Research article

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

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ABSTRACT: Brewing using enzyme rich 'green' (germinated, but not kilned) malt has the potential to unlock considerable energy savings in the malting and brewing chain. This paper examines the major quality issues associated with green malt, by monitoring lipoxygenase (LOX) activity and S-methyl methionine (SMM) levels through a micromalting cycle both with and without rootlets after 48 h of germination. The data suggest that rootlets are a major concern when brewing with green malt and that their influence on wort and beer quality needs to be further investigated. Lipoxygenase activity and nonenal potential were measured following treatment under varying conditions of pH, temperature and pre-treatment. Results indicated that lipoxygenase activity can be controlled to a substantial degree by manipulating these limiting factors, while preserving diastatic enzyme activity. Green malt worts were then prepared from (i) whole green malt immediately post-germination; (ii) heat treated green malt (65°C x 1 h); (iii) re-steeped green malt and (iv) endosperm-rich extracts of green malt after the husk and rootlets had been removed; using laboratory mashing with a 'LOX-hostile' mash schedule. Data were compared with mashing of kilned pale malt made from the same green malt, as a reference point. Based on the present data, re-steeping of green malt in combination with a LOX hostile mashing environment (63°C, pH 5.2) could help to control LOX activity and the trans-2-nonenal potential of green malt. The resultant brewing process would need to be optimised to deal with the elevated SMM levels in green malt worts. © 2020 The Authors. Journal of the Institute of Brewing published by John Wiley & Sons Ltd on behalf of The Institute of Brewing & Distilling

Keywords: Malting Science; Lipoxygenase; Green malt brewing; S-methyl methionine; Dimethyl sulfide precursor

Introduction

The malting process comprises three main steps: steeping, germination and kilning. The kilning step is by far the dominant user of energy *(1–4)* and has logically become the main target in attempts to reduce the carbon footprint of malting operations. The average energy requirement to kiln 100,000 tonnes of malt per annum is 80 GWh thermal and 9 GWh electrical *(4)*. Globally about 23 million tonnes of malt are produced annually, of which Europe contributes almost 9.7 million tonnes *(5)*. The agricultural production of malting barley accounts for about 241 kg CO_2eq/t, whilst the malting process adds an additional 217 kg CO_2eq/t, almost doubling the total malt carbon footprint *(6)*. Thus, the malting industry is always interested in improving its energy efficiency. Additionally, national-level energy or carbon taxes are being imposed by governments, making energy use reduction not just an environmental but also a finanical imperative.

If omitting the kilning process entirely, the brewer must brew with freshly germinated (green) malt, which introduces new technical challenges, but offers the reward of significantly lower energy and water usage. However, apart from being a dominant consumer of heat and electricity *(3)*, the kilning process has many beneficial quality impacts on malt quality, such as reduction of lipoxygenase activity *(7–10)*, regulation of S-methyl methionine (SMM) levels *(11–13)*, facilitating rootlet removal and most importantly in developing the characteristic colour and flavours which malt imparts to beer. On the other hand, green malt, rich in diastatic enzyme activity, can very efficiently convert the starch of unmalted grain into fermentable sugars *(14,15)*.

To our knowledge, the first detailed investigations into the use of green malt in brewing were carried out in the early 1960s *(14–16)*, showing that extracts of green malt with equal quality to those from kilned malt could be achieved by using a suitable mill and that acceptable beer could be produced. However, although wort and beer were analysed by standard analytical measures and compared to wort and beer made of a kilned malt control, there was no detailed sensory analysis of the beer or assessment of its flavour stability. Additionally, Duff et al. (1963) described experiments in which green malt was used for brewing stout, which could mask potential flavour defects and may thus be considered a less demanding beer matrix in terms of potential flavour defects.

One major quality concern when handling green malt is the elevated activities of both lipoxygenase isoenzymes (LOX-1 and LOX-2; *(7,10)*). Even relatively low activities of lipoxygenase in kilned malt are known to significantly influence flavour stability via enzymatic lipid oxidation *(17,18)*. LOX enzymes can oxidise unsaturated fatty acids, principally linoleic acid in barley, to hydroperoxy acids in the presence of oxygen. Hydroperoxy acids can be further transformed via several enzymatic pathways *(19)*

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to mono-, di- and trihydroxy fatty acids and can eventually be degraded non-enzymatically into flavour active carbonyls, such as trans-2-nonenal or hexanal, which are examples of beer staling compounds *(10,20,21)*. Furthermore, lipoxygenase worsens the foam stability of beer, possibly due to the production of trihydroxy octadecenoic acid (THOD), which is detrimental to foam stability *(22,23)*.

Furthermore, one important factor has been neglected in prior research – the rootlets of green malt. The rootlets of green malt are particularly rich in lipoxygenase *(9,20)* and SMM *(11)*. Rootlets of kilned malt are hygroscopic, due to their fibre content of up to 15 % *(24,25)*, can absorb oil and have emulsification capacities *(24)*. However, the antioxidant capacity of rootlets obtained from kilned malts has also been investigated *(26–28)*. The antioxidant potential, due to the high content of antioxidant phenolic compounds, could potentially reduce the formation of free radicals, thus becoming a source for natural antioxidants, favouring wort and beer flavour stability. Nevertheless, malt rootlets are considered to impair the flavour of beer, hence maltsters try to avoid excessive rootlet growth during germination (in order to minimise malting losses) and remove the rootlets, by abrading them after kilning, with an associated malting loss of around 4%. The rootlets are then usually sold as animal feed or organic fertiliser. Adequate removal of rootlets from green malt is problematic due to the high moisture content, meaning they will not form a malting loss and if untreated remain on the grain. Thus, rootlet composition needs to be considered before starting to brew with green malt.

