LTP1 and LOX-1 in barley malt and their role in beer production and quality

by

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Declaration

I, Melanie Nieuwoudt, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Melanie Nieuwoudt Date

Summary

Selection of raw materials for a consistent and high quality end product has been a challenge for brewers globally. Various different factors may influence quality and although a great number of methods for malt analysis exist today for the prediction of end product quality, some still do not accurately represent malt performance in beer. This research focussed on determining parameters in malts to predict two of the major beer quality determining factors namely, foam- and flavour stability. Specific biochemical markers in barley malt such as lipid transfer protein 1 (LTP1) lipoxygenase-1 (LOX-1), anti-radical/oxidant potential (AROP), free amino nitrogen and intact protein were determined and used in beer quality prediction from malt character. These biochemical quality predictions were then correlated with the end product beer quality as assessed in sensory analysis trials on micro-brewed beers.

Being such a multi-faceted factor in beer, LTP1 have already become an attractive field of study. LTP1 is primarily associated with stable beer foam, as a foam protein in its own right, and acting as a lipid scavenger. This protein is also theorised to play a role in the stability of beer flavour by possibly acting as anti-oxidant. Lastly LTP1 is known to have anti-yeast activity, which could negatively impact fermentation. In this study LTP1 and its lipid bound isoform LTP1b were successfully purified in an economical and easy five step protocol. Both isoforms showed temperature stability at temperatures >90°C and prefer more neutral and basic pH environments. Although the reported antioxidant activity was not observed, both purified LTP1 and LTP1b inhibited lipoxygenase-1 (LOX-1) activity, which is responsible for the enzymatic breakdown of linoleic acid to form 2(*E*)-nonenal. This is a novel finding that links LTP1 also to flavour stability. LTP1 exhibited anti-yeast activity whereas LTP1b lost most if not all the activity. However, since most of the LTP1 is converted to LTP1b and glycosylated isoforms during the brewing process fermentation will not be greatly influenced, while foam and flavour stability could still be promoted by the presence of LTP1b.

Flavour deterioration of the final packaged product is partially due to the enzymatic production of 2(*E*)-nonenal by LOX-1 and the presence of free oxygen radical species, limited anti-radical/oxidant potential (AROP) and LTP1. The development of two 96-well micro-assays

based on the ferrous oxidation-xylenol orange (FOX) assay for the determination of LOX-1 and AROP was successfully accomplished and compared well with established assays. The LOX-FOX and AROP-FOX assays were specifically developed for the on-site, high throughput comparative determination of LOX-1 and AROP in malt and other brewery samples.

The AROP-FOX and LOX-FOX micro-assays and a number of established assays were used to categorise malts in different predicted quality groups, various biochemical markers were measured which included LOX activity, LTP1 content, FAN values, intact protein concentration and AROP. An excellent trend (R^2=0.93) was found between FAN/LOX and LTP1/LOX which also correlated with the novel observation that LOX-1 activity is inhibited by LTP1 at various concentrations. These trends could assist brewers in optimal blending for not only high quality end products but also fermentation predictions.

To determine whether these biochemical markers selected for screening in barley malt are predictive of shelf life potential of the end product, sensory trials were performed. Three barley malt cultivars were selected for LOX, AROP, LTP1, protein and FAN content and used in micro-brewery trials at 0 and 3 months and evaluated using sensory analysis. Good correlation was found between the biochemical predictors and sensory trial for the best quality malt and beer. These parameters were therefore highly relevant for predicting shelf life potential, although additional research is required to elucidate the effect of LTP1 and LOX-1 on each other during the brewing process, since it seems that high LOX-1 concentrations could be leading to LTP1 decreases. With this study it is proposed that if more detailed protein or FAN characterisation is used together with the screening of LOX-1, LTP1 and AROP, an more accurate shelf life prediction, based on malt analysis, is possible and with the help of these parameters brewers can simply blend malts accordingly.

Opsomming

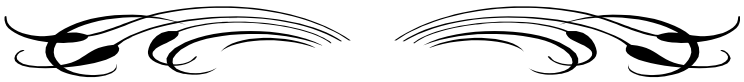
Die keuse van roumateriaal om ‟n konstante eindproduk van goeie kwaliteit te lewer, was nog altyd ‟n uitdaging vir brouers wêreldwyd aangesien verskeie faktore ‟n invloed het op die kwaliteit van die produk. Alhoewel daar tans verskeie metodes vir moutanalise bestaan wat die eindproduk–kwaliteit voorspel, is daar min wat werklik die eindproduk kwaliteit soos voorspel deur moutanalise verteenwoordig. Hierdie navorsing fokus op die bepaling van mout-eienskappe om twee van die belangrikste bierkwaliteitvereistes, naamlik skuim- en geurstabiliteit te voorspel. Spesifieke biochemiese eienskappe in garsmout soos lipiedtransportproteien-1 (LTP1), lipoksigenase-1 (LOX-1), antioksidant-antiradikaal potensiaal (AROP), vry aminostikstof (FAN) is geïdentifiseer en gebruik in voorspelling van bierkwaliteit vanaf moutkarakter. Hierdie biochemiese kwaliteit voorspellings is dan gekorreleer met die eindproduk soos ge-evalueer d.m.v sensoriese analise op mikro-gebroude bier.

Omdat LTP1 soveel fasette in bier beïnvloed, het dit reeds ‟n aanloklike studiefokus geword. LTP1 word hoofsaaklik geassosieer met stabiele skuimkwaliteit in bier en tree op as ‟n lipiedmop (“lipid scavenger”). Die proteien speel teoreties ook ‟n rol in die stabiliteit van bier geur deur moontlik as „n anti-oksidant op te tree. Laastens is LTP1 bekend vir sy antigis aktiwiteit wat moontlik ‟n negatiewe uitwerking op fermentasies het. Gedurende hierdie navorsing is LTP1 en sy lipiedbinding isoform LTP1b suksesvol gesuiwer met ‟n ekonomies en eenvoudige 5-stap protokol. Beide isoforme het stabiliteit by temperature >90°C en meer neutrale en basiese pH omgewings getoon. Alhoewel die voorheen gerapporteerde anti-oksidant aktiwiteit vir LTP1 nie bevestig kon word nie, is daar wel gevind dat beide LTP1 en LTP1b, LOX-1, wat verantwoordelik is vir die ensimatiese afbraak van linoleensuur na 2(*E*)-nonenal, se aktiwiteit inhibeer. Dit is ‟n unieke bevinding wat LTP1 ook koppel aan geurstabiliteit. LTP1 het antigis aktiwiteit getoon, maar LTP1b het die meeste, indien nie alle antigis-aktiwiteit verloor. Omdat die meeste van die LTP1‟s omgeskakel word na LTP1b‟s en geglikosileerde isoforme tydens die brouproses, sal fermentasie nie beduidend beinvloed word nie, maar die skuim- en geurstabiliteit sal steeds bevorder word deur die blote teenwoordigheid van die LTP1b.

Geurverval van die finale verpakte produk is gedeeltelik a.g.v die ensimatiese produksie van 2(*E*)-nonenal deur LOX-1 en die teenwoordigheid van vry suurstofradikaal spesies, beperkte AROP en LTP1. Die ontwikkeling van twee 96-putjie mikroessaïs, gebasseer op die yster oksidasie-xilenol oranje (FOX) essai vir die bepaling van LOX-1 en AROP, was suksesvol en het goed vergelyk met reeds gevestigde essaïs. Die LOX-FOX en AROP-FOX mikroessaïs is spesifiek ontwikkel vir die residente, hoë deurvloei vergelykende bepaling van LOX-1 en AROP in mout en ander brouery-monsters.

Die AROP-FOX en LOX-FOX mikroessaïs en ‘n paar gevestigde essaïs is gebruik om moute te kategoriseer in die verskillende voorspelde kwaliteitsgroepe. Die biochemiese merkers wat gemeet is het die volgende ingesluit: LOX aktiwiteit, LTP1 inhoud, FAN waardes, proteïen konsentrasie en AROP. ‘n Merkwaardige korrelasie (R^2=0.93) is gevind tussen FAN/LOX en LTP1/LOX wat ook ooreenstem met die waarneming dat LOX-1 aktiwiteit onderdruk word deur LTP1 by verskeie konsentrasies. Hierdie korrelasies kan brouers help met optimale versnitting van moute vir, nie net die hoogste kwaliteit eindproduk nie, maar ook vir fermentasie voorspellings.

Om te bepaal of hierdie geselekteerde biochemiese merkers in mout die potensieële raklewe van die eindproduk verteenwoordig, is sensoriese evaluerings uitgevoer. Drie gars-mout kultivars is geselekteer o.g.v LOX-, AROP-, LTP1-, proteïen- en FAN-inhoud en gebruik in mikro-brouery proewe en op 0 en 3 maande en is ge-evalueer deur sensoriese analise. Goeie korrelasie is gevind tussen die biochemiese voorspellers en sensoriese evaluering vir die beste kwaliteit mout en bier. Hierdie maatstawwe is daarom uiters relevant vir voorspelling van die potensiele rakleeftyd, alhoewel addisionele navorsing nodig is om die effek van LTP1 en LOX-1 op mekaar gedurende die brouproses te bepaal. Dit blyk dat ‘n hoë LOX-1 konsentrasies kan lei tot ‘n afname in LTP1. Met hierdie stuidie word dit voorstel dat, as meer gedetaileerde proteien of FAN karakterisering saam met LOX-1, LTP1, en AROP analise uitgevoer word, ‘n meer akkurate raklewe voorspelling moontlik is en met behulp van hierdie parameters kan brouers moute dienooreenkomstig versnit.



Dedicated with love to my family

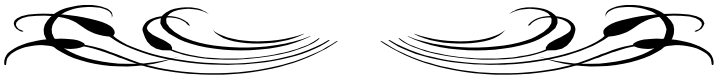


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Table of Contents

List of Abbreviations and Acronyms

°C	Degrees Celsius
°P	Degrees Plato
ACBP	Acyl-CoA
AOS	Allene oxide synthase
AROP	Anti-radical/oxidant potential
BSA	Bovine serum albumin
BHT	Butylated hydroxytoluene
CD	Circular dichroism
CIELAB	Method of colour measurement
CO_2	Carbon dioxide
CUPRAC	Cupric ion reducing antioxidant capacity
Da	Dalton
DA	Discriminant analysis
DSA	Descriptive sensory analysis
EBC	European brewery convention
ESMS	Electrospray mass spectrometry
ET/SET	Electron transfer/single electron transfer
FABP	Fatty acid binding proteins
FAN	Free amino nitrogen
FG	Final gravity
FOX	Ferrous oxidation-xylenol orange
HAT	Hydrogen atom transfer
HPL	Hydroperoxide lyase
HRP	Horse radish peroxidase
ISO	International Organization for Standardisation
IC_{50}	Inhibitory concentration for 50% inhibition of growth

JA	Jasmonic acid
K_d	Binding constant
KDa	Kilodalton
KOD	Keto-octadecadienoic acids
KOT	Keto-octadecatrienoic acids
L	Litre
LA	Linoleic acid
LOQ	Limit of quantisation
LOX	Lipoxygenase
LSD	Least Significant Difference
LTP	Lipid transfer protein
M	Molarity, moles/L
MaxEnt	Algorithm software used in combination with MS data to calculate macro molecules
M_r	Relative molecular mass
mL	Millilitre
MYGP	Yeast growth media
m/z	Mass over charge ratio
n	Number of samples
NFP	National Food products
ns	Not significant
OPDA	Phytodienoic acid
OD	Optical density
OG	Original gravity
P	Statistical value, indicating % confidence interval
PBS	Phosphate buffered saline
PCA	Principle component analysis
PUFA	Poly unsaturated fatty acids

R^2	Coefficient of determination
r	Correlation coefficient
RNP	Residual nonenal potential
RPM	Revolutions per minute
S. cereviseae	*Saccharomyses creviseae*
SABMiller	SAB Plc. Worldwide
SAB5	*S. cereviseae* strain
SAS™	Statistical analysis system
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate poyacrylamide gel electrophoresis
SEM	Standard error of the mean
Tris-HCl	2-amino-2-(hydroxymethyl)-1,3-propandiol-hydrochloride
UPLC	Ultra performance liquid chromatography
VSP	Vegetative storage protein
α-DOX	α-doxygenase
Σ-value	Sigma value for foam determination
2/3D	Two or three dimensional
9-HPOD	9(*S*)-hydroperoxy-10(*E*), 12(Z)-octadecadienoic acid
9-HPOT	9(*S*)-hydroperoxy-10(*E*), 12(*Z*), 15(*Z*)-octadecatrienoic acid
13-HOT	13(*S*)-hydroxy-9(*Z*), 11(*E*), 15(Z)-octadecatrienoic acid
13-HPOD	13(*S*)-hydroperoxy-9(*Z*), 11(E), 15(*Z*)-octadecatrienoic acid
13-HPOT	13(*S*)-hydroperoxy-9(*Z*), 11(E), 15(*Z*)-octadecatrienoic acid

Preface

Beer brewing is a form of biotechnology that has been practised by man for over 800 decades but it is due to research done during the last 150 years that a better understanding of its exact science was gained (Bamforth, 2000a). It is of utmost importance to the brewer to produce a beer that is of acceptable quality (Leisegang & Stahl, 2005) as well as one that stays flavour and foam consistent over a range of seasons and product sites (Van Nierop *et al.*, 2004).

One of the main quality determining factors of beer is stable, attractive beer foam (Bamforth, 2000b; Smythe *et al.*, 2002). Different theories surrounding the formation and stability of such a foam exists today, some of which includes the bitter substances derived from hops and surface active proteins and peptides to name but a few (Bamforth, 1985). The surface active protein that is proposed to be involved in the formation/stability of beer foam is lipid transfer protein 1 (LTP1) (Sorensen *et al.*, 1993; Lusk *et al.*, 1995). LTP1 is also known as an antimicrobial protein, which protects the grain in the field and during germination (Gorjanovic *et al.*, 2005). The protein, however, also inhibits the growth of *Saccharomyces cerevisiae* or common brewer‟s yeast, which could negatively impact the fermentation process (Gorjanovic *et al.*, 2004). The protein LTP1 together with its modified form LTP1b was characterised in Chapter 2.

Another protein that will influence beer quality, and in particular, beer flavour, is lipoxygenase 1 (LOX-1), an enzyme responsible for the breakdown of a poly-unsaturated fatty acids to yield flavour active components. High LOX-1 activity, fatty acid hydroxyperoxide lyase-like (HPL-like) activity and low antiradical/oxidant potential (AROP) in malts have been linked to this unwanted oxidation reactions, nonenal production and the loss of flavour stability. In order to ensure flavour stability in beer, malts/worts must be selected for low LOX activity, high AROP and low residual nonenal potential (NRP). A possible link became apparent in Chapter 5 when it was discovered that LTP1 can in some cases be covalently bound to lipid-like

adduct to form a structure known as LTP1b. This adduct was identified by Bakan *et al.,* (2006) to be an allene oxide, α-ketol-9-hydroxy-10-oxo-12(Z)-octadecadienoic acid, derived from linoleic acid.

Brewers are already well aware of the effects of LTP1, LOX-1 and AROP on beer quality, although quantifying these proteins are currently time consuming and complicated. With a better understanding of how these proteins influence the quality of the end product, brewers will be able to easily distinguish between malts with higher- to those with lower quality and brewing potential.

In the following chapter (Chapter 1) aspects surrounding beer quality are addressed with special emphasis on the crucial variables impacting flavour and foam head. Observations, based on pass, work and literature, will assist in building a platform from which the research done throughout this thesis will be based upon and validate the importance thereof.

This PhD research project was initiated to address the issue of reliably and consistent screening methods for malt blending. The main goal of this PhD study was to determine if screening methods focusing on the two barley proteins, LTP1 and LOX-1, could improve malt selection and subsequently beer quality. In order to reach the goal of this project the following objectives were set:

- *Optimise the purification and further characterise LTP1 and LTP1b from barley malt.* Foam is one of the most important quality determining aspects of beer. LTP1 is known to play an integral role in the stability of beer foam and recently beer flavour. A better knowledge of this protein‟s character is, however, needed to fully understand its relevance in the end product and its survival during brewing procedures. This study is reported in Chapter 2 which has been submitted for publication.

- *Development of medium throughput lipoxygenase (LOX-1) and antioxidant/antiradical power (AROP) assays for beer and evaluation of different malts.* Flavour stability is one of the major problems faced within the beer industry, mainly due to chemical reactions that occur long after bottling. Some of the main contributors to these reactions are the presence of oxygen (or lack of anti-oxidants) and lipoxygenase enzymes which are known to mediate the formation of stale flavour compounds. To be able to blend malts according to their shelf life potential could be of great benefit for brewers and can lead to a reduction in losses. In order to blend malts accordingly a simple, rapid and robust assay for the determination of LOX-1 and AROP is needed. A simple assay was developed that can be used for both LOX-1 and AROP determination at an on-site laboratory, needing little specialised equipment. It was successfully used for LOX-1 and AROP studies in malt and wort. These studies are reported in Chapters 3 and 4, which are both individually submitted for a two-part publication.

- *Assess correlations between positive beer fermentation and flavour factors and LOX-1 activity in different barley malt varieties.* In order to predict malt performance in terms of fermentability, foam head and flavour potential, certain biochemical analyses are required. Different local and imported barley malt cultivars were subjected to protein extraction and analysed on the basis of LTP1-, LOX-1-, AROP and free amino nitrogen (FAN) content and examined using multi-variant analysis techniques. Clear trends became apparent and emphasised the complexity of malt character and that, in order to choose the best malt for brewing, LOX activity and LTP1 content must be determined in conjunction with FAN. This study is reported in Chapter 5.

- *Assess the validity of pre-determined selection criteria using biochemical markers such as LTP1, LOX-1, AROP, intact protein content and FAN on quality and sensory aging of beer.* In Chapter 5 selective criteria in terms of LTP1, AROP and FAN as quality positive factors and LOX-1 were used to select malts for brewing. We investigated the criteria and impact of

these five variables on the quality of the product on a sensory level. This applied study is reported in Chapter 6 and will in future be submitted for publication.

The background of this study is given in Chapter 1 and the summation of all work done is given in Chapter 7 along with proposals for future research. To facilitate the intended publication of this research all experimental chapters (Chapters 2-6) were written, to some extent, in article format and form individual units. This structure therefore led to some unavoidable repetition, however, I tried to keep any unnecessary repetition to a minimum.

Most of the above mentioned objectives were completed at the department of Biochemistry, Stellenbosch University, while collaborating departments Food Science, Wine biotechnology and Process Engineering also played part in certain experimental procedures.

References

Bakan, B., Hamberg, M., Perrocheau, L., Maume, D., Rogniaux, H., Tranquet, O., Rondeau, C., Blein, J.P., Ponchet, M. & Marion, D. (2006). Specific adduction of plant lipid transfer protein by an allene oxide generated by 9-lipoxygenase and allene oxide synthase. *Journal of Biological Chemistry*, **281**, 38981-38988.

Bamforth, C.W. (1985). The foaming properties of beer. *Journal of the Institute of Brewing*, **91**, 370-383.

Bamforth, C.W. (2000a). Brewing and brewing research: Past, present and future. *Journal of the Science of Food and Agriculture*, **80**, 1371-1378.

Bamforth, C.W. (2000b). Perceptions of beer foam. *Journal of the Institute of Brewing*, **106**, 229-238.

Gorjanovic, S., Spillner, E., Beljanski, M.V., Gorjanovic, R., Pavlovic, M. & Gojgic-Cvijanovic, G. (2005). Malting barley grain non-specific lipid-transfer protein (ns-LTP): Importance for grain protection. *Journal of the Institute of Brewing*, **111**, 99-104.

Gorjanovic, S., Suznjevic, D., Beljanski, M., Ostojic, S., Gorjanovic, R., Vrvic, M. & Hranisavljevic, J. (2004). Effects of lipid-transfer protein from malting barley grain on brewers yeast fermentation. *Journal of the Institute of Brewing*, **110**, 297-302.

Leisegang, R. & Stahl, U. (2005). Degradation of a foam-promoting barley protein by a proteinase from brewing yeast. *Journal of the Institute of Brewing*, **111**, 112-117.

Lusk, L.T., Goldstein, H. & Ryder, D. (1995). Independent role of beer proteins, melanoidins and polysaccharides in foam formation. *Journal of the American Society of Brewing Chemists*, **53**, 93-103.

Smythe, J., O'Mahony, M. & Bamforth, C. (2002). The impact of the appearance of beer on its perception. *Journal of the Institute of Brewing*, **108**, 37-42.

Sorensen, S., Bech, L., Muldbjerg, M., Beenfeldt, T. & Breddam, K. (1993). Barley lipid transfer protein 1 is involved in beer foam formation. *Technical Quarterly*, **30**(4), 136-145.

Van Nierop, S.N.E., Evans, D.E., Axcell, B.C., Cantrell, I.C. & Rautenbach, M. (2004). Impact of different wort boiling temperatures on the beer foam stabilizing properties of lipid transfer protein 1. *Journal of Agricultural and Food Chemistry*, **52**, 3120-3129.

Outputs of PhD study

- M. Nieuwoudt (2012) LTP1 and LOX1 in barley malt and their role in beer production and quality (2012), Department of Biochemistry and Food Science, Stellenbosch University, Oral presentation
- M. Nieuwoudt (2011) Characterisation of barley malt LTP1 and LTP1b (2011), Department of Food Science, Stellenbosch University, Oral presentation
- M. Nieuwoudt (2010) Effects of barley LTP1 on beer foam stability and yeast growth Introduction to protein isolation and the isolation of LTP1 from barley (2010), Department of Food Science, Stellenbosch University, Oral presentation
- M. Nieuwoudt (2009) Optimisation of barley LTP1 isolation, Department of Food Science, Stellenbosch University, Oral presentation
- M. Nieuwoudt, N. Lombard, A de Beer, M Rautenbach (2012) Optimised purification and characterisation of two LTP1 species from barley malt, SASBMB/FASBMB conference, Champagne Castle, Kwazulu-Natal, SA, Poster presentation
- M. Nieuwoudt, N. Lombard, M. Rautenbach. Optimised purification and characterisation of lipid transfer protein 1 (LTP1) and its lipid-bound isoform LTP1b from barley malt. Manuscript submitted to *Food Chemistry* (FOODCHEM-S-13-04686, accepted with revisions)
- M. Nieuwoudt, M. Rautenbach. An optimised FOX assay for LOX activity in malt and brewery samples. Manuscript submitted to *Food Chemistry* (FOODCHEM-D-13-04775, in review)
- M. Nieuwoudt, M. Rautenbach. An adapted FOX assay for anti-radical/oxidant potential in barley malt. Manuscript submitted to *Food Chemistry* (FOODCHEM-D-13-04776, in review)
- M. Nieuwoudt, M. Rautenbach. LTP1, LOX1 antioxidant/antiradical power, FAN and intact protein in selection of malts with optimal fermentability, foam and flavour stability character for beer brewing, Manuscript in preparation for submission to *Journal of Agricultural and Food Chemistry*

CHAPTER 1: Literature review

Role of selected proteins in beer quality

Beer quality

Many new approaches in defining, analyzing, predicting and studying beer quality is constantly being developed and applied. Beer quality is overall interpreted by the paying consumer and is placed in the criteria of: flavour (and flavour stability), foam (stability in terms of retention and cling), haze, colour, alcohol content and CO_2 content (Bamforth, 1985a). During this sub-section the beer quality determinants contributing to foam and flavour will be addressed in detail.

Role of foam

Beer quality is to a great extent determined by the quality and stability of the beer foam. Beer foam's functional properties include the prevention of the emanation of flavour and the inhibition of oxidation by preventing direct contact between air and beer (Okada *et al.*, 2008). Beer foam also plays an important part in the overall aroma due it carrying aromas over the air-beer interface towards the drinker as well as adding to beer's mouth feel (Delvaux *et al.*, 1995).

Foam is a colloidal suspension of gas bubbles in a liquid. These gas bubbles increase the surface area of the liquid, while the surface tension is decreased. The stability of foam is thus maximised when surface tension is kept at a minimum and surface elasticity and -viscosity relatively high. The composition and viscosity of the liquid plays a vital role in the integrity of foam (Bamforth, 1985b). Proteins, for instance, can stabilise a foam by forming a visco-elastic and relatively stiff film between air and liquid (Clark *et al.*, 1994). The nature of the gas also plays a vital role in the stability of the foam bubble. Gas that is dissolved in a liquid facilitates movement through air bubbles. It is known that foams containing carbon dioxide have larger air

bubbles than those containing nitrogen or oxygen. Smaller bubbles are also more stable, since it rises slower to the surface giving the surface active substances time to associate within the bubble walls. The smaller bubbles will also take longer to be drained of liquid (Bamforth, 1985b). Beer foam is complex and multifaceted, thus a number of influencing factors should be taken into account when producing a beer with acceptable foam quality (Van Nierop, 2005).

The integrity of beer foam can be reduced by components competing for absorption. Such components are surface active, low molecular weight components, for instance lipids or detergents (Clark *et al.*, 1994). Surface active components can be classified on the basis of their size and hydrophobicity. Proteins with greater molecular weights (>5000Da) are more surface active, and the hydrophobic proteins share this character (Slack & Bamforth, 1983; Siebert & Knudson, 1989). Lipid transfer proteins (LTPs), together with protein Z, are two of the major role-playing proteins involved in beer and beer foam; the former being able to survive malting and brewing due to its resistance towards high temperatures, malt and yeast protease (Gorjanovic *et al.*, 2005). Other foam promoting or foam positive proteins include members of the hordein barley storage protein family (Asano *et al.*, 1982; Evans *et al.*, 2003). Hop acids and polysaccharide compounds are also foam promoting factors in beer (Jegou *et al.*, 2001).

Except for the stability and strength of beer foam, other very important aspects regarding foam quality includes, lacing (also known as adhesion or cling), whiteness of the foam, bubble size, foam density and foam viscosity (Bamforth, 1985b). A measure in controlling foam stability is through modification of the malt. The modification is negatively correlated to foam stability, since this results in the decrease of foam-positive proteins as well as viscosity due to the degradation of non-starch polysaccharides (e.g. β-glucan and arabinoxylan). If the malt modification is too low on the other hand, the malt extract will be insufficient and the beer filtration efficiency will be reduced (Okada *et al.*, 2008). Thus an optimum level of malt modification is necessary to generate beer with an acceptable foam head.

Foam positives

Components such as beer iso-α-acid from hops, metal ions (Bamforth, 1985b; Evans & Bamforth, 2009), barley grain and -malt surface active proteins and beer viscosity increasers, such as gums, dextrins and glycoproteins, that reduce the drainage of liquid from foam (Evans *et al.*, 1999), are regarded as factors that have a positive effect on the formation of beer foam. The gas composition of a particular beer is also considered to be a foam positive factor (Bamforth, 1985b). The hop resin, in particular isohumulone, has been shown to increase the degree of foam formation by lowering the surface tension of the beer. It also proved to enhance foam stability. The poor foam stability of "unhopped beer" has been shown to be significantly enhanced with the addition of α-acids, soft resins and hops (Bamforth, 1985b).

Metal ions promote the formation of foam due to its cross-linking action. It can, however, only be achieved when the beer is hopped. Presumably iso-α-acid in the bubble walls will bind with the metal ions and polypeptides, after which it will precipitate and promote the adhesion to bubble walls (Bamforth, 1985b).

Other foam positive factors include melanoidins, derived from monosaccharide and amino acids, which is formed during the kilning of malt (Bamforth, 1985b). It is known that proteins with higher molecular mass ($M_r > 5000$) are foam promoting factors and thus enhance the foam's stability (Bamforth, 1985b; Evans & Bamforth, 2009). Foam positive proteins identified so far are LTP1, protein Z and hordein fragments. Protein Z and LTP1 from barley are able to survive the malting and brewing process partly due to their protease inhibiting properties (Evans & Hejgaard, 1999). Although LTP1 plays a role in foam formation it is only responsible for foam stability in combination with other foam proteins, such as protein Z4 (Evans & Hejgaard, 1999). The effect of LTP1 on beer foam stability and quality will be discussed in detail later. Kapp and Bamforth (2002) also discovered that albumin and hordein protein fractions are also foam stability contributing factors and even more so in a denatured state.

The concentration of alcohol in beer is also known to play a role in the stability of foam. Too low (<1%) or too high (>3%) concentrations of ethanol can be detrimental to the beer foam (Bamforth, 1998). Possible explanations may be that ethanol lowers surface tension and can also interact with polypeptides. It has also been suggested that the presence of ethanol reduces CO_2 solubility in beer, leading to a more viscous and lacy beer foam (Bamforth, 1985b).

As previously reported (Bamforth, 1985b), the gas inside the bubbles comprising the beer foam is an important aspect in foam biochemistry. For a good foam head when dispensing, high levels of CO_2 in beer is recommended, although lower levels are suitable at higher temperatures.

Foam negatives

Yeast proteinase A, lipids, high concentrations of ethanol, detergents, and basic amino acids are considered to be foam negative factors (Evans & Bamforth, 2009). Detergents from manufacturing and cleaning procedures and lipids from malt or yeast can disrupt interactions between proteins in the lamellae surrounding bubbles. Ethanol at the concentration found in most beer is detrimental to the stability of foam, although at levels of <1% (*v/v*) it may enhance the foam. The reason for this may be because of ethanol's impact on surface tension and carbon dioxide solubility (Bamforth, 1985b). Another important detrimental influence on beer foam is the level of malt modification; an over-modified malt will cause a decrease in foam stability (Okada *et al.*, 2008).

Flavour stability

To date several hundred flavour components have been identified in beer, some contributing more to the overall beer flavours and aromas than others (De Keukeleire, 2000; Igyor *et al.*, 2001; Lodolo *et al.*, 2008). The major contributors to beer flavour are malt, hops and yeast (Lustig, 1999; Vanderhaegen *et al.*, 2006). The Maillard reaction's by-products and other sulphur containing substances are introduced into beer via the malt (Vanderhaegen *et al.*, 2006), while the bitter and other aroma compounds are due to the hops added (Vanderhaegen *et al.*,

2006; Intelmann & Hofmann, 2010). The yeast is responsible for introducing sulphur substances (Bamforth, 2000; Vanderhaegen *et al.*, 2006), carboxylic acids, higher alcohols and esters (Bamforth, 2000). It is very important to have the correct balance of flavours; since some desirable flavours can be undesirable when in abundance and some undesirable flavours will not be noticed when under a certain threshold. Besides beer flavour, brewers should also take into account the "drinkability" and mouth feel of the end product (Bamforth, 2000).

