Effect of Mashing and Malt Types on Yeast Assimilable Nitrogen Content in Wort

Bachelor thesis

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Meskimise ja linnaste mõju pärmile omastatavale lämmastiku kontsentratsioonile õllevirdes

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Declaration

Hereby I declare that I have compiled the paper independently and all works, important standpoints and data by other authors have been properly referenced and the same paper has not been previously been presented for grading.

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Contents

Abstract

Fermentation is an integral stage in the production of beer, influencing many of the quality parameters of the final product. Therefore, for brewer's yeast to be able to perform a successful fermentation, an ample supply of nutrients in beer wort is important. One of those essential nutrients is nitrogen which is present in wort in different forms, not all of which yeast is able to metabolise. The nitrogenous compounds that can be consumed by yeast are collectively termed yeast assimilable nitrogen (YAN).

Nine different worts were prepared to assess the impact that three different mashing regimes and three malt types had on wort's YAN concentrations in the form of free (FAA) and bound amino acids (BAA). The FAA content was analysed by high-performance liquid chromatography coupled with UV detection with pre-column derivatisation. BAA content was determined indirectly as there are no established direct analysis methods. For this, acid hydrolysis was performed to break peptides and proteins down to individual amino acids prior to their quantification. However, a significant problem arises when performing acid hydrolysis in a medium such as beer wort. The high reducing sugar content of wort results in a significantly lower yield of amino acids due to the Maillard reaction. To avoid the interference caused by sugars, a novel method for wort pre-treatment was tested. The worts were fermented with a peptide transporters knockout (PepKO) yeast strain to reduce the sugar content prior to acid hydrolysis. Subsequently, the total amino acid (TAA) content was determined with the method described above for FAA analysis. BAA content was calculated by subtracting the FAA from the TAA.

The malt types used responded differently to different mashing regimes regarding FAA content – Pilsen malt worts maintained a relatively steady FAA content, however including a protease and peptidase activity incubation step (50°C) in the mashing regime increased the FAA content of Pale Ale and Distilling malt worts by 26% and 30% respectively.

The only effect of mashing on the fermentable sugar content in wort was that the mashing regimes which included the optimal temperature range of β-amylase (60-65°C) resulted in a higher sugar content. Between the three malts used, Pilsen malt based worts consistently had the highest sugar contents. On average the PepKO fermentations decreased the reducing sugar content of the worts by 95.7%, leaving behind 3.5 g/L of residual sugars. The PepKO fermented worts demonstrated a 33% increase in total BAA content when compared to the original worts, indicating that wort pre-treatment was successful in increasing the recovery of amino acids during acid hydrolysis. When analysing the <3 kDa peptide fraction, contrary results were obtained – PepKO treated worts resulted in a 35% lower BAA content. These results suggest that the small fraction of reducing sugars is still interfering with the analysis and that further optimisation of the PepKO method is needed to analyse smaller fractions of BAA.

The Biuret test which quantifies protein and peptides indicated that both the total and <3 kDa BAA contents of worts stayed relatively stable despite different mashing regimes and malt types being used.

Annotatsioon

Fermentatsioon ehk kääritamine on oluline osa õlletootmise protsessist, mis mõjutab mitmeid õlle kvaliteedinäitajaid. Eduka fermentatsiooniprotsessi eelduseks on see, et õllevirre sisaldab küllaldaselt pärmile vajalikke toitaineid. Üks neist hädavajalikest toitainetest on lämmastik. Lämmastikku esineb õllevirdes mitmel erineval kujul, kuid pärm ei ole võimeline neid kõiki tarbima. Seda hulka lämmastikuühenditest, mida pärm suudab omastada, nimetatakse pärmile omastatavaks lämmastikuks.

Erinevaid linnaseid ja meskimisrežiime kasutades valmistati üheksa õllevirret, milles uuriti pärmile omastatava lämmastiku sisaldust vabade ja peptiidsidemetega seotud aminohapete kujul. Vabade aminohapete sisalduse määramiseks kasutati kõrgefektiivset vedelikkromatograafiat UV detektsiooni ja kolonnieelse derivatiseerimisega. Peptiidide sisalduse analüüsimiseks otsese meetodi puudumise tõttu määrati neid kaudselt, kasutades eelnevalt happelist hüdrolüüsi peptiidide ja valkude lagundamiseks vabadeks aminohapeteks. Happelise hüdrolüüsi kasutamisel õllevirdes kerkib esile üks oluline probleem, nimelt kaasneb õllevirde kõrge taandavate suhkrute sisaldusega Maillard'i reaktsiooni toimumine, mistõttu vabade aminohapete saagis väheneb oluliselt. Selle probleemi lahendamiseks katsetati uut meetodit õllevirde töötlemiseks enne happelist hüdrolüüsi. Suhkrusisalduse vähendamiseks viidi läbi fermentatsioonid peptiiditransporterite knock-out (PepKO) pärmitüvega. Seejärel määrati virrete kogu aminohappesisaldus ülalkirjeldatud meetodiga ning peptiidsidemetega seotud aminohapete sisalduse leidmiseks lahutati vabad aminohapped kõigist aminohapetest.

Vabade aminohapete sisalduse sõltuvus meskimisrežiimidest varieerus erinevate linnaste puhul. Pilsen linnastest valmistatud virrete vabade aminohapete sisaldused olid erinevate meskimisreziimide puhul sarnased. Pale Ale ja Distilling linnastest valmistatud virrete vabade aminohapete sisaldused suurenesid aga vastavalt 26% ja 30% siis, kui meskimisrežiimile lisati etapp proteaaside ja peptidaaside aktiivsuse optimaalsel temperatuuril (50°C).

Meskimisel suurendasid virde suhkrusisaldust need režiimid, mis sisaldasid β-amülaasi optimaalset temperatuurivahemikku (60-65°C). Võrreldes erinevaid linnaseid olid kõrgeimad suhkrusisaldused Pilsen linnastest valmistatud virretes. PepKO fermentatsioonid vähendasid virrete taandavate suhkrute sisaldust keskmiselt 95.7%, jättes alles 3.5 g/L jääksuhkruid. Esialgsete virretega võrreldes oli PepKO fermenteeritud virrete peptiidsidemetega seotud aminohapete sisaldus kolmandiku võrra suurem, millest võib järeldada, et meetod suurendas aminohapete saagist happelise hüdrolüüsi protsessis. Alla 3 kDa molekulmassiga peptiidide fraktsiooni analüüsimisel oli PepKO fermentatsioonide efekt aga vastupidine, vähendades tuvastatud peptiidide hulka 35% võrra. Sellest tulenevalt võib arvata, et kindla peptiidifraktsiooni analüüsimiseks vajab PepKO meetod optimeerimist.

Biureedi test, mida kasutatakse valkude ja peptiidide määramiseks, näitas et kõigist peptiidsidemetega seotud aminohapetest ega ka pärmile omastatavast fraktsioonist pärit lämmastiku sisaldus virretes ei sõltunud oluliselt erinevate meskimisrežiimide ja linnaste kasutamisest.