To avoid an increased staling potential in the final beer, a minimum requirement when brewing with green malt is that mashing needs to occur in a lipoxygenase hostile environment, mashing in at $>$ 63 °C, at a pH in the region of 5.2 under oxygenlimited conditions *(19,29–31)*. Most certainly, the usage of green malt for conventional brewing processes requires alternative techniques to reduce total LOX activity.

The present research aims to evaluate the feasibility of brewing using freshly germinated (green) malt, with omission of the kilning step. Here, the laboratory scale development of such a process is reported to enable evaluation of the significant quality impacts on the brewing process and finished beer. Attention was first directed to control lipoxygenase activities through its limiting factors: heatsensitivity, pHsensitivity *(7,8,32)* and the availability of oxygen as a substrate. Those 'weaknesses' could help to control LOX when brewing with green malt. Additionally, the quality of wort has been evaluated and compared to wort produced from kilned malt, with a special focus on SMM levels. This knowledge will enhance our understanding of key quality concerns as well as potential benefits of using green malt and will form the basis for subsequent pilot-scale brewing trials.

Materials and Methods

Materials

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

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

Malt and wort preparation methodology

Barley (500 g) was screened over a 2.2 mm sieve and put into a micro malting cage and malted in a Custom Lab micromaltings K steep germinator and kiln (Curio Malting, Milton Keynes, UK). Typical process parameters were as follows: Barley was steeped at 16°C using an automated program of alternating wet (immersed) and air rests designed to reach a steep-out moisture content of 46%. A '3-wet' steep cycle was used with the following cycle times (43 h in total): 7 h wet stand, 12 h air rest, 8 h wet stand, 12 h air rest and 4 h wet stand. Germination was conducted for 5 days at 12°C with automatic turning of the sample cages set at 1 min in every 10 min. Kilning: The air-on temperature during drying was programmed as follows: 55°C for 12h, 72°C for 4 h and 80°C for 4 h. Malt rootlets were removed using a benchtop deculmer (Curio Malting, Milton Keynes, UK).

Production of 'endosperm-rich' extracts of green malt

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

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

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

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

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

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

Malt analysis

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

Determination of Alpha- and Beta- Amylase Activity

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

Determination of the total lipoxygenase activity in malt

In this study, the LOX activity was determined by a spectrophotometric technique based on a combination of the methods of Guido et al. (2005) *(33)* and De Buck et al. (1997) *(7,32)*. The oxidation of linoleic acid by LOX increases the production of conjugated diene which absorbs at 234 nm. Milled barley or malt (5 g) was dispersed in acetate buffer (0.1 M, pH 5), containing the non-ionic detergent Brij 99 (0.1%) and stirred for 30 min. The homogenate was centrifuged (9632 x *g*, 5 minutes, 4°C) and the total LOX activity was determined spectrophotometrically using the supernatant as crude extract. To prepare substrate solution, 250 μL linoleic acid was dispersed by homogenisation in 5 mL borate buffer (25 mM, pH 9.0) with Tween20 (0.25% v/v), NaOH (1 M, 0.65 mL) and cold RO water (3.85 mL) to facilitate dispersion. The total LOX activity was determined spectrophotometrically by adding 50 μL of the enzyme extract to 50 μL of the airsaturated substrate solution in 2.90 mL of sodium phosphate buffer (0.1 M, pH 6.8), equilibrated at 30 °C in a total volume of 3 mL in UV-cuvettes (Plastibrand disposable Macro plastic 2.5 mL, Fisher Scientific, UK). The formation of a conjugated diene of the hydroperoxide as a result of LOX oxidation of linoleic acid was determined by measuring the absorption at 234 nm, using a UV/Vis Spectrophotometer (7315 UV/visible Spectrophotometer, Jenway, UK) and absorption was measured exactly 2 minutes after the addition of the enzyme, and then after 6 minutes. For the reference cell, the enzyme solution was replaced by buffer (2950 μL buffer and 50 μL substrate solution). The LOX activity correlates to the absorbance and is expressed as enzyme U/per gram of malt on a dry basis (U/g d.b.).

Determination of DMS in grain samples

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

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

Determination of S-methyl methionine in grain

The SMM determination was based on the original method proposed by White and Wainwright *(12)* following the altered protocol described by De Rouck et al. *(34)* without the usage of NaOH to avoid side formation of oxidised products such as DMSO and $DMSO_2$. Since SMM is heat labile, its content in malt is commonly measured by the subtraction of free DMS from total DMS. During heating the non-volatile DMS precursor was converted into DMS which allows its indirect quantification. Additionally, in contrast to the proposed ASBC method, the internal standard was added after (rather than before) heat treatment. Preliminary tests indicated a loss of almost 50 % in EMS peak area, which did not occur when EMS was heated in water, suggesting that heating EMS in wort leads to side reactions.

Wort analysis

Wort specific gravity and density were analysed using an Anton Paar DMA 4500 (UK). Extract yield was calculated according to Analytica EBC Method 4.4. Wort FAN (Free Amino Nitrogen) was determined according to Analytica EBC 8.10.

Determination of DMS and S-methyl methionine in wort

DMS and indirectly SMM in wort were determined according to the above SPME-GC-PFPD methodology for malt analysis. Samples were prepared in a total volume of 5 mL in a headspace vial (20 mL; Agilent, UK), using an appropriate dilution factor to remain within the calibration curve. DMS concentrations were determined based on the external calibration series (0.1-10 μg/L) and the internal standard, ethyl methyl sulphide (EMS, 1 μg/L).