During storage other factors than those responsible for flavour development during production will influence the overall flavour of beer, since it is during this time that beer can be exposed to factors that might negatively influence beer aroma and taste. During ageing a slow decrease in bitterness is observed, together with an increase in sweet taste, toffee-like, caramel and burnt sugar aromas (Dalgliesh, 1977; Vanderhaegen *et al.*, 2006). A sharp increase of *ribes* (similar to blackcurrant leaves) is also observed, but this decreases after long periods of storage (Dalgliesh, 1977). The characteristic cardboard flavour development constantly increases during storage to reach a maximum, but will then decrease (Vanderhaegen *et al.*, 2006). Flavours associated with fruity floral flavour are known to steadily decrease in aged beers (Bamforth, 1999b). All these changes are circumstantial and will vary between different beer types as well as between beers that differ in raw material. It is, however, true for any beer that oxidation will occur in the presence of oxygen leading to the deterioration of flavour compounds. It has been observed that flavour will also deteriorate even when oxygen levels are at a minimum which suggests non-oxidative reactions are also present (Bamforth, 1999b; Vanderhaegen *et al.*, 2006). The possible pathways for flavour related compounds are as follows; melanoidin-type oxidation; Strecker degradation of amino acids; oxidation of isohumulones; enzyme-mediated degradation of lipids; aldol condensation of aldehydes (short chain); and secondary oxidation of aldehydes (long-chain) (refer to a review by Takashio and Shinotsuka, (1998)). The effect of temperature will affect the rate of chemical reactions inherent to beer (Vanderhaegen *et al.*, 2006) and the presence of anti-oxidants in beer will reduce beer staling by scavenging free radicals responsible

for oxidative breakdown of compounds to form unwanted flavour components (Takashio & Shinotsuka, 1998).

In general, the deterioration of beer flavour during storage is due to the formation and degradation of compounds. If compounds are formed to levels above the desired taste threshold it will impact overall flavour, while if other compounds are broken down the beer might lose its initial fresh-beer flavour. Of all negative associated flavour compounds, carbonyl compounds have probably received most attention and includes the formation of (*E*)-2-nonenal, which will be discussed in more detail later on in this chapter. Other compounds that will effect flavour include cyclic acetals, heterocyclic compounds, esters, sulphur compounds and non-volatile compounds such as the bitterness and astringency contributors of beer (Vanderhaegen *et al.*, 2006).

Proteins and beer quality

Beer quality can be attributed to factors regarded as important by the consumer such as flavour (De Keukeleire, 2000), colour and clarity (Shellhammer, 2009) as well as foam stability (Bamforth, 1985b). Various polypeptides and proteins in beer play an integral part in the formation of a stable foam head (Bamforth, 1985b). Colour is also influenced by protein reactions and interactions for example, the production of colour components that are formed during the Maillard reaction and polyphenol-protein interactions (Shellhammer, 2009). Flavour is also greatly influenced by proteins present and will be discussed in more detail. Proteins play an integral part in the quality of beer. In this chapter the discussion will be focussed on the impact of LTP1 and lipoxygenase-1 (LOX-1) on beer quality and in particular foam formation and flavour stability.

Lipid transfer protein 1

Structure and Expression

In plants there are two major lipid transport protein (LTP) families. LTPs are polypeptides that consist of 90 to 95 amino acids and are characterised by a basic p*I* (Gorjanovic et al., 2005). They are known as LTP1 and LTP2 and are composed of proteins of molecular masses, 9.7 kDa and 7 kDa respectively. These proteins are also referred to as non-specific LTP's (ns-LTP) due to their lack of substrate specificity (Kader, 1996). Both families, although different in structure, are characterised by a pattern of cystine residues (disulphide bonded Cys). Eight Cys residues, located at conserved positions (Kader, 1996), are linked by intramolecular disulphide bonds (Douliez et al., 2000). In the case of LTP1, Cys^{3} is paired with Cys^{50} and Cys^{48} with Cys^{87}. In the case of LTP2, Cys^{3} and Cys^{35} are paired and Cys^{35} pairs with Cys^{68}. It is thus clear that there is a mismatch in the cysteine motif (Carvalho & Gomes, 2007). Tryptophan residues are lacking in both families and phenylalanine residues are rare in the sequence of LTP, while two tyrosine residues are located at the N-terminal, as well as the C-terminal of the polypeptide backbone (Douliez *et al*., 2000).

The tertiary structure of the LTP protein family consists of four α-helices (Fig. 1). The helices are linked by flexible loops and form a hydrophobic cavity (Heinemann *et al.*, 1996). Disulphide bonds stabilise this folding while the cavity provides a potential binding site for one fatty acid chain (Douliez *et al.*, 2000). The structure and size of the cavity can vary between different types of LTP. In some cases the cavity can even be replaced with a tunnel alongside the long axis of the protein (Douliez *et al.*, 2000). Due to differences in cavity composition, LTP1 is able to bind linear lipids, while LTP2 can additionally bind planar sterols (Stanislava, 2007).

Since its discovery in plants by Kader (1975), LTPs have been isolated from numerous plants, including barley (Sorensen *et al.*, 1993; Evans & Hejgaard, 1999; Douliez *et al.*, 2000; Garcia-Casado *et al.*, 2001; Jegou *et al.*, 2001; Lindorff-Larsen *et al.*, 2001; Gorjanovic *et al.*,

2005; Perrocheau *et al.*, 2006; Mills *et al.*, 2009). LTP1 can be located outside cells, associated with cell walls, as well as secreted into the culture medium of embryogenic cells. The genes of LTPs are mainly expressed in the epidermal tissue of plants and have also been isolated from surface waxes. In cereal kernels, these proteins make up about 5-10% of the total soluble proteins (Stanislava, 2007). In barley seeds, most of the LTP gene expression is limited to aleurone layer around the starchy endosperm (Kalla *et al.*, 1994).

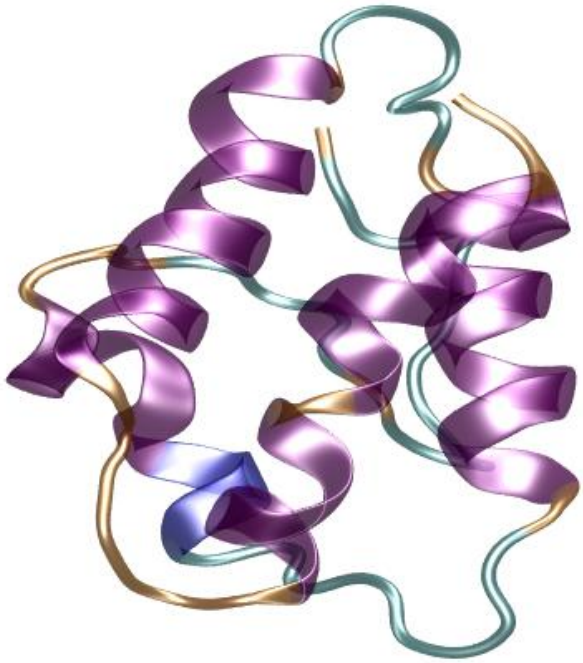

Fig. 1. A three-dimensional structure of LTP1 from barley (*Hordeum vulgare*) seeds (Heinemann *et al.*, 1996) (PBD ID: 1LIP).

Functions of LTP1

As previously described, LTPs are involved in the transport of fatty acids, fatty alcohols and hydroxy-fatty acids. Waxy and polymeric cutin layers of most organs, such as seeds, are composed of these monomers (Douliez *et al.*, 2000), thus limiting most of the LTP gene expression to the peripheral cell layers (Kalla *et al.*, 1994).

LTP1 can also be found in a form covalently bound to α-ketol, 9-hydroxy-10-oxo-12(*Z*)-octadecenoic acid. During the germination of the barley seed adduction of the α-ketol takes place and the complex is then known as LTP1b (Bakan *et al.*, 2009) firstly reported by Evans and Hejgaard (1999). During this time allene oxide synthase and 9-lipoxygenase oxidises linoleic acid to 9,10-allene oxide. This is then further broken down via a nucleophilic attack by Asp^{7}'s carboxylate group and bound to LTP1 to form LTP1b (Bakan *et al.*, 2009). The addition of this lipid like adduct results in LTP1b having a 294 Da higher molecular weight than LTP1 (Matejkova *et al.*, 2009). It was proven by Wijesinha-Bettoni *et al.* (2007) that this modification does not alter the secondary or tertiary structure of LTP1, which points to it being bound within the hydrophobic cavity. The dynamics of the protein was, however, found to be altered, giving it a more loosely packed structure, enabling more molecules to bind in the cavity and increasing its surface activity.

Barley LTP1 has in past studies been characterised on the basis of its antimicrobial qualities, which forms part of the seed's defence system (Molina *et al.*, 1993; Gorjanovic *et al.*, 2005; Yang *et al.*, 2008; Van Nierop *et al.*, 2009). Not only will it be up-regulated as a defence against possible pathogens but is also known to be involved in various other plant stress responses such as drought, chemical shock and temperature changes (Lindorff-Larsen *et al.*, 2001). These small proteins are also suggested intermembrane transporters of lipids, possibly playing a role in the transport of cutin monomers and subsequent assembly of cutin layers as well as in flowering (Lindorff-Larsen *et al.*, 2001; Gorjanovic *et al.*, 2005).

Lipoxygenase 1

Structure and Expression

Lipoxygenase (LOX) enzymes fall in a class of non-heme iron-containing dioxygenases found in numerous animals and plants (Porta & Rocha-Sosa, 2002). LOX catalyses the oxygenation of polyunsaturated fatty acids (PUFAs) containing a (*Z, Z*)-1,4-pentadiene structure

(Porta & Rocha-Sosa, 2002), such as linoleic-, α-linoleic- and arachidonic acids (Liavonchanka & Feussner, 2005) to yield unsaturated fatty acid hydroperoxides (Loiseau *et al.*, 2001; Porta & Rocha-Sosa, 2002). Several structures of LOX have been identified to date and numerous classification systems have been proposed (Loiseau *et al.*, 2001; Liavonchanka & Feussner, 2005). An older classification system divides LOX species into categories depending on catalytic behaviour, i.e. pH for optimal activity. Here, type 1-LOX has an optimal activity pH of 9-10 and type 2 an optimal pH of 6-7 (Loiseau *et al.*, 2001). Position on the fatty acid hydrocarbon backbone where oxygenation occurs is another way of classifying different LOX types. The oxygenation of linoleic acid and α-linoleic acid will either take place at carbon atom 9 or 13 when catalysed by 9-LOX and 13-LOX respectively (Liavonchanka & Feussner, 2005) which in turn will also respectively produce 9-hydroperoxylinoleic acid and 13-hydroperoxylinoleic acid (Loiseau *et al.*, 2001). More recently LOX have been classified on the basis of amino-acid sequence similarity. If the enzyme is harbouring a plastidic transit peptide it is classified as LOX-2 (in some literature also referred to as type 2-LOX) and if no such peptide is present it is known as LOX-1 (in some literature also referred to as type 1-LOX) (Loiseau *et al.*, 2001; Liavonchanka & Feussner, 2005). Both these types of LOX belong to the linoleate 13-LOX subfamily.

LOX-1 in barley seeds are localised in the germ and mainly yields 9-hydroperoxides. It has a molecular mass of about 90 kDa and an isoelectric point of 5.2. The enzyme's pH for optimum activity is ±6.5 (Loiseau *et al.*, 2001). Of all LOX enzymes, LOX-1 from soybean has been the most thoroughly studied with regard to structure. This enzyme possesses two domains namely domain I and II. Domain I comprises of a 146 amino residues less than the 693 residues of domain II's. Domain II also contains the active site involved in the binding of substrate (Nelson & Seitz, 1994). It is also LOX-1 that is predominantly responsible for the oxygenation breakdown of poly-unsaturated fatty acids in barley to form among others some flavour active

compounds (Kuroda *et al.*, 2003). LOX-2, however, is also present in barley but have been proven to only be present after germination (Yang *et al.*, 1993).

Functions of LOX

The precise *in vivo* functionality of LOX is still relatively unclear due to the diversity of the isoenzymes and end-products produced. It has been suggested to play a role in stress response, defence against insects and pathogens (Prost *et al.*, 2005; Zhu-Salzman *et al.*, 2005), wounding (Liavonchanka & Feussner, 2005), growth (Porta & Rocha-Sosa, 2002), development (Porta & Rocha-Sosa, 2002) and senescence. Some isoenzymes are also known to play a role in vegetative storage (Loiseau *et al.*, 2001; Porta & Rocha-Sosa, 2002).

A detailed description of LOX effect on microbial attack will be given below. Various plant defence mechanisms initiated by LOX are present in plants, which are all characterised by an increase in LOX activity (Gardner, 1991a). It has been observed that when a plant is wounded a number of compounds with signalling activity are present, which is then a function of LOX that becomes present and oxylipins are produced as a response to wounding (Porta & Rocha-Sosa, 2002). Some other LOX pathway compounds/products that play a vital role in signalling on wound response as well as attack by insects and animals are jasmonic acid (JA) and phytodienoic acid (OPDA) (Porta & Rocha-Sosa, 2002). It should be noted that when referring to "jasmoids", it groups both JA and other related C_{12} cyclopentanone derivatives (Grechkin, 1998). Other signalling compounds which are up regulated upon wounding are aldehydes, C_6 volatiles and alcohols produced *via* the hydroperoxide lyase (HPL) pathway. Numerous other mechanisms exist in plants as defence against wounding and insect attack, such as the up regulation of volatiles through the LOX pathway induced by specific herbivore traits. Furthermore, the production of volatiles differ between types of infestation and wounds (Porta & Rocha-Sosa, 2002).

LOX enzymes are mainly localised in the cytosol of various cells and as growth precedes this positioning shifts to the vascular bundle surroundings, epidermis and hypodermis (Loiseau *et al.*, 2001). LOX can be seen as a vegetative storage protein (VSP), which plays a role in the regulation of a seed's nitrogen storage and is enhanced by high nitrogen levels as well as sink shortages, wounding, water deficit and JA (Porta & Rocha-Sosa, 2002). Jasmonate, a product of the LOX pathway and growth hormone, which increases during germination, accumulates in sink tissues and is possibly responsible for the regulation of accumulation of storage proteins. This indicates that LOX may be involved in the storage and synthesis of proteins during germination. Evidence also indicates that the various enzymes and intermediates involved in conversion of JA from α-linolenic play a cardinal role in germination and growth (Loiseau *et al.*, 2001). It has been observed that increased amounts of LOX are present in rapidly growing tissue of plants (Terp *et al.*, 2006).

LOX enzymes are extremely important in the food industry due to its involvement in off-flavours and -aroma production (Loiseau *et al.*, 2001). It also plays a vital role in the bread production industry by contributing to the improvement of dough rheology and acting as a bleaching agent (Robinson *et al.*, 1995; Cumbee *et al.*, 1997). In this study the focus will be on the generation of stale flavours of beer during storage, as well as investigating if there is a link between LOX-1 and LTP1. Very little work has been done on the role of LOX in the formation of LTP1b. Bakan *et al.* (2006) identified the reactive oxylipin adduct bound to LTP1 to form LTP1b as α-ketol 9-hydroxy-10-oxo-12(*Z*)-octadecenoic acid (9-HPOD). 9-HPOD is formed by the oxygenation of linoleic acid by LOX-9. In the presence of allene oxide synthase (AOS) hydroperoxides generates an unstable allene oxide which then produces structures such as this α-ketol. The produced α-ketol is specifically trapped by LTP1 to form LTP1b by the consecutive actions of LOX-9 and AOS. In higher plants, the main function of AOS is to supply an allene oxide, derived from linoleic acid which will undergo cyclization in the presence of allene oxide

cyclase to form 12-oxo-10, 15-phytodienic acid, a precursor of the jasmonate oxylipins (Bakan *et al.*, 2006).

Lipid activity of LTP1 and LOX-1

Many of LOX and LTP1 functions seem to overlap. Both are involved in the plant or seed's responses to stressful conditions, both have antimicrobial qualities, both are involved in lipid metabolism and also to some extent synthesis of certain components in seeds and plants (Bakan *et al.*, 2006).

Lipid binding and transport by LTP1

In plant seeds, such as barley kernels, lipids fulfil different key functions. These functions range from the storage of energy to the control of exchanges with environmental constituents (Douliez *et al.*, 2000). Specific lipid-binding proteins are partly responsible for the intra- and extracellular transport of these lipids. These macromolecules include fatty acid binding proteins (FABP) and acyl-coA binding proteins (ACBP) that are capable of binding monoacyl lipids. They also include LTPs that binds monoacyl and diacyl lipids (Douliez *et al.*, 2000).

Although LTP has the ability to enhance, *in vitro*, inter-membrane lipid transfer action, the method of transfer remains to some extent unclear (Douliez *et al.*, 2000). One of the proposed lipid transfer systems involves a shuttle-like mechanism where complexes are formed with LTP to facilitate transport of lipids (Kader, 1996). It is impossible to interpret the binding capacity of plant LTP on the basis of the free protein's tunnel volume. When polar lipids bind to LTP, a hydrogen bond between the tyrosine (on the C-terminal region) and a lipid phosphate or carboxylate group will stabilise the complex (Douliez *et al.*, 2000). Douliez *et al.* (2000) proposed that the mechanism of binding involves the exposure of the hydrophobic cavity due to the opening of the C-terminal region. The crossing of the lipid's polar head with the LTP1 will result in the lipid being sucked up within the protein. The lipid would then also be able to exit the protein with a reverse version of this process or just continue across the protein and be

expulsed on the opposite side (Douliez *et al.*, 2000). It is still unclear if in fact the protein undergoes conformational changes when interacting with membranes or lipids, but a reduction of the disulfide bonds in the protein will inhibit its lipid transfer ability, emphasising the importance of the disulfide bonds in the protein's structure (Kader, 1996). The binding of LTP1 to lipids have been investigated in the past. By understanding the protein's ability to bind different lipids, a better understanding of its functionality can be obtained. Previous studies indicated a binding constant (K_d) of 10^{-2}-10^{-4} M for LTP1 with fatty acids and lysophosphatidylcholine and a K_d of 10^{-6} M for acyl-CoA. These values indicate a very low affinity between the molecules. Douliez *et al.* (2001) found that LTP1 lacks specificity when binding fatty acids and various chain lengths of phospholipids. A K_d value of around 10^{-6} M was determined for these binding equilibria.

Lipid peroxidation by LOX-1

LOX are enzymes responsible for the dioxygenation of *cis, cis*-1,4-pentadiene containing polyunsaturated fatty acids to form *cis, trans*-diene hydroperoxy derivatives (Yang *et al.*, 1993). Various different types of LOX enzymes have been characterised throughout the plant kingdom of which LOX-1 and LOX-2 have been characterised from germinated barley embryos (Schmitt & VanMechelen, 1997). The presence of LOX in barley is an extremely important factor to take into consideration by brewers, since some of the products formed via LOX related pathways will impact the flavour stability of beer (Yang *et al.*, 1993). LOX-1 and LOX-2 forms 9-HPOD and 13-HPOD from linoleic acid respectively and during germination both these enzymes illustrated similar expression patterns. However, it has been proven that LOX-1 accounts for the majority of lipoxygenase activity in the mature barley grain (Schmitt & VanMechelen, 1997).

From Fig. 2 it is apparent that there are three possible enzymatic pathways that the products of lipoxygenase activity can be fed into.

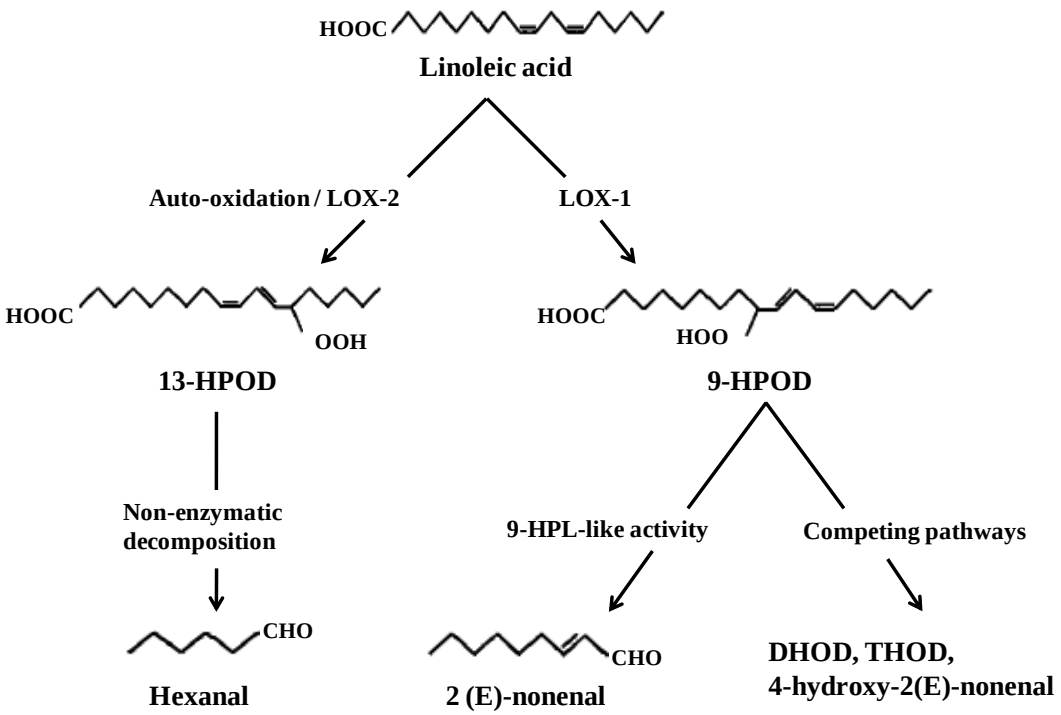

Fig. 2. Pathways associated with the oxidation of linoleic acid to form hydroxyperoxides and aldehydes (Zimmerman & Vick, 1970; Gardner, 1991b; Kuroda *et al.*, 2003).

Each pathway will lead to different sets of lipid-breakdown products (Schmitt & VanMechelen, 1997) of which the 2(*E*)-nonenal, 2,4(*E, E*)-decadienal, hexenal and hexanal are the flavour-active compounds. The product 2(*E*)-nonenal is known to be associated with a cardboard-like flavour even when present at extremely low concentrations (Kuroda *et al.*, 2002). Linoleic acid will be converted to preferably 9-HPOD by LOX-1 during mashing procedures. 13(*S*)-hydroperoxy-9(*Z*), 11(E), 15(*Z*)-octadecatrienoic acid (13-HPOD) will also be produced from linoleic acid to a lesser degree by auto-oxidation (Kuroda *et al.*, 2003) and/or 2-LOX (Schmitt & VanMechelen, 1997). The 2(*E*)-nonenal will be produced from 9-HPOD by the cleavage of the latter by HPL-like activity (Kuroda *et al.*, 2003).

Plant adaption to environmental conditions

A plant's ability to modify and regulate its metabolism under stressful circumstances is of utmost importance for survival. Plants utilise a series of low molecular weight proteins and peptides as their innate defence system to cope with stress factors other than microbial infection.

Other stress factors include chemical exposure, drought and cold (Kader, 1996; Van Nierop *et al.*, 2009). The regulatory patterns of LTP expression in barley under stressful environmental circumstances are extremely complex. Kader (1996) in a review of LTP concluded that LTP production is significantly up-regulated under circumstances involving water deprivation such as salt stresses, drought and cold. This statement is justified by the involvement of LTP in cutin formation under low water availability.

More or less the same up-regulation was observed for LOX and LOX pathway derived products in plants that are under microbial attack (Grechkin, 1998). This defence is aided by the liberation of linoleic acid which is broken down via the LOX pathway to either components that possess antimicrobial qualities or compounds that act as stress signalling molecules (Prost *et al.*, 2005).

Role of LTP1 and LOX-1 in plant defence

Antimicrobial activity of LTP1

Barley's resistance to microbial infection is of great importance for the malting and brewing industry (Gorjanovic *et al.*, 2004). The presence of LTP related proteins has an inhibitory effect on bacterial pathogens and fungi. It was shown that LTPs combined with thionins have a synergistic, inhibitory effect against fungi (Kader, 1996). The antifungal activity of LTPs, however, vary between different pathogens and the extent of infection (Kader, 1996). LTP presumably causes damage to yeast cell membranes which leads to leakage of cell constituents (Gorjanovic *et al.*, 2004); most likely due to its high isoelectric point (Kader, 1996). The basic groups present on LTP molecules appear to be necessary for the proteins to detach from the cell membrane, while it is hypothesised that the hydrophobic domains are inserted into the cell membrane bilayer. According to Gorjanovic *et al*. (2004), it seems possible that LTP forms pores when inserted into the fungal cell membrane and in this way causes cell leakage. The vitality of cells is directly related to membrane integrity and optimal functionality.

Effect of LTP1 on brewer's yeast

Previous research showed that LTP1 inhibits fermentation by *Saccharomycess cereviseae* (Gorjanovic *et al.*, 2005; Stanislava, 2007) by preventing respiration and incorporation of sugars into the yeast cell membrane. It proved to be membrane active, causing ruptures in the yeast cell membrane that results in leakage of certain cell constituents. At high enough concentrations (4 µg/mL) it causes cell death. This theory was later verified for LTP1 that had not been exposed to high brewing temperatures, but contradicted when taking the high temperatures' effect on the protein into account (Van Nierop *et al.*, 2006). Gorjanovic *et al.* (2005) found that vital cell functions of brewer's yeast, *S. cerevisiae,* were impaired in the presence of LTP1 due to its inhibitory effect on the yeast's respiration. The concentration of LTP required, according to Gorjanovic *et al*. (2005), for 50% inhibition after a 24 hour incubation period (IC_{50}) is 100 and 80 µg/mL for *S. cerevisiae* and *Fusarium solani,* respectively. Gorjanovic *et al*. (2004) also found that LTP1 loses its ability to inhibit yeast growth after the mashing process, although it has been proven that this protein only completely denatures at temperatures above 100°C (Mills *et al.*, 2009). Van Nierop *et al.* (2005*;* 2006)*,* however, showed that the antimicrobial effect of LTP1 on brewer's yeast stayed intact throughout the brewing process and the most recent work by (Jiang *et al.*, 2011) showed that the inhibition of LTP1 towards yeast still occur after a 100°C treatment.

LOX-1 in plant defence

Plants are perpetually exposed to attack by various microorganisms and have therefore developed mechanisms of preventing, or at least limiting, such attacks. When a pathogen is present the plant will recognise it by pathogen-derived-molecules binding to receptors. This binding triggers defence-signalling pathways which activates various defence responses (Laxalt & Munnik, 2002). It is known that certain LOX pathway intermediates and products possesses antimicrobial properties (Gardner, 1991a; Grechkin, 1998) due to the various signalling functions of some (Porta & Rocha-Sosa, 2002). The breakdown of signalling components is

possibly due to the liberation of linoleic acid when plants are confronted with a stressful circumstance (Grechkin, 1998). LOX pathway products, 9(*S*)-hydroperoxy-10(*E*), 12(Z)-octadecadienoic acid (9-HPOD), 9(*S*)-hydroperoxy-10(*E*), 12(*Z*), 15(*Z*)-octadecatrienoic acid (9-HPOT),13(*S*)-hydroperoxy-9(*Z*), 11(*E*)-octadecadienoic acid (13-HPOD) and 13(*S*)-hydroperoxy-9(*Z*), 11(E), 15(*Z*)-octadecatrienoic acid (13-HPOT) are those possibly responsible for the signalling activities (Prost *et al.*, 2005) and anti-microbial action (Grechkin, 1998). Other such compounds include oxylipins, generated by α-doxygenase (α-DOX), and various 13-LOX derived components, which includes Jasmonic acid (JA), 12-Oxo-PDA, methyl jasmonate, C_6 aldehydes derived from 13-HPL, 13(*S*)-hydroxy-9(*Z*), 11(*E*), 15(Z)-octadecatrienoic acid (13-HOT), 13(*S*)-hydroperoxy-9(*Z*), 11(*E*), 15(*Z*)-octadecatrienoic acid (13-HPOT) and keto-octadecatrienoic acids (KOT's) and keto-octadecadienoic acids (KOD's) (Prost *et al.*, 2005). JA is synthesised as a product of a 13-hydroperoxide derived from linoleic acid (Terp *et al.*, 2006).

Oxylipins, 13-HPOT and 13-HOT, colnelenic acid, colneleic acid and some epoxy- or polyhydroxylated fatty acids are produced to serve as defence against pathogenic attack, in particular fungal infections (Prost *et al.*, 2005). Although the role of LOX in pathogen resistance have been recognised, some aspects still remain unclear and thus needs further characterisation (Loiseau *et al.*, 2001).

LTP1, LOX-1 and AROP in beer brewing

Influence of brewing temperature on LTP1 and LOX-1

Temperature stability of LTP1

The influence of temperature on LTP1 is directly linked to the quality of beer foam, making this field of study of great importance for brewers (Van Nierop *et al.*, 2004). Brewing of beer consist of steps where heat is necessary to activate or inactivate certain processes. Barley LTPs have been proven to survive the germination step, where it is exposed to protease (Evans &

Hejgaard, 1999). The malting step can be altered or modified to the brewer's desire to control foam stability (Okada *et al.*, 2008). It is, however, important to insure optimum malt modification, since an under-modified malt can increase foam head as well as viscosity (Bamforth, 1985b). Partially modified malt can also result in insufficient malt extract yield (Okada *et al.*, 2008).