Abbreviations

AA	Amino acid
AQC	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
BAA	Bound amino acids
BSA	Bovine serum albumin
FAA	Free amino acids
FAN	Free amino nitrogen
PDA	Photo diode array
TAA	Total amino acids
YAN	Yeast assimilable nitrogen
YPD	Yeast peptone dextrose

Introduction

Nitrogen is an important element for yeast during beer fermentation. In the fermentation industry, the amount of nitrogen in the fermentation feedstock that can be assimilated by yeast is defined by the term yeast assimilable nitrogen (YAN). YAN includes free amino acids, small peptides and ammonia (Hill & Stewart, 2019). In all grain beer wort these YAN-sources make up a total of 190 to 470 mg/L (Thompson-Witrick & Pitts, 2020).

An adequate YAN level in wort is a prerequisite for yeast growth and fermentation performance as it is required for the synthesis of yeast cellular proteins, including enzymes (Koller & Perkins, 2022; Lekkas et al., 2005). However, the level of nitrogen does not only have an effect on fermentation performance but also affects the beer organoleptic properties (Ferreira & Guido, 2018, p. 23). Esters, diacetyl (vicinal diketones), and sulphur compounds are all side products in the amino acid synthesis pathways, and off-flavour compounds such as higher alcohols and aldehydes are formed with amino acids as precursors (Ferreira & Guido, 2018; Hazelwood et al., 2008; Pires et al., 2014). The amount and source of nitrogen in the fermentation feedstock is therefore important for the final product quality. Reliable quantitative measurement for some of these compounds exists. Ammonia and free amino acids can be quantified using ultra-high performance liquid chromatography (UPLC) coupled to an ultraviolet (UV) detector after derivatization with e.g. 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) using a standard curve (Fiechter & Mayer, 2011; John et al., 2004). Although most of the nitrogen assimilated by yeast comes from ammonia and free amino acids, it has been shown that approximately 40% of the oligopeptides present in beer wort are also utilised by yeast (Lekkas et al., 2009). Quantification of peptides is much harder mainly due to the high number of different peptides (>3 million amino acid combinations for di- to pentapeptides) with a large variety in chemical properties (John et al., 2004). The peptide content is therefore usually indirectly quantified, e.g. by first hydrolysing the peptide to individual amino acids with a strong acid (Moore & Stein, 1948, 1951; Mustățea et al., 2019) and subsequently measuring the resulting amino acids using the aforementioned method. However, the high temperatures that acid hydrolysis requires, and the high reducing sugar content of fermentation feedstocks interfering with amino acid analysis through the Maillard reaction, makes this method unsuitable to follow the nitrogen consumption from the peptide fraction in by yeast quantitatively without first purifying the sample (John et al., 2004).

This thesis aims to study the effect of different malts and mashing regimes on the fermentable sugars and YAN content in beer wort, and to test a novel method to remove interfering reducing sugars from the samples.

1. Literature review

1.1. Barley

1.1.1. Grain structure and composition of barley

Beer is primarily made from barley (*Hordeum vulgare*) malt. The barley grain consists of the endosperm and the embryo which are separated by the scutellum and covered by several layers: the testa, the pericarp and the husk. The grain coverings serve a protective role and provide nutrients to the embryo at the start of germination (Lynch et al., 2016).

The endosperm is the largest part of the barley grain and consists of the starchy endosperm and the aleurone layer surrounding it. As the name suggests, the cells of the starchy endosperm contain starch granules in a matrix of storage proteins and provide the necessary nutrients for the developing embryo during germination (Holopainen-Mantila, 2015). Starch granules make up around 60% of the dry weight of the barley grain and consist of amylose and amylopectin in varying proportions (Fox, 2010; Holtekjølen et al., 2006). The cell walls of the barley grain are made up of β-glucan and arabinoxylan in varying ratios depending on the part of kernel. (Holopainen-Mantila, 2015; Kok et al., 2019).

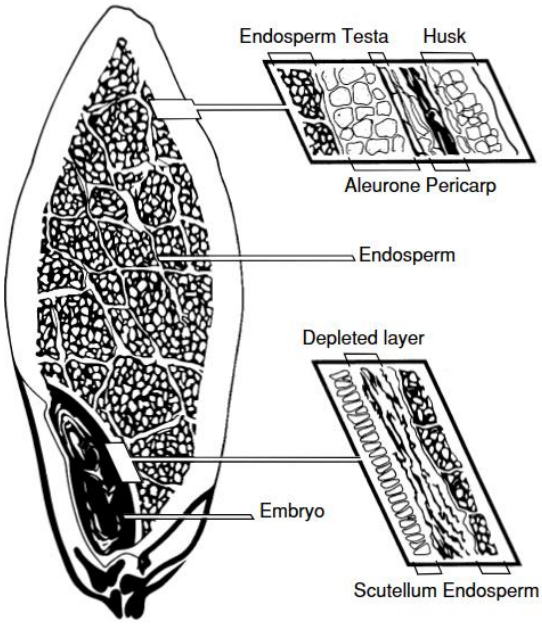

Figure 1. Structure of the barley grain (MacGregor, 2003)

1.1.2. Barley proteins

Barley proteins can be categorised by different attributes. Based on their biological function barley proteins are classified as storage proteins, structural and metabolic proteins, and protective proteins (Bahmani et al., 2021). Additionally, barley proteins are divided into four different fractions based on their solubility: albumins, which are soluble in water, globulins in salt solutions, hordeins in aqueous alcohol solutions and glutelins in alkali (Rani & Bhardwaj, 2021). This thesis focuses mainly on the

proteins most important from a brewer's standpoint, namely storage proteins and the metabolic proteins that play a role in the brewing process.

The protein content of barley grains can vary as a result of different cultivars, growth environments and available nitrogen. Although barley protein content usually falls in the range of 8-15% (Gupta et al., 2010; MacGregor, 2003), up to 30% can be achieved with nitrogen supplementation during growth (Qi et al., 2006). However, the optimal protein content of barley for malting is 10-12%. A higher protein content has been observed to be negatively correlated with the starch content and extractable sugars (Fox, 2010) and contributes to haze formation in beer (Ye et al., 2016). On the other hand, barley with a lower protein content might not provide sufficient YAN for a successful fermentation (Luo et al., 2019).

Storage proteins serve as a source of nutrients and energy for the developing embryo during germination, providing it with nitrogen, sulphur and carbon. The major storage proteins of the barley endosperm are hordeins and glutelins, making up 30-50% and 35-45% of the whole protein content of the grain respectively (Jaeger et al., 2021). Hordeins belong to the prolamins class of proteins that are characterised by being rich in proline and glutamine (Baxter, 1981; Yupsanis et al., 1990). Although the hordeins present in barley belong to subsections that differ in their extractability, over half of the total hordein content is solubilised in malt (Gupta et al., 2010; Steiner et al., 2011). Glutelins are characterised by a high proportion of nonpolar amino acids, e.g. proline and glycine, which makes them very poorly soluble (Steiner et al., 2011; Zhao et al., 2011). Similarly to hordeins, they are also rich in glutamine (Jaeger et al., 2021).

Barley globulins, also known as edestin, and barley albumins, also known as leucosin, are similar in that they mainly serve metabolic functions, although some globulins are storage proteins (Holopainen-Mantila, 2015; Steiner et al., 2011). Globulins and albumins form around 15% and 11% of the total protein content respectively.

1.1.3. Barley malting process

Malting is the controlled germination of the grain with the purpose of producing hydrolytic enzymes in the grain and the partial enzymatic hydrolysis of the cell walls, storage proteins and starchy endosperm by those enzymes (Celus et al., 2006). The process is divided into three stages: steeping, germination and kilning, during all of which the moisture content and temperature of the grains are controlled.