Determination of the nonenal potential

The nonenal potential, an indicator of how a beer will release t-2-nonenal during storage, was determined using gas chromatography–mass spectrometry (GC-MS) based on the method of Drost et al. *(30)* and the protocol described by Guido et al. *(33)*. Filtered wort (150 mL, adjusted to pH 4, using 5% orthophosphoric acid) was purged for 5 min with nitrogen (99.5 %, Air Liquide, BOC, UK) to reduce the oxygen level. The sample was subsequently heated at 100°C for 2 h under constant nitrogen purging and then placed on ice. The liquid–liquid extraction of nonenal was performed on a 70 mL aliquot of the wort, using carbon disulphide (3 mL), as well as 7 g of NaCl for a salt-induced phase separation. The mixture was shaken for 30 min on a rotary action shaker. The sample was placed in ice for around 10 minutes to condense the carbon disulphide, subsequently transferred into a separating funnel (100 mL, Fisher Scientific, UK), and set aside for 15 minutes to allow for the complete separation of the two immiscible solvent phases. The lower (solvent) layer was separated into a 10 mL glass vial with cap, placed into a 50 mL falcon tube and centrifuged at 4704 x *g* for 10 min. The resultant carbon disulphide extract was removed using a glass syringe and analysed using an ISQ 7000 GC-MS system (Thermo Fisher Scientific, UK), fitted with an instant Connect SSL Injector for TRACE 1300 GC Series (Thermo Scientific) and a ZB-wax polar column (Phenomenex, Macclesfield, UK; 30 m x 0.25 mm ID with a 1 μm film thickness) was used. The carrier gas was helium (BOC, UK) at a set pressure of 18 psi. The mass data were collected in full scan mode with a scan range from m/z 35 to m/z 250. Compounds were analysed using selected ion mode and quantified by comparing the peak area of the selected compounds with the peak area of the internal standard 3-heptanone, as well as an external standard series run for trans-2-nonenal and hexanal (0.01 – 10 mg/L). The selected ions were as follows: trans-2-nonenal m/z 70, 96, 111; 3-heptanone m/z 57, 114; and additionally, hexanal m/z 56, 82.

Statistical analysis

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

Results and Discussion

Grain analysis

As a result of water removal during kilning the rootlets became brittle and could be removed using the desktop deculmer. Rootlets from green malt, however, did not form part of the malting loss. As shown in Table 1, wellgerminated (120 h) green malt consisted of about 14% rootlets (fresh weight), and the rootlets had a moisture content of around 66%. Malt rootlets, are considered to impair the flavour of beer, mainly due to their high content of lipoxygenase isoenzyme 2 *(9)* and their SMM content *(11)*. Therefore, in subsequent experiments the relative merits of mashing with or without rootlets present were investigated, by developing a laboratory protocol for preparing endosperm-rich extracts of green malt, separated from the husk and rootlet fraction (Section 2.2).

Table 1. Proportions by mass of kernel and rootlets with the moisture contents of each in green malt after 120 h germination.

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

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

Enzymatic activity in well germinated green malt

The development of lipoxygenase activity (Figure 1) and SMM levels (Figure 2) were monitored across the malting process. In a parallel experiment under identical malting conditions, a sample of germinating malt was taken daily and the developing rootlets were excised by hand prior to analysis so that the impact of the rootlets on the development of LOX activity and SMM levels in the germinating grain could be ascertained. Figure 1 shows that incoming barley had a total lipoxygenase activity of 4.8 ± 0.3 U/g d.b., which relates to the activity of LOX-1, which is already present in unmalted barley *(9,10,35)*. During malting the lipoxygenase activity started to increase significantly after 24 h of germination. Both isoenzymes are known to increase in activity during germination *(20,36)*. After 120 h of germination a total LOX activity of 27.5 ± 2.5 U/g d.b. was determined, which reduced by kilning to 1.6 ± 0.2 U/g d.b. After removing the rootlets of the well germinated (120 h germination time) green malt (18.9 ± 1.2 U/g d.b) a significant reduction of about 30 % in lipoxygenase activity was measured. According to previous research *(9,10)*, only LOX-2 is in the malt rootlets, thus a large proportion of LOX-2 would be removed with the rootlets before mashing if such a procedure was applied. A similar pattern was observed when monitoring the SMM development (Figure 2). The amount of SMM, (expressed as DMS equivalents), increased significantly between 24 h (2.5 ± 0.2 mg/L) and 48 h (11.1 ± 1.1) of germination. Levels further increased to 12.6 ± 2.5 mg/L after 120 h of germination and significantly dropped after kilning (2.6 ± 0.5 mg/L). When removing the rootlets, the DMS precursor in green malt significantly reduced to 7.3 ± 1.2 mg/L, an average decline of about 40 % by removing the rootlets. These data for LOX and SMM, suggest that rootlets are a major concern when brewing with green malt and that their influence on quality needs to be further investigated.