Other steps in the brewing process involving heat exposure include the **(1)** mashing step, where the ground malt is mixed with water and heated (60-70°C) to activate enzymes that continue the breakdown of endosperm reserves, a process that initially started during malting. Sweet wort is produced during **(2)** lautering, which separates the insoluble fraction from the hot mash. Another high temperature involved step in the brewing process is **(3)** wort boiling (±100°C). Here, wort is boiled in a kettle to inactivate enzymes, sterilize the wort, remove any undesirable flavour compounds, precipitate haze-forming proteins and polyphenols and isomerise hop α-acids. During **(4)** fermentation, which takes place at about 11°C, wort sugar and nutrients are converted by yeast cells to alcohol, carbon dioxide and flavour components. The **(5)** maturation process allows the final yeast and haze component settlement and the removal of undesirable flavour components formed during secondary fermentation. It involves a temperature decrease to 2°C. The final temperature involved processes in beer making are the **(6)** filtration and packaging steps. Filtration produces a clear, bright beer, which is then anaerobically packaged into sterile containers. LTP1 is not only resistant toward protease attack, but is relatively heat stable (Lindorff-Larsen & Winther, 2001) and only undergoes a phase change around 100°C (Mills *et al.*, 2009). The denaturation of LTP1 is due to the reduction of its disulphide bridges (Perrocheau *et al.*, 2006). Although LTP1 undergoes heat denaturation, necessary for its foam promoting functions, there seems to be a limit to which this denaturation is desirable (Van Nierop *et al.*, 2004).

Under circumstances where barley is exposed to high temperatures for long periods of time, such as during malting, starch is degraded yielding high amounts of monosaccharides. Due to the Maillard reaction the monosaccharide, D-glucose, reacts with a free ε-amino group from lysine and the guanidine group from arginine in a process called glycation (Petry-Podgórska *et al.*, 2010). This non-enzymatic glycation reaction yields a compound known as a Schiff base that rearranges itself to form a more stable amino acid complex called an Amadori compound (Petry-Podgórska *et al.*, 2010). The glycation of LTP1 prevents its precipitation and unfolding to some extent during wort boiling. According to Jegou *et al*. (2001), this precipitation should ensure better adsorption of the protein at the air-water interface of the beer foam by increasing the amphiphilicity. Only a fraction of the LTP1s are denatured or exposed to glycation (Jegou *et al.*, 2001). Mills *et al.* (2009) verified this hypothesis by indicating that after extensive heating, a portion of the protein did not become denatured and retained its native form, although the majority unfolded to become surface active. In its native form, LTP1 displays poor foam promoting qualities (Jegou *et al.*, 2001) and are weak emulsifiers (Mills *et al.*, 2009), therefore only contributes to this quality aspect in its unfolded form which occurs after wort boiling (Jegou *et al.*, 2001).

It can be concluded that temperature control during the brewing process is highly influential on the final product's quality. During wort boiling LTP1 undergoes a molecular mass shift from 9.633 kDa to 9.6-9.99 kDa (Jin *et al.*, 2009). It was found that wort boiling temperatures between 103 and 110°C accelerated protein coagulation, hop-α-acid isomerisation and also increased the rate of dimethyl sulphide stripping in wort. Higher wort boiling temperatures (~102°C) also reduces the level of LTP1 (Van Nierop *et al.*, 2004; Jin *et al.*, 2009). The same is true for the opposite, since lower wort boiling temperatures at ~96°C yielded higher LTP1 levels (Jin *et al.*, 2009). It is therefore recommended that wort boiling temperatures should not exceed 103-104°C (Van Nierop *et al.*, 2004).

Only a few studies conducted on the thermal unfolding of LTP1 take glycation into account (Perrocheau *et al.*, 2005; Perrocheau *et al.*, 2006; Petry-Podgórska *et al.*, 2010). Another reaction that occurs during mashing procedures is acylation (Jin *et al.*, 2009). Acylation is the grafting of acyl chains on LTP1, which may influence its lipid transfer properties. According to Pato *et al*. (2002) this increases the hydrophobicity of the protein surface and in effect increases LTP1's lipid transfer ability (Pato *et al.*, 2002)

It was found by Lindorff-Larsen *et. al.* (2001) and Bakan *et. al.* (2006) that the lipid modified version of LTP1, known as LTP1b was more heat stable due to the presence of oxilipin and these species had a 15°C higher melting point than LTP1.

Temperature stability of LOX-1

Temperature differences between different brewing practices will influence LOX activity as observed by Kobayashi *et al.,* (1993). The optimum LOX enzyme activity has been reported to be at a pH of 6 and a temperature of 47°C, although the enzyme is stable up to 50°C, but completely inactivated at 65°C. Therefore as the mashing temperature rises it will lead to a decrease in lipoxygenase activity until it becomes completely inactive at 65°C. It was also shown that malt with higher lipoxygenase activity will produce more hydroperoxides during the mashing procedures and that the oxidation via enzymatic pathways is preferred to auto-oxidation (Kobayashi *et al.,* (1993). It was, however, shown that the formation of hexanal was completed non-enzymatic, since no concentration differences were observed prior and post mashing (Kuroda *et al.*, 2003). According to findings by Kuroda *et al.* (2003), LOX-2 is mainly inactivated by the kilning process leaving LOX-1 as the main lipoxygenase in the system. This then leads to 9-HPOD being the major product formed due to oxidation by LOX-1, although some 13-HPOD will still be formed possibly via auto-oxidation. 9-HPOD serves as a precursor for among others, 2(*E*)-nonenal, an undesirable flavour component. It was suggested that the use of heat labile LOX or 9-HPL-like (enzyme responsible for the conversion of 9-HPOD to the undesirable flavour component) activity will reduce the production of 2(*E*)-nonenal (Kuroda *et*

al., 2003). It is also during this process where the use of anti-oxidants could help prevent the flavour degradation (Vanderhaegen *et al.*, 2006).

LTP1, LOX-1 and AROP in beer quality

LTP1 as beer quality enhancer

The stability and quality of beer foam is an essential part in beer quality evaluation (Evans & Bamforth, 2009). A variety of factors play a role in the stabilisation and destabilisation of beer foam. Foam primarily stabilised by proteins is extremely prone to disruptions such as lipid destabilisation (Clark et al., 1994). LTP1 contributes to the stabilisation of beer foam in more than one way. Firstly it is proposed that LTP1, together with protein Z, are the key foam proteins. An alternative theory is that a wider variety of polypeptides contributes to the formation of foam, due to their hydrophobic nature. Van Nierop et al. (2004) also stated that LTP1 is responsible for foam formation in conjunction with other proteins. This theory is supported by the fact that the denaturation of LTP1 leads to the increase in internal hydrophobicity (Kapp & Bamforth, 2002) and amphiphilicity (Marion et al., 2007) of the protein structure and so improves its foam stabilising qualities (Kapp & Bamforth, 2002). During malting the protein is subjected to acylation and glycation, while during brewing the protein unfolds (Marion *et al*., 2007). LTP1 can be present in beer in conjunction with a lipid like adduct and is then known as LTP1b. Mills *et al.* (2009) showed that both these forms of LTP1s are surface active after heating, although LTP1b shows higher surface activity then LTP1. It was proposed that the lipid like adduct on LTP1b increases the molecular flexibility of the molecule that in turn is able to change internal stress. This highlights the fact that both the hydrophobicity of the surface and molecular flexibility plays a vital role in surface activity (Mills et al., 2009).

The *in vitro* functions of LTP1 include the intermembrane transfer of lipids. This function makes this protein of great biotechnological importance (Douliez *et al.*, 2000). In the brewing industry this quality contributes to the protein's ability to stabilise beer foam, by binding foam

destabilising lipids. This protein, however, must be subjected to mild modification before it becomes foam-stabilising. Gorjanovic *et al*. (2005) found that beer-LTP1 displayed better foaming properties than barley-LTP1, with the only difference between the two LTP1s being the heat exposure in the case of beer-LTP1.

It has never been observed that the presence of LTP1 will limit the formation of 2(*E*)-nonenal *via* the LOX-1 pathway, but it seems to be highly likely. LTP1 binds a linoleic acid derivative, which if broken down further will form 2(*E*)-nonenal (Bakan *et al.*, 2006). If a high amount of LTP1 is available, it could inhibit the formation of the stale cardboard flavour in beer by holding on to the necessary intermediates, i.e. α-ketol 9-hydroxy-10-oxo-12(Z)-octadecenoic acid. Besides from possibly being involved in the LOX pathway, LTP1 has recently also been observed to have antioxidant activity (Wu *et al.*, 2011), which strengthens the belief of its positive impact on flavour.

LOX-1 and beer quality

Numerous factors can play a role in the flavour stability of beer, but in more recent times the focus has shifted towards the effect raw materials and malting techniques have on flavour stability (Guido *et al.*, 2005). The staling of aged beers are mainly contributed to the formation and/or presence of long chain aldehydes, ketones and esters (Guido *et al.*, 2005). The substance in aged beer responsible for the characteristic stale, cardboard flavour is known as 2(*E*)-nonenal (Kuroda *et al.*, 2002) and is especially a topic of interest due to its very low flavour threshold of 0.035 µg/L (Guido *et al.*, 2005). This compound is formed due to the oxidation of lipids, especially linoleic acid, naturally present in malt, and only becomes apparent in beer after storage for a long period of time at ambient temperatures or higher (Kuroda *et al.*, 2005). The development of 2(*E*)-nonenal in aged beer has been correlated to the enzymatic oxidation of lipids by malt LOX-1. During mashing malt LOX-1 produces a compound, 9-hydroperoxy-10(*E*), 12(*Z*)-octadecadienoic acid (9-HPOD), which can then be further degraded to 2(*E*)-

nonenal and some other aldehydes by 9-fatty acid hydroperoxide lyase (9-HPL-like activity) (Kuroda *et al.*, 2003).

It is necessary for the brewer to determine whether certain malts are more prone to staling due to oxidised lipid breakdown when aged, since this will lead to the product having an undesired cardboard/fatty/glassy odour and taste. To determine to what extent a product has the potential to stale, a number of factors should be taken into consideration. In the past, LOX-1 activity determination has been used as an indication of the potential formation of the nonenal compound

The testing of LOX-1 activity alone is, however, not the only determining factor that plays a role in the formation of nonenal compounds (Guido *et al.*, 2005). The natural presence of anti-oxidants in beer will also affect the degree of lipid oxidation, since these will scavenge any radicals formed during lipid peroxidation and so promote a more stable flavour (Takashio & Shinotsuka, 1998; Guido *et al.*, 2005). For the measurement of total anti-radical/oxidant potential (AROP) a number of existing assays are also available (Re *et al.*, 1999) (these will be mentioned in Chapter 4).

It can be deducted that if a specific malt has a high LOX-1 activity it does not necessarily mean that it will produce a product with a shortened shelf life, since this enzyme's activity could be countered if that malt should contain a high AROP concentration (Bamforth, 1999a). This will then lead to a low potential to form nonenal compounds or a lower residual nonenal potential (RNP). The same is true for the opposite. It is clear from the last statement that it will not be sufficient to assess only one parameter to determine the potential flavour stability of a malt.

AROP in beer quality

One of the major problems faced in the brewing word today is beer shelf life. Oxygen in the packaged product is one of the main causes for flavour deterioration, by initiating numerous

chemical reactions (Vanderhaegen *et al.*, 2006). Antiradical/antioxidant species are known to delay such oxygen mediated reactions (Samaras *et al.*, 2005b), such as the LOX mediated reaction to form the cardboard taint of 2(*E*)-nonenal (Goupy *et al.*, 1999). These antiradical/antioxidant species are being considered by researchers as a means to lengthen shelf-life by increasing its endogenous concentration in the product (Bamforth, 1999a; Zhao *et al.*, 2010). Not only is the AROP in the product beneficial for the stability of flavour, but it also contributes to beer's astringent mouth feel and colour. It also plays a role in browning and chill haze formation (Goupy *et al.*, 1999). The AROP of beer is introduced by the raw materials, primarily by the malt, but also by the hops, yeast and additives (Zhao *et al.*, 2008). Various components present in malt and beer can contribute to the total AROP of the product. These include vitamins (e.g. ascorbic acid), cartenoids, thiols (Samaras *et al.*, 2005a), phenolic compounds, sulphite, melanoidins (from Maillard reactions) and chelating agents (such as phytic acid) (Vanderhaegen *et al.*, 2006). The AROP profiles of beers are also affected by the process of brewing (Maillard & Berset, 1995; Lu *et al.*, 2007). It is thus safe to say that AROP in beer will vary significantly depending on the raw material and brewing practices (Zhao *et al.*, 2010).

The screening of malts with high AROP could possibly insure a beer with a high radical scavenging potential and could extend shelf-life (Zhao *et al.*, 2008). It would therefore be of great benefit to have such a screening method designed to be applicable in a brewery laboratory setup. Such an assay should be economic, fast, reliable, robust and should not require overly-specialised equipment.

Malt proteins LTP1 and LOX-1 together with the natural antioxidants present play a vital role in the stability of beer and foam. By including these factors in the biochemical evaluation of malt to predict end product quality or at least elucidate mode of action and impact could be extremely beneficial for brewers.

Reference

Asano, K., Shinagawa, K. & Hashimoto, N. (1982). Characterization of haze-forming proteins of beer and their roles in chill haze formation. *American Society of Brewing Chemists*, **40**, 147-154.

Bakan, B., Hamberg, M., Larue, V., Prangé, T., Marion, D. & Lascombe, M.B. (2009). The crystal structure of oxylipin-conjugated barley LTP1 highlights the unique plasticity of the hydrophobic cavity of these plant lipid-binding proteins. *Biochemical and Biophysical Research Communications*, **390**, 780-785.

Bakan, B., Hamberg, M., Perrocheau, L., Maume, D., Rogniaux, H., Tranquet, O., Rondeau, C., Blein, J.P., Ponchet, M. & Marion, D. (2006). Specific adduction of plant lipid transfer protein by an allene oxide generated by 9-lipoxygenase and allene oxide synthase. *Journal of Biological Chemistry*, **281**, 38981-38988.

Bamforth, C.W. (1985a). Cambridge prize lecture biochemical approaches to beer quality. *Journal of the Institute of Brewing*, **91**, 154-160.

Bamforth, C.W. (1985b). The foaming properties of beer. *Journal of the Institute of Brewing*, **91**, 370-383.

Bamforth, C.W. (1999). Bringing matters to a head: The status of research on beer foam. In: *Monograph-European Brewery Convention Monograph*, XXVII, Amsterdam, pp. 10-23.

Bamforth, C.W. (1999a). Enzymatic and non-enzymatic oxidation in the brewhouse: A theoretical consideration. *Journal of the Institute of Brewing*, **105**, 237-242.

Bamforth, C.W. (1999b). The science and understanding of the flavour stability of beer: A critical review. *Brauwelt International*, **17**,98-110.

Bamforth, C.W. (2000). Brewing and brewing research: Past, present and future. *Journal of the Science of Food and Agriculture*, **80**, 1371-1378.

Carvalho, A.d.O. & Gomes, V.M. (2007). Role of plant lipid transfer proteins in plant cell physiology - a concise review. *Peptides*, **28**, 1144-1153.

Clark, D.C., Wilde, P.J. & Marion, D. (1994). The protection of beer foam against lipid-induced destabilization. *Journal of the Institute of Brewing*, **100**, 23-25.

Cumbee, B., Hildebrand, D. & Addo, K. (1997). Soybean flour lipoxygenase isozymes effects on wheat flour dough rheological and breadmaking properties. *Journal of Food Science*, **62**, 281-283.

Dalgliesh, C. (1977). Flavour stability of beer. In: *Proceedings of the 16th congress-European Brewery Convention.* Dordrecht, Amsterdam (Netherlands), pp. 623-659.

De Keukeleire, D. (2000). Fundamentals of beer and hop chemistry. *Quimica Nova*, **23**, 108-112.

Delvaux, F., Daems, V., Vanmechelen, H. & Neven, H. (1995). Retention of beer flavours by the choice of an appropriate glass. In: P*roceedings of Congress-European Brewery Convention*, Brussels. **25**. pp. 533-533.

Douliez, J.P., Jegou, S., Pato, C., Molle, D., Tran, V. & Marion, D. (2001). Binding of two mono-acylated lipid monomers by the barley lipid transfer protein, LTP1, as viewed by fluorescence, isothermal titration calorimetry and molecular modelling. *European Journal of Biochemistry*, **268**, 384-388.

Douliez, J.P., Michon, T., Elmorjani, K. & Marion, D. (2000). Structure, biological and technological functions of lipid transfer proteins and indolines, the major lipid binding proteins from cereal kernels. *Journal of Cereal Science*, **32**, 1-20.

Evans, D.E. & Bamforth, C.W. (2009). *Beer foam: Achieving a suitable head.* In: Handbook of alcoholic beverages: Beer, a quality perspective, C. W. Bamforth, Russel, I., & Stewart, G. G. Ed, Elsevier: Burlington, MA, pp. 1-60.

Evans, D.E. & Hejgaard, J. (1999). The impact of malt derived proteins on beer foam quality. Part I. The effect of germination and kilning on the level of protein Z4, protein Z7 and LTP1. *Journal of the Institute of Brewing*, **105**, 159-169.

Evans, D.E., Robinson, L.H., Sheehan, M.C., Tolhurst, R.L., Hill, A., Skerritt, J.S. & Barr, A.R. (2003). Application of immunological methods to differentiate between foam-positive and haze-active proteins originating from malt. *Journal of the American Society of Brewing Chemists*, **61**, 55-62.

Evans, D.E., Sheehan, M.C. & Stewart, D.C. (1999). The impact of malt derived proteins on beer foam quality. Part II: The influence of malt foam-positive proteins and non-starch polysaccharides on beer foam quality. *Journal of the Institute of Brewing*, **105**, 171-177.

Garcia-Casado, G., Crespo, J.F., Rodriguez, J. & Salcedo, G. (2001). Isolation and characterization of barley lipid transfer protein and protein Z as beer allergens. *Journal of Allergy and Clinical Immunology*, **108**, 647-649.

Gardner, H.W. (1991). Recent investigations into the lipoxygenase pathway of plants. *Biochimica et Biophysica Acta*, **1084**, 221-239.

Gorjanovic, S., Spillner, E., Beljanski, M.V., Gorjanovic, R., Pavlovic, M. & Gojgic-Cvijanovic, G. (2005). Malting barley grain non-specific lipid-transfer protein (ns-LTP): Importance for grain protection. *Journal of the Institute of Brewing*, **111**, 99-104.

Gorjanovic, S., Suznjevic, D., Beljanski, M., Ostojic, S., Gorjanovic, R., Vrvic, M. & Hranisavljevic, J. (2004). Effects of lipid-transfer protein from malting barley grain on brewers yeast fermentation. *Journal of the Institute of Brewing*, **110**, 297-302.

Goupy, P., Hugues, M., Boivin, P. & Amiot, M.J. (1999). Antioxidant composition and activity of barley (hordeum vulgare) and malt extracts and of isolated phenolic compounds. *Journal of the Science of Food and Agriculture*, **79**, 1625-1634.

Grechkin, A. (1998). Recent developments in biochemistry of the plant lipoxygenase pathway. *Progress in Lipid Research*, **37**, 317-352.

Guido, L.F., Boivin, P., Benismail, N., Goncalves, C.R. & Barros, A.A. (2005). An early development of the nonenal potential in the malting process. *European Food Research and Technology*, **220**, 200-206.

Heinemann, B., Andersen, K.V., Nielsen, P.R., Bech, L.M. & Poulsen, F.M. (1996). Structure in solution of a four-helix lipid binding protein. *Protein Science*, **5**, 13-23.

Igyor, M.A., Ogbonna, A.C. & Palmer, G.H. (2001). Effect of malting temperature and mashing methods on sorghum wort composition and beer flavour. *Process Biochemistry*, **36**, 1039-1044.

Intelmann, D. & Hofmann, T. (2010). On the autoxidation of bitter-tasting iso-α-acids in beer. *Journal of Agricultural and Food Chemistry*, **58**, 5059-5067.

Jegou, S., Douliez, J.P., Molle, D., Boivin, P. & Marion, D. (2001). Evidence of the glycation and denaturation of LTP1 during the malting and brewing process. *Journal of Agricultural and Food Chemistry*, **49**, 4942-4949.

Jiang, X.L., Dong, X.Y., Yao, J., Dong, L.A., Chen, X., Li, M.D. & Zhao, C.X. (2011). Barley protein function as growth inhibitor in *Saccharomyces cerevisiae*. *Journal of Plant Biochemistry and Biotechnology*, **20**, 134-137.

Jin, B., Liu, G.-Q., Zhu, Y.-K. & Liao, L.-N. (2009). Structural changes of malt proteins during boiling. *Molecules*, **14**, 1081-1097.

Kader, J. (1975). Proteins and the intracellular exchange of lipids: I. Stimulation of phospholipid exchange between mitochondria and microsomal fractions by proteins isolated from potato tuber. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, **380**, 31-44.

Kader, J.C. (1996). Lipid-transfer proteins in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, **47**, 627-654.

Kalla, R., Shimamoto, K., Potter, R., Nielsen, P.S., Linnestad, C. & Olsen, O.-A. (1994). The promoter of the barley aleurone-specific gene encoding a putative 7 kDa lipid transfer protein confers aleurone cell-specific expression in transgenic rice. *The Plant Journal*, **6**, 849-860.

Kapp, G.R. & Bamforth, C.W. (2002). The foaming properties of proteins isolated from barley. *Journal of the Science of Food and Agriculture*, **82**, 1276-1281.

Kobayashi, N., Kaneda, H., Kano, Y. & Koshino, S. (1993). The production of linoleic and linolenic acid hydroperoxides during mashing. *Journal of Fermentation and Bioengineering*, **76**, 371-375.

Kuroda, H., Furusho, S., Maeba, H. & Takashio, M. (2003). Characterization of factors involved in the production of 2(E)-nonenal during mashing. *Bioscience, Biotechnology, and Biochemistry*, **67**, 691-697.

Kuroda, H., Kobayashi, N., Kaneda, H., Watari, J. & Takashio, M. (2002). Characterization of factors that transform linoleic acid into di- and trihydroxyoctadeconoic acids in mash. *Journal of Bioscience and Bioengineering*, **93**, 73-77.

Kuroda, H., Kojma, H., Kaneda, H. & Takashio, M. (2005). Characterization of 9-fatty acid hydroperoxide lyase-like activity in germinationg barley seeds that transform 9(S)-hydroperoxy-10(E), 12(Z)-oktadecadienoic acid into 2(E)-nonenal. *Bioscience, Biotechnology, and Biochemistry*, **69**, 1661-1668.

Laxalt, A.M. & Munnik, T. (2002). Phospholipid signalling in plant defence. *Current Opinion in Plant Biology*, **5**, 332-338.

Liavonchanka, A. & Feussner, I. (2005). Lipoxygenases: Occurrence, functions and catalysis. *Journal of Plant Physiology*, **163**, 348-357.

Lindorff-Larsen, K., Lerche, M.H., Poulsen, F.M., Roepstorff, P. & Winther, J.R. (2001). Barley lipid transfer protein, LTP1, contains a new type of lipid-like post-translational modification. *Journal of Biological Chemistry*, **276**, 33547-33553.

Lindorff-Larsen, K. & Winther, J.R. (2001). Surprisingly high stability of barley lipid transfer protein, LTP1, towards denaturant, heat and proteases. *FEBS Letters*, **488**, 145-148.

Lodolo, E.J., Kock, J.L.F., Axcell, B.C. & Brooks, M. (2008). The yeast *Saccharomyces cerevisiae* - the main character in beer brewing. *Federation of European Microbiological Societies*, **8**, 1018-1036.

Loiseau, J., Vu, B.L., Macherel, M.H. & Le Deunff, Y. (2001). Seed lipoxygenases: Occurrence and functions. *Seed Science Research*, **11**, 199-211.

Lu, J., Zhao, H., Chen, J., Fan, W., Dong, J., Kong, W., Sun, J., Cao, Y. & Cai, G. (2007). Evolution of phenolic compounds and antioxidant activity during malting. *Journal of Agricultural and Food Chemistry*, **55**, 10994-11001.

Lustig, S. (1999). Analytical tools and practical options to improve flavour stability. *Ferment*, **12**, 25-29.

Maillard, M.-N. & Berset, C. (1995). Evolution of antioxidant activity during kilning: Role of insoluble bound phenolic acids of barley and malt. *Journal of Agricultural and Food Chemistry*, **43**, 1789-1793.

Marion, D., Bakan, B. & Elmorjani, K. (2007). Plant lipid binding proteins: Properties and applications. *Biotechnology Advances*, **25**, 195-197.

Matejkova, M., Zidkova, J., Zidek, L., Wimmerova, M., Chmelik, J. & Sklenar, V. (2009). Investigation of thermal denaturation of barley nonspecific lipid transfer protein 1 (ns-LTP1b) by nuclear magnetic resonance and differential scanning calorimetry. *Journal of Agricultural and Food Chemistry*, **57**, 8444-8452.

Mills, E.N.C., Gao, C., Wilde, P.J., Rigby, N.M., Wijesinha-Bettoni, R., Johnson, V.E., Smith, L.J. & Mackie, A.R. (2009). Partially folded forms of barley lipid transfer protein are more surface active. *Biochemistry*, **48**, 12081-12088.

Molina, A., Segura, A. & Garciaolmedo, F. (1993). Lipid transfer proteins (nsLTPs) from barley and maize leaves are protein inhibitors of bacterial and fungal plant-pathogens. *FEBS Letters*, **316**, 119-122.

Nelson, M.J. & Seitz, S.P. (1994). The structure and function of lipoxygenase. *Current Opinion in Structural Biology*, **4**, 878-884.

Okada, Y., Limure, T., Takoi, K., Kaneko, T., Kihara, M., Hayashi, K., Ito, K., Sato, K. & Takeda, K. (2008). The influence of barley malt protein modification on beer foam stability and their relationship to the barley dimeric alpha-amylase inhibitor-1 (BDAI-1) as a possible foam-promoting protein. *Journal of Agricultural and Food Chemistry*, **56**, 1458-1464.

Pato, C., Tran, V., Marion, D. & Douliez, J.P. (2002). Effects of acylation on the structure, lipid binding, and transfer activity of wheat lipid transfer protein. *Journal of Protein Chemistry*, **21**, 195-201.

Perrocheau, L., Bakan, B., Boivin, P. & Marion, D. (2006). Stability of barley and malt lipid transfer protein 1 (LTP1) towards heating and reducing agents: Relationships with the brewing process. *Journal of Agricultural and Food Chemistry*, **54**, 3108-3113.

Perrocheau, L., Rogniaux, H., Boivin, P. & Marion, D. (2005). Probing heat-stable water-soluble proteins from barley to malt and beer. *Proteomics*, **5**, 2849-2858.

Petry-Podgórska, I., Zídková, J., Flodrová, D. & Bobálová, J. (2010). 2D-HPLC and MALDI-TOF/TOF analysis of barley proteins glycated during brewing. *Journal of Chromatography*, **878**, 3143-3148.

Porta, H. & Rocha-Sosa, M. (2002). Plant lipoxygenases. Physiological and molecular features. *Plant Physiology*, **130**, 15-21.

Prost, I., Dhondt, S., Rothe, G., Vicente, J., Rodriguez, M.J., Kift, N., Carbonne, F., Griffiths, G., Esquerre-Tugaye, M.T., Rosahl, S., Castresana, C., Hamberg, M. & Fournier, J. (2005).

Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defense against pathogens. *Plant Physiology*, **139**, 1902-1913.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine*, **26**, 1231-1237.

Robinson, D.S., Wu, Z., Domoney, C. & Casey, R. (1995). Lipoxygenases and the quality of foods. *Food Chemistry*, **54**, 33-43.

Samaras, T.S., Camburn, P.A., Chandra, S.X., Gordon, M.H. & Ames, J.M. (2005a). Antioxidant properties of kilned and roasted malts. *Journal of Agricultural and Food Chemistry*, **53**, 8068-8074.

Samaras, T.S., Gordon, M.H. & Ames, J.M. (2005b). Antioxidant properties of malt model systems. *Journal of Agricultural and Food Chemistry*, **53**, 4938-4945.

Schmitt, N.F. & VanMechelen, J.R. (1997). Expression of lipoxygenase isoenzymes in developing barley grains. *Plant Science*, **128**, 141-150.

Shellhammer, T.H. (2009). *Beer colour.* In: Handbook of alcoholic beverages: Beer, a quality perspective, C. W. Bamforth, Russel, I., & Stewart, G. G. Ed, Elsevier: Burlington, MA, pp. 213-228

Siebert, K. & Knudson, E. (1989). The relationship of beer high molecular weight protein and foam. *Technical quarterly-Master Brewers Association of the Americas*, **26(4)**, 139-146.

Slack, P. & Bamforth, C. (1983). The fractionation of polypeptides from barley and beer by hydrophobic interaction chromatography: The influence of their hydrophobicity on foam stability. *Journal of the Institute of Brewing*, **89**, 397-401.

Sorensen, S., Bech, L., Muldbjerg, M., Beenfeldt, T. & Breddam, K. (1993). Barley lipid transfer protein 1 is involved in beer foam formation. *Technical Quarterly*, **30(4)**, 136-145.

Stanislava, G. (2007). Barley grain non-specific lipid-transfer proteins (ns-LTPs) in beer production and quality. *Journal of the Institute of Brewing*, **113**, 310-324.

Takashio, M. & Shinotsuka, K. (1998). Preventive production of beer against oxidation - recent advances in brewing technology. *Food Science and Technology International Tokyo*, **4**, 169-177.

Terp, N., Goebel, C., Brandt, A. & Feussner, I. (2006). Lipoxygenases during brassica napus seed germination. *Phytochemistry*, **67**, 2030-2040.

Van Nierop, S.N.E. (2005). Investigation of malt factors that influence beer production and quality. PhD Department Biochemistry, University of Stellenbosch, South Africa.

Van Nierop, S.N.E., Axcell, B.C., Cantrell, I.C. & Rautenbach, M. (2009). Quality assessment of lager brewery yeast samples and strains using barley malt extracts with anti-yeast activity. *Food Microbiology*, **26**, 192-196.

Van Nierop, S.N.E., Evans, D.E., Axcell, B.C., Cantrell, I.C. & Rautenbach, M. (2004). Impact of different wort boiling temperatures on the beer foam stabilizing properties of lipid transfer protein 1. *Journal of Agricultural and Food Chemistry*, **52**, 3120-3129.

Van Nierop, S.N.E., Rautenbach, M., Axcell, B.C. & Cantrell, I.C. (2006). The impact of microorganisms on barley and malt quality - a review. *Journal of American Society of Brewing Chemists*, **64**, 69-78.

Vanderhaegen, B., Neven, H., Verachtert, H. & Derdelinckx, G. (2006). The chemistry of beer aging - a critical review. *Food Chemistry*, **95**, 357-381.