The steeping process involves subsequent immersion and air rest cycles of the grains. Once the moisture content of the grains is high enough, the embryo resumes metabolic activity, and induces the production and activation of hydrolytic enzymes which take part in the modification of the grain (Rani & Bhardwaj, 2021).

The hydrolytic enzymes produced during germination are divided into three main groups according to their substrates: cell wall hydrolases, starch hydrolases and proteolytic enzymes. The main cell wall hydrolases are β-glucanases and arabinoxylanases (Jamar et al., 2011). Sufficient degradation of the cell walls is essential for the starch hydrolases and proteases to access the starch and protein reserves (Habschied et al., 2020). The starch hydrolases group includes α-amylase, β-amylase, limit-dextrinase and α-glucosidase, all of which participate in the degradation of starch into fermentable sugars

(Bamforth, 2009). Proteolytic enzymes are divided into endopeptidases that hydrolyse the internal peptide bonds, and exopeptidases that hydrolyse the terminal peptide bonds at either the carboxy or amino terminus of peptides and proteins (Schmitt et al., 2013). Over 40 different endopeptidases have been found in barley, belonging into four main classes: cysteine proteases, aspartic proteases, serine proteases and metalloproteases class (Jones, 2005).

Once optimal modification is achieved, the process is terminated by kilning, during which the germinated grains (green malt) are dried and cured by a stream of heated dry air being passed through them (Izydorczyk & Edney, 2003; Schwarz & Li, 2010). During kilning the grain moisture content is reduced below 5% for stability during transport and storage (Paramithiotis et al., 2023). The specific temperatures used in the kilning process depend on the desired characteristics of the malt being produced, e.g. in case of base malts preserving enzymatic activity is important which requires that at the start of the kilning process while the grain moisture content is still high, kiln temperatures are kept low (Izydorczyk & Edney, 2003; Kreisz, 2009). During the curing phase the malt obtains it colour and flavour as a result of the Maillard reaction (Kreisz, 2009).

1.2. Mashing

Mashing is a critical step in the beer production process. During mashing fermentable sugars and YAN necessary for fermentation and other compounds that influence beer quality are released from malt as the result of enzymatic hydrolysis of starch, proteins and barley cell walls (Laus et al., 2022). In essence, mashing is a continuation of the same processes that started during malting. Many of the enzymes active during germination survive the kilning step and are reactivated during mashing (Osman et al., 2002).

Prior to mashing the malt is milled to allow easier access for the hydrolytic enzymes. The milled malt is added to hot water and thoroughly mixed to ensure uniform distribution. Extraction of various different compounds is dependent on the temperature and duration of mashing, therefore different mashing regimes are employed in the brewing process (Wunderlich & Back, 2009). The optimal temperature ranges of enzymes relevant in the brewing process are presented in table 1.

Some mashing regimes include a single saccharification rest in the optimum temperature range of the two most important amylolytic enzymes present in malt: α-amylase and β-amylase. They contribute the most to starch saccharification as α-amylase breaks the starch polymers down into oligosaccharides and β-amylase releases maltose from the non-reducing ends of those starch polymers and saccharides (Evans et al., 2018).

Including rests in the optimal activity ranges of cytolytic and proteolytic enzymes allows for additional degradation of the cell walls and protein matrix in case of poorly modified malts or unmalted grains being used (Krottenthaler et al., 2009). Mashing regimes that include rests at different temperatures need to be heated between rests. This can be done by either the decoction method, where part of the wort is removed, boiled and subsequently added back, or infusion method where the whole mash is heated (Mikyška et al., 2023).

Table 1. Substrates, products and optimal temperatures of relevant enzymes during mashing. Adapted from Wunderlich & Back, 2009

	Enzyme	Substrate	Product	Optimum temperature in mash (°C)
Cytolysis	β-glucan-solubilase	Matrix bound β-glucan	Soluble, high molecular weight β-glucan	62-65
	Endo-1,3-β-glucanase	Soluble, high molecular weight β-glucan	Low molecular weight β-glucan, cellobiose, laminaribiose	40-45
	Endo-1,4-β-glucanase	Soluble, high molecular weight β-glucan	Low molecular weight β-glucan, cellobiose, laminaribiose	40-45
	Endo-1,3–1,4-β-glucanase	Soluble, high molecular weight β-glucan	Low molecular weight β-glucan, cellobiose, laminaribiose	40
	Exo-β-glucanase	Cellobiose, laminaribiose	Glucose	40
Proteolysis	Endopeptidase	Proteins	Peptides, FAN	45-50
	Carboxypeptidase	Proteins, peptides	FAN	50
	Aminopeptidase	Proteins, peptides	FAN	45
	Dipeptidase	Dipeptides	FAN	45
Amylolysis	α-amylase	High and low molecular weight α-glucan	Oligosaccharides	65-75
	β-amylase	α-glucan	Maltose	60-65
	Maltase	Maltose	Glucose	35-40
	Limit dextrinase	Limit dextrinase	Dextrins	55-60
	α-glucosidase	α-glucan	Glucose	< 45

1.3. Analysis methods for nitrogen determination

Different methods exist to assess the nitrogen content in fermentation samples. The most used method in the fermentation industry is Free Amino Nitrogen (FAN) analysis. This method makes use of a reaction between the free amino group of amino acids, peptides and proteins with ninhydrin, which creates a dark blue/purple colour that can be measured using a spectrophotometer (Lie, 1973). The FAN (mg FAN/L) is then calculated from a glycine standard curve. Because this analysis is biased toward smaller molecules, wort and beer FAN are correlated with the total amino acid concentration. However, this method does not take into account those amino acids that contain multiple nitrogen atoms or the nitrogen that is contained within the rest of the peptide or protein chain, and thus does not provide a true value for the contained nitrogen in the sample (Lekkas et al., 2005).

Similarly, the Biuret test is a colorimetric method based on measuring the absorption of a coloured complex that is formed when peptide bonds react with copper (II) ions in an alkaline solution. The absorption is proportional to the amount of protein and peptides that contain at least two peptide bonds (Bianchi-Bosisio, 2005). The Biuret method thus does not take into account amino acids and

dipeptides. The amount of peptides and proteins (g/L) is calculated from a standard curve of bovine serum albumin (BSA) and the amount of nitrogen is then subsequently calculated using the Jones' factor (Mariotti et al., 2008). The main advantages of these colorimetric methods are the speed, low cost and simple analysis procedure, however, both methods described come with some inaccuracy. On the one hand, the measurement of FAN does not provide a true value for the amount of nitrogen contained in the sample. On the other hand, the biuret test is specific to peptides and proteins with at least two peptide bonds. Furthermore, the biuret test is hindered by presence of other substances like reducing sugars, which is a problem if using it to determine protein content in wort (O'Hara, 1968).

Amino acid analysis is the only method that can determine the protein content directly from amino acid residues, therefore avoiding possible interference from other substances. It is the recommended method of measuring food protein content by the Food and Agricultural Organization of the United Nation (Food and Agriculture Organization of the United Nations, 2003). Compared to the Biuret test, amino acid analysis is much more time consuming and the expensive equipment that is required can make the method inaccessible (Hayes, 2020).