Diastatic enzyme activities are of key concern to the brewer. Figure 3 displays the development of α- and β-amylase activities

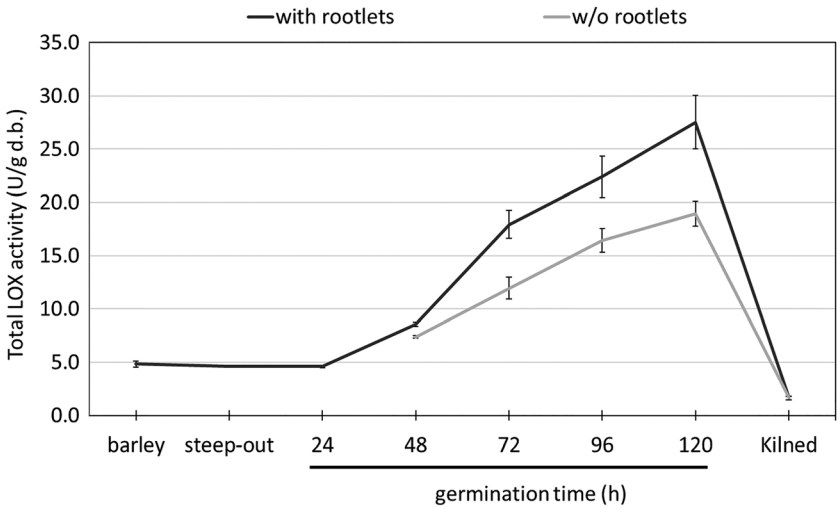

Figure 1. Lipoxygenase activity monitored during germination in the malting barley variety Flagon with/without rootlets. Data are the mean ±SD of 3 biological and 3 technical replicate measurements.

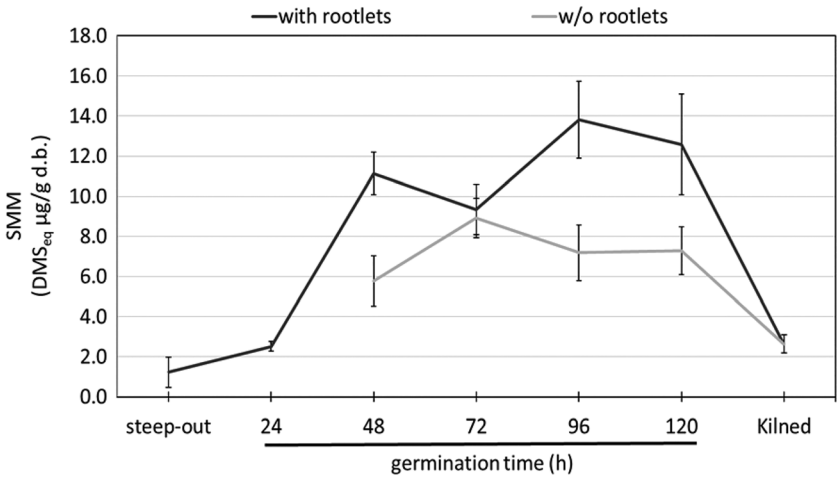

Figure 2. S-methyl methionine, expressed as DMS equivalents (µg/g d.b.). monitored during germination in the malting barley variety Flagon with/without rootlets. Data are the mean ±SD of 3 biological with each 3 technical replicate measurements.

through the same micromalting process. β-amylase, which is present in bound form in unmalted barley, suffered a considerable loss during kilning from 15.3 ± 0.3 to 9.7 ± 0.5 BU after kilning, whereas α-Amylase, in accordance with the literature *(37)*, was more thermostable with little loss in enzyme activity across kilning. These results support the hypothesis *(14–16)*, that there is a good potential to generate highly fermentable worts using green malt.

Approaches to limit the lipoxygenase activity in green malt

Whilst the LOX activity in kilned malt is already low, previous research indicated that even this residual activity accelerated beer staling *(17)*. The increased LOX activity in green malt, as illustrated in Figure 1, is a primary concern in terms of beer flavour and flavour (in)stability. Thus, the usage of green malt for conventional brewing processes requires alternative techniques to reduce total LOX activity. If LOX activity can be minimised at source, through adequate malting and/or mashing conditions, significant off-flavours in the resultant beer could be avoided. The hypothesis was to control lipoxygenase via its limiting factors: heat sensitivity, pH sensitivity, oxygen availability.

Short heat treatment of green malt

Green malt was subjected to short heat treatments in a convection oven. Experimental design software (Design-Expert v 11), was used to produce a response surface design with two numerical variables (time, 5-60 min; temperature, 65-90°C). The design consisted of 18 heat treatments of green malt, arranged into three blocks (according to different batches of green malt). Resulting data for LOX activity, α- and β-amylase were modelled across the design space resulting in either quadratic or two factor interaction (2FI) predictive models. Contour plots of these models are shown in Figure 4.

Figure 4A shows a 2D contour plot of the derived model for lipoxygenase activity as a function of time (min) and temperature (°C). Both heating time and temperature were significant factors in the model for LOX activity across the design space ($p < 0.0001$). Not surprisingly the LOX activity decreased as both temperature and the time of heat treatment of the green malt

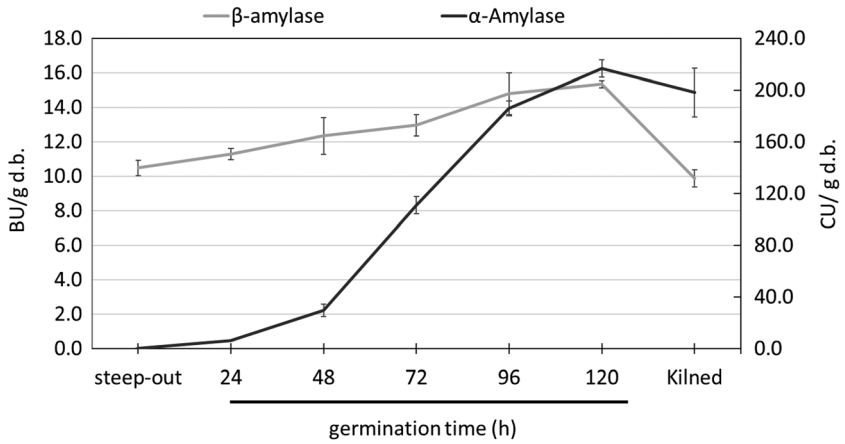

Figure 3. α- and β-amylase activity monitored during germination in the malting barley variety Flagon with rootlets β-amylase (Beta – Units; BU) is displayed on the primary y-axis whereas α-amylase (Ceralpha-Units; CU) is displayed on the secondary y-axis. Data are the mean ±SD of 3 biological and 2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