Wijesinha-Bettoni, R., Gao, C., Jenkins, J.A., Mackie, A.R., Wilde, P.J., Mills, E. & Smith, L.J. (2007). Post-translational modification of barley LTP1b: The lipid adduct lies in the hydrophobic cavity and alters the protein dynamics. *FEBS Letters*, **581**, 4557-4561.

Wu, M.J., Clarke, F.M., Rogers, P.J., Young, P., Sales, N., O'Doherty, P.J. & Higgins, V.J. (2011). Identification of a protein with antioxidant activity that is important for the protection against beer ageing. *International Journal of Molecular Sciences*, **12**, 6089-6103.

Yang, G.S., Schwarz, P.B. & Vick, B.A. (1993). Purification and characterization of lipoxygenase isoenzymes in germinating barley. *Cereal Chemistry*, **70**, 589-595.

Yang, X.Y., Wang, X.W., Li, X.B., Zhang, B.B., Xiao, Y.H., Li, D.M., Xie, C.J. & Pei, Y. (2008). Characterization and expression of an nsLTPs-like antimicrobial protein gene from motherwort (leonurus japonicus). *Plant Cell Reports*, **27**, 759-766.

Zhao, H., Chen, W., Lu, J. & Zhao, M. (2010). Phenolic profiles and antioxidant activities of commercial beers. *Food Chemistry*, **119**, 1150-1158.

Zhao, H., Fan, W., Dong, J., Lu, J., Chen, J., Shan, L., Lin, Y. & Kong, W. (2008). Evaluation of antioxidant activities and total phenolic contents of typical malting barley varieties. *Food Chemistry*, **107**, 296-304.

Zhu-Salzman, K., Bi, J.L. & Liu, T.X. (2005). Molecular strategies of plant defense and insect counter-defense. *Insect Science*, **12**, 3-15.

Zimmerman, D.C. & Vick, B.A. (1970). Hydroperoxide isomerase a new enzyme of lipid metabolism. *Plant Physiology*, **46**, 445-453.

CHAPTER 2

Five step purification and characterisation of two LTP1 species from barley malt

Introduction

One of the most important contributing factors towards beer quality is beer foam (Bamforth, 1985). Beer foam consists of interactions and relations between a variety of factors such as hop acids, proteins, polysaccharides, the presence of metal ions, foam destabilising factors, ethanol, yeast protease activity and more (Bamforth, 1985; Evans & Bamforth, 2009).

It is already widely known that protein Z and lipid transfer protein 1 (LTP1) are two of the major contributors to the formation and stability of beer foam (Evans & Bamforth, 2009). Both proteins can withstand and tolerate the high temperatures used during brewing (Lindorff-Larsen & Winther, 2001). LTP1 is a 9 kDa basic protein (Jegou *et al.*, 2000) with a pI range >9 (Lindorff-Larsen & Winther, 2001). This protein can bind a wide range of lipids non-specifically in its modified form (Stanislava, 2007) and so doing prevent lipids from destabilising beer foam. Modification, such as glycocylation, of LTP1 occurs when the protein is exposed to the high temperatures of the brewing process (Jegou *et al.*, 2000). Post-translational modification of LTP1 due to a lipid-like adduct covalently bound to it (Perrocheau *et al.*, 2006), leads to the formation of LTP1b, which is 294 kDa larger than LTP1 (Mills *et al.*, 2009). This adduct of LTP1b was identified to be α-ketol 9-hydroxy-10-oxo-12(Z)-octadecenoic acid (Bakan *et al.*, 2006) and is formed when linoleic acid is oxygenised to form a hydroperoxide that is subsequently dehydrated (Matejkova *et al.*, 2009).

LTP1 has numerous proposed biological functions of which one is acting as an antimicrobial protein for protecting the barley grain from infections in the field (Lindorff-Larsen

et al., 2001). It is theorised that LTP1 acts on the membranes of pathogens and other microbes (Kader, 1996). It is of importance to the brewing industry to fully understand the effect of the presence of these proteins or peptides during processing. LTP1 has been shown to have inhibitory effects on the common lager brewing yeast, *Saccharomyces cerevisiae* (Gorjanovic *et al.*, 2004; van Nierop *et al.*, 2008) as well as some fungal pathogens occurring in the field, which can be detrimental to beer quality.

Due to the LTP1s resistance to high temperatures, it will survive the mashing process and still be present during fermentation (Gorjanovic *et al.*, 2004). The peptides are thus not only important to brewers as a major contributor towards foam formation but will also influence fermentation. Previous research indicated that neither LTP1 nor LTP1b unfolded completely at temperatures reaching 100°C (Perrocheau *et al.*, 2006; Matejkova *et al.*, 2009) and will thus be necessary to take into account throughout the brewing process.

LTP1s account for 5-10% of all soluble proteins (Stanislava, 2007) in grains and have, in past, been isolated from barley (Sorensen *et al.*, 1993; Lusk *et al.*, 1995; Douliez *et al.*, 2001; Garcia-Casado *et al.*, 2001; Lindorff-Larsen *et al.*, 2001; Gorjanovic *et al.*, 2004; Gorjanovic *et al.*, 2005; Matejkova *et al.*, 2009; Mills *et al.*, 2009), wheat (Tassin-Moindrot *et al.*, 2000) and various other plant materials (Molina *et al.*, 1993; Zoccatelli *et al.*, 2007; Oshchepkova *et al.*, 2009; Zaman & Abbasi, 2009). During this study optimisation of the existing purification methods for barley LTP1 and LTP1b were broadly based on the method described by Sorensen *et al.* (1993). Purified proteins were characterised with SDS-PAGE, immunological detection and analysis, electrospray mass spectrometry, circular dichroism as well as assessed in terms of its activity towards strains of *S. cereviseae*.

Materials and Methods

Materials

Raw material and Chemicals: Barley (*Hordeum vulgare*), cultivar SSG 506 and SAB5 *S. cereviseae* strain were supplied by SABMiller (Caledon, South Africa). *S. cereviseae* NFP was from National Food Products (Emmarentia, Johannesburg, South Africa). Chemicals and other materials were obtained from the following companies: tri-sodium citrate from B&M Scientific cc (Cape Town, South Africa); ammonium sulphate and acetic acid from Kimix (Eppindust, South Africa); Filtraflow 4252 from INFIGRO Natural Technologies (Pty)Ltd (Olifantsfontein, South Africa); Celetom FW-13, Eagle Pitcher Filtration & Minerals Inc. (Reno, Nevada, USA); Sephadex G25 and G50 from Pharmacia Fine Chemicals (Uppsala, Sweden); CM Sepharose from Amersham Pharmacia Biotech AB (Uppsala, Sweden); Tris from Melford Laboratories Ltd (Ipswich, UK); Coomassie blue R-250, SDS sample buffer and low molecular weight markers from Sigma Aldrich (Missouri, USA);; nitrocellulose membranes from Pall Corporation (Florida, USA); goat anti-rabbit HRP-conjugate from Bio-Rad (Parklands, South Africa); immunoblotting substrate from Pierce (Thermo Scientific, Rockford, Illinois, USA); propidium iodide were from Sigma-Aldrich (St. Louis, USA). LTP1 generated primary antibodies were generously donated by Prof. Evan Evans (School of Plant Science, University of Tasmania). Analytical quality water was prepared by filtering water from a reverse osmoses plant through a Millipore Milli Q® water purification system (Milford, USA).

LTP1 Purification

The five step purification protocol for LTP1 and lipid associated analogue LTP1b from barley is depicted in Fig. 1 and described in detail hereafter. One hundred grams barley was milled using a Cyclotec 1093 sample mill from Foss Tecator (Hillerod, Denmark) and extracted for 20 hours with 300 mL analytical quality water at 4^{o}C. In the case of isolating unmodified

LTP1, barley was defatted first as was recommended by Bakan *et al*., 2006 by washing the kernels with a 60% ethanol solution and subsequently drying it in a vacuum dryer overnight. For LTP1b isolation the ethanol wash step was omitted. The extracted milled barley slurry was centrifuged at 4˚C for 20 minutes at 17 600 × *g* (10000 RPM using a Beckman JA-10 rotor).

The supernatant was saturated to 40% ammonium sulphate and filtered using filter aids: 1.0 g Filtraflo 4251/10 cm^2 filtration surface and 2 g Celetom FW-13 per 100 mL filtrate. The filtrate was further saturated with ammonium sulphate up to 80% and centrifuged at 17 600 × *g* at 4˚C for 20 minutes. The 80% ammonium sulphate precipitated was solubilised in analytical quality water and desalted using a 1.6 × 18 cm Sephadex G25 column connected on an AKTA purifier (Amercham Pharmacia Biotech, Upsala, Sweden). A 1% acetic acid solution was used to elute the protein fractions at a flow speed of 0.5 mL/min. The protein containing fractions collected were concentrated by freeze drying.

The LTP1 containing fraction, as determined with SDS-PAGE and dot immune blot, was solubilised in analytical quality water and applied to a CM-Sepharose column (4 mL bed volume) equilibrated with 5 mM Tris-HCl (pH 7.0) and a flow rate of 0.5 mL/minute. Contaminating proteins were separated from LTP1 (or LTP1b) by running a stepwise gradient. After loading the protein fraction, 5 mM Tris-HCl (pH 7.0) was applied for five column volumes, followed by 50 mM Tris-HCl at pH 9.0 for five column volumes and finally a step gradient of 50 mM Tris-HCl, 0.5 M NaCl at pH 9.0 for 3 column volumes.

The LTP1 containing fraction, as determined by SDS-PAGE and western blot, was concentrated by freeze drying. The freeze-dried LTP1 fraction was further purified on a 1.5 × 36 cm Sephadex G50 column and eluted with 1% acetic acid at a flow speed of 0.5 mL/min. Protein fractions collected were analysed using SDS-PAGE, electrospray mass spectrometry (ESMS), circular dichroism (CD) and antiyeast activity assays.

Analysis of protein fractions

SDS-PAGE analysis: Fractions were pre-boiled at 90°C for 10 minutes with an equal amount of sample buffer and subsequently subjected to SDS-PAGE. The gels were cast by making use of Hoefner mighty small dual system from Hoefner Inc. (Holliston, USA). A 15% SDS-polyacrylamide stacking gel and 5% separating gel was used to analyse fractions as described by Laemmli (Laemmli, 1970). Ultra low range molecular weight markers were used as size indicators. The gel was run under a constant current of 20 mA. The gel was removed and stained for 1 hour using the staining solution, 0.125% Coomassie blue R-250, 50% methanol and 10% acetic acid. Thereafter it was destained with destain solution 1, 50% methanol and 10% acetic acid for another hour and lastly placed in destain solution 2, 7% acetic acid and 5% methanol until the gel was completely clear. The gel was subsequently digitised using UN-SCAN-IT gel 1.6 software package (Silk Scientific Inc, Orem, Utah).

Dot and western Blot analyses: Immunological assays were performed using anti-LTP1 polyclonal antibodies. For the dot immuno-blot, 2 μL of each fraction was placed on a strip of nitrocellulose membrane. The membrane was incubated with casein buffer (154 mM NaCl, 0.5 casein, 10 mM Tris-HCl and 0.02% thiomersal, pH 7.2) for 20 minutes while slowly shaken. This was followed by the incubation of the membrane with whole serum (1:5000 *v/v*) and further incubated for 60 minutes at room temperature. The membrane was then washed four times for 5 minutes with PBS-Tween (0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO_4, 0.02% KH_2PO_4, 0.1% Tween 20, pH 7.2) after which it was placed in casein buffer with goat anti-rabbit horse radish peroxidase-conjugate for another 60 minutes at room temperature. The membrane was then washed again four times for 5 minutes with PBS-Tween and incubated for 1 minute with Pierce ECL western blotting substrate after which it was exposed and developed.

A western blot was performed using the same antibodies as for the dot immuno-blot. The proteins seperated on the SDS-PAGE gel were transferred onto a nitrocellulose membrane at 0.8

mA/cm^2 gel. The membrane with the transferred proteins was incubated with 20 mL casein buffer (154 mM NaCl, 0.5 casein, 10 mM Tris-HCl and 0.02% thiomersal, pH 7.2) for 20 minutes under slight agitation. This was followed by the incubation of the membrane with whole serum (1:5000 *v/v*) and further incubation for 60 minutes at room temperature. The membrane was then washed four times for 5 minutes with 20 mL PBS-Tween (0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO_4, 0.02% KH_2PO_4, 0.1% Tween 20, pH 7.2) after which it was placed in 20 mL casein buffer with goat anti-rabbit HRP-conjugate (1:10 000 dilution) for another 60 minutes at room temperature. The membrane was then washed again four times for 5 minutes with 20 mL PBS-Tween and incubated for 1 minute with Pierce ECL western blotting substrate after which it was exposed 15 minutes and developed.

Mass spectrometry: Samples were prepared by diluting peptide in analytical quality water to yield a final concentration of 200 µg/mL. Electrospray mass spectrometry (ESMS) was performed using a Waters Quadrupole Time-of-Flight Synapt G2 mass spectrometer. A Waters Acquity UPLC injected 2µL of each sample into a Z-spray electrospray ionization source in positive mode to analyse direct mass. A capillary voltage of 3.0 kV and cone voltage of 15 V were applied at a source temperature of 120˚C. Data acquisition was in the positive mode with scanning from *m/z* 150 to 2000. Molecular masses from the multi-protonated spectra's were calculated over 2000-40000 Da mass range with 20 charges set as maximum and automatic peak width detection using the MaxEnt 3 algorithm from MassLynx V4.1.

Characterisation

Heat and pH stability: LTP1/1b fractions (0.200 mg/mL) were analytically prepared in analytical quality water for temperature stability determination and in 40 mM phosphate-buffered saline adjusted with either 6M NaOH or 6M HCl, to the desired pH, for the pH stability. The pH range buffers at which LTP1 and LTP1b enriched preparations were tested included pHs

1, 3, 5, 7, 9, and 11. Solvents were individually scanned for each respective pH and subtracted as baseline from each of their respective samples.

The temperature and pH stability of LTP1 and LTP1b preparations were obtained on a Chirascan Plus CD spectrometer (Applied Photophysics, UK). All analyses were conducted at room temperature using a quartz cell with a path length of 0.5 mm. CD scans were from 190 to 260 nm at a band width of 1 nm. Each scan was repeated in triplicate at 0.5 seconds per scan. For the temperature related experiments, a metal probe was inserted into the sample cell to record the sample temperature. During the temperature ramping, each scan was repeated 5 times and each point was scanned for 0.2 sec at the varying temperatures. Scans were performed, starting at 25°C and with 5°C increases until a temperature of 95°C was reached. The solvent used was scanned and subtracted as baseline from each respective temperature spectrum.

Anti-yeast activity: A dose response was performed for each of the peptide enrichments towards two different *S. cereviseae* strains (SAB5 and NFP), as previously described by Van Nierop (2005). The yeast strains were both grown in MYGP broth (3% each of malt and yeast extract, 5% peptone, 10% glucose) at 30°C from OD 0.01 to 0.5 at 595 nm and diluted 200 fold before inoculation. Doubling dilutions were made with analytical quality water of both peptide enrichments starting at 800 µg/mL and decreasing to 12.5 µg/mL. Gramicidin S was used as a positive control and was also applied in a doubling dilution series starting at 8 µg/mL, decreasing to 0.125 µg/mL. For each peptide, the assay was done in duplicate with triplicate technical repeats per assay. Plates were incubated at 30°C for 16 hours and yeast growth was determined at 595 nm in a microtitre plate reader after being shaken on medium speed for 5 seconds. The results were analysed as described by Du Toit and Rautenbach (2000).

Lytic activity: To determine whether the high temperatures of the brewing process would result in a loss of anti-yeast activity LTP1 was boiled for 5 and 10 minutes at 100°C and evaluated by a membrane permeability assay as previously described by Bink *et al.* (2012) using

a membrane impermeable dye propidium iodide. Yeast was prepared in a similar manner for the membrane permeability assay as for the dose response assays. LTP1 was used in concentrations five and ten times greater than its IC_{50} (75 and 150µM) and subjected to the various heat treatments. Assays were performed in a 96 well florescent micro-titre plate. All samples, growth controls and blanks were treated with 1% propidium iodide. Gramicidin S (17.5 µM) and 1% Triton X-100 served as lytic controls. The propidium iodide emitted fluorescence was recorded using a Varioskan (Thermo Scientific, Waltham, MA, USA).

Results and discussion

Purification

The optimised five step purification protocol for LTP1 and LTP1b is depicted in Fig.1. Bakan *et al.* (2006) found that barley malt washed with a 60% ethanol solution yielded more LTP1 while unwashed barley would yield a higher amount of LTP1b. This washing principle was thus applied to the current study as the first step. An extract was made from barley flour by precipitating it at 40%, followed by an 80% ammonium sulphate in the second step. In the third step the solubilised ammonium sulphate precipitate was desalted on a Sephadex G25 column and this method of desalting proved to be highly efficient as very little protein was lost during this step.

The desalted protein fraction was further purified via cation exchange chromatography on a caboxymethyl-modified resin in the fourth step. The pH 7 buffer was used to elute all proteins with pI≤7 from the column ("void" in Fig. 2A). Since LTP1 has a pI of 8.19, it was expected to elute with a buffer at pH >8, but two protein fractions (fractions 1 and 2) eluted at pH 9 and one fraction at pH 9 with high [NaCl] (Fig. 2A). Fractions 1 and 2 were evaluated using SDS-PAGE (results not shown) and dot immuno-blotting using a polyclonal anti-LTP1 antibody preparation contained LTP1 like proteins (Fig. 2A).

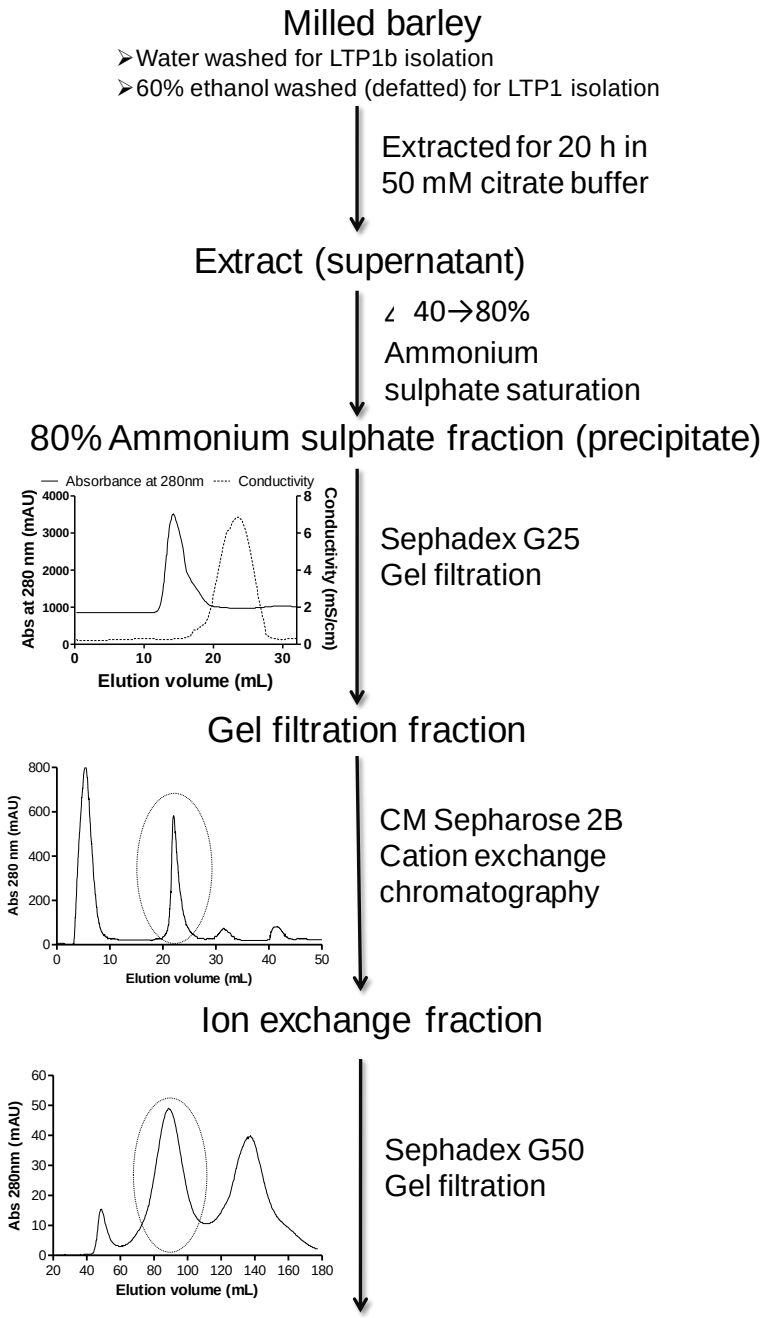

Fig. 1. A Stepwise flow diagram of the LTP1 and LTP1b isolation procedure from barley. Circled elution peaks indicates LTP1 positive fractions.

It is clear from Fig. 2 that fraction 1 contained most of the proteins reactive to the LTP1 antibody, although fraction 2 also had a similar sized protein at 9-10 kDa, but less prominent reactivity towards the anti-LTP antibodies. To further purify the LTP containing, fraction 1, the final purification step entailed another size exclusion chromatography step (Fig. 2B). Three fractions were found (Fig. 2B) which correlated with the SDS-PAGE results of fraction 1

indicating a mixture of different sized proteins apart from the LTP1 (SDS-PAGE results not shown). ESMS of peak fraction 2 confirmed that the fraction contained about 70% pure LTP1 or LTP 1b, depending if ethanol wash was included or not (Table 1).

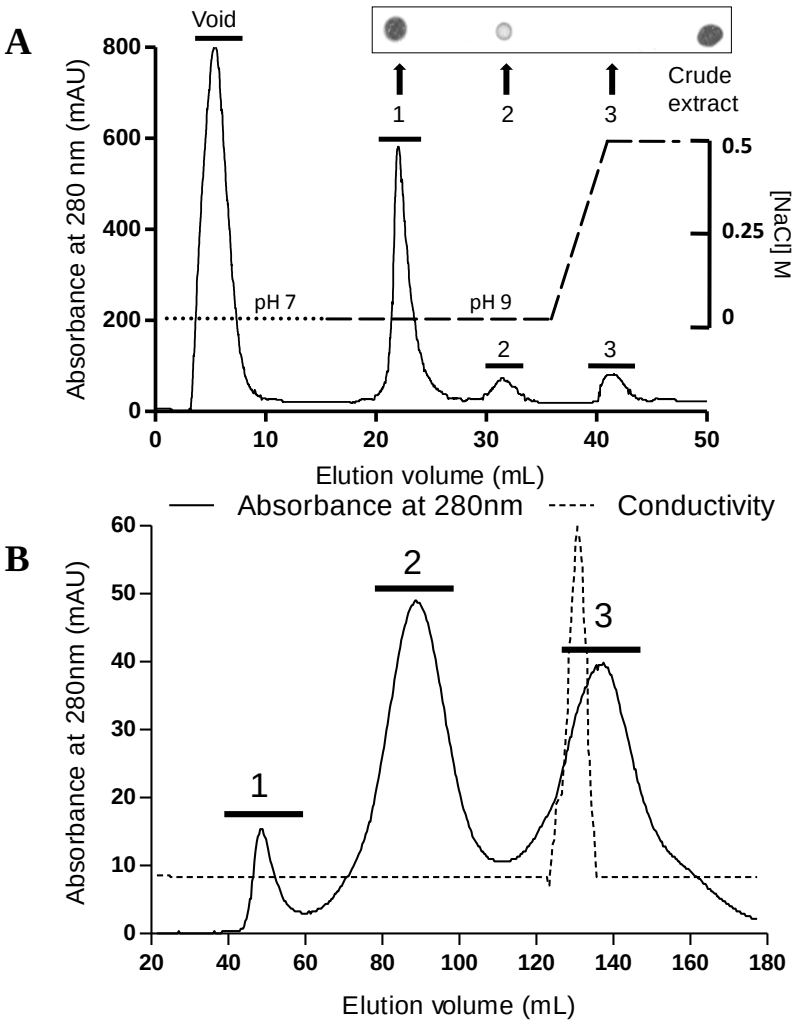

Fig. 2. A A representative cation exchange chromatogram obtained from a CM Sepharose column separation of the desalted 80% ammonium sulphate fraction as developed with a stepwise gradient of 5 mM Tris-HCl (pH 7), 50 mM Tris-HCl (pH 9) and 50 mM Tris-HCl + 0.5 M NaCl (pH 9). The bold lines above the peaks indicate the pooled peak factions. Dot immuno-blot analysis, using anti-LTP1 antibodies of the three different fractions (1-3), showing bands correlating to LTP1, as well as the original crude extract. **B.** A representative Sephadex G50 size exclusion chromatogram of the final purification step of the LTP1 containing fraction 1 from cation-exchange chromatography step depicted in A, using 1% acetic acid as eluant. The bold lines above the peaks indicate the pooled factions.

The purified protein fraction from the defatted barley predominantly consisted of the unmodified LTP1 isoform with an experimentally determined M_r of 9680.85 (expected 9680.53) and minor contributions of LTP1b and other isoforms (Fig. 3A, Table 1). These M_r-values for

LTP1 and LTP1b compared well with those determined by (Jegou *et al.*, 2000):(Jegou *et al.*, 2001) and (Van Nierop *et al.*, 2004) using ESMS . A small amount of a protein with an M_r of 9517.82 was also detected, which could be LTP1b without its C-terminal tyrosine residue (see insert Fig 3A). When the purification protocol was repeated using barley malt not defatted, purification yielded LTP1b as major component. ESMS revealed that the amounts of the two major isoforms reversed, yielding more of the LTP1b isoform with experimentally determined M_r of 9973.75 (expected 9974.74) and less LTP1 (Fig. 3B, Table 1). A putative dimer of LTP1b was also detected at M_r =19356.66, as well as hydroxylated forms at 9991.75 and 10007.15 (Fig. 3B).

Table 1 Summary purification data for LTP1 and LTP1b from barley.

Fraction from protein purification step	**Enriched fractions containing**	
	LTP1	**LTP1b**
Fraction 1: Gel filtration with Sephadex G25[1]	776 mg	688 mg
Fraction 1: Cation-exchange chromatography[2]	593 mg	413 mg
Fraction 2: Gel filtration with Sephadex G50[3]	88 mg	72 mg
Experimental M_r*[$] (Theoretical M_r*)	9680.85 (9680.53)	9973.75 (9974.74)

*Monoisotopic mass; [$]Experimental Mr determined with MaxEnt 3 algorithm from MassLynx V4.1.
[1.] Amount of salt-free protein extract from 80% ammonium sulphate step.
[2.] Amount of salt-containing protein fraction 1.
[3.] Amount of salt-free purified LTP1 and LTP1b enriched fractions.

The experimental monoisotopic M_r values for the two protein preparations were almost identical to the expected theoretical M_r of each LTP isoform indicating that the correct proteins were isolated. Furthermore, this also indicated that the four native disulphide bonds and all the peptide bonds (primary structure) of the two analysed proteins in the fractions were intact, even though rather harsh isolation procedures were used. The protein extract yield in this study was determined by weighing the protein after the samples from each chromatography run was freeze dried. A total yield of 880 mg/kg and 720 mg/kg was obtained from barley malt for LTP1 and LTP1b respectively (Table 1).

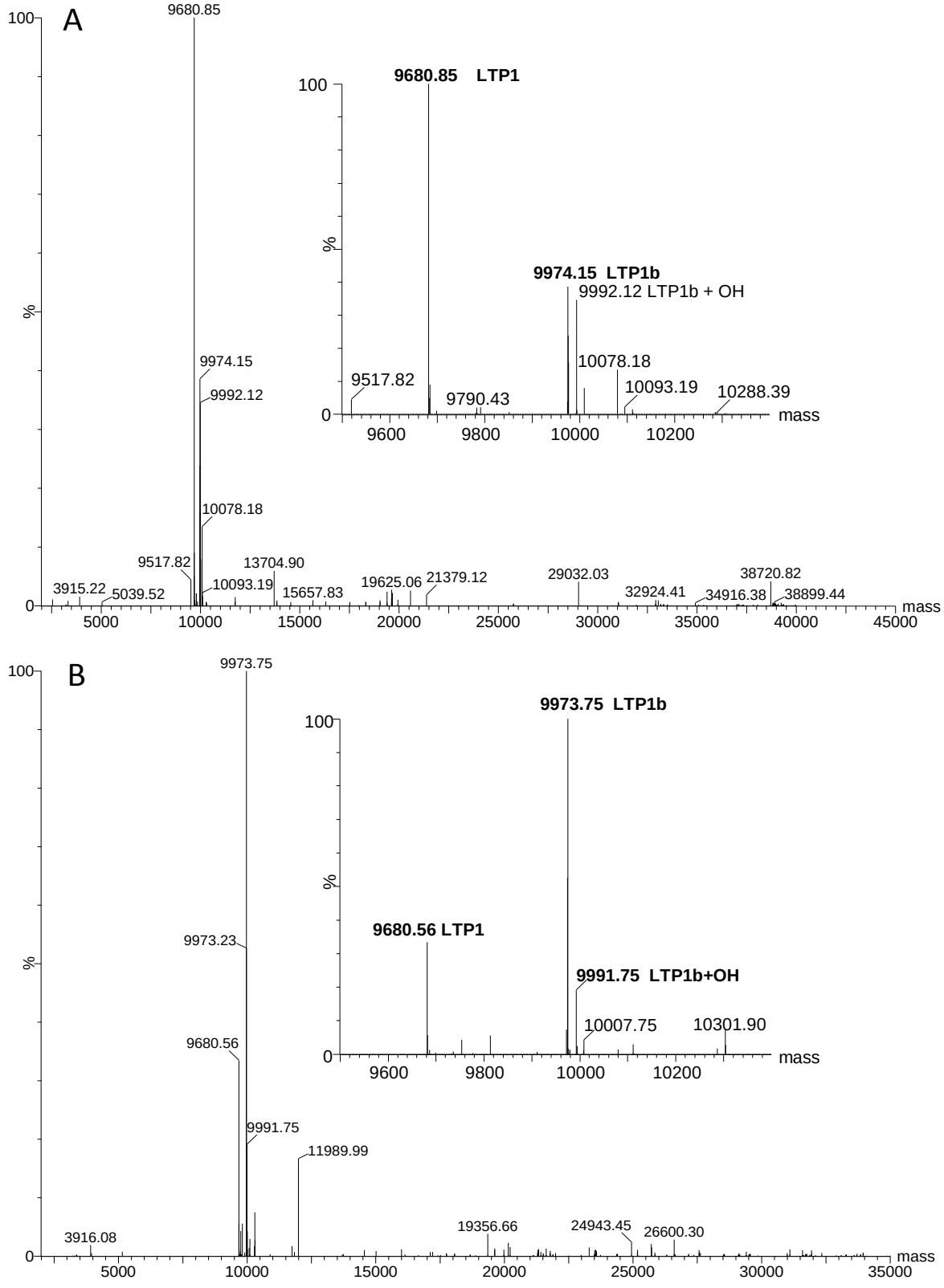

Fig. 3. Positive mode ES-MS mass generated* spectra of the Sephadex G50 purified fraction 2 from (A) defatted malt extract and (B) from malt which was not defatted. The LTP1 isoform is indicated at 9680.85 Da (major peak) and the LTP1b isoform is indicated at 9974.15 Da in A and the LTP1b isoform is indicated 9973.74 Da (major peak) and the LTP1 isoform is indicated at 9680.85 Da in B. *Mass spectra were generated with the MaxEnt 3 algorithm from MassLynx V4.1. The inserts shown in each of the two larger spectra show the 9500-10500 Da region.