Before performing amino acid analysis to determine protein content hydrolysis of the peptide bonds is required. Acid hydrolysis is used to release all amino acids except tryptophan (Davidson, 2003). During the standard time of hydrolysis some of the amino acids are partially destroyed whereas others are not completely released yet, meaning that the total amino acid content may be underestimated. The hydrolysis and destruction rates of different amino acids can be taken into account to improve the accuracy of amino acid analysis (Mæhre et al., 2018; Rutherfurd, 2009). In addition, released amino acids will react with reducing sugars through the Maillard reaction, which creates interference with the analysis. Because of the high reducing sugar content in fermentation feedstocks this method cannot be applied without first purifying the sample (John et al., 2004).

The main aim of this thesis is to quantify the YAN of nine worts prepared from different malts using different mashing regimes. The Biuret test and amino acid analysis were chosen to determine wort YAN content, while also testing a novel method for the reduction of wort reducing sugars content and the interference they cause during the analysis.

2. Materials and Methods

2.1. Preparation of worts

Nine worts were prepared using different malts and mashing regimes. The malts used were Château Pilsen 2RS, Château Pale Ale and Château Distilling (La Malterie du Château S.A., Belgium). Each malt was mashed using three different mashing regimes. The simplest mashing regime (A) was a single rest infusion mash at 67°C for 60 minutes. For regime B a 20-minute rest at 50°C (protease/peptidase activity) was added, and regime C included another 20-minute rest at 40°C (β-glucanase activity) prior to the previously mentioned ones. Taking into consideration the temperature of the malt and the ratio of water to malt, the strike temperature at which the malt was added to the mash tun had to be higher than the first rest temperature: 71°C for mash A, 53°C for mash B and 42°C for mash C. The wort sample numbers that were used are presented in table 2.

Table 2. Malt types, mashing regimes, and respective sample numbers

	Mash A 67°C 60 min	**Mash B** 50°C 20 min, 67°C 60 min	**Mash C** 40°C 20 min, 50°C 20 min, 67°C 60 min
Malt 1: Pilsen Malt	1.A	1.B	1.C
Malt 2: Pale Ale Malt	2.A	2.B	2.C
Malt 3: Distilling Malt	3.A	3.B	3.C

The worts were prepared using 2 L of MilliQ water and 440 g of crushed malt. Once the water reached the strike temperature, the malt was added and the mixture was stirred thoroughly. The temperature of the mash was monitored and extra heat was added when necessary to maintain a steady temperature. For the multi rest regimes the mash was heated between rests while stirring constantly. After the saccharification rest at 67°C, the temperature was increased to 78°C to stop all enzymatic activity. The mash was then strained to separate the wort and grains. An additional 0.2 L of room temperature (22°C) MilliQ was used to rinse the spent grains. The acquired wort was then boiled for 60 minutes and set to cool in a fridge. The cooled worts were subsequently centrifuged at 4667 x g to remove insoluble debris. The wort was then brought up to volume with distilled water in a 2 L volumetric flask.

2.2. Fermentations with peptide transporter knock-out strain

The worts were fermented with a strain of *Saccharomyces cerevisiae* in which the genes encoding for all peptide transporters were knocked out (Becerra-Rodríguez et al., 2021) to reduce the sugar content while preserving the original peptide content of wort. The fermentations were performed by Hidde Yaël Berg.

Each yeast inoculum was prepared from a single colony that was pitched to a shake flask containing yeast peptone dextrose (YPD) medium (1% yeast extract, 2% bacteriological peptone, 2% glucose). The shake flask was incubated overnight at 30°C and 150 rpm. Cells were washed twice with equal volumes of sterile 0.9% NaCl prior to inoculation into fermenters to deliver 5×10^6 cells/mL in a final volume of

100 mL of wort. Fermentations were performed at 24°C in 100 mL Pyrex™ bottles equipped with a GL45 open top PBT screw cap and PYREX™ Media Bottle Septum (Corning Inc., Corning, NY, USA). A gas outlet was installed to prevent overpressure by piercing the septum with a Sterican® Ø 0.8 x 40 mm single-use hypodermic needle (B. Braun, Melsungen, Germany) attached to a Millex-FG 0.2 µm hydrophobic PTFE filter (Merck KGaA, Darmstadt, Germany). As the yeast strain used is derived from a wine strain, it was needed to hydrolyse dextrins with glucoamylase (0.1% w/w, MEGA PACIFIC TECHNOLOGY INC, Arcadia CA, USA).

The fermentations ran for 168 hours. Samples were taken every 24 hours for the first 96 hours of fermentation. A final sample was taken at the end of the fermentation (168h). The Brix values of these samples were used to choose a suitable sample for further analysis and was determined with an Atago PAL-α Digital Pocket Refractometer (Atago Corporation, Tokyo, Japan).

2.3. Sugar content analysis

Concentration of glucose, fructose, maltose and maltotriose were determined on a Waters Alliance 2695 HPLC system (Waters Corporation, United States) equipped with a Bio-Rad HPX-87H column (Bio-Rad Laboratories Inc, United States) and refractive index detector. The mobile phase was 0.005M H2SO4 with a flow rate of 0.6 mL/min. Sample temperature was 10°C and the column temperature was 35°C. The original wort samples and PepKO-treated wort samples were diluted 10x with MilliQ. The standard solution contained 10 g/L of each sugar and was diluted 2, 4, 8, 16, 32 and 64x to create a standard curve.

2.4. Fractionation

The analysis of nitrogen was performed for the whole and <3 kDa fraction for each wort and PepKO-fermented wort. The <3 kDa fraction was chosen to study the content of peptides that yeast is able to absorb (Mo et al., 2013). Samples were first filtered through a 0.22 µm syringe filter (Sartorius, Göttingen, Germany) to obtain the whole fraction without solubles. Subsequently, 1 mL of wort was filtered with an Amicon Ultra-0.5 Centrifugal Filter 3 kDa MWCO (Merck – Millipore, Burlington, MA, USA) to obtain the <3 kDa fraction.

2.5. Amino acid analysis

Amino acid analysis was used to analyse the whole and <3 kDa fractions of the original worts and PepKO-fermented worts. The bound amino acid (BAA) content was calculated by subtracting the free amino acid (FAA) content from the total amino acid (TAA) content of the samples. The FAA content in the <3 kDa and whole fraction was used to calculate the dilution factor according to which the results from the <3 kDa fraction were adjusted.

For the AA analysis, 10x dilutions of the wort samples and 2x dilutions of the PepKO-fermented wort samples were prepared and derivatized with AQC from the AccQ-Fluor Reagent Kit (Waters Corporation, United States). AQC reacts with primary and secondary amino acids to form stable derivatives that are both UV-absorbing and fluorescent. All samples were analysed in triplicate.

The FAA content was determined in the soluble fraction of the mash. The Acquity UPLC I Class plus (Waters) system with the AccQ-TagTM Ultra column (2.1x100 mm) connected to a PDA detector (λ = 260 nm) was used for the analysis. The solutions necessary for the analysis were seal wash

(methanol:MilliQ 50%:50% v:v), strong needle wash (acetonitrile:MilliQ:formic acid 50%:50%:0.1% v:v:v), weak needle wash (acetonitrile:MilliQ:formic acid 5%:95%:0.1% v:v:v), eluent A (AccQTag Ultra eluent) and eluent B (99% acetonitrile + 1% formic acid). The column and sample temperatures were 55°C and 8°C respectively and a 10 min gradient elution from 0.1 to 59.6% eluent B with a flow rate of 0.7 ml/min was used to separate the amino acids. FAA concentrations were calculated with a standard curve created from a standard solution containing all 20 amino acids (0.1M), diluted 2, 4, 8, 16, 32 and 64x.