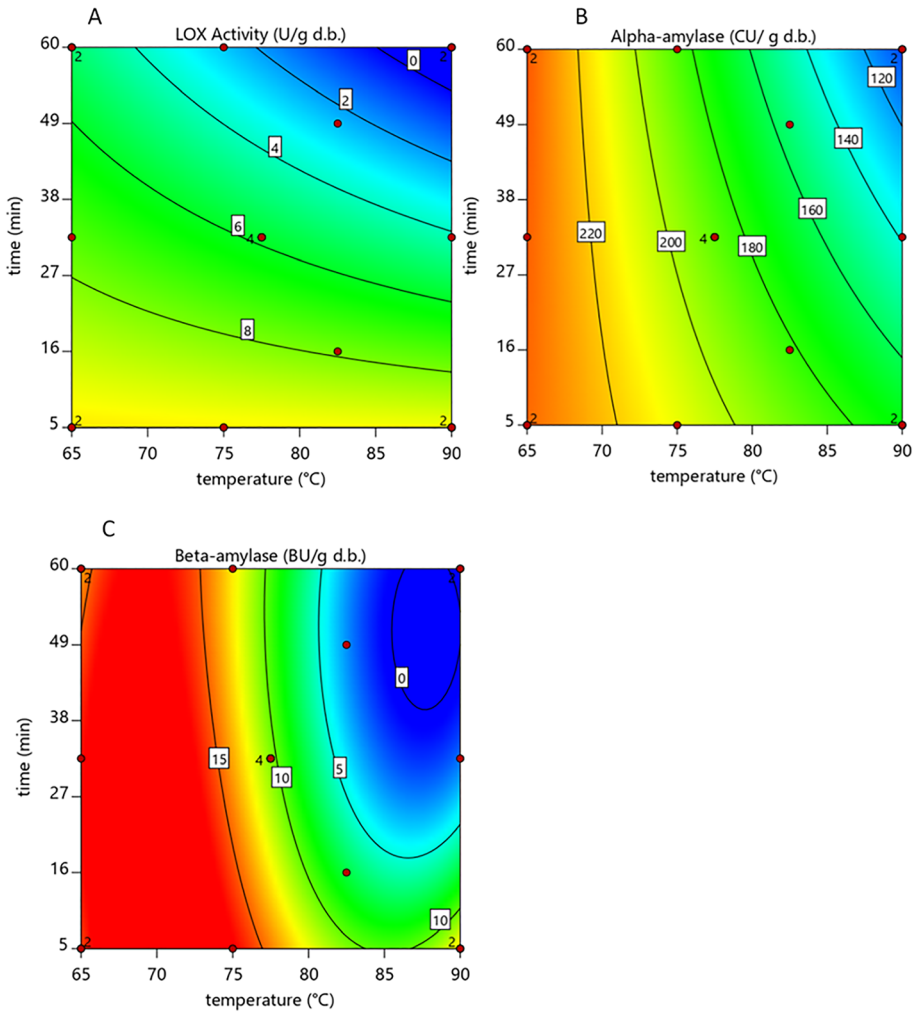

Figure 4. Contour plots modelling the influence of a short (5-60 min) heat treatment (65-90 °C) on the activities of (A) lipoxygenase (0-12.9 U/g d.b.), (B) α-amylase (96.7 – 251.0 CU/g d.b.) and (C) β-amylase (0.4 – 16.5 BU/g d.b.) in green malt. Colour legend: red (high) – blue (low). Plots show the predictive models fitted to data from 18 data points (red dots) across each design space. Model fit statistics: α-amylase: $p<0.0001$; $R^2=0.8205$; LOX: $p<0.0001$; $R^2=0.8715$; β-amylase:$p=0.0001$; $R^2=0.8544$. [Colour figure can be viewed at wileyonlinelibrary.com]

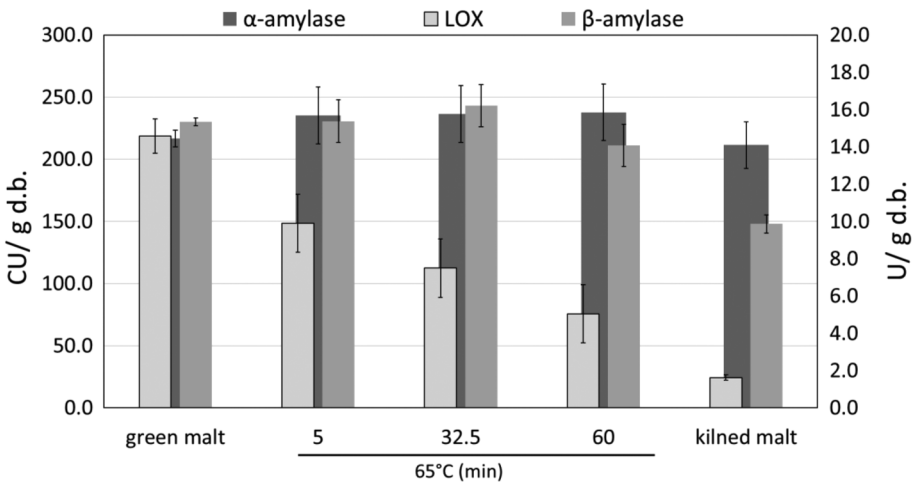

Figure 5. Model data showing the impacts of heating green malt at 65°C for periods of up to 1 h.The primary y-axis displays α-amylase activity (ceralpha units/ g d.b); the secondary y-axis displays β-amylase (Betamyl-2 Units/ g d.b.) and lipoxygenase activity (U/g d.b). Data are predicted responses given by the model fitted to experimental results.