Reported LTP1 yields from literature were 200 mg LTP1/kg and 100 mg LTP1b/kg barley malt (Douliez *et al.*, 2001), 103.3 mg LTP1/kg and 82.7 mg LTP1b/kg (Jegou *et al.*, 2000) and 187.5 mg LTP1b/kg barley malt (Mills *et al.*, 2009). These methodologies had three to six chromatography steps, some incorporating dialysis and/or ultrafiltration steps, all of which can lead to cumulative losses. In our experience dialysis and ultrafiltration can lead to large losses of amphipathic peptides and proteins. High yields were obtained because losses were limited using only five purification steps and by elimination of dialysis and untrafiltration steps. The five step optimised LTP1 and LTP1b purification protocol can also be easily upscaled for commercial or industrial purification of LTP1.

Temperature and pH stability as determined by circular dichroism

To probe the structure and better understand the thermal changes undergone by LTP1 and LTP1b during the brewing process, both peptides were evaluated over a wide temperature range using circular dichroism (CD). Due to LTP1's major contribution towards beer foam, thermal studies on its secondary structure have in the past been performed, also by making use of CD (Jegou *et al.*, 2000; Jegou *et al.*, 2001; Pato *et al.*, 2002; Van Nierop *et al.*, 2004; Perrocheau *et al.*, 2006; Mills *et al.*, 2009). Although we analysed mixtures of LTP1 and LTP1b, each preparation was highly enriched and the dominant protein would have the largest influence on the spectra. The spectra of both the preparations showed a typical CD spectrum of a protein containing predominantly α-helices (Heinemann *et al.*, 1996), as found in LTP1. From the spectra (Fig. 4) two minima's are observed at 208 and 222 nm respectively for both isoforms. Similar minima's were observed by Van Nierop *et al.* (2004), Mills *et al.* (2009) and Jegou *et al.* (2001). These minima indicate the presence of α-helixes, known to be an integral part of the secondary structure of LTP1 and LTP1b. This indicated that the secondary structure of the proteins remained intact during the isolation. However, LTP1 showed a much more pronounced 208 nm minimum with 208 nm/222 nm ratio of 1.20, while LTP1b had a 208 nm/222 nm ratio of

1.15, indicating the isoforms have secondary structure differences, possibly induced by the fatty acyl modification of LTP1b.

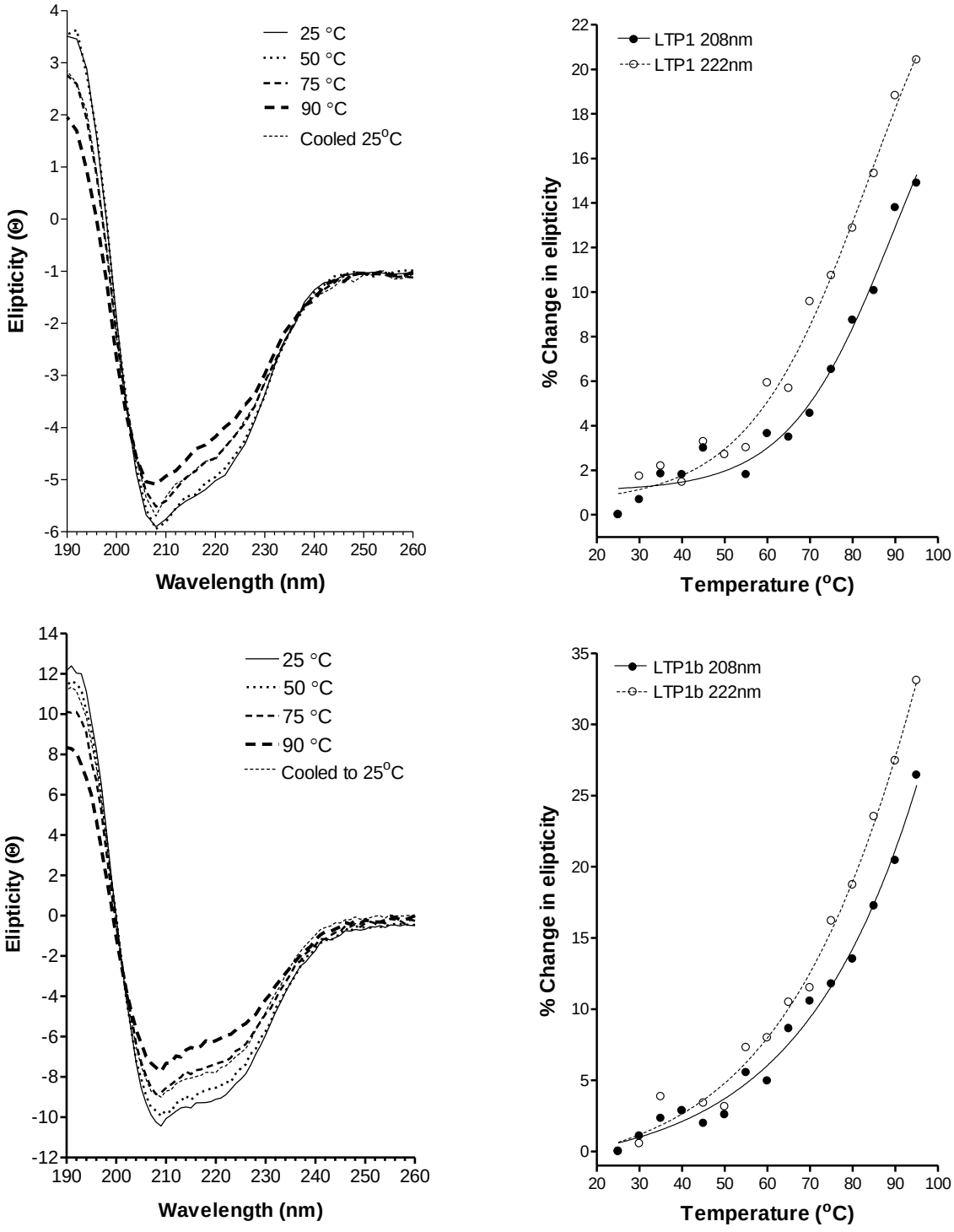

Fig. 4. CD spectra for LTP1 (top left) and LTP1b (bottom left) over a temperature range of 25°C-95°C. Percentage change in ellipticity of the α-helical structures found in LTP1 (top right) and LTP1b (bottom right) at 208 and 222 nm on the CD spectra over temperature increase from 25-95°C.

Upon heating most of the changes in ellipticity for each LTP1 isoform took place, as expected for a protein, at the far-UV CD region between 200 and 260 nm. A decrease of ellipticity and slight shift in the two minima's was observed upon heating, which indicated that both peptides started to partially unfold and lose some of its α-helical structure. Although it seemed that some secondary structure were lost, the proteins did not completely unfold at temperatures reaching 95°C.

Results obtained for the thermal studies of LTP1 compared well to previous CD results when heated up to 95°C (Jegou *et al.*, 2000; Van Nierop *et al.*, 2004) and also for LTP1b, previously isolated from barley (Mills *et al.*, 2009) and wort (Jegou *et al.*, 2001). To better assess how the peptide's α-helixes react to a temperature increase, the percentage change in elipticity was plotted against temperature for both wavelengths, recorded at 208 and 222 nm respectively (Fig. 5). It was observed that an increase in temperature resulted in a decrease in elipticity, indicating that some α-helical structure were lost. The secondary structure of LTP1b seemed to change more over the temperature range from 25^{o}C to 90^{o}C with an overall change of 24% at 208 nm and 33% at 222 nm, while there was only a 15% and 20% change for LTP1 under identical conditions. This is comparable to the 25% loss of structure observed by Mills *et al.* (2009) for both LTP1 and LTP1b and the 10-15% loss for LTP1 structure as found by Van Nierop *et al*. (2004). However, when samples were cooled down to the original starting temperatures of 25°C, the CD spectra of both LTP1 and LTP1b CD, indicated that both are able to fold back close to their original state, with the refolded protein spectra differing less that 10% and 16% from the original spectra for LTP1 and LTP1b, respectively.

When the spectra of the LTP1 isoform preparations were recorded under different pH conditions, both isoforms, again, showed two minima's at 208 and 222 nm characteristic of α-helical structures. A decrease in ellipticity was observed for both LTP1- and LTP1b enrichments as the pH moved away from neutral (Fig. 5; top right for LTP1 and bottom right for LTP1b). The

secondary structure of LTP1b seemed to change more from pH 7 to pH 1 with an overall change of 26% at 208 nm and 33% at 222 nm, while there was only a 15% and 20% change for LTP1 respectively.

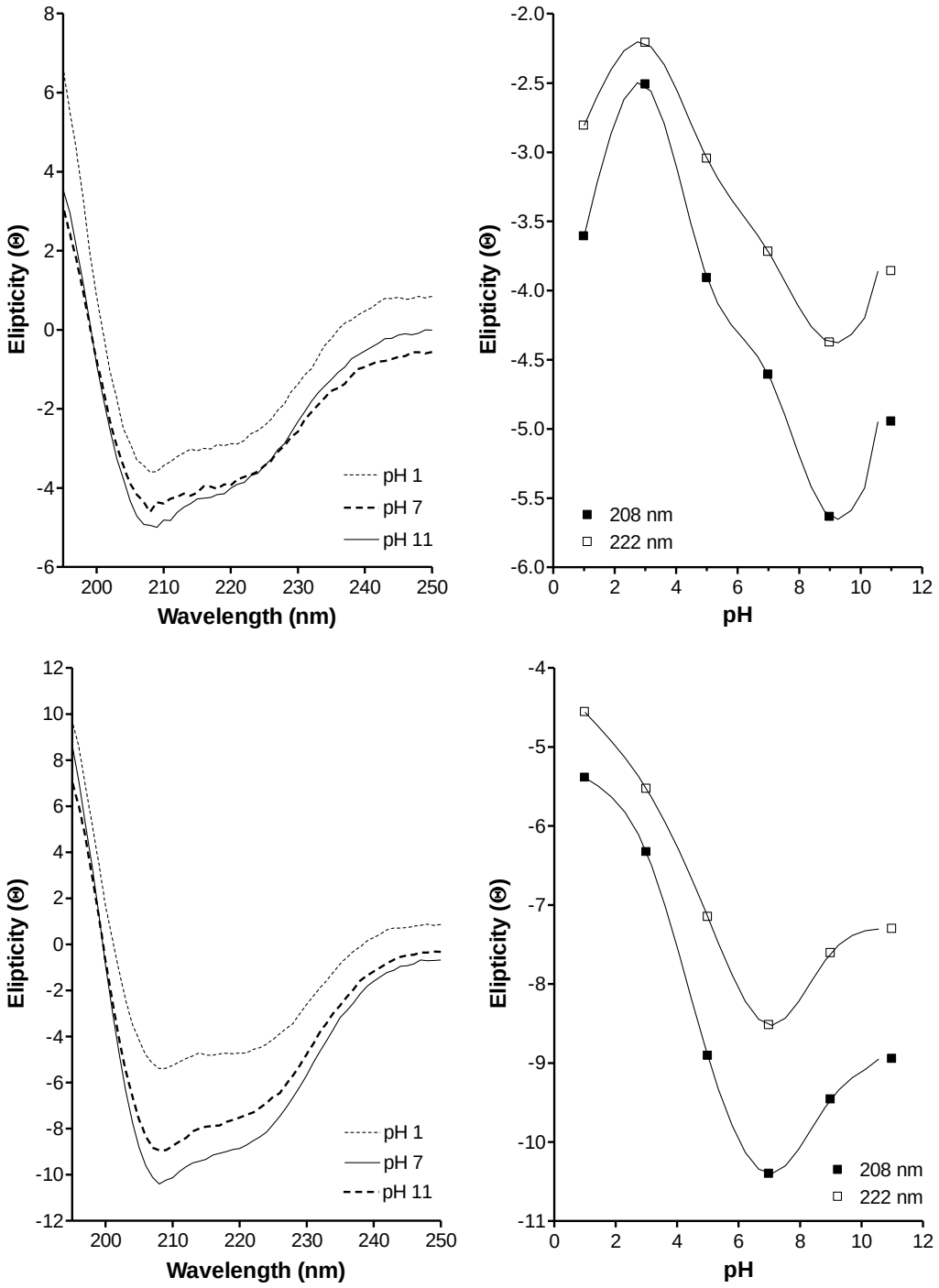

Fig. 5. CD spectra for LTP1 (top left) and LTP1b (bottom left) at pH range of 1, 7 and 11. The left hand graphs show the relationship between ellipticity at 208 and 222 nm on the CD spectra of the LTP1 and LTP1b preparations over the pH range of 1-11.

However, when pH was increased from pH 5 to pH 11, LTP1 and LTP1b CD spectra's indicated that both are able to remain close to their original state, with the states differing less that 22% and 10% from the structure for LTP1 and LTP1b at neutral pH, respectively (Fig. 5). It seems that the LTP isoforms are most stable in neutral environments, but are still relatively stable in basic environments. A clear decrease of stability takes place at more acidic conditions with loss of secondary structure.

Antiyeast activity

Gorjanovic *et al.* (2004) illustrated that LTP possesses antiyeast activity and therefore as the maintenance of the expected LTP1 antimicrobial activity is a good indicator of three-dimensional structural integrity and identity, this activity was probed in the LTP preparations. LTP1 and LTP1b were evaluated for their ability to inhibit the growth of two different strains of *S. cereviseae*. The strains chosen were a commercially available brewing strain, further on referred to as NFP and the brewing yeast currently being used in the industry, referred to as SAB5. From observation SAB5 grew slightly slower than NFP and it seemed to be inhibited slightly less by LTP1 than the NFP strain (Table 2). The activity of the LTP1 were in the low micromolar range (15-25 μM), only 3- to 4-fold less active that gramicidin S, a highly antifungal peptide (Troskie *et al.*, 2012), indicating a potent antiyeast activity and retention of this activity in our purified fraction.

We observed, using light microscopy, that LTP1 probably interacts with its yeast target membrane leading to lyses within a few minutes (results not shown) correlating with previous observations by Gorjanovic *et al.*(2004) and Cvetković *et al.*(1997). This activity is most likely due to the positively charged LTP surface binding to the negatively charged yeast cell surface (Stanislava, 2007). To determine whether the high temperatures of the brewing process would impact LTP1s anti-yeast activity, a membrane permeability assay was performed as described by Bink *et al.* (2012) using the membrane impermeable dye propidium iodide (PI) which forms

fluorescent complexes with nucleotides when it enters damaged cells. The lytic activity of LTP1, as detected *via* PI leakage into treated yeast cells, was maintained after heating to 100°C for 10 minutes and subsequently cooled (Table 2). This correlated well with our observations of the LTP1 temperature stability using CD.

Table 2 Summary of activity data for LTP1 and LTP1b from barley and gramicidin S, used as control lytic peptide.

Compound	IC_{50}, Antiyeast activity against SAB5 strain	IC_{50}, Antiyeast activity against NFP strain	Lytic activity ito TritonX100 ([compound])
LTP1[#]	23±1.2 μM[#]	15±1.3 μM[#]	117±11% (75 μM)
LTP1[#] heated for 10 minutes at 100°C	nd	nd	97±16% (75 μM)
LTP1b[#]	>80 μM	>80 μM	nd
Gramicidin S	5.8±1.0 μM	5.2±0.5 μM	95±20% (17.5 μM)

[#]Concentrations calculated from the assumption that the preparations contained >90% LTP isoforms. The values are the average of duplicate experiments with at least triplicate technical repeats ± the standard error of the mean).

Gorjanovic *et al.* (2004) illustrated that LTP1 does not exhibit its inhibitory effect on fermentation in a brewing setup. Conversely, Van Nierop *et al.* (2006) suggested that even after the mashing process some of the antiyeast activity of LTP1 stays intact. Our results also indicated that LTP1 maintains it activity after boiling (Table 2). However, it was found that most of the LTP1 was converted into LTP1b during the 20 hour malt extraction. Similarly, ESMS analysis of a wort extract prepared using the ammonium sulphate precipitation and desalting step of our optimised protocol, showed that it contained a large proportion of LTP1b, as well as LTP1 and glycosylated LTP1 and LTP1b (Fig. 6). LTP1b was unable to inhibit the growth of any of the two yeast strains up to 80 μM (Table 2), while at 80 μM LTP1 no live NFP yeast cells were present. This might be due to the fact that since LTP1b is already covalently bound to a lipid-like adduct it would not attach itself to the yeast cell membrane to cause cell lyses.

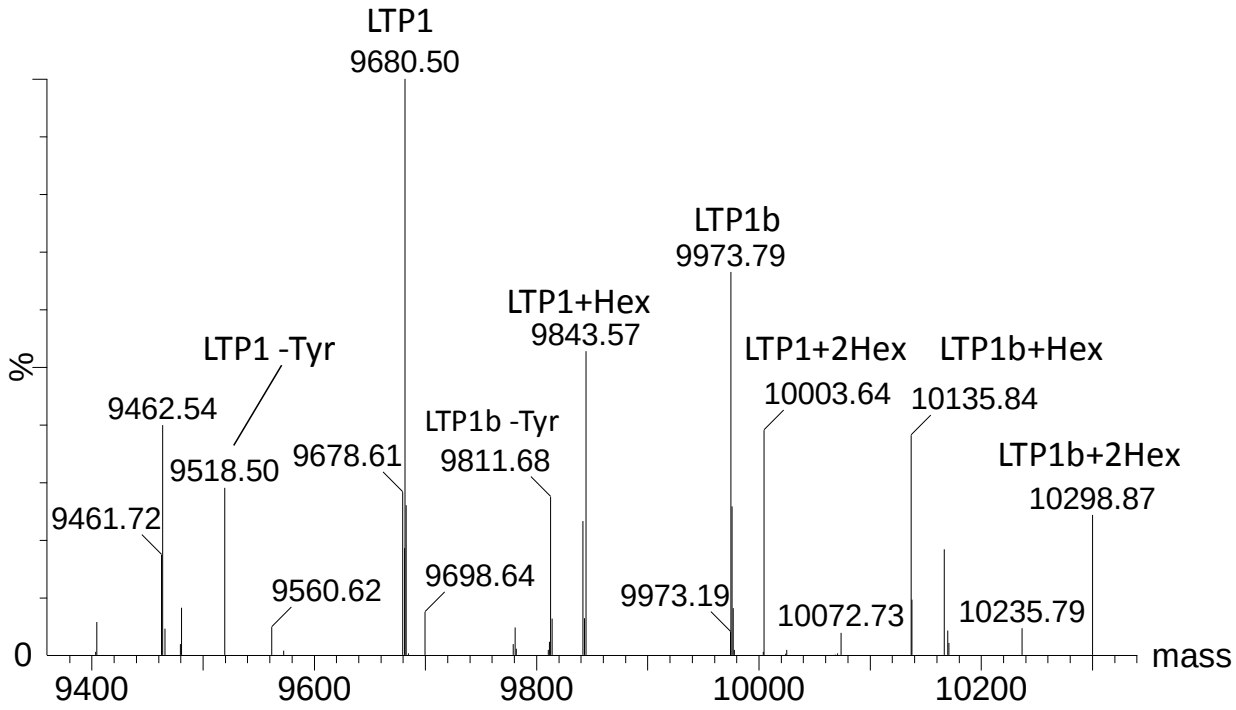

Fig. 6. Positive mode ES-MS mass generated* spectra of a desalted LTP-fraction from a wort extract. The denotation "Hex" indicates that a putative hexose unit was added to the protein structure to form the glycosylated LTP isoform. *Mass spectra were generated with the MaxEnt 3 algorithm from MassLynx V4.1.

Conclusions

LTP1 and LTP1b were successfully purified using only five purification steps with a high purified protein yield. The CD characterisation of LTP1 and LTP1b showed that both proteins are extremely tolerant to the high temperatures involved in the brewing process. At temperatures reaching >90°C LTP1 and LTP1b still remained relatively intact and refolds to almost its original state upon cooling back to 25°C. The pH stability studies indicated that these proteins are more stable at neutral to a more basic pH.

LTP1 exhibited antiyeast activity depending on lysis indicating membrane activity, while this activity was lost in LTP1b, indicating that the fatty acid moiety compromised the antimicrobial activity of LTP1. LTP is found in beer in concentrations of <35 µg/mL (Van Nierop *et al.*, 2004; Evans & Bamforth, 2009), depending on the severity of the boil, thus, the LTP1 antiyeast activity may be problematic in brewing as this could lead to yeast inhibition and

stuck fermentations. However, it was found that most of the LTP1 was converted into LTP1b during extraction and that this structural change could lead to a loss in activity, as seems to be the case for LTP1b. Furthermore an ESMS analysis of a wort extract indicated that this is probably the case in a brewery setting. LTP1 exhibited antiyeast activity, while this activity was lost in LTP1b, indicating that the fatty acid moiety compromised the antimicrobial activity of LTP1. Gorjanovic *et al.* (2004) observed that LTP1 does not exhibit an inhibitory effect on fermentation in a brewing setup. They theorised that the presence of wort components antagonise LTPs inhibitory effect and/or that glycation of LTP during mashing leads to inactivation. Our study further indicated that the formation of LTP1b and its glycosylated forms may also account for the lack of overt antiyeast activity in the brewery setting.

The information provided by this study could shed light on brewing related issues, in particular problems involving beer foam and problematic fermentations. The lack of this protein could result in poor foam stability, while a high concentration of unmodified LTP1 could negatively influence the fermentation process.

References

Bakan, B., Hamberg, M., Perrocheau, L., Maume, D., Rogniaux, H., Tranquet, O., Rondeau, C., Blein, J.P., Ponchet, M. & Marion, D. (2006). Specific adduction of plant lipid transfer protein by an allene oxide generated by 9-lipoxygenase and allene oxide synthase. *Journal of Biological Chemistry*, **281**, 38981-38988.

Bamforth, C.W. (1985). The foaming properties of beer. *Journal of the Institute of Brewing*, **91**, 370-383.

Bink, A., Kucharíková, S., Neirinck, B., Vleugels, J., Van Dijck, P., Cammue, B.P. & Thevissen, K. (2012). The nonsteroidal antiinflammatory drug diclofenac potentiates the in vivo activity of caspofungin against candida albicans biofilms. *Journal of Infectious Diseases*, **206**, 1790-1797.

Cvetković, A., Blagojević, S., Hranisavljević, J. & Vučelić, D. (1997). Effects of pathogen-related proteins from barley grain on brewers yeast. *Journal of the Institute of Brewing*, **103**, 183-186.

Douliez, J.P., Jegou, S., Pato, C., Larre, C., Molle, D. & Marion, D. (2001). Identification of a new form of lipid transfer protein (LTP1) in wheat seeds. *Journal of Agricultural and Food Chemistry*, **49**, 1805-1808.

Du Toit, E. & Rautenbach, M. (2000). A sensitive standardised micro-gel well diffusion assay for the determination of antimicrobial activity. *Journal of Microbiological Methods*, **42**, 159-165.

Evans, D.E. & Bamforth, C.W. (2009). *Beer foam: Achieving a suitable head.* In: Handbook of alcoholic beverages: Beer, a quality perspective, C. W. Bamforth, Russel, I., & Stewart, G. G. Ed, Elsevier: Burlington, MA, pp 1-60.

Garcia-Casado, G., Crespo, J.F., Rodriguez, J. & Salcedo, G. (2001). Isolation and characterization of barley lipid transfer protein and protein Z as beer allergens. *Journal of Allergy and Clinical Immunology*, **108**, 647-649.

Gorjanovic, S., Spillner, E., Beljanski, M.V., Gorjanovic, R., Pavlovic, M. & Gojgic-Cvijanovic, G. (2005). Malting barley grain non-specific lipid-transfer protein (ns-LTP): Importance for grain protection. *Journal of the Institute of Brewing*, **111**, 99-104.

Gorjanovic, S., Suznjevic, D., Beljanski, M., Ostojic, S., Gorjanovic, R., Vrvic, M. & Hranisavljevic, J. (2004). Effects of lipid-transfer protein from malting barley grain on brewers yeast fermentation. *Journal of the Institute of Brewing*, **110**, 297-302.

Heinemann, B., Andersen, K.V., Nielsen, P.R., Bech, L.M. & Poulsen, F.M. (1996). Structure in solution of a four-helix lipid binding protein. *Protein Science*, **5**, 13-23.

Jegou, S., Douliez, J.P., Molle, D., Boivin, P. & Marion, D. (2000). Purification and structural characterization of LTP1 polypeptides from beer. *Journal of Agricultural and Food Chemistry*, **48**, 5023-5029.

Jegou, S., Douliez, J.P., Molle, D., Boivin, P. & Marion, D. (2001). Evidence of the glycation and denaturation of LTP1 during the malting and brewing process. *Journal of Agricultural and Food Chemistry*, **49**, 4942-4949.

Kader, J.C. (1996). Lipid-transfer proteins in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, **47**, 627-654.

Laemmli, U.K. (1970). Cleavage of structural proteins during assembly of head of bacteriophage-T4 *Nature*, **227**, 680-685.

Lindorff-Larsen, K., Lerche, M.H., Poulsen, F.M., Roepstorff, P. & Winther, J.R. (2001). Barley lipid transfer protein, LTP1, contains a new type of lipid-like post-translational modification. *Journal of Biological Chemistry*, **276**, 33547-33553.

Lindorff-Larsen, K. & Winther, J.R. (2001). Surprisingly high stability of barley lipid transfer protein, LTP1, towards denaturant, heat and proteases. *FEBS Letters*, **488**, 145-148.

Lusk, L.T., Goldstein, H. & Ryder, D. (1995). Independent role of beer proteins, melanoidins and polysaccharides in foam formation. *Journal of the American Society of Brewing Chemists*, **53**, 93-103.

Matejkova, M., Zidkova, J., Zidek, L., Wimmerova, M., Chmelik, J. & Sklenar, V. (2009). Investigation of thermal denaturation of barley nonspecific lipid transfer protein 1 (ns-LTP1b) by nuclear magnetic resonance and differential scanning calorimetry. *Journal of Agricultural and Food Chemistry*, **57**, 8444-8452.

Mills, E.N.C., Gao, C., Wilde, P.J., Rigby, N.M., Wijesinha-Bettoni, R., Johnson, V.E., Smith, L.J. & Mackie, A.R. (2009). Partially folded forms of barley lipid transfer protein are more surface active. *Biochemistry*, **48**, 12081-12088.

Molina, A., Segura, A. & Garciaolmedo, F. (1993). Lipid transfer proteins (nsLTPs) from barley and maize leaves are protein inhibitors of bacterial and fungal plant-pathogens. *FEBS Letters*, **316**, 119-122.

Oshchepkova, Y.I., Veshkurova, O.N., Rogozhin, E.A., Musolyamov, A.K., Smirnov, A.N., Odintsova, T.I., Egorov, T.A., Grishin, E.V. & Salikhov, S.I. (2009). Isolation of the lipid-transporting protein ns-LTP1 from seeds of the garden fennel flower (nigella sativa). *Russian Journal of Bioorganic Chemistry*, **35**, 315-319.

Pato, C., Tran, V., Marion, D. & Douliez, J.P. (2002). Effects of acylation on the structure, lipid binding, and transfer activity of wheat lipid transfer protein. *Journal of Protein Chemistry*, **21**, 195-201.

Perrocheau, L., Bakan, B., Boivin, P. & Marion, D. (2006). Stability of barley and malt lipid transfer protein 1 (LTP1) towards heating and reducing agents: Relationships with the brewing process. *Journal of Agricultural and Food Chemistry*, **54**, 3108-3113.

Sorensen, S., Bech, L., Muldbjerg, M., Beenfeldt, T. & Breddam, K. (1993). Barley lipid transfer protein 1 is involved in beer foam formation. *MBAA Technical Quarterly*, **30,** 136-145.

Stanislava, G. (2007). Barley grain non-specific lipid-transfer proteins (ns-LTPs) in beer production and quality. *Journal of the Institute of Brewing*, **113**, 310-324.

Tassin-Moindrot, S., Caille, A., Douliez, J.P., Marion, D. & Vovelle, F. (2000). The wide binding properties of a wheat nonspecific lipid transfer protein - solution structure of a complex with prostaglandin B-2. *European Journal of Biochemistry*, **267**, 1117-1124.

Troskie, A.M., Vlok, N.M. & Rautenbach, M. (2012). A novel 96-well gel-based assay for determining antifungal activity against filamentous fungi. *Journal of Microbiological Methods*, **91**, 551-558.

Van Nierop, S.N.E. (2005). Investigation of malt factors that influence beer production and quality. PhD Department Biochemistry, University of Stellenbosch, South Africa.

Van Nierop, S.N.E., Axcell, B.C., Cantrell, I.C. & Rautenbach, M. (2008). Optimised quantification of the antiyeast activity of different barley malts towards a lager brewing yeast strain. *Food Microbiology*, **25**, 895-901.

Van Nierop, S.N.E., Evans, D.E., Axcell, B.C., Cantrell, I.C. & Rautenbach, M. (2004). Impact of different wort boiling temperatures on the beer foam stabilizing properties of lipid transfer protein 1. *Journal of Agricultural and Food Chemistry*, **52**, 3120-3129.