Acid hydrolysis was applied to prepare both the original wort and PepKO-fermented wort for TAA analysis. The Eldex H/D workstation (Eldex Laboratories Inc, United States) was used. The samples were hydrolysed with a HCl/phenol solution (6 M HCl + 1% phenol) at 105°C for 22 hours, hydrolysis of all samples was performed in duplicate. The hydrolysed samples were dissolved in methanol and diluted with MilliQ, after which they were derivatized and analysed as described for the FAA analysis.

2.6. Biuret test for protein and peptides

The Biuret test was applied to quantify the <3 kDa and whole fractions of bound amino acids (BAA) in the PepKO-fermented worts. The Synergy H1 microplate reader (Agilent Technologies Inc, United States) and a 96 well plate was used for the analysis. 100 µl of sample and 100 µl of the Biuret reagent (6mM $CuSO_4$; 29mM $KNaC_4H_4O_6$; 0.75M NaOH) was pipetted into each well and the absorbance at 540 nm was measured. The samples were analysed in duplicate. BSA solutions with concentrations of 1 g/L to 0.1 g/L were used to create a standard curve. The BSA solutions were analysed in triplicate. The Jones factor 5.83 was used for converting protein content to nitrogen content (Food and Agriculture Organization of the United Nations, 2003).

3. Results

3.1. Fermentations with peptide transporter knock-out strain

The fermentations with PepKO strain were performed to remove fermentable sugars from the worts, which otherwise would have interfered with TAA analyses. The sugar contents of the worts were monitored over the course of the fermentations and based on the gravity readings of samples taken, suitable samples were chosen for further analysis. The fermentable sugar content decreased rapidly for the first 48 h and was nearly depleted by 72 h for all 9 worts (Figure 2). The 72 h samples were chosen because further incubation in a nutrient poor environment can result in yeast autolysis, causing the intracellular components of yeast cells to be released into wort interfering with the AA analysis.

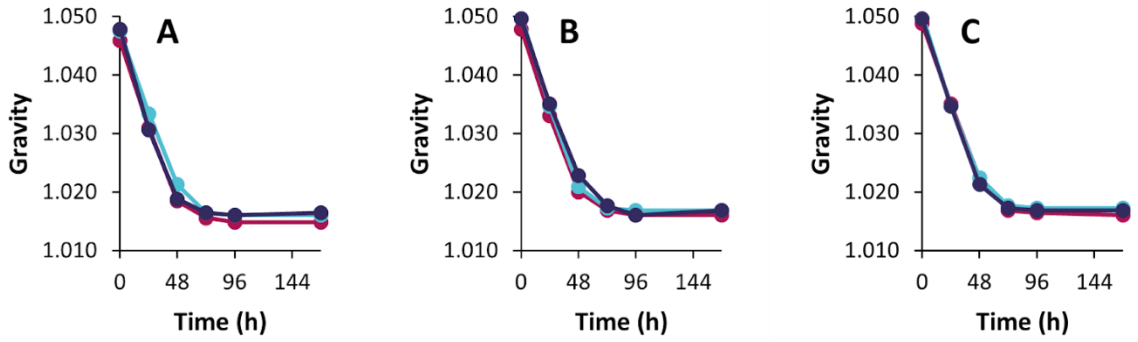

Figure 2. Gravity readings of fermentations of 9 different worts over 168h with a strain of *Saccharomyces cerevisiae* in which the genes encoding for all peptide transporters were knocked out (PepKO). A: Pilsner Malt; B: Pale Ale Malt; C: Distilling Malt. Mash 1-3 are the colours burgundy, light blue and dark blue, respectively.

3.2. Sugars content in the worts

The reducing fermentable sugar contents of all nine worts were determined as they were important in estimating the effectiveness of PepKO fermentations. In addition, the effect of using different malts and mashing regimes was assessed (Figure 3). Mashing regimes in which additional incubation breaks were used at 50°C (regime B) or 40°C and 50°C (regime C) both produced a higher total fermentable sugar content than regime A which included a single temperature (67°C). On average, mash B increased the sugar content of the worts by 9% and mash C by 8%. Comparing all three malts, Pilsen malt produced a higher fermentable sugar concentration than Pale Ale and Distilling malts.

The fermentations with PepKO strain removed 95.7% of the reducing sugars in the original wort. The PepKO-fermented worts contained on average 3.5 g/L of residual sugars, of which 65.4% (w/w) were maltose and maltotriose.

Figure 3. Sugar contents of 9 different worts before (pink) and after (blue) 72 h fermentation with PepKO strain. Malt 1: Pilsen; malt 2: Pale Ale; malt 3: Distilling; mash A: 67°C 60 min; mash B: 50°C 20 min + 67°C 60 min; mash C: 40°C 20 min + 50°C 20 min + 67°C 60 min.

3.3. Free amino acids content in the worts

The aim of the analysis was to assess whether the three different mashing regimes had an effect on the FAA content of worts. The results are illustrated in figure 4 and the individual amino acid values are presented in Appendix A tables 3-5.

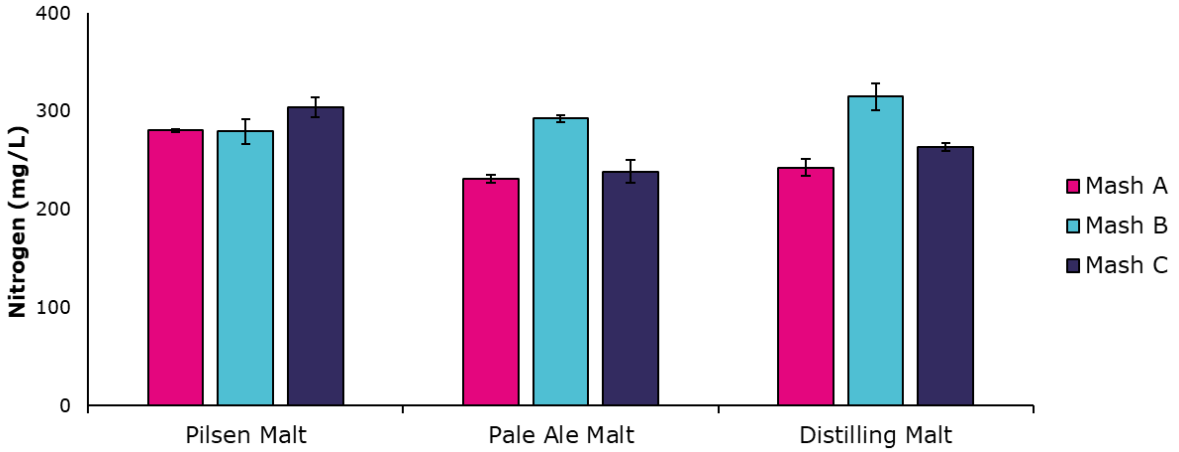

Figure 4. YAN contents from free amino acids in 9 different worts. Mash A: 67°C 60 min; mash B: 50°C 20 min + 67°C 60 min; mash C: 40°C 20 min + 50°C 20 min + 67°C 60 min.

