in levels of hordein, without problems with beer quality.

Also in chapter 6, tannic acid demonstrated a dose dependent reduction in the levels of beer hordeins. Unlike previous studies, tannic acid was applied at a single point in the brewing process and at varied dose to look at impact on levels of beer

hordeins and beer quality. Like silica gel, high doses of tannic acid were very effective at reducing beer hordeins. However, the impact on beer quality was not acceptable.

This thesis also provided detailed information on how mTG reduces beer hordeins, which was not previously available. The patented method for reducing beer hordeins using mTG outlines several points of application. This study focused on a single point of mTG application at filtration, and demonstrates a dose dependent reduction in beer hordeins. Using a single dose of mTG at the correct level can produce a beer below 10 mg/kg hordein. There are no other published research papers on the application of mTG in beer as of yet.

This work has raised some interesting topics to study. In chapter three, we showed that the barleys grown at Glanbia had significantly higher levels of hordein in single cultivar beers than those produced from barley grown by Seedtech. It is likely this is due to the level of nitrogen applied as fertilizer during the growth of the barley, in our study we had no control over fertilizers applied to the developing barley. Further studies will hopefully look into the link between types and amounts of fertilizer and how it can affect the level of hordein that makes it into the beer. This would give a better understanding how farmers can influence the quality of their crop with a focus on producing low hordein beer. Some very interesting experiments should be possible on how environmental conditions could be used to control beer hordeins. Another interesting area to study would be how certain cultivars respond to the different levels of nitrogen during growth with regard to beer hordeins.

Possible future work which would likely result in very low hordein beer is to use a combination of the results from chapter three with the methods from chapter four. By choosing the cultivar that produced the lowest hordein model beer (Overture) and extending the malting process to perhaps seven days the resultant beer would more than likely be below the 10mg/kg hordein threshold. This means that by screening a crop of barley and optimizing the malting process a brewer could make gluten-free beer.

By using AN-PEP during malting, beer hordein could be reduced even further, although this is likely to be a very expensive method for producing low hordein beer. Further work optimizing the method and timing of AN-PEP application during malting perhaps with combinations of other enzymes may result in low-hordein malt which may have uses in other food applications.

The obvious future work that will be interesting to look at is the larger scale production of low hordein beer using cultivar selection and optimization of malting process. These beers could be tested with regard to long term stability, foam and flavour quality and general consumer acceptance.

Some of these beer quality tests were performed on beers stabilized with silica gel and tannic acid using pilot scale processes with some positive results. This method of hordein reduction is the easiest to implement in most breweries, it could easily be combined with a selected cultivar to produce low hordein and even without using the optimized malting process you could produce a beer below 10 mg/kg hordein. We showed that high levels of tannic acid could result in poor quality beer in chapter six, if a dose of between 2 – 6 g/hl tannic acid was used in a beer that

was already low in hordein, it would probably be a very effective method for producing beer below 10 mg/kg hordein without the issues with beer quality.

Silica gel has shown great potential as tool for producing beers below 10 mg/kg hordein. Interesting future work would be to look at the use of stabilising agents on wheat beers to see if there is any hope for reducing the gluten content. Perhaps by combining the mTG treatment outlined in chapter seven with silica gel stabilisation alongside tannic acid stabilisation you could reduce the levels of gluten in a wheat based beer. The impacts on beer quality would be important to look at here as both mTG and tannic acid had effects on beer colour and so many stabilisation treatments combined is likely to have some effect on the proteins involved in foam. However, it could be an option for some beer styles.

One of the most important things that should be looked at in future studies is the validity of the calculation for beer gluten levels. There are good arguments against applying a factor of two to calculate gluten after performing a direct measurement of beer hordein in 100% barley malt beers. The factor of two is applied to account for insoluble particles that are removed during brewing and filtration steps. The results might be more valid if there was no factor applied. Obviously the safety of the consumer is of primary concern, but the threshold levels should be clear and easy to understand.

Overall, all of the methods shown in this thesis could be combined in many ways with the potential for huge reductions in beer hordeins. There is potential for future work showing the effect of combining the different methods.

The main outcome of this work is the description of a set of practical tools for reducing beer hordeins outlining some of the potential impacts on malt and beer

quality. By building upon previous knowledge and developing novel new techniques, this toolbox of hordein reduction methods outlines effective ways to help brewers and maltsters reduce beer hordeins.