Van Nierop, S.N.E., Rautenbach, M., Axcell, B.C. & Cantrell, I.C. (2006). The impact of microorganisms on barley and malt quality - a review. *Journal of American Society of Brewing Chemists*, **64**, 69-78.

Zaman, U. & Abbasi, A. (2009). Isolation, purification and characterization of a nonspecific lipid transfer protein from cuminum cyminum. *Phytochemistry*, **70**, 979-987.

Zoccatelli, G., Dalla Pellegrina, C., Consolini, M., Fusi, M., Sforza, S., Aquino, G., Dossena, A., Chignola, R., Peruffo, A., Olivieri, M. & Rizzi, C. (2007). Isolation and identification of two lipid transfer proteins in pomegranate (*Punica granatum*). *Journal of Agricultural and Food Chemistry*, **55**, 11057-11062.

CHAPTER 3

An optimised FOX micro-assay for screening barley lipoxygenase l activity in malt and beer brewery samples

Introduction

During the malting process barley malt is exposed to high temperatures and oxygen, leading to a variety of oxidative breakdown product from lipids and fatty acid in the seed. This oxidation leads to conversion of polyunsaturated fatty acids, in particular linoleic acid, to 9- and 13-hydroxyoctadecadienoic acid (9-HPOD; 13-HPOD) by enzymatic and non-enzymatic pathways respectively (Kuroda *et al.*, 2002; Kuroda *et al.*, 2003; Guido *et al.*, 2005; Kuroda *et al.*, 2005). The enzymatic oxidation of linoleic acid is catalyzed by lipoxygenase 1 (LOX-1), after which it is further degraded by hydroxyperoxide lyase–like (HPL-like) enzyme to 2*(E)*-nonenal and *via* competing pathways to form other aldehydes (Kuroda *et al.*, 2002; Kuroda *et al.*, 2003; Kuroda *et al.*, 2005). LOX-2, which is also present in barley and malt, is also responsible for the breakdown of linoleic acid to form aldehydes (Hugues *et al.*, 1994). Oxidised lipid breakdown products such as 2*(E)*-nonenal, 2,4*(E,E)*-decadienal and hexenal lead to unwanted odours and tastes in aged beer, often described as "cardboard/fatty". Nonenal, linked to the stale "cardboard" flavour of aged beer have a very low flavour threshold at about 0.1 ppb (Drost *et al.*, 1990; Varmuza *et al.*, 2002) and the formation of nonenal in malts and worts under defined experimental parameters, termed residual nonenal potential (RNP), has been shown to directly correlate with beer flavour degradation during aging (Guido *et al.*, 2007).

The determination of LOX-1 can be indicative of a malt's ability to produce these off-flavours (Zimmerman & Vick, 1970; Guido *et al.*, 2005) and various methods in the past have been employed to do so (Lilius & Laakso, 1982; Auerbach *et al.*, 1992; Nakayama *et al.*, 1995;

Suda *et al.*, 1995; Waslidge & Hayes, 1995; Romero & Barrett, 1997; Anthon & Barrett, 2001; del Carmen Pinto *et al.*, 2007; Guido *et al.*, 2007; Whent *et al.*, 2010; Sun *et al.*, 2012). A chemiluminescence-based assay by Lilius & Laakso (1982) rests on the principle that chemiluminescent light is emitted during the lipoxygenase reaction in the presence of luminol. Some spectrophotometric assays have a tendency to become turbid due to the low solubility of fatty acid substrates, while this assay, although not as sensitive, is not influenced by turbidity. A high-throughput fluorescence assay, also not influenced by tubidity, was developed for the determination of LOX-1 in soybean samples and has a sensitivity advantage, although it would have problems estimating LOX-1 content in samples containing radical scavenging properties (Whent et al., 2010). Other methods for the determination of LOX-1 includes methylene blue based assays (Suda *et al.* 1995, Romero & Barrett 1997, Ayerbach *et al*., 1991 and Whent *et al*., 2010) that are based on the bleaching ability of LOX-1 and is measured spectrophotometrically at 660 nm. The spectrophotometric monitoring of the enzymatic oxygenation of linoleic acid by LOX-1 was reported by Nakayama *et al*. (1995). This method directly measures the amount of HPOD formed at 660-750 nm after the addition of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) or ABTS to the solution. One of the generally used assays for determination of LOX-1 measures the conjugated diene produced by LOX-1 spectrophotometrically as an increase of absorbance at 234 nm over time as reported by Guido *et al*. (2005) and Sun *et al*. (2012). This method is simple and sensitive, but has little potential for up-scaling sample throughput. Another highly sensitive colorometric method is the ferrous oxidation-xylenol orange (FOX) assayIn short, the FOX assay is based on the ability of xylenol orange to change colour in the presence of Fe^{3+} oxidised from Fe^{2+} by hydroxyperoxides (Waslidge & Hayes, 1995). Thus, the higher the LOX activity, the more Fe^{2+} will be oxidised to Fe^{3+} and a more intense purple/blue colour will develop from an initial yellow colour. The change in colour can be measured spectrophotometrically. Previously, the FOX chemistry was applied for the determination of LOX in malt (Yang & Schwarz, 1995; Kuroda *et al.*, 2002; Li & Schwarz,

2012), soybean (Fukuzawa *et al.*, 2006) general plant extracts (DeLong *et al.*, 2002; del Carmen Pinto *et al.*, 2007), biological samples (Waslidge & Hayes, 1995; Gay & Gebicki, 2003; Fukuzawa *et al.*, 2006).

LOX activities, particularly LOX-1, are central to the oxidation of unsaturated fatty acids and the potential for nonenal formation. It is complex to discriminate between the quantification of LOX-1 and LOX-2 and most of the assays do not fully discriminate between these two enzyme activities. LOX-2, however, is much less thermo-stable than LOX-1, particularly at >pH 5.5 and will thus denature at a greater rate than LOX-1 during brewing procedures (Hugues *et al.*, 1994). It was also found that LOX-1 contributed to a much greater fraction of the total LOX activity in brewing samples than LOX-2 (Yang *et al.*, 1993) and during this study it is assumed that most of the LOX activity detected is mainly due to LOX-1.

LOX activity showed a very good correlation with nonenal potential in malts and worts (Guido *et al.*, 2007) and it is therefore essential to determine this type of activity in malts before brewing. During this study a reliable FOX micro-assay in 96-well micro titre plates was developed to measure LOX content in different malts and worts. Our LOX-FOX micro-assay is based on the FOX assay described by (Waslidge & Hayes, 1995).

The optimised LOX-FOX micro-assay was compared to a widely used spectrophotometric method used for barley malts (Zimmerman & Vick, 1970; Guido *et al.*, 2005; Gökmen *et al.*, 2007; Sun *et al.*, 2012). The formation of 9- and 13-hydroperoxides by LOX is measured as an increase in absorbance at 234 nm (Gökmen *et al.*, 2007). The 234 nm assay, as it will be referred to from here on onwards, however, relies on the principle that the increase of 0.001 nm at 234 nm is equal to one LOX unit. During this study a standard curve was generated in order to determine the LOX activity in a sample for further comparison to results obtained using the LOX-FOX micro-assay. This newly developed assay was used to investigate the effect kilning position on the LOX content in barley malt samples, compile a LOX activity library of

the 2009 harvest year's barley malts and monitor the LOX content in certain malts at different stages in the brewing process.

Materials and methods

All barley malt and wort samples were graciously supplied by Xolani Mthembu from SAB-Miller (Caledon, South Africa). All malt was freshly milled (0.75 mm sieve) on the same day of analysis and brewery samples were analysed within 6 hours after sampling. For the validation and comparison between the LOX-FOX micro-assay and the 234 nm spectrophotometric assay malt M, was randomly selected and analysed. To determine the effect kilning position has on AROP content malt N1, N2, S1 and S2 were analysed. To monitor the effect the brewing procedures have on LOX barley malts C, Co and M were analysed together with each malt's respective wort. For the determination of LOX content in barley malts harvested during the 2009, 17 malts were analysed, coded as malt 5, Be, C, Cl, E, H, He, M, N, P, S-06, S-11, S, S-4, S-5, Se and T. Assay reagents namely sulphuric acid and iron(II)sulphate were from Merck (Merck, Darmstadt, Germany), lipoxidase type 1-B (LOX-1) from *Glycine max* (soybean), 221700 U/mg solid (Enz-no: 1.13.11.12, lot #050M1910), xylenol orange tetrasodium salt (CAS: 3618-43-7, Lot #: BCBF8231V), linoleic acid and methanol (>99.9% pure) from Sigma Aldrich, (Missouri, USA), and Tris-HCl from Melford Laboratories Ltd (Ipwich, UK)

LOX-FOX micro-assay

The assay was conducted as previously described by Waslidge and Hayes (1995) with modifications to adapt it to 96-well microtiter plate format, as well as optimising the linoleic acid concentration and reaction time to get maximum sensitivity. The FOX reagent comprised of 25 mM sulphuric acid, 100 µM xylenol orange, 100 µM iron(II)sulphate and methanol, 50 mM Tris-HCl, pH 7.4 in a 9:1 methanol to Tris-HCl ratio. It is advisable to make up the iron(II) sulphate together with the sulphuric acid as to prevent iron oxidation.

For the standard curve, a dilution of pure lipoxygenase 1 (LOX-1) from soybean (Sigma Aldrich, Missouri, USA) was tested against an excess linoleic acid. The standard soybean lypoxygenase was made up in doubling dilutions in triplicate starting with 1000 units and ending with 0.98 units. The definition of LOX-1 activity is described by the Sigma Aldrich catalogue and states that 1 LOX-1 unit will cause an 0.001 increase of absorbance at 234nm per minute (pH: 9.0 at 25°C) when linoleic acid is used as substrate in a 3 mL volume and a 1cm lightpath. One A_{234} unit is equivalent to the oxidation of 0.12 µmole of linoleic acid. The linoleic acid was used in excess, to prevent the reactions taking place from being limited by the amount of substrate available. For the standard ranges of 0.98-250 LOX units/mL and 31.25-1000 LOX units/mL 70 µM and 0.7 mM linoleic acid were used, respectively. The standard curves were set up with LOX units/mL (ie mmole linolenic acid oxidised per minute per millilitre sample) on the x-axis in order to allow easy calculation of LOX activity for the liquid extracts.

To determine LOX activity the reaction was initiated by adding 50 µL of 70 µM of a linoleic acid solution to the LOX standards in the chilled 96-well microtiter plate, with exclusion of the controls, before incubation for 15 minutes at 37°C. After incubation 100 µL FOX reagent was added to each well, including that of the controls. Some controls also received 50 µL of a 70 µM linoleic acid solution after the FOX reagent addition. The controls enabled the determination of any possible activity and background colour development in the absence of substrate during

incubation. The colour development in the reaction mixtures was then determined at 620 nm, for 30 minutes at 1 minute intervals using a Bio-Rad microtiter plate reader after the addition of the FOX reagent.

The formation of a purple/blue colour from an initial yellow acidified xylenol orange indicated the oxidation of Fe^{2+} to Fe^{3+}, as the latter reacted with the dye. The higher intensity of the purple colour indicated that more lipid peroxide was formed via the LOX reaction.

Spectrophotometric 234 nm assay

In order to standardise results obtained with the FOX micro-assay one of the earlier, more frequently used, methods for LOX-1 determination was used. The conjugated diene produced by LOX-1 in the presence of linoleic acid is spectrophotometrically measured at 234 nm over a certain period of time. It was previously estimated and defined by several investigators (Aziz *et al.*, 1999; Gökmen *et al.*, 2002; Elkahoui *et al.*, 2005; Guido *et al.*, 2005) that an increase of 0.001 in absorbance per minute at 234 nm is equal to one LOX-1 unit. One A_{234} unit is equivalent to the oxidation of 0.12 µmole of linoleic acid. The method reported in this study is based on one reported by Guido *et al*. (2005) and adapted to also include a standard curve for the determination of LOX-1. By including a standard curve, the 234 nm assay and its kinetic LOX-1 determination equation was validated, as well as the newly developed FOX micro-assay. For the standard curve, a dilution range was made with pure LOX-1 from soybean in 0.1 M acetate buffer (0.1 M NaCl, pH 5) and stored at 4°C.

For determination of malt LOX-1 activity a malt sample (malt M) was extracted by adding 1 g of milled barley malt to 10 mL of the same buffer for 2 hours at 4°C, after which it was centrifuged for 20 minutes at 3000 x *g* and the supernatant kept on ice for LOX-1 determination. Both the pure enzyme and barley extracts' LOX signals were determined in the presence of linoleic acid as substrate. Linoleic acid was dissolved in a borate buffer under N_2 saturation/atmosphere as far as possible. Linoleic acid (1.0 M) was dispersed in 2 mL borate

buffer (25 mM, pH 9.0) which also contained 0.25% (*v/v*) Tween. Two millilitres of NaOH (1.0 M) solution was added to assist in dispersion and to clarify the solution. To determine the LOX-1 activity in each standard and the sample, 50 µL of each respective enzyme containing solution (standards and sample extract) and 10 µl of the linoleic acid solution, was added to 2.94 mL 0.1 M sodium phosphate buffer (pH 6.8) at room temperature. The absorbance was measured in glass cuvettes over a period of 10 minutes in 10 second intervals at 234 nm using a Cary 60 UV-Vis spectrophotometer. The blank contained all components except for the enzyme. LOX-1 activity in the sample was determined by making use of the standard curve generated. All samples and standards were measured in triplicate and the spectrophotometer was calibrated between every triplicate measurement.

LOX-1 activity of barley malt and wort samples

The LOX activity of a malt and wort sample was determined using the LOX-FOX micro-assay. Five grams of malt flour was extracted in 20 mL ice cold water for 2 hours at 4 ^{o}C with agitation. The ratio of sample to extraction media of 1:4 was implemented to mimic the mash ratio of malt to water. The malt cultivar samples, as well as the wort samples were supplied by SABMiller, Caledon, South Africa. The wort samples were centrifuged and the supernatants used as the extracts. Six doubling dilutions of each sample (malt and wort) were prepared in an ice cold microtiter dilution plate. The undiluted extracts were used as the highest concentration in each case, diluting it down to a 32-fold dilution as the lowest concentration. For the negative control, a portion of a random malt extract was boiled for 10 minutes at 90 °C and subsequently centrifuged. Fifty microliters of each diluted extract was then transferred in triplicate into a chilled flat-bottom 96-well microtiter plate. In the case of the control, the extract was only added after incubation and FOX addition. The LOX-FOX micro-assay was conducted as describe above for determination of the standard soy bean LOX-1 activity.

Results and Discussion

A reliable and medium-throughput method for determination of LOX activity was needed for the analysis of barley malts and worts, specifically to assist brewers in predicting a beer's potential shelf life. Another prerequisite for this assay was that it should be easily adaptable in a brewery setup/laboratory and not require any major specialised equipment. Different assays were put to trial, but it was found that the LOX-FOX assay and the spectrophotometric measurement at 234 nm of the conjugated diene formed by LOX in the presence of a substrate gave the best results for all the samples tested. The latter 234 nm assay being more time consuming when performed with biological- and technical repeats and also leaving little room for modifying into a faster and higher throughput format. The LOX-FOX assay, however, proved to be sensitive and quickly assembled for a medium-throughput assay and easily adaptable to an onsite laboratory, requiring only a spectrophotometer and incubator as specialised equipment.

The FOX-LOX micro-assay in this study, as well as numerous other FOX-based methods to determine hydrogen peroxide or LOX activity in samples are generally based on the FOX-2 method, as is reviewed by (Bou *et al.*, 2008). A comparable FOX assay to our FOX-LOX micro-assay was developed by Li & Schwarz (2012) for the analysis of LOX activity in malt samples. Li & Schwarz (2012) determined sample extraction efficiency by varying the sample coarseness, extraction temperature and volumes. It was found that finely ground barley gave higher extraction rates in combination with a sample to buffer ratio of 1:20, extracted at 4°C or lower for 30 minutes. This assay also included butylated hydroxy toluene (BHT) as to limit peroxidation. Other investigators, however, found that BHT can interfere with colour formation, causing lower sensitivity and is therefore not advisable (Hermes-Lima *et al.*, 1995; Grau *et al.*, 2000). In the FOX-LOX micro-assay described in this study, malt was finely milled and extracted for two hours at 4°C at a sample:extraction media ratio equivalent to that used for mashing, to enable comparable extraction of enzymes.

Li & Schwarz (2012) incubated samples with high concentrations of linoleic acid (8 mM) as substrate. However, in this study it was found that such high linoleic acid concentrations lead to rapid colour development and loss of detection and quantitation sensitivity at higher LOX1 activities. We found that 70 µM or 0.7 mM linoleic acid was optimal depending on the LOX activities that were quantified. This correlated with the assay of Waslidge & Hayes as they incubated their samples (blood platelets) with 70 µM arachidonic acid as fatty acid substrate.

The assay of Waslidge & Hayes (1995) was followed for the FOX-working solution by using 100 µM iron(II)sulphate, rather than 250 µM ammonium ferrous sulphate used by Li & Schwarz (2012).. Neither of the assays described by Waslidge & Hayes (1995) nor Li & Schwarz (2012) incorporates the use of a standard LOX-1 activity curve, but the investigators utilised specific equations to calculate LOX activity.

We opted to utilise standard curves to compensate for variation in each assay, as it was found that xylenol orange from different manufacturers and batches may have different responses (Gay *et al.*, 1999). The standard curves were set up with commercial high purity soy bean LOX-1 and the activity enzyme preparation was validated with the 234 nm assay. To compensate for the matrix effect (Bou *et al.*, 2008) on the xylenol orange one set of blanks contained the boiled malt extract.

The time-dependence of the FOX assay was determined by evaluating the change in colour of the solution after the addition of the FOX reagent as is observed in Fig. 1. Absorbance was measured in one minute intervals for 30 minutes following the addition of the FOX reagent to the standard soy LOX-1 concentrations. It was determined that after 15 minutes only a slight increase of absorbance took place and it was also observed that the colour development remained stable in the microtiter plate when sealed and stored at 4°C for over 48 hours (also refer to photo in Fig. 3 discussed later). The FOX assay used by Waslidge and Hayes (1995) only allowed 10 minutes reaction time at 37°C, after which the FOX reagent was added. Their assay was adapted

from one that of (Jiang *et al.*, 1991) in which substrate and sample was incubated for 30 minutes at room temperature after which the FOX reagent was added. According to Jiang *et al.*(1991), the reaction is essentially complete after 5 minutes and the colour stable for hours to follow. Li & Schwarz (2012) recommended 5 minute reaction time between samples and substrate and 10 minutes reaction time with this complex in combination with the FOX reagent. These result correlated with our observations It is clear from that the first order reaction for colour development is completed within 5-7 minutes (Fig. 1C). The colour was measured at either 15 or 30 minutes at the zero order plateau, which was also reflected in the linear regression parameters of the LOX-1 standard curves over time (Fig. 1A en B).

To determine the absolute LOX activity in malt samples a commercial soy bean LOX-1 enzyme preparation was used to set up standard curves (Fig. 2). To evaluate the repeatability of the FOX assay used for LOX determination, the procedure for generating the assay's standard curve was repeated numerously. Examples of standard curves are given in Fig. 2A and B. The LOX-FOX assay yielded a highly linear response over a broad range of LOX activities (0.98 to 250 soybean LOX1 units) with a 30 minute incubation time. With a 15 minute assay time the standard range could also be shifted to 31.25-625 LOX-1 units with the use a ten time higher linoleic acid concentration, with almost identical linear regression line to that of the lower range (Fig. 2B). In both cases highly repeatable standard curve results were obtained as indicated by the 95% prediction interval, particularly for the lower ranges of LOX activity (Fig. 2A and B).

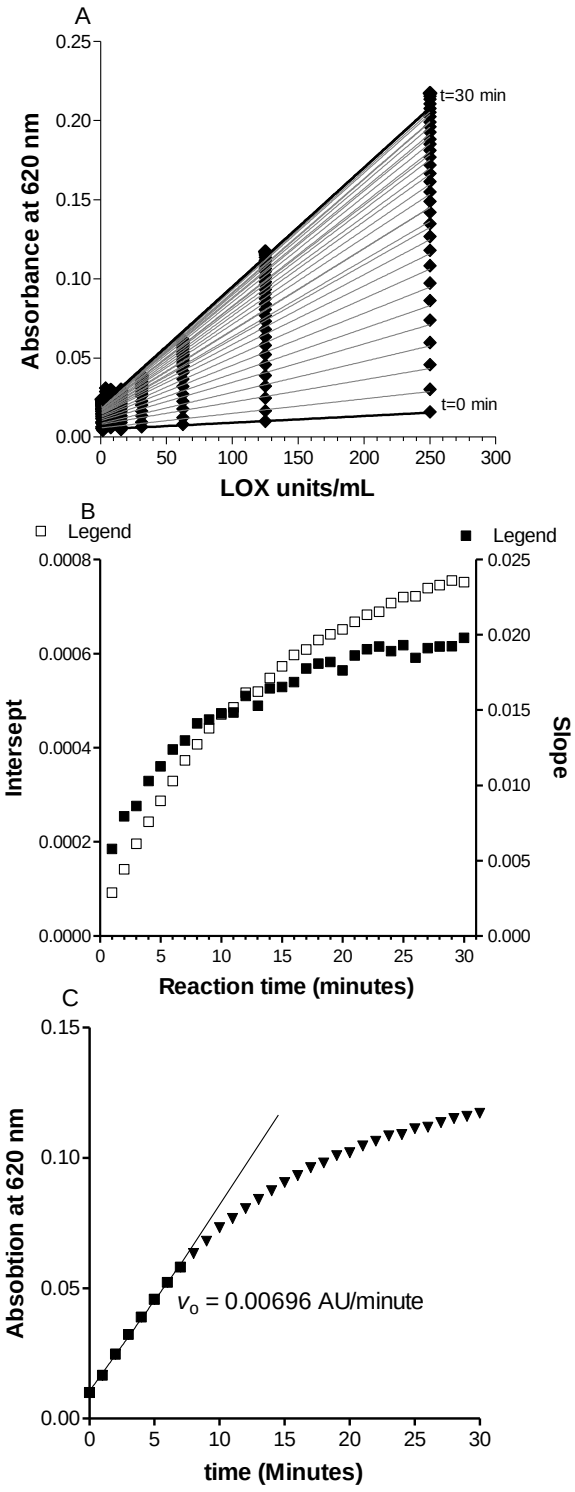

Fig. 1. Time dependence of FOX micro-assay for LOX-1 activity. **A.** Change in standard curve (n=7) for the determination of LOX-1 activity of standard soy bean LOX-1 with linoleic acid as substrate. **B.** Change in the linear regression line fit parameters of the standard curve over 30 minutes. **C.** The kinetics of assay colour development as absorption increase for 6.25 LOX-1 activity units.

The lowest limit of quantisation (LOQ) in our assays was 16 LOX units and the highest LOQ was found to be 625 LOX units. Loss of sensitivity was observed above 1000 LOX-units as hyperbolic standard curves were obtained (results not shown), but this could probably be prevented with higher amounts of substrate and FOX reagent. As mentioned before, a too great excess of linoleic acid influenced the colour development of the assay leading to a loss of sensitivity (loss of standard curve linearity) at high LOX activities.

In order to validate the optimised FOX micro-assay for the determination of LOX activity, an alternative, more widely applied 234 nm assay was used. The 234 nm assay is based on the ability of LOX-1 to produce 9- and 13-hydroperoxides and is measured as an increase in absorbance at 234 nm (0.001 increase in A_{234} per minute = 1.0 LOX-1 unit = 0.21 μmole linolenic acid converted by LOX-1). An increase of absorbance did take place at the estimated rate, but a standard curve was compiled for the change in absorbance at each respective dilution of LOX-1 units (Fig. 2C). It was determined that the best linear trend existed between minutes 3 and 7, so the change in absorbance that occurred in this period of time for the sample was considered, thus the conversion rate as the change in absorbance at 234 nm from 3-7 minutes was plotted over the LOX-1 concentration as LOX units/mL (Fig. 2C). The lowest fairly reliable LOQ that was found for this assay, using the standard curve was 125 soybean LOX-1 units.

A barley malt sample (malt M) and soy bean LOX-1 standards were used to compare the ability of the two assays to determine the LOX activity in a malt sample (Table 1). No significant difference ($p>0.05$) was found in the determination of LOX using the 234 nm assay with either a standard curve or the kinetic equation (increase in 0.001 absorbance units/min = 1 LOX unit) and the LOX-FOX micro-assay. Both assays showed similar results when determining a known LOX concentration as well as for a malt sample, malt M.

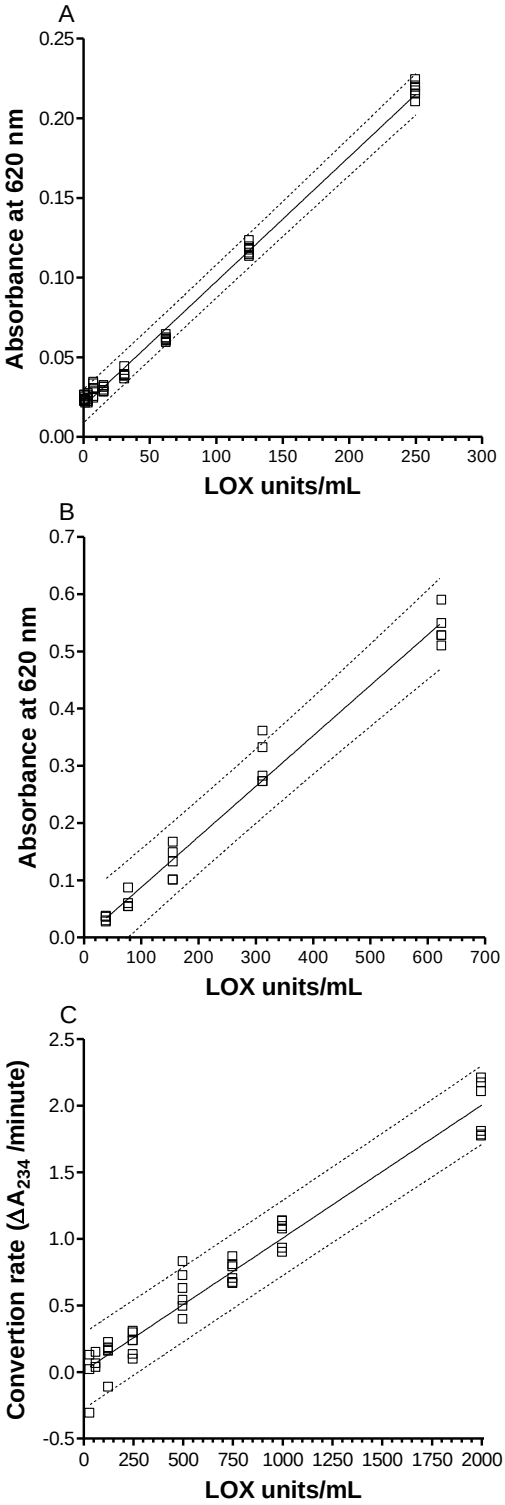

Fig. 2. Comparison of standard curves for the determination of LOX-1 activity of standard soy bean LOX-1 with linoleic acid as substrate. **A.** Example of standard curve over 0.98-250 LOX units used in the FOX-LOX micro-assay. The regression line (y = 0.0008x+ 0.0193) fitted to the individual points (n=7) of the graph with R^2 = 0.995. **B.** Example of a standard curve over 31.25-625 LOX-1 units used in the FOX-LOX micro-assay. The regression line (y = 0.0009x–0.0006) fitted to the individual points (n=5) of the graph with R^2 = 0.994. **C.** A representative example of a standard curve used in the 234 nm spectrophotometric assay. The standard curve compiled with the change in absorbance (conversion rate) at 234 nm from 3-7 minutes for each respective standard LOX-1 concentration. The regression line fit to the individual points (n=3) of the graph was R^2 = 0.959. The dotted lines in both graphs represent the 95% prediction interval.

Table 1 Actual LOX units from soybean lipoxygenase 1 and LOX units (± standard deviation) as determined with the 234 nm assay using the kinetic equation or standard curve and with the FOX assay.

Standard [LOX-1] units/mL[a] (μmole linolenic acid converted/min/μg)	**Estimated [LOX-1] by standard kinetic calculation**	**Estimated [LOX-1] by standard curve using 234 nm assay[c]**	**Estimated [LOX-1] by standard curve using FOX assay**
125	218 ± 37[#]	104 ± 26	115 ± 26
(0.56)	(0.98)	(0.47)	(0.52)
1000	921 ± 124	1056 ± 136	1005 ± 124[$]
(4.5)	(4.15)	(4.76)	(4.5)
Unknown- Malt M	14395 ± 1026[$]	16017 ± 1468[$]	16868 ± 1654[$]
	(64.9)	(74.5)	(76.1)

[a] LOX-1 units as supplied on Sigma product
[b] increase in absorbance measured from 3-7 minutes
[c] Significantly different from other two determinations, $P<0.05$
[d] Determined for dilutions

The development of the purple colour sample with high LOX activity also enabled us to visually discriminate between malt samples with high LOX and low LOX activity when using the LOX-FOX assay. A photo of such an example result is given in Fig. 3A. The comparative LOX activities of malts from cultivars harvested in the 2009 season are shown in Fig 3B. Note the difference in purple colour between for example malt M and malt B, and the corresponding difference of these malts and other malts in the 2009 season (Fig. 3B). The variation in LOX in barley malt throughout the different localities, harvest seasons and genotypes was expected as it was previously reported (Schwarz & Pyler, 1984). The LOX activities of the malts compared to LOX activity levels as reported by Doderer *et al.* (1992), Kaukovirta-Norja *et al.* (1998) and Douma *et al.*(2002). Malt LOX activity from this study, however, differed a factor of 10^3-10^4 from previous findings by Yang & Schwarz (1995) and Schwarz & Pyler (1984), but this may be due to different definitions of a LOX unit.