For Pilsen malt, mash A and mash B both produced a YAN content of about 280 mg/L and mash C resulted in a slightly higher YAN content of 300 mg/L. Mash A and mash C resulted in comparable FAA contents for Pale Ale malt around 235 mg/L. For Distilling malt, the FAA content of mash C was slightly higher – 260 mg/L compared to 240 mg/L for mash A. Mash B resulted in a 26% increase in FAA content compared to mash A for Pale Ale malt and a 30% increase for Distilling malt.

The SD values of the analysis results for some amino acids were quite high, so no definitive conclusions can be made, however, arginine and lysine seemed to contribute the most to the increase in the Pale Ale wort (Appendix A, Table 3), whereas proline accounted for most of the increase in the Distilling

wort (Appendix A, Table 4). Proportionally, the amounts of glutamine, lysine, methionine for Pale Ale malt and glycine, lysine, methionine for Distilling malt increased the most.

3.4. Bound amino acids content in the worts

3.4.1. Amino acid analysis with and without fermentation with PepKO strain

To determine the amount of peptide-derived nitrogen in the worts, acid hydrolysis was performed on both the original worts and PepKO-fermented worts and the BAA contents of both hydrolysates were determined. The results are presented in figure 5.

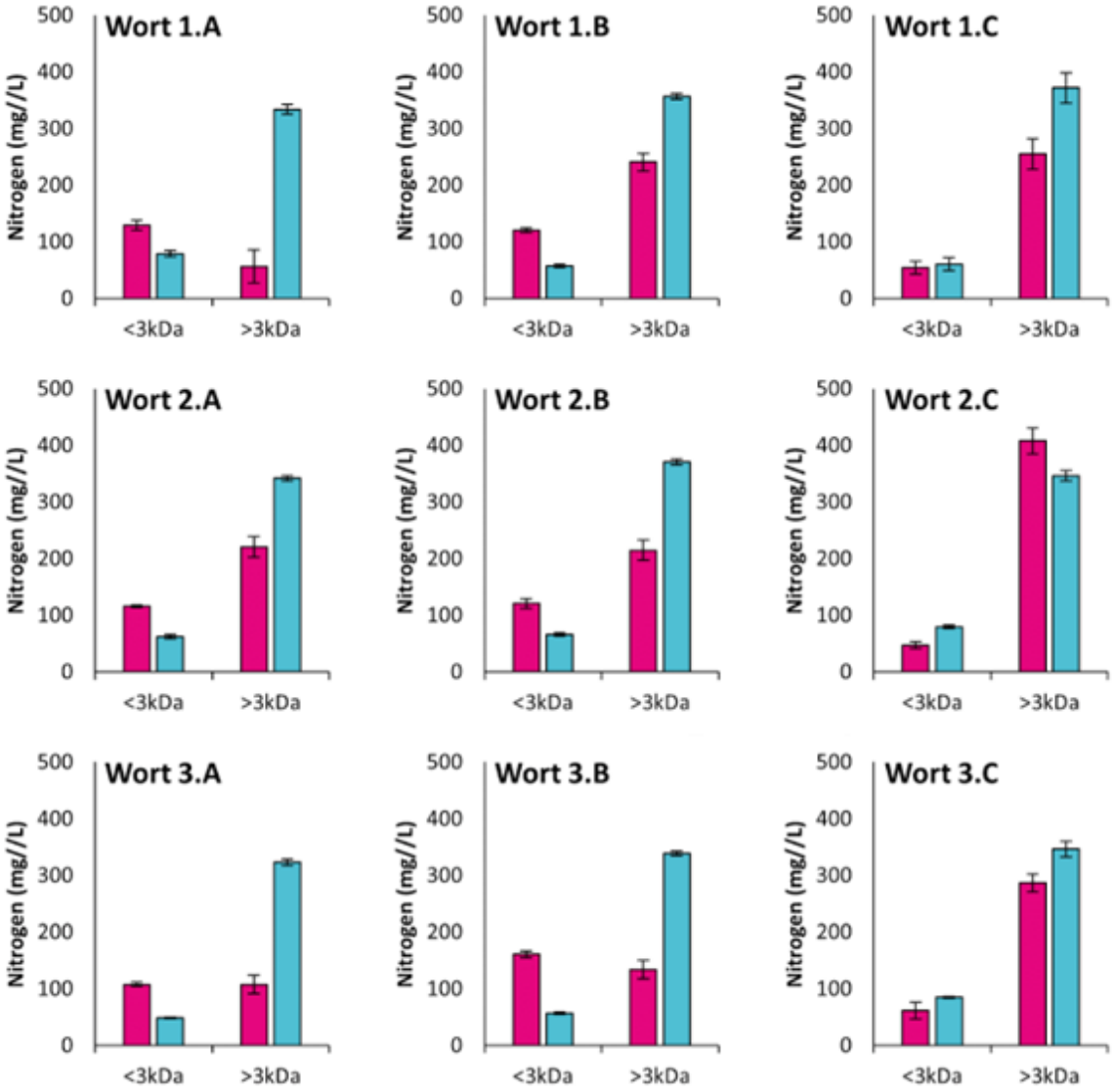

Figure 5. The amount of nitrogen from the <3 kDa and >3 kDa BAA fractions of 9 different worts determined by acid hydrolysis of the original worts (pink) and PepKO-fermented worts (blue). Malt 1: Pilsen; malt 2: Pale Ale; malt 3: Distilling; mash A: 67°C 60 min; mash B: 50°C 20 min + 67°C 60 min; mash C: 40°C 20 min + 50°C 20 min + 67°C 60 min.

The total BAA content was determined to be higher when fermentation with PepKO strain was used for all worts, except 2.C, for which no difference was observed. This can be explained by removal of

sugars, which interfere with the bound amino acids analysis. However, for unknown reasons the amount of <3 kDa peptides were significantly lower when fermentation with PepKO strain was used in six of the nine worts. The small peptide content increased in two PepKO-fermented worts and no difference was observed in one wort. The average nitrogen contents of all malt types were similar, in all three malts an average close to 100 mg/L was observed in the original worts and around 65 mg/L in the PepKO fermented worts. When comparing the nitrogen contents coming from the fraction assimilable by yeast (<3 kDa peptides) produced by the different mashing regimes, different results were obtained between the original worts and PepKO fermented worts. For the original worts, regime C produced the lowest nitrogen contents. However, in the PepKO fermented worts regime C resulted in the highest nitrogen content for Pale Ale and Distilling malts. These results suggest that the PepKO method did not improve the analysis of the <3 kDa fraction of peptides.

3.4.2. Biuret test

The Biuret test was used as an additional method to determine the BAA contents of the PepKO-fermented worts. The analysis results are presented in figure 6. The total BAA content was relatively stable between different mashing regimes for Pilsen, Pale Ale and Distilling malts. The <3 kDa BAA content was also similar between all mashing regimes for Pilsen, Pale Ale and Distilling malts. All combinations resulted in close to 230 mg/L of nitrogen that can be potentially available to yeast.