## Literature cited

- Asano, K., Shinagawa, K., & Hashimoto, N. (1982). Characterization of haze-forming proteins of beer and their roles in chill haze formation. *J. Am. Soc. Brew. Chem*, 40(4), 147-154.
- Briggs, D. E. (1998) *Malts and malting* (1st ed., pp. 628, 175). London, Blackie Academic.
- Briggs, D. E., & Hough, J. S. (1981). Malting and brewing science (2nd ed., pp. 82, 83, 112). London, Chapman and Hall.
- Chiya, K., Jiro, S., & Takahiko, S. (1996). *The Usefulness of Transglutaminase for Food Processing* (Vol. 637). Washington, U.S.A.: American Chemical Society.
- Dostalek, P., Hochel, I., Mendez, E., Hernando, A., & Gabrovska, D. (2006). Immunochemical determination of gluten in malts and beers. *Food Additives and Contaminants*, 23(11), 1074-1078. doi:Doi 10.1080/02652030600740637
- Grujic, O. (1998). Application of a commercial enzyme preparation in the barley malting process. *Journal of the Institute of Brewing*, 104(5), 249-253.
- Guerdrum, L. J., & Bamforth, C. W. (2011). Levels of gliadin in commercial beers. *Food Chemistry*, 129(4), 1783-1784. doi:10.1016/j.foodchem.2011.06.021
- Guerdrum, L. J., & Bamforth, C. W. (2012). Prolamin Levels Through Brewing and the Impact of Prolyl Endoproteinase. *Journal of the American Society of Brewing Chemists*, 70(1), 35-38. doi:10.1094/Asbcj-2012-0130-01
- He, Q., Shi, B., & Yao, K. (2006). Interactions of galloylannins with proteins, amino acids, phospholipids and sugars. *Food Chemistry*, 95(2), 250-254. doi:DOI 10.1016/j.foodchem.2004.11.055
- Kuntz, R. J., & Bamforth, C. W. (2007). Time course for the development of enzymes in barley. *Journal of the Institute of Brewing*, 113(2), 196-205.
- Kunze, W. (2010). *Technology brewing and malting*, Beer production: Improving colloidal stability of beer (pp. 562 - 564): VLB, Berlin, Germany.
- Lewis, M., & Bamforth, C. W. (2006). *Essays in brewing science*: Springer. New York, U.S.A.
- Shewry, P. R., Faulks, A. J., Parmar, S., & Mifflin, B. J. (1980). Hordein Polypeptide Pattern in Relation to Malting Quality and the Varietal Identification of Malted Barley-Grain. *Journal of the Institute of Brewing*, 86(3), 138-141.

- Shewry, P. R., & Halford, N. G. (2002). Cereal seed storage proteins: structures, properties and role in grain utilization. *Journal of Experimental Botany*, 53(370), 947-958. doi:DOI 10.1093/jexbot/53.370.947
- Shewry, P. R., Tatham, A. S., & Halford, N. G. (2001). Nutritional control of storage protein synthesis in developing grain of wheat and barley. *Plant Growth Regulation*, 34(1), 105-111. doi:Doi 10.1023/A:1013382803849
- Siebert, K. J., Carrasco, A., & Lynn, P. Y. (1996). Formation of protein-polyphenol haze in beverages. *Journal of Agricultural and Food Chemistry*, 44(8), 1997-2005. doi:Doi 10.1021/Jf950716r
- Siebert, K. J., & Lynn, P. Y. (1997). Mechanisms of beer colloidal stabilization. *Journal of the American Society of Brewing Chemists*, 55(2), 73-78.
- Siebert, K. J., Troukhanova, N. V., & Lynn, P. Y. (1996). Nature of polyphenol-protein interactions. *Journal of Agricultural and Food Chemistry*, 44(1), 80-85. doi:Doi 10.1021/Jf9502459
- Tanner, G. J., Blundell, M. J., Colgrave, M. L., & Howitt, C. A. (2015). Creation of the first ultra-low gluten barley (*Hordeum vulgare* L.) for coeliac and gluten-intolerant populations. *Plant Biotechnol J*. doi:10.1111/pbi.12482
- Ullrich, S. E. (2011). Barley, production, improvement, and uses (pp. 228, 514). Chichester, West Sussex, UK ; Ames, Iowa: Wiley-Blackwell.
- Van Landschoot, A. (2011). Gluten-free barley malt beers. *Cerevisia*, 36(3), 93-97.
- Van Zandycke, S. (2013, Nov/Dec). GLUTEN-REDUCED BEERS. *The New Brewer*, 79-84.
- Walter, T., Wieser, H., & Koehler, P. (2014). Production of gluten-free wheat starch by peptidase treatment. *Journal of Cereal Science*, 60(1), 202-209. doi:10.1016/j.jcs.2014.02.012