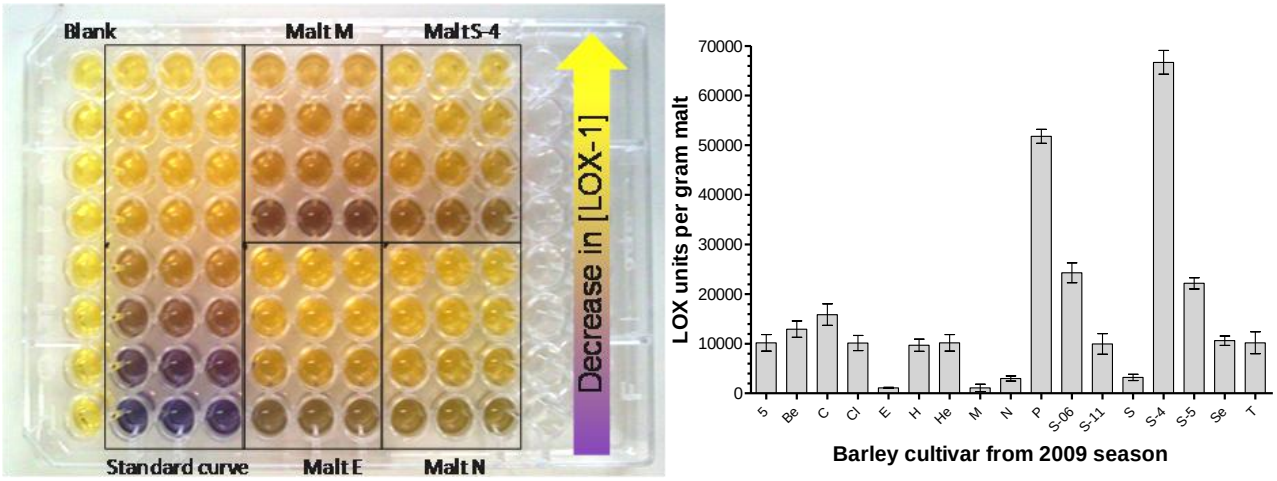

Fig. 3. A. Photograph of a representative FOX-LOX assay in a 96-well plate showing the colour development of the standards ad slected malt samples in the presence of linoleic acid. **B.** Comparison of LOX activity of different barley malt samples from the 2009 season as determined with the LOX-FOX assay. Cultivar names are coded and bars represent the average of duplicate or triplicate determinations with SEM.

Different malted barley varieties were analysed for LOX content when sampled at different kilning positions (Fig. 4). From these results it became apparent that sampling position does have an influence on LOX-1 content in samples with relatively high activity. For example, sample (N1) showed significantly higher LOX-1 activity in samples from the top than samples collected from the bottom of the kiln. This could be due to positional temperature differences leading to LOX activity loss in the bottom samples. Samples with low LOX activity were not influenced by die sampling position. Protein concentration stayed constant irrespective of where the sample was collected from in the kiln (results not shown).

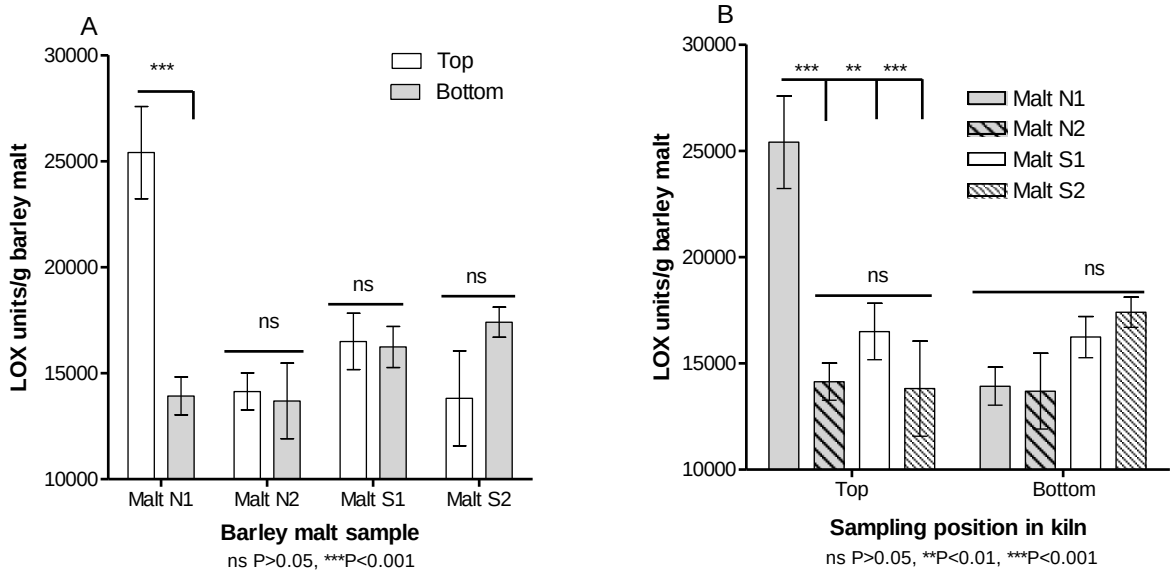

Fig. 4. LOX-1 units/g barley malt of four different malted barley cultivars showing **A**. the influence of sampling position in the kiln (top and bottom) and **B**. the comparison of the four samples with each other at the two sampling positions. Cultivar names are coded and bars represent the mean of triplicate determinations with standard error of the mean (SEM).

A LOX activity library of malts can aid brewers in blending malts for optimal product quality with regards to shelf-life. In Fig. 3B such an example library for different mixed barley malt varieties produced from 2009 barley season is presented. Analyses of two cultivars from the 2010 season, malt S and malt S-4, with malt S showed >2-fold decrease and S-4 >9-fold increase in LOX activity (results not shown). Through this comparison it is clear that LOX content varies significantly among cultivars from the same season, but also within the same cultivar over different harvesting seasons and/or localities, corresponding to previous research (Schwarz & Pyler, 1984).

The LOX activity was also monitored for three barley cultivars (Co, C and M) in their and malts and worts. It was found that the LOX-FOX assay worked well for malt, brewery extracts and wort samples (Table 2).

According to Mei *et al.*(2008), plant LOX seemed to be more tolerant to high temperatures than mammalian LOX and could partially explain the results. However, due to the high temperatures involved in the brewing process, is has been observed that LOX activity is

almost completely inactivated during mashing (Schwarz & Pyler, 1984). As expected due to the temperature sensitivity of LOX (Kobayashi *et al.*, 1993; Ludikhuyze *et al.*, 1998), the worts exhibited a ~99% reduction in LOX activity compared to that in the malts. The apparent presence of LOX activity in beer contradicts findings by Schwarz & Pyler (1984). Artefact LOX activity, if detected with the LOX-FOX assay, could be due to radical formation as the assay is dependent on Fe^{2+} oxidation to Fe^{3+}. For example, if Maillard reactions leading to radical formation (McMurrough *et al.*, 1983; Woffenden *et al.*, 2002) took place during the assay period or if stabilised radicals were present, it will influence the FOX assay results giving a false high LOX activity. The auto-oxidative reactions of linoleic acid could also lead to radicals, but all precautions were taken to remove molecular oxygen from our assay solvents and reagents. Conversely, the radicals could be quenched *via* the antioxidants by the time the assays are conducted and that oxygen dependent radical reactions are limited in the assay as N_2 flushing was used to protect reagents and solvent from oxidation. Also, many of the Maillard reaction products have been observed to possess antioxidant activity (McMurrough *et al.*, 1983; Woffenden *et al.*, 2002; Samaras *et al.*, 2005) and these compounds and remaining antioxidants will suppress LOX-activity. However, in the light of putative interference by radicals and the Mailard reaction with the FOX-LOX micro-assay it is not possible to confirm that some of the LOX-1 protein did survive in the micro-brew samples. We therefore decided to utilise this assay only for malt samples.

Table 2 Estimated LOX activity (units/g malt) of three different malted barley cultivars and respective brewery samples in terms of soybean LOX-1. Cultivar names are coded and values are the average of triplicate determinations ± standard deviation.

Process	Malt Co	Malt C	Malt M
Laboratory 4°C malt extract	36164 ± 5600	24531 ± 8165	16365 ± 1458
Wort (brewery extract after boil)	711 ± 9	417 ± 14	385 ± 54

Conclusions

The FOX assay as described by (Waslidge & Hayes, 1995) for the determination of LOX content in plant materials were successfully adapted to a medium-throughput assay in 96-well microtiter plates. It proved to be highly reliable and sensitive with lowest LOQ of at least 16 soybean LOX units. Although the LOX-FOX assay does not discriminate between LOX-1 and LOX-2 activities, LOX-2 reactions have been shown only have a minor contribution in barley (Yang *et al.*, 1993), therefore it was assumed that most of the activity measured was due to LOX-1. This method was also successfully validated and can therefore be regarded as a repeatable, robust, medium-throughput assay for the determination of LOX-1 in barley malts. It also does not require highly specialised equipment besides a spectrophotometer or microplate reader and could therefore be a user friendly solution for an onsite brewery laboratory. A possible limiting factor for this assay, however, would be the analysis of highly modified malts with darker colours absorbing at 620 nm and care must take to include the appropriate blanks.

It is important to remember that the presence of LOX and reactive oxygen species in beer goes hand-in-hand when it comes to the reduction in shelf life (Vanderhaegen *et al.*, 2006). It would therefore be beneficial to have a single, high-throughput assay available for the determination of both LOX activity and antioxidant/antiradical potential (AROP) in a brewery setup. This notion led to the development of a similar FOX-based assay for the determination of AROP of malts and worts as reported in Chapter 4. If both the LOX and AROP content of a malt or wort is known, a more calculated prediction regarding ageing potential could be made.

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References

Anthon, G.E. & Barrett, D.M. (2001). Colorimetric method for the determination of lipoxygenase activity. *Journal of Agricultural and Food Chemistry*, **49**, 32-37.

Auerbach, B.J., Kiely, J.S. & Cornicelli, J.A. (1992). A spectrophotometric microtiter-based assay for the detection of hydroperoxy derivatives of linoleic acid. *Analytical Biochemistry*, **201**, 375-380.

Aziz, S., Wu, Z. & Robinson, D.S. (1999). Potato lipoxygenase catalysed co-oxidation of β-carotene. *Food Chemistry*, **64**, 227-230.

Bou, R., Codony, R., Tres, A., Decker, E.A. & Guardiola, F. (2008). Determination of hydroperoxides in foods and biological samples by the ferrous oxidation–xylenol orange method: A review of the factors that influence the method's performance. *Analytical Biochemistry*, **377**, 1-15.

del Carmen Pinto, M., Tejeda, A., Duque, A.L. & Macías, P. (2007). Determination of lipoxygenase activity in plant extracts using a modified ferrous oxidation-xylenol orange assay. *Journal of Agricultural and Food Chemistry*, **55**, 5956-5959.

DeLong, J.M., Prange, R.K., Hodges, D.M., Forney, C.F., Bishop, M.C. & Quilliam, M. (2002). Using a modified ferrous oxidation-xylenol orange (fox) assay for detection of lipid hydroperoxides in plant tissue. *Journal of Agricultural and Food Chemistry*, **50**, 248-254.

Doderer, A., Kokkelink, I., van der Veen, S., Valk, B.E., Schram, A. & Douma, A.C. (1992). Purification and characterization of two lipoxygenase isoenzymes from germinating barley. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, **1120**, 97-104.

Douma, A.C., Doderer, A., Cameron-Mills, V., Skadhauge, B., Bech, L.M., Schmitt, N., Heistek, J.C. & Van Mechelen, J.R. (2002). Low lipoxygenase 1 barley. WIPO Patent No. 2002053721. Geneva, Switzerland: World Intellectual Property Organization.

Drost, W., B., Berg, V.d., R., Freijee, M., F.J., Velde, V.d., G., E., Hollemans & M. (1990). *Flavor stability*. Pp. 8. St. Paul, MN, ETATS-UNIS: American Society of Brewing Chemists.

Elkahoui, S., Hernández, J.A., Abdelly, C., Ghrir, R. & Limam, F. (2005). Effects of salt on lipid peroxidation and antioxidant enzyme activities of catharanthus roseus suspension cells. *Plant Science*, **168**, 607-613.

Fukuzawa, K., Fujisaki, A., Akai, K., Tokumura, A., Terao, J. & Gebicki, J.M. (2006). Measurement of phosphatidylcholine hydroperoxides in solution and in intact membranes by the ferric–xylenol orange assay. *Analytical Biochemistry*, **359**, 18-25.

Gay, C., Collins, J. & Gebicki, J.M. (1999). Determination of iron in solutions with the ferric–xylenol orange complex. *Analytical Biochemistry*, **273**, 143-148.

Gay, C.A. & Gebicki, J.M. (2003). Measurement of protein and lipid hydroperoxides in biological systems by the ferric–xylenol orange method. *Analytical Biochemistry*, **315**, 29-35.

Gökmen, V., Bahçeci, S. & Acar, J. (2002). Characterization of crude lipoxygenase extract from green pea using a modified spectrophotometric method. *European Food Research and Technology*, **215**, 42-45.

Gökmen, V., Serpen, A., Atli, A. & Köksel, H. (2007). A practical spectrophotometric approach for the determination of lipoxygenase activity of durum wheat. *Cereal Chemistry*, **84**, 290-293.

Grau, A., Codony, R., Rafecas, M., Barroeta, A.C. & Guardiola, F. (2000). Lipid hydroperoxide determination in dark chicken meat through a ferrous oxidation-xylenol orange method. *Journal of Agricultural and Food Chemistry*, **48**, 4136-4143.

Guido, L.F., Boivin, P., Benismail, N., Goncalves, C.R. & Barros, A.A. (2005). An early development of the nonenal potential in the malting process. *European Food Research and Technology*, **220**, 200-206.

Guido, L.F., Curto, A.F., Boivin, P., Benismail, N., Gonçalves, C.R. & Barros, A.A. (2007). Correlation of malt quality parameters and beer flavor stability: Multivariate analysis. *Journal of Agricultural and Food Chemistry*, **55**, 728-733.

Hermes-Lima, M., Willmore, W.G. & Storey, K.B. (1995). Quantification of lipid peroxidation in tissue extracts based on fe(III) xylenol orange complex formation. *Free Radical Biology and Medicine*, **19**, 271-280.

Hugues, M., Boivin, P., Gauillard, F., Nicolas, J., Thiry, J. & Richard-Forget, F. (1994). Two lipoxygenases from germinated barley-heat and kilning stability. *Journal of food science*, **59**, 885-889.

Kaukovirta-Norja, A., Reinikainen, P., Olkku, J. & Laakso, S. (1998). Influence of barley and malt storage on lipoxygenase reaction. *Cereal Chemistry*, **75**, 742-746.

Kobayashi, N., Kaneda, H., Kano, Y. & Koshino, S. (1993). The production of linoleic and linolenic acid hydroperoxides during mashing. *Journal of Fermentation and Bioengineering*, **76**, 371-375

Kuroda, H, Hisao, Maeba, Hideo, Takoshio & Masachika (2003). *Enzymes that transform linoleic acid into di- and trihydroxyoctadecenoic acids in malt.* Pp. 6. Madison, WI, ETATS-UNIS: Master Brewers Asociation of the Americas.

Kuroda, H., Kobayashi, N., Kaneda, H., Watari, J. & Takashio, M. (2002). Characterization of factors that transform linoleic acid into di- and trihydroxyoctadeconoic acids in mash *Journal of Bioscience and Bioengineering*, **93**, 73-77.

Kuroda, H., Kojma, H., Kaneda, H. & Takashio, M. (2005). Characterization of 9-fatty acid hydroperoxide lyase-like activity in germinationg barley seeds that transform 9(S)-hydroperoxy-10(E), 12(Z)-oktadecadienoic acid into 2(E)-nonenal. *Bioscience, Biotechnology, and Biochemistry*, **69**, 1661-1668.

Li, Y. & Schwarz, P.B. (2012). Use of a ferrous oxidation-xylenol orange (FOX) assay to determine lipoxygenase activity in barley and malt. *Journal of the American Society of Brewing Chemists*, **70**, 287-289.

Lilius, E.-M. & Laakso, S. (1982). A sensitive lipoxygenase assay based on chemiluminescence. *Analytical Biochemistry*, **119**, 135-141.

Ludikhuyze, L., Indrawati, Van den Broeck, I., Weemaes, C. & Hendrickx, M. (1998). Effect of combined pressure and temperature on soybean lipoxygenase. 1. Influence of extrinsic and intrinsic factors on isobaric-isothermal inactivation kinetics. *Journal of Agricultural and Food Chemistry*, **46**, 4074-4080.

McMurrough, I., Hennigan, G.P. & Loughrey, M.J. (1983). Contents of simple, polymeric and complexed flavanols in worts and beers and their relationship to haze formation. *Journal of the Institute of Brewing*, **89**, 15-23.

Mei, G., Di Venere, A., Nicolai, E., Angelucci, C.B., Ivanov, I., Sabatucci, A., Dainese, E., Kuhn, H. & Maccarrone, M. (2008). Structural properties of plant and mammalian lipoxygenases. Temperature-dependent conformational alterations and membrane binding ability. *Biochemistry*, **47**, 9234-9242.

Nakayama, T., Takeura, Y. & Ueda, T. (1995). Visible spectrophotometric assay, purification, and molecular properties of a lipoxygenase from eggplant (solanum melongena linne) fruits. *Biochemical and Biophysical Research Communications*, **214**, 1067-1072.

Romero, M.V. & Barrett, D.M. (1997). Rapid methods for lipoxygenase assay in sweet corn. *Journal of Food Science*, **62**, 696-700.

Samaras, T.S., Camburn, P.A., Chandra, S.X., Gordon, M.H. & Ames, J.M. (2005). Antioxidant properties of kilned and roasted malts. *Journal of Agricultural and Food Chemistry*, **53**, 8068-8074.

Schwarz, P.B. & Pyler, R.E. (1984). Lipoxygenase and hydroperoxide isomerase activity of malting barley. *Journal of the American Society of Brewing Chemists*, **42**, 47-53.

Suda, I., Hajika, M., Nishiba, Y., Furuta, S. & Igita, K. (1995). Simple and rapid method for the selective detection of individual lipoxygenase isozymes in soybean seeds. *Journal of Agricultural and Food Chemistry*, **43**, 742-747.

Sun, W., Du, J., Jin, Y., Liu, J. & Kong, L. (2012). Preliminary research on wheat lipoxygenase during malting. *Journal of the Institute of Brewing*, **118**, 192-197.

Vanderhaegen, B., Neven, H., Verachtert, H. & Derdelinckx, G. (2006). The chemistry of beer aging - a critical review. *Food Chemistry*, **95**, 357-381.

Varmuza, K., Steiner, I., Glinsner, T. & Klein, H. (2002). Chemometric evaluation of concentration profiles from compounds relevant in beer ageing. *European Food Research and Technology*, **215**, 235-239.

Waslidge, N.B. & Hayes, D.J. (1995). A colorimetric method for the determination of lipoxygenase activity suitable for use in a high throughput assay format. *Analytical Biochemistry*, **234**, 354-358.

Whent, M., Ping, T., Kenworthy, W. & Yu, L. (2010). High-thoughput assay for detection of soybean lipoxygenase-1. *Journal of Agricultural and Food Chemistry*, **58**, 12602-12607.

Woffenden, H.M., Ames, J.M., Chandra, S., Anese, M. & Nicoli, M.C. (2002). Effect of kilning on the antioxidant and pro-oxidant activities of pale malts. *Journal of Agricultural and Food Chemistry*, **50**, 4925-4933.

Yang, G. & Schwarz, P.B. (1995). Activity of lipoxygenase isoenzymes during malting and mashing. *Journal of the American Society of Brewing Chemists*, **53**, 45-49.

Yang, G.S., Schwarz, P.B. & Vick, B.A. (1993). Purification and characterization of lipoxygenase isoenzymes in germinating barley. *Cereal Chemistry*, **70**, 589-595.

Zimmerman, D.C. & Vick, B.A. (1970). Hydroperoxide isomerase a new enzyme of lipid metabolism. *Plant Physiology*, **46**, 445-453.

CHAPTER 4

An adapted FOX micro-assay for screening anti-radical/oxidant potential in malt samples

Introduction

One of the greatest challenges faced in the brewing industry today is the beer quality after prolonged storage, especially since the recent growth of beer export (Takashio & Shinotsuka, 1998). The development of a stale flavour over time varies between beer types, -brands and the level of staling (Lustig, 1999) and can be attributed to the formation and degradation of different compounds (Vanderhaegen *et al.*, 2006). Oxygen in beer is a major contributing factor towards staling. Carbonyls, such as 2*(E)*-nonenal are responsible for the characteristic cardboard flavour in stale beer, and are essentially an oxidative breakdown product of an unsaturated fatty acid through among others, the lipoxygenase pathway and the enzyme lipoxygenase-1 (LOX-1) (Drost *et al.*, 1990; Kuroda *et al.*, 2005; Vanderhaegen *et al.*, 2006; Guido *et al.*, 2007). Essentially all stale flavour carbonyls are produced by any one or more of the following reactions: (1) Strecker degradation of amino acids, (2) oxidation of higher alcohols by melanoidins (Hashimoto, 1972), (3) oxidative degradation of iso-alpha acids (Hashimoto & Kuroiwa, 1975), (4) enzymatic and non-enzymatic oxidation and auto-oxidation respectively of fatty acids (Drost *et al.*, 1990; Intelmann & Hofmann, 2010), (5) aldol condensation of short chain aldehydes and finally (6) secondary oxidation of aldehydes (Narziss *et al.*, 1985).

Antioxidants in beer are believed to prevent flavour degradation to a certain extent by scavenging free radicals and metal ions (some antioxidants act as chelating agents) (Vanderhaegen *et al.*, 2006). Raw plant materials such as the hops and malt provide the main supply of antioxidants in the finished product. The antioxidant/antiradical potential (AROP)

varies between different varieties of barley and is also dependent on the malting process (Takashio & Shinotsuka, 1998). In order to possibly predict a malt's or wort's staling potential it will be necessary to measure certain parameters such as the antioxidant potential.

Many evaluation methods for AROP exists today and are classified as either determining hydrogen atom transfer (HAT) or electron transfer/single electron transfer (ET/SET) (Prior *et al.*, 2005; Sun & Tanumihardjo, 2007). HAT methods are based on an antioxidant's ability to donate hydrogen and quench free radicals, while ET methods are based on the ability of an antioxidant to transfer a single electron and so doing reduce radicals, carbonyls or metals. The endpoint of such an ET reaction is indicated by a colour change of the oxidant and includes assays such as the ferrous oxidation-xylenol orange (FOX) (Waslidge & Hayes, 1995) and cupric ion reducing antioxidant capacity (CUPRAC) assays (Prior *et al.*, 2005; Apak *et al.*, 2007; Sun & Tanumihardjo, 2007).

During this study a reliable micro-assay for the screening of AROP in beer malts was developed. An optimised FOX micro-assay, originally adapted from one previously described by Waslidge & Hayes (1995) was implemented for determining lipoxygenase-1 (LOX-1) in malts (Chapter 3). The idea behind using the FOX assay to determine antioxidant/antiradical potential or AROP was to simplify the testing for both LOX-1 and AROP in malt and brewery samples, as both are possible determinants of flavour stability. In short, the FOX assay is based on the ability of xylenol orange to change colour in the presence of Fe^{3+} oxidised from Fe^{2+} by hydroxyperoxides (Waslidge & Hayes, 1995). Therefore, the lower the AROP activity, the more Fe^{2+} will be converted to Fe^{3+} and a more intense purple/blue colour will develop from an initial bright yellow colour. Hydrogen peroxide was used to assess the scavenging capacity of the malt extracts. The lower the antioxidant concentration of the extract the more free peroxide will be in the solution to react with the Fe^{2+} in the FOX reagent and the more intense the colour change. Samples with high AROP will remain yellow, while those with low AROP turn purple.

In order to validate the newly developed AROP-FOX assay it was compared with the CUPRAC assay, since this method has proved to be highly reliable and, according to Apak *et al*., (2007), to a certain extent superior over other antioxidant determination methods. The CUPRAC assays is also a ET based method and measures the colour development at 450 nm that occurs when copper(II)-neocuproine (light blue) is reduced to copper(I)-neocuproine chelate (yellow/orange) when oxidising antioxidant compounds (Özyürek *et al.*, 2011). A standard CUPRAC test tube assay procedure (Güçlü *et al.*, 2006; Alpinar *et al.*, 2009) was modified and adapted to be used in a 96-well microtiter plate format (CUPRAC micro-assay) for comparison. The AROP-FOX assay, similar to the LOX-FOX assay, requires little specialised equipment and could thus easily be used as an onsite AROP determination method in brewery laboratories.

With this newly developed method the effect kilning position had on the AROP content of different and randomly selected barley malts could be determined.

Materials and Methods

All barley malt cultivars, wort and brewery samples were graciously supplied by Xolani Mthembu from SAB-Miller (Caledon, South Africa). For the validation and comparison between the AROP-FOX micro-assay and the CUPRAC assay malts M, N, E and S-4 were analysed. To determine the effect kilning position has on AROP content malts N1, N2, S1 and S2 were analysed.

AROP-FOX micro-assay

The FOX reagent comprised of the following solutions: 25 mM sulphuric acid (Merck, Wadeville, Gauteng, South Africa), 100 µM xylenol orange (Sigma Aldrich, Missouri, USA), 100 µM iron(II)sulphate (Merck, Darmstadt, Germany) and methanol (>99.9% pure, Sigma Aldrich, Missouri, USA) , 50 mM Tris-HCl (Melford Laboratories Ltd Ipwich, UK), pH 7.4 in a 9:1 methanol to Tris-HCl ratio.

The assay was conducted as previously described by Waslidge and Hayes (1995) with minor modifications. For the standard curve, a dilution of pure L(+) ascorbic acid (Merck, Darmstadt, Germany) was tested against a constant concentration (0.5 mM) of hydrogen peroxide. The ascorbic acid standard curve was constructed using doubling dilutions with 50 mM Tris-HCl buffer (pH 7.4) over the concentration range of 0.3125–40 mM. Fifty microlitres of each dilution was carried over to an ice cold micro-titre plate and each dilution was done in triplicate. The AROP-FOX assay was conducted as described above for determination of AROP.

AROP of barley malt samples

For determining the AROP in malt samples (malt M, S, S-4 and E), 5.0 g of freshly milled malt flour was extracted in 20 mL ice cold water for two hours with agitation. Six doubling dilutions of each sample were prepared in an ice cold micro-titre dilution plate. The undiluted extracts were used as the highest concentration in each case, diluting it down to a 32-fold dilution as the lowest concentration and as blank control, sample solvent was used. Fifty microlitres of each diluted extract and control was then transferred in triplicate into a chilled flat-bottom 96-well microtiter plate.

To determine AROP, the reaction was initiated by adding 50 µL of 0.5 mM hydrogen peroxide (Alpha Pharm, Allied drug Company, Durban, South Africa) solution to the extracts, with exclusion of the controls and incubated for 10 minutes at 37°C. After incubation 100 µL FOX reagent was added, followed by 50 µL of 50 mM Tris-HCl (pH 7.4). The coloured reaction product was then measured on a Biorad microtiter plate reader from 0-30 minutes at 620 nm. The formation of a purple/blue colour from an initial yellow acidified xylenol orange indicated the oxidation of Fe^{2+} to Fe^{3+}, the latter reacting with the dye.

CUPRAC antioxidant micro-assay

The CUPRAC assay as described by Alpinar *et al*., 2008 and Güçlü *et al.*, 2006 was modified to be performed in a microtiter plate. The CUPRAC solution was mixed prior to each

experiment and per single plate experiment consisted of the following solutions: 10 mL 10 mM $CuCl_2$ (Sigma Aldrich, Missouri, USA), 10 mL 7.5 mM neocuproine (Sigma Aldrich, Missouri, USA) and 10 mL 1.0 M ammonium acetate buffer, pH 7 (Merck, Wadeville, Gauteng, South Africa). Ascorbic acid was used as standard over a concentration range of 2.44 µM to 5.0 mM, prepared using doubling dilutions. The barley malt samples were extracted in analytical quality water (5 g per 20 mL) and subsequently centrifuged to yield a clear supernatant from which a dilution range was created. Hundred microlitres of the sample/standard was pipetted into each well followed by 300 µL CUPRAC solution. For each dilution of each sample/standard, three repeats were done. The plate was incubated at room temperature for 30 minutes and spectrophotometrically measured at 450 nm. A standard curve was generated by plotting the absorption values of ascorbic acid at the different ascorbic acid concentrations and afterwards used to determine the antioxidant potential relating to ascorbic acid in the unknown samples.

In order to directly compare the CUPRAC assay with the AROP-FOX assay, four different and randomly selected barley malt varieties were analysed and will be referred to as malt M, S, S-4 and E.

Results and discussion

Guido *et al.*(2007), by determining AROP with the DPPH method, found that AROP in malt correlates positively with the taste scores of aged beers and their polyphenolic content as characterised by Goupy *et al.* (1999). This observation indicated that the presence of a high AROP would retard the development of oxidised flavours in beer (Drost *et al.*, 1990). It was also found that LOX activity is inhibited by high AROP. This information would prove valuable for maltsters and brewers, since malt can be selected with high AROP for the brewing of beer of extended shelf life High AROP could lead to the inhibition of oxidation taking place during malting and in the final packaged product (Huige, 1993).

Previously, a FOX assay was optimised to determine LOX-1 ((Waslidge & Hayes, 1995) to a micro-assay for LOX-1 (Chapter 3)). This FOX micro-assay was then further adapted to assess AROP in standardised samples containing different amounts of ascorbic acid (vitamin C) as antioxidant and in malt extracts. In the adapted AROP-FOX micro-assay it is possible to visually discriminate between samples with high and low AROP, with high AROP samples showing less purple colour development (compare malt M with the three other malts in Fig. 1A). The modified CUPRAC micro-assay was similarly utilised to assay samples containing ascorbic acid and malt samples. In this assay a yellow colour is indicative of high ascorbic acid or AROP (Fig. 1B).