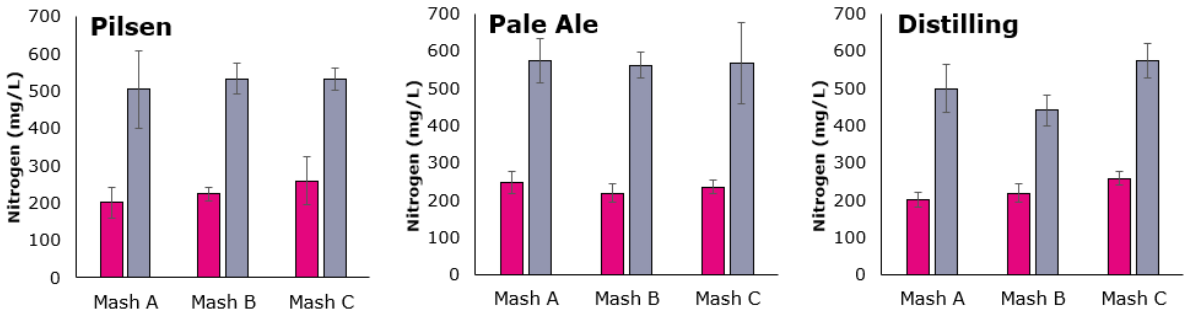

Figure 6. The <3 kDa (pink) and total (grey) BAA contents of nine different PepKO-fermented worts determined by the Biuret test. Mash A: 67°C 60 min; mash B: 50°C 20 min + 67°C 60 min; mash C: 40°C 20 min + 50°C 20 min + 67°C 60 min.

Figures 7 and 8 illustrate the differences between wort BAA contents determined by the three different methods used. The results of the Biuret test were consistently higher than amino acid analysis results, suggesting recovery losses during acid hydrolysis. On average, using fermentation with PepKO resulted in a significantly higher total BAA content (78% of Biuret) compared to the normal acid hydrolysis results (58% of Biuret). However, a significantly lower <3 kDa BAA content was observed when using the PepKO method when compared to normal acid hydrolysis (29% and 46% of Biuret results respectively).

The part that <3 kDa BAA fraction made up in the total BAA content depended on the analysis method used. According to the normal acid hydrolysis results, <3 kDa BAA accounted for 37% of the total BAA on average. The Biuret results were similar with 43% of all BAA coming from <3 kDa BAA. PepKO acid hydrolysis reported a significantly lower content of <3 kDa BAA, only 16%.

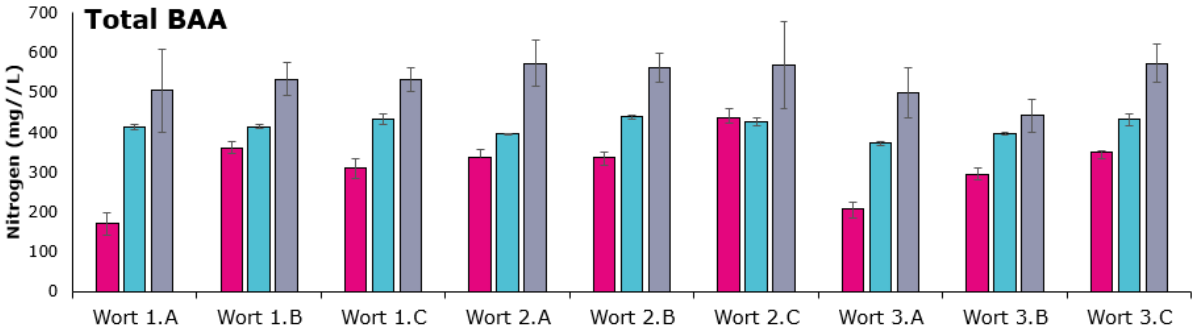

Figure 7. The total BAA contents of nine different worts measured by the Biuret test (grey), amino acid analysis with acid hydrolysis of original worts (pink) or acid hydrolysis of PepKO fermented worts (blue). Malt 1: Pilsen; malt 2: Pale Ale; malt 3: Distilling; mash A: 67°C 60 min; mash B: 50°C 20 min + 67°C 60 min; mash C: 40°C 20 min + 50°C 20 min + 67°C 60 min.

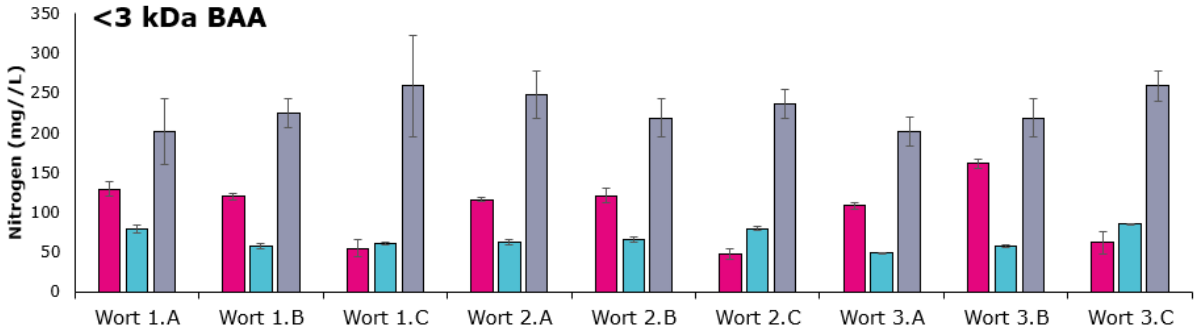

Figure 8. The <3 kDa BAA contents of nine different worts measured by the Biuret test (grey), amino acid analysis with acid hydrolysis of original worts (pink) or acid hydrolysis of PepKO fermented worts (blue). Malt 1: Pilsen; malt 2: Pale Ale; malt 3: Distilling; mash A: 67°C 60 min; mash B: 50°C 20 min + 67°C 60 min; mash C: 40°C 20 min + 50°C 20 min + 67°C 60 min.

4. Discussion

The sugar analysis results show that different mashing regimes affected the fermentable sugar content for all malts tested. Mashing regimes B and C both resulted in a higher fermentable sugar content than mash A. The increase can be explained by differences in β-amylase activity between the mashing regimes. While mash A is a single-temperature mash at 67°C, mashing regimes B and C both include a gradual heating of the mash from 50°C to 67°C. This temperature range includes the optimal temperature for β-amylase activity: 60°C-65°C (Sammartino, 2015). Additionally, because there is some β-amylase activity at lower temperatures, the increased sugar content can be attributed to a longer mashing time.

Including a protein rest at 50°C in the mashing regime increased the FAA contents of worts prepared from Pale Ale and Distilling malts by around 25% when compared to a single-temperature mash at 67°C. This is due to the optimal temperature range for barley protease activity being 40°C-55°C (Sammartino, 2015). However, including a third rest at 40°C resulted in about the same FAA content as the single rest mash. Previous research has found that on average 71% of the free amino acids present in wort are produced during malting and only 29% are a product of proteolysis during mashing (Lekkas et al., 2014). However, this ratio significantly varies between amino acids. For example, only half of free aspartate, glutamate, methionine and lysine is formed during malting, the rest comes from mashing (Lekkas et al., 2014). This is partly in agreement with the results of this analysis: the amounts of lysine and methionine increased by over 50% between mashing regimes A and B for both Pale Ale and Distilling malts, however the amount of aspartate increased much less and no significant change was observed in the amount of glutamate. In the case of Pilsen malt, the different mashing regimes resulted in relatively stable wort FAA contents. This suggests that the malt is very well modified and a single-rest mash is sufficient for extracting the FAA (Castle Malting, 2022; Kühbeck et al., 2005).