increased. Figures 4B and 4C indicate the potential to reduce diastatic enzyme activities, suggesting that care must be taken not to destroy desirable enzyme activities by using excessive temperatures. Thus, the most promising results were achieved at the more moderate temperature of 65°C. As summarised in Figure 5, LOX activity decreased after just 5 minutes at 65°C, further decreasing when heated for 60 minutes. Most importantly diastatic enzyme activities were not substantially affected by this heat treatment. Additionally, after only 60 minutes the moisture content of the grain (including rootlets) was reduced to 29% and rootlets could be removed from the grain using a benchtop deculmer. Thus, this procedure could offer the added benefit of rootlet removal, should it prove scaleable.

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

Oxygen levels and oxygen pick-up need to be controlled throughout the malting and brewing process, to avoid LOX initiated enzymatic oxidations *(20,31,32,38)*. Previous studies *(20)* showed a decline in LOX activity during steeping, which was related to the dissolved oxygen in the steep water being used up quickly if the water is not aerated sufficiently. Consequently, the aim was to control lipoxygenase by removal of its substrate, opting to re-steep the well-germinated green malt in deaerated water (grist:water, 1:3) for one hour. Furthermore, the pH of the water used for re-steeping was varied within the range pH 4 – 7. The optimum pH for LOX activity has been reported to be 6.5, with LOX-2 being more pH sensitive than LOX-1 *(7,10,32)*. The most striking result (Figure 6), is that by re-steeping the grain for 1 h lipoxygenase activity decreased by around 50%.

Contrary to expectations, the pH of the water used for re-steeping did not influence the resulting lipoxygenase activity. However, it should be noted that the lipoxygenase activity is assayed in a buffer solution at a pH of 6.8. Therefore, the results need to be interpreted with care. LOX-1 showed in previous studies only 50% activity remaining at pH 5 whereas LOX-2 shows an activity rate close to zero *(7,8,32,39)*, suggesting that LOX-1 might have been inactive at an acidic pH, however, reactivated in the buffer solution. Further analysis will be necessary to obtain clearer

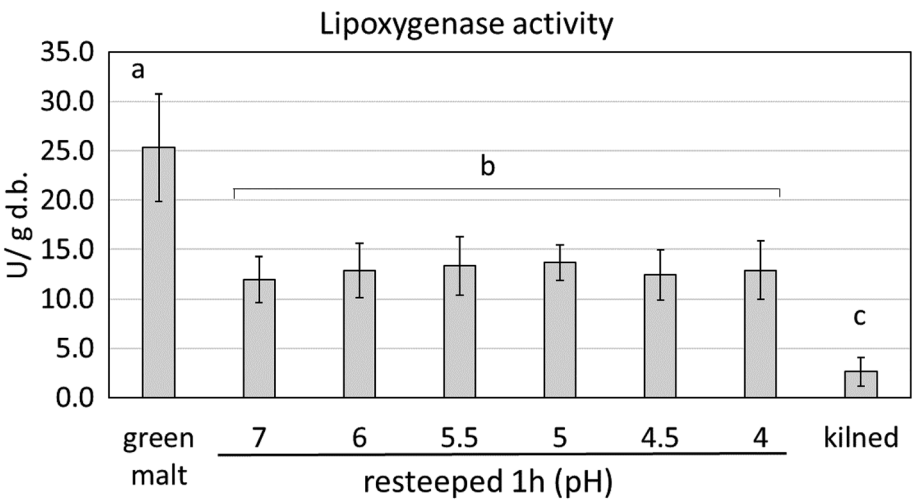

Figure 6. Influence of re-steeping at different water pH on the lipoxygenase activity in green malt. One-way ANOVA with Holm-Sidak post hoc test. Results are presented as mean values ± SD; n=3, tr=3.

information on lipoxygenase activity after re-steeping at a different water pH values. Moreover, residual water samples after re-steeping were analysed to help understand the reasons for loss of LOX activity. It was also important to know how much LOX activity was transferred to the re-steep water as it would be important for water use efficiency to be able to re-use this water elsewhere in the process. About 13.4 Units/g d.b. in activity were lost by re-steeping the green malt, however only 2.2 Units (16.7%) could be measured in the re-steep water. The remaining activity loss could at this stage not be further explained and requires more research but could be related to the onset of grain asphyxiation. The moisture content after re-steeping increased from the average 44.7% to 52.7%, which needs to be considered in terms of the subsequent brewing protocol. Re-steeping experiments (1 hour) demonstrated that LOX activity could be decreased by around 50%, whilst simultaneously preserving amylase activities (Figure 7).

Table 2 provides a summary of the impact of the main treatments developed to minimise green malt LOX activity, on the LOX and diastatic enzyme activities as well as levels of SMM. Reference values for kilned malt and for green malt with and without rootlets are provided by way of comparison. Either re-steeping or a heat treatment at 65°C for periods of 1 hour were effective in reducing LOX activity by around 50 % in the green malt. However, residual levels were still 3-8 fold higher than in finished kilned malt. Furthermore, the SMM levels were elevated 2.5-4.5 fold relative to kilned malt and were only significantly reduced by the short heat treatment, but unaffected by re-steeping.

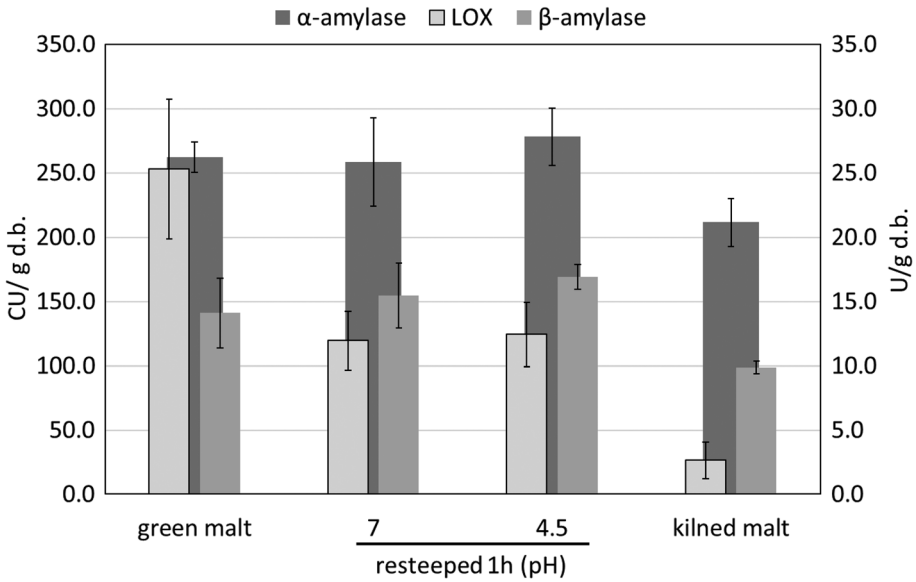

Figure 7. Lipoxygenase and amylase activities in green malt, re-steeped (at pH 7 or pH 4.5) as well as kilned malt.The primary y-axis displays α-amylase activity (ceralpha units/g d.b); the secondary y-axis displays β-amylase (Betamyl-2 Units/g d.b.) and lipoxygenase activity (U/g d.b.). Data are the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

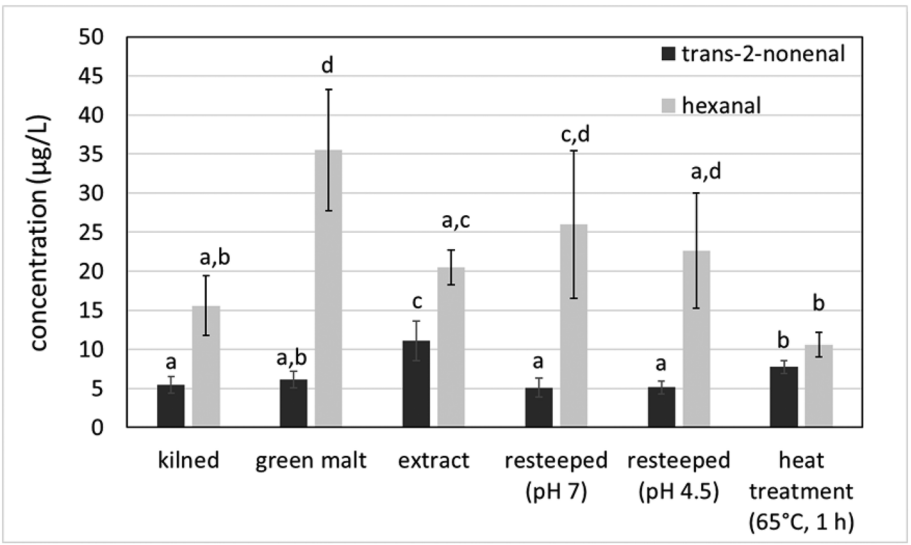

Figure 8. trans-2-nonenal and hexanal potentials (µg/L) after forced aging of wort samples. Data are the mean ±SD of 4 biological and 2-4 technical replicate measurements. Statistics: One-Way ANOVA on the ranks with Dunn's posthoc test.

Table 2. Enzymatic activities and S-methyl methionine concentration in malt; with or without pre-treatment.

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

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

Wort analysis

The main treatments developed in terms of lipoxygenase control (Table 2) were then compared in terms of the resultant wort quality following laboratory mashing (Table 3). This Table also features comparable results for mashing with the endosperm-rich extract of green malt so that the potential impacts of rootlet inclusion or removal are apparent. Wort was prepared under lipoxygenase hostile conditions *(29,31,38,40)*: mashing-in temperature 63°C, at low mash pH (e.g. pH 5.2), under oxygenlimited conditions to further investigate the properties of green malt by nitrogen purging the water used for mashing prior to processing. Data were compared with mashing of kilned pale malt (equivalent mass on a dry weight basis) made from the same green malt. The extract yields of all 'intact' green malt mashes (90.0 ± 2.9%), were greater than that for kilned malt (82.3 ± 3.3%) and wort FAN values were equivalent to or greater than the 188 mg/L in kilned malt wort. The endosperm rich extract yield was significantly lower, although extract values up to 71% as-is were achieved. Additionally, colour and FAN levels were significantly lower in worts prepared from the endosperm rich extract. Based on the present data it is not possible to definitively explain the reason for the lower FAN levels in these worts. However, it is logical to suggest that the endosperm extraction process left behind some of the aleurone and sub-aleurone tissues associated with the outer layers of the barley grain and that these layers contain a significant proportion of grain nitrogen and

Table 3. Analytical measures of wort of kilned malt or green malt – with or without pre-treatment.