The results of the whole BAA fraction analysis indicate that fermentation of the samples with the peptide transporter knock-out strain (PepKO) was successful in improving the accuracy of the analysis, which is expected since it removed most of the reducing sugars that interfere with the analysis by reacting with FAA and BAA during hot acid hydrolysis (Tester & Karkalas, 2003). On the other hand, the yield of <3 kDa BAA was negatively affected by the PepKO method. This could be explained by the fact that in addition to reducing wort sugar content, the yeast metabolised some of the FAA during the PepKO fermentations. In the original wort the reducing sugars reacted with both FAA and amino acids being released from BAA during acid hydrolysis. However, with less FAA present and the higher molecular weight BAA being filtered out the leftover reducing sugars might have disproportionately affected the <3 kDa BAA fraction. This suggests that optimisation of the PepKO method is necessary to be able to analyse a certain fraction of BAA.

The <3 kDa BAA fraction analysis results obtained by amino acid analysis were conflicting when comparing the effect of mashing regimes in original worts and PepKO fermented worts. According to the Biuret test results neither different mashing regimes or malt types had a significant effect on the <3 kDa BAA content of the worts as all combinations of mashing regimes and malt types produced a similar nitrogen content from <3 kDa BAA around 230 mg/L. Amino acid analysis results also found no difference between the malt types, however the nitrogen content from <3 kDa BAA was significantly

lower than observed by the Biuret test, around 100 mg/L in the original worts and 65 mg/L in the PepKO fermented worts.

Conclusion

This thesis aimed to describe the effects of different malts and mashing regimes on wort composition, with yeast assimilable nitrogen being the main focus. Whether the FAA content of wort was influenced by different mashing regimes depended on malt type. The amount of nitrogen from FAA extracted from Pilsen malt stayed around 280-300 mg/L for all mashing regimes, which is by far more than needed for yeast to complete the fermentation of the mash with the given gravity. The nitrogen from FAA from Pale Ale and Distilling malt worts varied more, ranging from about 240 mg/L to 300 mg/L, the highest concentrations were produced by a mashing regime that included two rests: protease and saccharification. The nitrogen content from <3kDa BAA was similar between Pilsen, Pale Ale and Distilling malts. The different mashing regimes also had similar results regarding nitrogen from <3 kDa BAA.

A novel method that used fermentation with a peptide transporter knockout yeast strain was tested to decrease the interference that reducing sugars cause during protein/peptides hydrolysis and the Biuret test. Sugar concentrations of the worts were also investigated as they were important in assessing the PepKO method. The results showed that sugar concentrations were influenced by different mashing regimes, being higher when the temperature range optimal for β-amylase activity was included. The PepKO method removed over 95% of the reducing sugars and was successful in increasing the recovery of BAA by 33% when the total BAA content was measured. However, the method still needs optimisation to analyse lower molecular weight BAA fractions.

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Appendix A. Results of FAA analysis

Table 3. Individual amino acid contents of Pilsen malt worts

	Mash A		Mash B		Mash C	
AA	N (mg/L)	SD	N (mg/l)	SD	N (mg/l)	SD
Trp	6.13	0.04	6.1	0.8	6.7	0.7
Phe	9.46	0.07	9.7	0.9	10.9	0.6
Ile	12.7	0.2	14.6	0.9	17.2	0.7
Leu	5.88	0.08	6.4	0.6	7.5	0.2
Val	11.5	0.1	12.6	0.9	14.5	0.4
Met	1.73	0.01	2.1	0.2	2.5	0.1
Tyr	6.08	0.05	6.5	0.5	7.3	0.3
Lys	12.5	0.7	14.6	0.9	18	2
Cys	0	0	0	0	0	0
Pro	81	1	66	12	67	8
Ala	15.8	0.4	18.4	0.5	21	2
Thr	5.51	0.04	6.3	0.3	7.3	0.1
Glu	10.9	0.4	9	1	8.3	0.2
Asp	7.6	0.3	6.8	0.6	7.5	0.2
Gly	4.47	0.03	5.3	0.5	6.4	0.3
Arg	32.2	0.6	39	2	43	4
Gln	12.9	0.2	12	2	11	2
Ser	7.42	0.08	8.2	0.7	9.5	0.3
Asn	25.4	0.6	24	2	24	1
His	11.3	0.6	12.4	0.8	13.5	0.3
TOTAL	**280**	**2**	**279**	**13**	**304**	**10**

Table 4. Individual amino acid contents of Pale Ale malt worts

	Mash A		Mash B		Mash C	
AA	N (mg/l)	SD	N (mg/l)	SD	N (mg/l)	SD
Trp	5.13	0.01	6.4	0.5	5	1
Phe	8.02	0.07	10.2	0.6	9	2
Ile	11.3	0.7	17	1	15	3
Leu	5.0	0.3	7.3	0.7	6	1
Val	10.4	0.6	14.1	0.9	12	2
Met	1.48	0.06	2.3	0.2	2.1	0.4
Tyr	5.47	0.06	6.9	0.3	6	1
Lys	10	2	18	1	14	5
Cys	0	0	0	0	0	0
Pro	54	3	60.8	0.4	49	6
Ala	16	1	20.9	0.8	18	3
Thr	5.3	0.2	7.0	0.4	6	1
Glu	8.3	0.6	8.9	0.4	6	1
Asp	6.0	0.4	7.5	0.5	6	1
Gly	4.1	0.2	5.6	0.2	5.0	0.9
Arg	35	1	44	1	36	6
Gln	7.7	0.3	13	2	9	2
Ser	6.7	0.3	9.1	0.6	8	1
Asn	20.6	0.5	22	1	17	1
His	10.7	0.4	12.9	0.9	10	2
TOTAL	**231**	**4**	**292**	**4**	**239**	**12**

Appendix A cont. Results of FAA analysis

Table 5. Individual amino acid contents of Distilling malt worts

	Mash A		Mash B		Mash C	
AA	N (mg/l)	SD	N (mg/l)	SD	N (mg/l)	SD
Trp	5.6	0.2	7.3	0.5	6.0	0.3
Phe	8.5	0.4	11.4	0.6	9.9	0.4
Ile	12.3	0.6	17.3	0.5	16.2	0.9
Leu	5.6	0.2	7.9	0.4	7.1	0.4
Val	11.0	0.7	14.9	0.7	13.4	0.7
Met	1.6	0.1	2.6	0.2	2.3	0.1
Tyr	5.7	0.4	7.5	0.4	6.5	0.3
Lys	11	1	17.4	0.9	16	2
Cys	0	0	0	0	0	0
Pro	54	6	76	13	52	3
Ala	17	2	19.9	0.4	19	1
Thr	5.5	0.4	7.4	0.3	6.6	0.3
Glu	8.8	0.8	8.9	0.3	7.0	0.3
Asp	6.6	0.5	7.7	0.4	6.8	0.4
Gly	4.2	0.4	6.7	0.8	5.6	0.3
Arg	36	4	40	2	40	2
Gln	9.9	0.5	13.8	0.3	10.7	0.4
Ser	7.3	0.5	10.0	0.6	8.7	0.4
Asn	20	4	25	3	17.8	0.7
His	12	2	13.7	0.9	11.5	0.6
TOTAL	**243**	**9**	**315**	**14**	**263**	**4**

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