Type of malt	Colour* (EBC)	Specific gravity***	Density*** (kg/m^3)	Extract corrected*** for weight used (%)	FAN*** (mg/L)	SMM*** (μg/L) DMS_{eq}	$DMS^{n.}$ $^{s.}$(μg/L)
kilned malt	3.4 ± 0.4[a]	1.0339[a]	1.0318[a]	82.3 ± 3.3[a]	188.4 ± 28.0[a]	587.5 ± 45.6[a]	88.1 ± 39.2
green malt (including rootlets)	3.3 ± 0.5[a]	1.0390[a]	1.0371[a]	90.0 ± 2.9[b]	224.5 ± 21.0[a]	1082.8 ± 88.2[b]	40.5 ± 10.5
green malt endosperm-rich extract	1.3 ± 0.5[b]	1.0271[b]	1.0258[b]	67.2 ± 4.6[c]	125.4 ± 8.1[b]	897.3 ± 21.7[b]	31.4 ± 6.1
re-steeped (pH 7)	2.6 ± 0.6[a]	1.0364[a]	1.0331[a]	84.3 ± 5.7[a,b]	189.3 ± 11.3[a]	911.9 ± 50.3[b]	40.9 ± 23.5
re-steeped (pH 4.5)	2.8 ± 0.5[a]	1.0361[a]	1.0345[a]	83.5 ± 4.4[a,b]	183.5 ± 4.8[a]	858.1 ± 44.8[b]	45.2 ± 20.7
heated at 65°C,1 h	2.9 ± 0.8[a]	1.0376[a]	1.0357[a]	86.9 ± 2.1[a,b]	215.3 ± 23.8[a]	858.0 ± 62.5[b]	25.9 ± 11.4

Indirect determination of S-methyl methionine from: (Total DMS – free DMS)
[a-e] superscripts represent the ANOVA post-hoc groupings. In each column treatments differed significantly from one another if they have a different ANOVA group letter.
asterisks represent the P-value significance
* $P<0.05$
** $P<0.01$
*** $P<0.001$
Data are the mean ±SD of 3 biological and 3-4 technical replicate measurements. Statistics: One-Way ANOVA with Fisher's LSD post-hoc test.

proteolytic activity. Additionally, SMM levels in worts prepared from green malt were relatively high and were not significantly altered by the proposed processing techniques. Hence, due to the increased DMS potential, special care must be taken during wort boiling to remove the DMS deriving from its precursor. Therefore, further research and brewing trials are required to evaluate the quality implications of the elevated precursor levels.

Nonenal potential

Increased trans-2-nonenal levels, as a result of enzymatic lipid oxidation, can become a major concern when using green malt for the brewing process. Trans-2-nonenal is an unsaturated aldehyde which is known to contribute cardboard stale flavours to beer. It has a very low flavour threshold in the low ppb range (0.035 μg/L; *(7,41,42)*. The nonenal potential was determined to further investigate if the proposed malt treatments, combined with lipoxygenase hostile mashing parameters, successfully controlled LOX activities and thus the formation of trans-2-nonenal in wort. Hexanal was also monitored as a marker of lipid oxidation. Hexanal is perceived as a green type odour and this flavour note decreases with increasing malt colour *(43)*. It can be found in pale malt worts at up to 50% higher concentrations than in wort made from dark malts.

Evaluation of the trans-2-nonenal and hexanal potentials of the worts (Figure 8) revealed that the separation technique used to prepare the endosperm-rich green malt extract caused increased trans-2-nonenal potential relative to all other treatments. This could result from the coldwater extraction, meaning that mashing in temperatures were initially lower than 63°C. Thus, enzymatic lipid oxidation could proceed until the required temperatures to destroy the enzyme were reached, which emphasizes the importance of temperature control during mashing. Re-steeping treatments resulted in trans-2-nonenal potentials which were not significantly different to that of kilned malt, but with an elevated hexanal potential. Furthermore, there was no difference between re-steeping the grain at pH 4 versus pH 7, indicating that the pH of re-steeping did not significantly impact on the trans-2-nonenal or hexanal potentials. Heating the green malt at 65°C for one hour decreased hexanal concentrations relative to those in wort prepared from kilned malt, but increased trans-2-nonenal levels. Interestingly, the trans-2-nonenal potential of the green malt wort without treatment was not significantly higher than for kilned malt, indicating that by mashing in under LOX hostile conditions, lipid oxidation in the mash was already controlled to a sufficient extent. However, hexanal levels remained a concern for the untreated green malt mash and would need to be regulated through wort boiling or stripping. The coefficient of variation (CV) of the trans-2-nonenal measurements was considerably lower than that for hexanal. This appeared to arise from variations between biological replicates, since the CV for technical replicates (instrumental analysis) was of the order of just 5.5%.

Conclusions. Development of processes to brew directly with green malt would represent disruptive technology and this approach is unlikely to be widely implemented in present day breweries. This forwardlooking project aims to develop proof-of-principle and enabling technology, with the potential to influence designs for the 'brewery of the future' when presumably operational and environmental pressures will prevail and force the malting and brewing chain to implement more energyefficient processes. Green malt production is a sustainable way of developing diastatic enzyme activities without moving to brewing with the use of unmalted grist materials and exogenous enzyme cocktails. Furthermore, proportions of green malt might be used in a mash to digest unmalted cereal adjuncts, rather than envisaging a process using solely green malt.

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

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

Author Contributions

Celina Dugulin: PhD student. Conducted all research and formal analysis in this manuscript. Writing – original draft.

Susan Clegg: Developed the laboratory methodology for producing an enzyme rich extract of green malt and trained Celina Dugulin in relevant protocols.

Gert De Rouck: Funding acquisition, conceptualisation and writing – review and editing.

David Cook: Funding acquisition, supervision of PhD, conceptualisation and writing – review and editing.

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Conflicts of Interest

The authors declare there are no conflicts of interest.

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