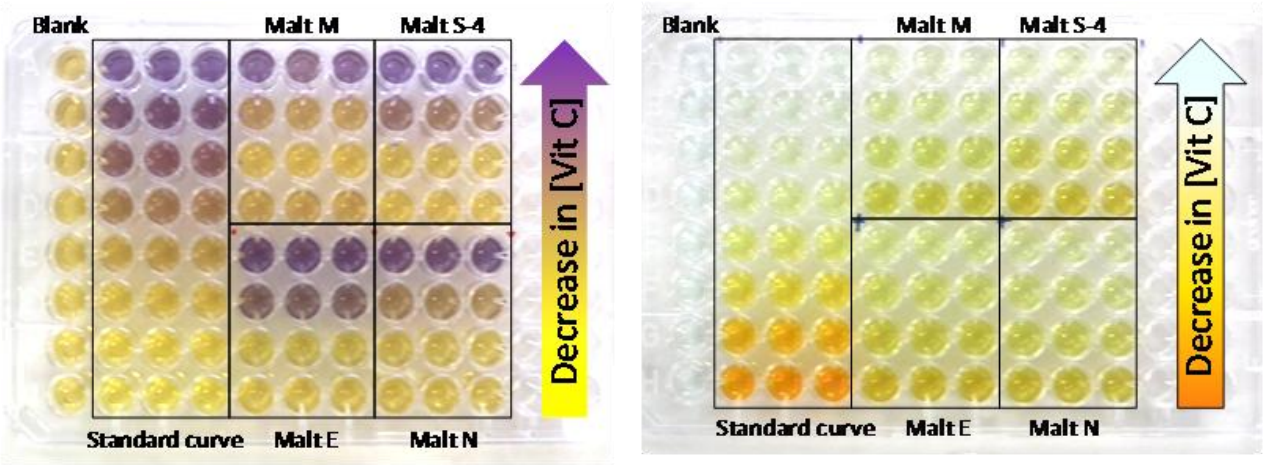

Fig. 1. Photographs of a representative **A)** FOX-AROP assay in 96 well plates showing the colour development in the presence of hydrogen peroxide and **B)** a modified CUPRAC assay showing the colour development from a light blue $Cu(Nc)_2^{2+}$ to a yellow/orange $Cu(Nc)^{2+}$.

It was essential to determine the FOX-micro assay's robustness by monitoring at which point in time absorbance should be recorded and for how long the colour remains stable. Fig. 2 depicts the change in absorbance of the standards over a 30 minute time period after the FOX reagent has been added. After 15 minutes, little change is observed, which makes it viable to read the plate 15 minutes after the FOX reagent's addition. It was observed that the colour development stayed visually stable for at least 48 hours if the plates are sealed and stored at 4^{o}C.

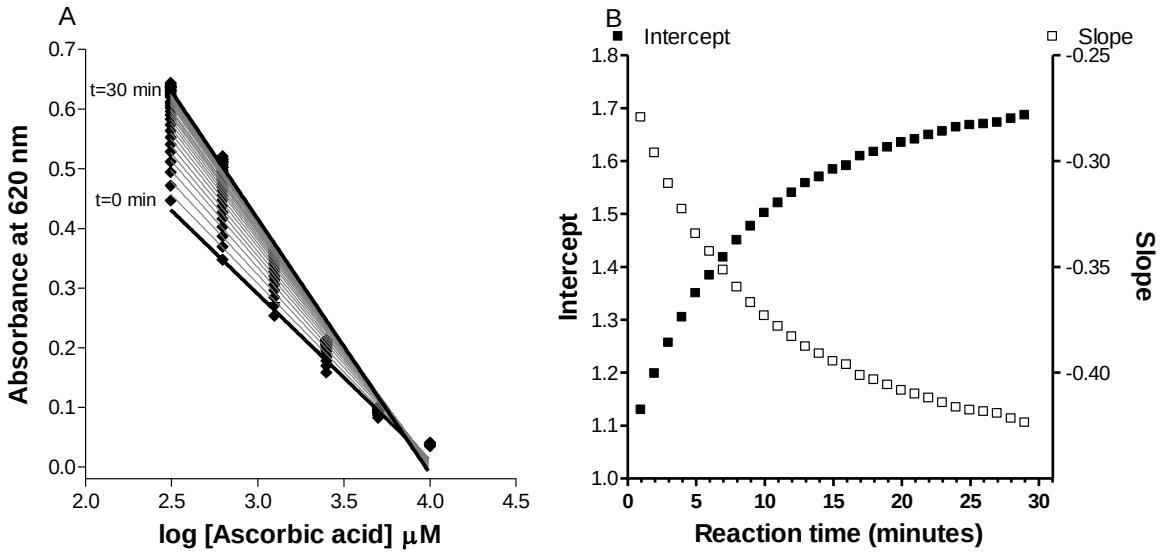

Fig. 2. Time dependence of FOX micro-assay for AROP. **A.** Change in standard curve (n=3) for the determination of AROP concentration using ascorbic acid *versus* hydrogen peroxide over 30 minutes. **B.** Change in the linear regression line fit parameters of the standard curve over 30 minutes.

The repeatability of the adapted FOX assay for determining AROP was established using its standard curves generated by incubating ascorbic acid with hydrogen peroxide to induce oxidation. Fig. 3 illustrates representative examples of standard curves generated using the AROP-FOX micro-assay and the CUPRAC assay adapted in 96-well plates. An exponential decrease in absorption with the increase in ascorbic acid was observed for the AROP-FOX-micro assay (Fig. 3A), and therefore the linear regression line was plotted using a semi-log graph. For the CUPRAC micro-assay (Fig. 3B) an increase in absorption was observed with an increase in ascorbic acid. From the highly repeatable standard curves of both assays it is clear that both these methods of AROP determination are equally repeatable (Fig. 3). With regards to the sensitivity and different ranges of quantisation of the two assays, the CUPRAC micro-assay have much higher sensitivity at lower AROP, with an upper limit of quantisation (LOQ) of 156 µM and well within range of the 95% prediction interval at concentrations as low as such as 2.44 µM. Results were comparable to the 5.6 µM and 85 µM linear range previously determined (Apak *et al.*,

2005). Although the AROP-FOX micro-assay was less sensitive at the lower ascorbic acid concentration, it showed a 64-fold higher upper LOQ of 10 mM, illustrating higher sensitivity at the high concentration ranges where the CUPRAC would no longer be reliable. As plant samples generally are expected to contain high antioxidant levels the higher upper LOQ of the AROP-FOX assay may be very useful for industrial food analysis (Vanderslice *et al.*, 1990; Szeto *et al.*, 2002).

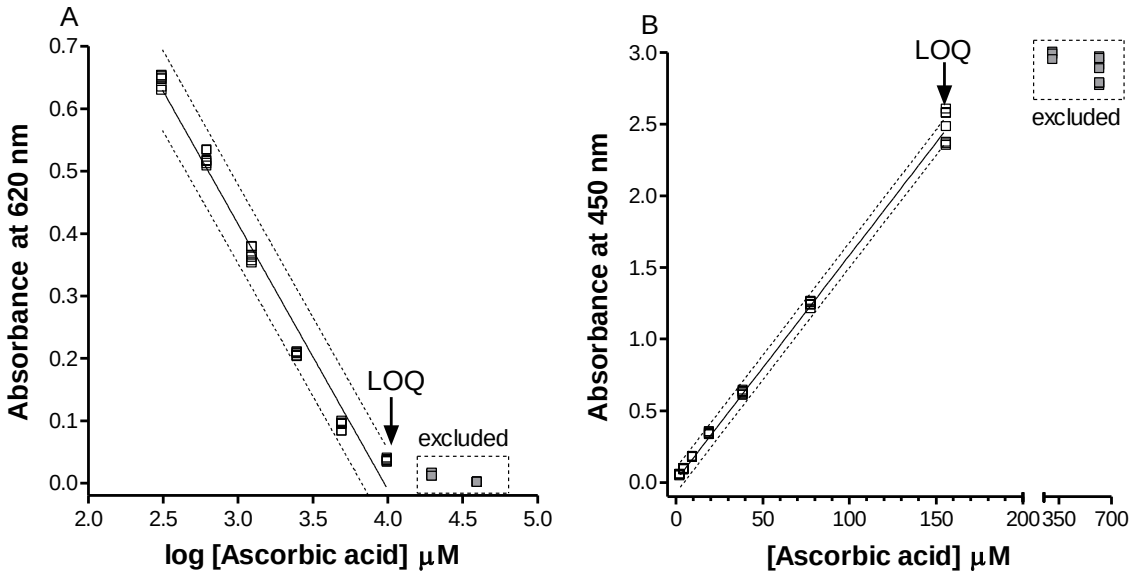

Fig. 3. Comparison of standard curves for the determination of AROP with ascorbic acid as antioxidant. **A.** A representative example of a standard curve (30 minute reaction time) used in the FOX-AROP micro-assay . The regression line fit to the individual points (n=6) of the semi-log graph was R^2 = 0.982 with the maximum limit of quantisation (LOQ) at 10 mM. **B.** A representative example of a standard curve used in the CUPRAC micro-assay. The regression line fit to the individual points (n=6) of the graph was R^2 = 0.998 with the maximum limit of quantisation (LOQ) at 156 µM. The dotted lines in both graphs represent the 95% prediction interval. The boxed grey-shaded data points in both graphs represent concentrations above the LOQ of the assay.

Numerous researchers have attempted to select or identify a standard method for the determination of AROP in different foodstuffs (Apak *et al.*, 2005; Huang *et al.*, 2005; Prior *et al.*, 2005; Apak *et al.*, 2007; Sun & Tanumihardjo, 2007) and this has proven to be a tedious challenge. The CUPRAC assay has been thoroughly compared to various other antioxidant

determination assays (Apak *et al.*, 2007) and has been highly recommended. When an ascorbic acid solution of 0.156 mM was analysed for AROP, highly similar results were obtained with both the AROP-FOX and CUPRAC with both assays closely predicting the ascorbic acid concentration as 0.156 mM (Table 1). For the malts CUPRAC assay predicted significantly higher AROP (19-84%) than the FOX-AROP assay.

Table 1 Comparison of antioxidant concentration and AROP as estimated by the adapted AROP-FOX and the CUPRAC micro-assays.

Sample	**[Antioxidant] and AROP equivalent to ascorbic acid (mM)**[$]	
	AROP-FOX assay	CUPRAC assay
0.156 mM ascorbic acid	0.161 ± 0.03	0.156 ± 0.01
Malt E	9.47 ± 0.74	17.4 ± 1.31***
Malt S-4	13.8 ± 1.26	19.2 ± 1.28**
Malt N	17.2 ± 0.86	21.8 ± 1.40**
Malt M	21.6 ± 1.61	25.8 ± 1.80*

[$] Data are the mean of triplicate determinations with standard deviation. Malt samples for assays were diluted as required by reliable standard range.
Significant difference according to one-tailed Student t-test:
***P<0.0005; **P<0.005; *P<0.05

It is, however, difficult to directly compare assays due the nature or mechanism of the reaction being measured (Prior *et al.*, 2005). The AROP-FOX assay measures the ability of the antioxidant to prevent oxidation of Fe^{2+} to Fe^{3+} in the presence of hydroperoxide, while the CUPRAC assay measures the ability of Cu^{2+} to be reduced to Cu^{+} by any antioxidants present. It was stated by Prior *et al*. (2005) that it is not likely to obtain similar results using methods over a wide range of plant extracts, especially if the method's reaction mechanisms are different. Although the AROP prediction were significantly different, a putative linear correlation (R^2=0.97) was observed between AROP in barley malt samples analysed using the modified AROP-FOX micro-assay and the CUPRAC micro-assay as is depicted in Fig. 4. From either set of assay results it was thus possible to rank the four malts in terms of their AROP or antioxidant status, namely malt M > N > S-4 > E (Fig. 4). The CUPRAC is already a standardised method

for determining antioxidant capacity in foodstuff (Prior *et al.*, 2005) and this result supports the applicability of the FOX-AROP micro-assay in malt analyses.

We then used the newly developed AROP-FOX method for determining AROP in different malt samples collected at different kilning positions (Fig. 5). This overall significantly higher AROP was observed in the samples closer to the bottom of the kiln. The positional variation of AROP within a specific barley malt variety could be due to the exposure to oxygen in the more exposed samples taken from the top of the kiln (Fig. 5A).

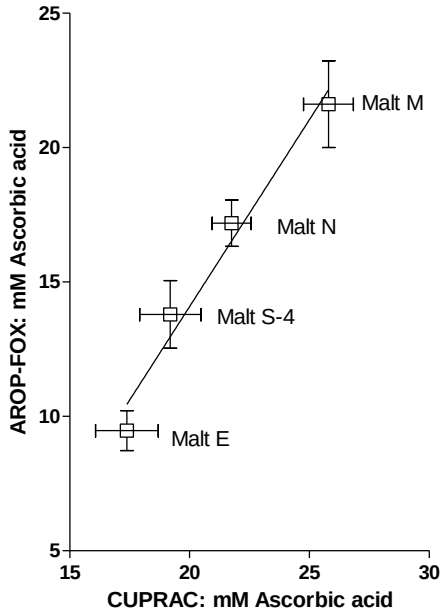

Fig. 4. Comparison of the AROP in terms of ascorbic acid units determined by the FOX-AROP micro-assay and the CUPRAC micro-assay in four different barley malt samples. A putative linear relationship is indicated by the linear regression line fit of R^2=0.97 with absolute sum of squares = 7.08 to the data points. Data points are a mean (± standard error of the mean) of triplicate determinations with each of the assays.

Significant AROP differences between the different malts were found in both the top and bottom samples, with more significant differences between the top samples. These differences between samples collected from the same kilning position are mainly due to the varieties showing different AROP (Fig. 5B).

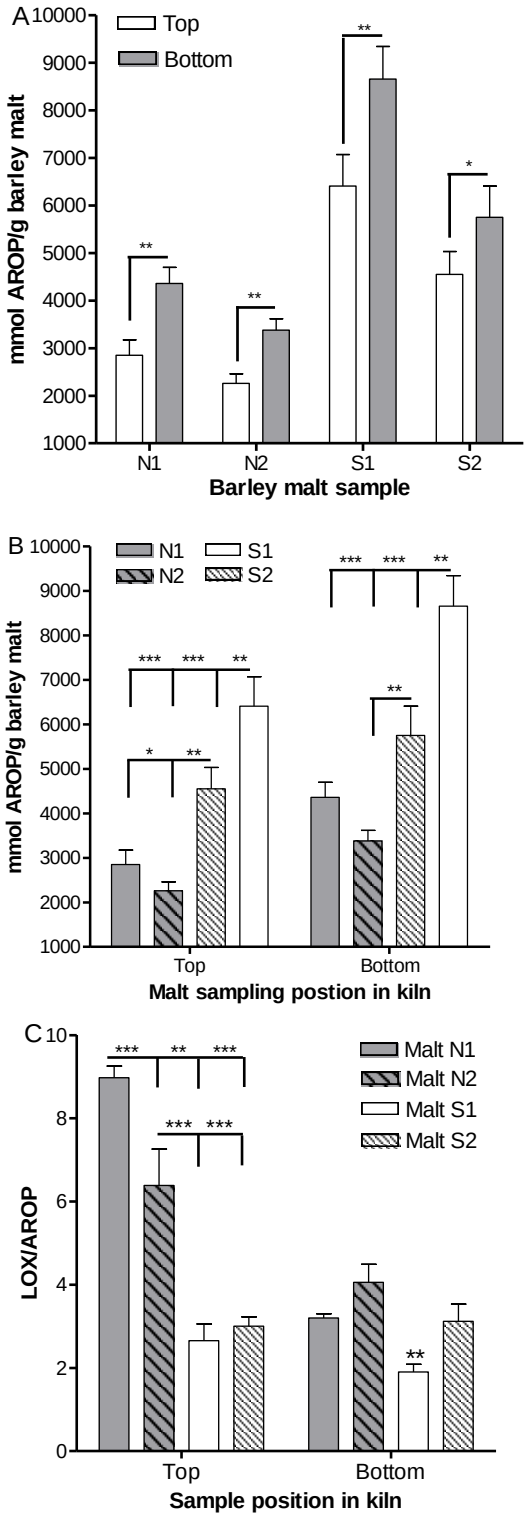

Fig. 5. Comparison of barley malt of four different malted barley cultivars in terms of **A:** influence of sampling from three different locations (top, middle and bottom) on mmol AROP/g (statistical analysis performed with the Student t-test with **P<0.01; *P<0.05), **B**: comparison of mmol AROP/g content with each other at the two sampling positions and **C:** comparison of LOX/AROP ratio of the four different malted barley cultivars collected at the two kilning positions. LOX activity was determined by the adapted LOX-FOX micro-assay (Chapter 3). Statistic analyses in B and C were performed with One-way ANOVA using Bonferroni multiple comparison test with ***P<0.001, **P<0.01; *P<0.05.

When evaluating the ratio of LOX to AROP of samples collected at different kilning positions (Fig. 5C) it was found that significant differences existed between the samples taken from the top. Malt N1 and N2 differed significantly from each other and the other two malts, S1 and S2, which were similar and displayed significantly lower LOX:AROP ratios. Such properties in malts N1 and N2 would be undesirable, as it is expected that a malt with a high LOX content in combination with a low AROP content could result in a shortened beer shelf life due to oxidised off-flavour compounds (Drost *et al.*, 1990). Malts taken from the bottom of the kiln only showed significant variation between malt S1 and the rest.

Conclusions

The FOX assay as described by Waslidge and Hayes (1995) for the determination of LOX in plant materials was successfully adapted for use as AROP determinant for malt extracts. The method also compared favourably to the popular CUPRAC method and can therefore be regarded as an alternative medium-throughput assay for the determination of AROP in barley malts. The reason for specifically using an adapted version of the FOX assay was to develop an assay that can be used for both LOX and AROP determination. In a previous report the FOX assay was adapted for a rapid, medium throughput determination of LOX in malt and wort samples (Chapter 3). The LOX-FOX and AROP-FOX assays can thus be used with the same colour reagents and malt factor influencing the colour reaction to determine LOX-1 activity and AROP, which are both important factors to be considered in the possible prediction of barley/wort's beer shelf-life (Vanderhaegen *et al.*, 2006).

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References

Alpinar, K., Özyürek, M., Kolak, U., Güçlü, K., Aras, Ç., Altun, M., Çelik, S.E., Berker, K.I., Bektasoğlu, B. & Apak, R. (2009). Antioxidant capacities of some food plants wildly grown in ayvalik of turkey. *Food Science and Technology Research*, **15**, 59-64.

Apak, R., Güçlü, K., Demirata, B., Özyürek, M., Çelik, S.E., Bektaşoğlu, B., Berker, K.I. & Özyurt, D. (2007). Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. *Molecules*, **12**, 1496-1547.

Apak, R., Güçlü, K., Özyürek, M., Karademir, S.E. & Altun, M. (2005). Total antioxidant capacity assay of human serum using copper (II)-neocuproine as chromogenic oxidant: The CUPRAC method. *Free Radical Research*, **39**, 949-961.

Drost, W., B., Berg, V.d., R., Freijee, M., F.J., Velde, V.d., G., E., Hollemans & M. (1990). Flavor stability. *American Society of Brewing Chemists*, **48**, 124–131.

Goupy, P., Hugues, M., Boivin, P. & Amiot, M.J. (1999). Antioxidant composition and activity of barley (hordeum vulgare) and malt extracts and of isolated phenolic compounds. *Journal of the Science of Food and Agriculture*, **79**, 1625-1634.

Güçlü, K., Altun, M., Özyürek, M., Karademir, S.E. & Apak, R. (2006). Antioxidant capacity of fresh, sun-and sulphited-dried malatya apricot (prunus armeniaca) assayed by CUPRAC, ABTS/TEAC and folin methods. *International Journal of Food Science &Technology*, **41**, 76-85.

Guido, L.F., Curto, A.F., Boivin, P., Benismail, N., Gonçalves, C.R. & Barros, A.A. (2007). Correlation of malt quality parameters and beer flavor stability: Multivariate analysis. *Journal of Agricultural and Food Chemistry*, **55**, 728-733.

Hashimoto, N. (1972). Oxidation of higher alcohols by melanoidins in beer. *Journal of the Institute of Brewing*, **78**, I972.

Hashimoto, N. & Kuroiwa, Y. (1975). Pathways for the formation of volatile aldehydes during storage of bottled beer. *Report of the Research Laboratories of Kirin Brewery Co., Ltd*, **18**, 1-11.

Huang, D., Ou, B. & Prior, R.L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*, **53**, 1841-1856.

Huige, N.J. (1993). Analysis, characterization and technological advances. In: *Beer and wine production* (edited by B.H. Gump). Chapter 5, pp 67. Washington, DC: American Chemical Society.

Intelmann, D. & Hofmann, T. (2010). On the autoxidation of bitter-tasting iso-α-acids in beer. *Journal of Agricultural and Food Chemistry*, **58**, 5059-5067.

Kuroda, H., Kojma, H., Kaneda, H. & Takashio, M. (2005). Characterization of 9-fatty acid hydroperoxide lyase-like activity in germinationg barley seeds that transform 9(S)-hydroperoxy-10(E), 12(Z)-oktadecadienoic acid into 2(E)-nonenal. *Bioscience, Biotechnology, and Biochemistry*, **69**, 1661-1668.

Lustig, S. (1999). Analytical tools and practical options to improve flavour stability. *Ferment*, **12**, 25-29.

Narziss, L., Miedaner, H. & Graf, H. (1985). Carbonyls and ageing of beer. Part 2: Influence of some technical parameters. *Monatsschrift fuer Brauwissenschaft*, **38,** 346-353.

Özyürek, M., Güçlü, K. & Apak, R. (2011). The main and modified CUPRAC methods of antioxidant measurement. *Trends in Analytical Chemistry*, **30**, 652-664.

Prior, R.L., Wu, X. & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, **53**, 4290-4302.

Sun, T. & Tanumihardjo, S. (2007). An integrated approach to evaluate food antioxidant capacity. *Journal of Food Science*, **72**, 159-165.

Szeto, Y.T., Tomlinson, B. & Benzie, I.F. (2002). Total antioxidant and ascorbic acid content of fresh fruits and vegetables: Implications for dietary planning and food preservation. *British Journal of Nutrition*, **87**, 55-59.

Takashio, M. & Shinotsuka, K. (1998). Preventive production of beer against oxidation - recent advances in brewing technology. *Food Science and Technology International Tokyo*, **4**, 169-177.

Vanderhaegen, B., Neven, H., Verachtert, H. & Derdelinckx, G. (2006). The chemistry of beer aging - a critical review. *Food Chemistry*, **95**, 357-381.

Vanderslice, J.T., Higgs, D.J., Hayes, J.M. & Block, G. (1990). Ascorbic acid and dehydroascorbic acid content of foods-as-eaten. *Journal of Food Composition and Analysis*, **3**, 105-118.

Waslidge, N.B. & Hayes, D.J. (1995). A colorimetric method for the determination of lipoxygenase activity suitable for use in a high throughput assay format. *Analytical Biochemistry*, **234**, 354-358.

CHAPTER 5

Correlations between positive beer fermentation and flavour factors and LOX-1 activity in different barley malt varieties

Introduction

It is difficult to select barley varieties according to specific characteristics responsible for the production of a high quality product (Fox *et al.*, 2003). Barley cultivars used for malting should have high yields in the field, as well as in the brew house and should ideally be free of any harmful organisms (Linko *et al.*, 1998; Van Nierop *et al.*, 2006). To test the quality of end products from each line is impractical due, to among others, the high costs involved. It would therefore be recommended to perform molecular and biochemical tests on the raw material to predict malting and brewing qualities of barley during the breeding process as well as to adequately describe the quality of a certain malt (Fox *et al.*, 2003).

Barley varieties are classified on a molecular basis and must fulfil certain specifications depending on its end use. Some of these traits include the concentrations of different types of proteins (grain proteins, storage proteins, non-storage proteins, hordeins, protein Z and lipid transfer proteins), barley carbohydrate composition, starch concentration and non-starch polysaccharides. Physiological traits include grain size, dormancy and grain hardness. For processing purposes, barley can be specified on the basis of its hot water extract, diastatic power and wort viscosity (Fox *et al.*, 2003). All these traits will assist in predicting the end product quality, but for this study the focus will only be on the free amino nitrogen (FAN), protein and antioxidant contributions.

Over the years there have been numerous attempts to predict more than one quality aspect of the end product by evaluating a number of physical or biochemical markers in the raw materials. A statistical approach using malt quality parameters such as malt extract, moisture, colour and total nitrogen, modification index, hardness and β-glucanase activity in the prediction of beer production indicated that the particular malt extract gave the most information about end product quality (Hyde & Brookes, (1978). In a review Bamforth (1985) concluded that fermentable sugars, foam proteins, radicals/oxygen, „pseudo"-hazes and β-glucan content each contribute to the beer quality aspects of alcohol content, foam, colour, flavour and haze. Guido *et al.*(2007) evaluated malts for diastic power, β-glucan content, friability, lipoxygenase activity, antiradical– and reducing power, as well as nonenal potential and statistically evaluated these parameters with sensory results. It was found that antiradical power in malt and barley correlates well with polyphenolic content and was also a major contributor to beer flavour stability. Iimure *et al.*(2010) constructed a proteome map by analysing beer using two-dimensional electrophoresis in order to determine the influence of barley cultivar and degree of malt modification on beer quality. The beer protein concentration and composition correlated with different quality beers, indicating the major role of proteins.

In this study some of the most influential determinants of beer quality will be assessed to determine a possible method for malt selection and quality prediction of beer made from a specific malt. The different quality determining aspects of a library of malted barley varieties under examination will include lipoxygenase (LOX,) activity specifically LOX-1 activity, representing a flavour negative factor, the anti-radical/oxidant potential (AROP), representing a flavour positive factor, lipid transfer protein 1 (LTP1) content, representing both a flavour and foam positive factor, and lastly protein concentration and FAN content as fermentation positive factors.

Lipoxygenase 1 (LOX-1) is an enzyme present in barley responsible for the deoxygenation of fatty acids and specifically linoleic acid to form conjugated diene hydroperoxides (Nelson & Seitz, 1994; Loiseau *et al.*, 2001) which are precursors for *(E)*-2-nonenal, a compound detrimental to the flavour of beer (Hirota *et al.*, 2006) and associated with a "cardboard" flavour in aged beers (Vanderhaegen *et al.*, 2006). It is, however, believed that the presence of antioxidants/radicals can have a stabilising effect on beer flavour (Guido *et al.*, 2007). It would therefore be beneficial to use raw materials low in LOX-1 and high in AROP to possibly extend shelf life. For brewers to inspect the LOX-1 concentration and AROP in different malts a reliable, robust and high throughput assay for these two factors is needed, which will not require too much specialised equipment. With this goal in mind it was decided to implement a single, simple assay for the measurement of both LOX-1 and AROP (refer to chapters 3 and 4).

LTP1 is a major water soluble (Perrocheau *et al.*, 2006), amphiphilic protein located in the aleurone layers of barley kernels (Mundy & Rogers, 1986) and is known to play a role in the seed"s defence reaction when under stressful circumstances such as microbial infestation, drought, chemical shock or dehydration (Kader, 1996). With regard to brewing; LTP1 is extremely tolerant towards denaturation brought on by extreme temperature (Jiang *et al.*, 2011) and pH fluctuations and will thus be present throughout the brewing process (Jegou *et al.*, 2001). LTP1 is known as an antimicrobial protein and has been observed to have inhibitory effects on the brewer"s yeast, *Saccharomyces cerevisiae* (Gorjanovic *et al.*, 2004; Jiang *et al.*, 2011). However, it was found that most of the LTP1 is converted, during malt extraction, to LTP1b that does not have significant anti-yeast activity, therefore it is not considered as a negative factor in fermentation (refer to Chapter 2). This 9.6 kDa protein is known to be involved in the stability of beer foam (Sorensen *et al.*, 1993) and has recently been identified as having anti-radical/oxidant activity (Wu *et al.*, 2011) and possibly protecting beer against ageing and can therefore also be regarded as a flavour positive factor. LTP1 can be found covalently bound to a fatty acid moiety

which Bakan *et al.,* (2003) identified to be a certain hydroxyoctodecenoic acid. Interestingly this compound is also known to be an LOX pathway intermediate which raises the question of whether LTP1 can bind to it and so doing inhibit the formation of (E)-2-nonenal.

FAN, consisting mainly of proteins, peptides, amino acids and amino sugars, serves as a crucial nutrient source for yeast during fermentation (Yano *et al.*, 2008). The measurement of FAN assists the brewer by indicating how well the nitrogen containing biomolecules present can be utilised by brewing yeast during the fermentation process (Hammond & Smart, 2000). It is, however, not only the FAN that play a role in efficient yeast metabolism, but the proteolytic enzymes which are responsible for the breakdown of larger nitrogen sources to be utilisable yeast nutrients (Agu & Palmer, 2001). A lengthened malting step will lead to increased FAN, total soluble nitrogen and Kolbach index values due to a longer exposure to proteolytic enzymes (Nie *et al.*, 2010). Total nitrogen refers to the sum total of all nitrogenous compounds, regardless if it can serve as nutrient source for yeast, while FAN refers to the free amino nitrogen portion. Little consensus have been reached regarding a specific variety‟s FAN value towards its modification potential, extract yield (Agu & Palmer, 2001) and fermentability (Abernathy *et al.*, 2009). This is mainly due to the difference in nitrogen i.e. amino acids (Nie *et al.*, 2010) and enzyme (Yano *et al.*, 2008) composition across various barley varieties.

For this study, 28 different malt samples were evaluated on the basis of their LOX-1 activity, AROP, protein content and composition (FAN content), as well as LTP1 content. The malts were selected by SABMiller and represents malts representing a wide range of fermentation character. With the data collected during this report we will provide a method for predicting a certain malt‟s performance in order to simplify and improve malt blending practices for brewers.

Materials and methods

LOX activity determination

LOX activity was measured using the LOX-FOX micro-assay as previously described in Chapter 3. The FOX (ferrous oxidation-xylenol orange) reagent comprised of 25 mM sulphuric acid (Merck chemicals, Wadeville, Gauteng, South Africa), 100 µM xylenol orange (Sigma Aldrich, Missouri, USA), 100 µM iron(II)sulphate (Merck, Darmstadt, Germany) and methanol (>99.9% pure, Sigma Aldrich, Missouri, USA): 50 mM Tris (Melford Laboratories Ltd Ipwich, UK) -HCl, pH 7.4 in a 9:1 methanol to Tris-HCl ratio.

Five grams of malt flour was extracted in 100 mL ice cold water for 2 hours with agitation. SABMiller (Caledon, South Africa) supplied 28 barley malt cultivar samples spanning the 2008-2010 seasons (Table 1) that were extracted and analysed. Six doubling dilutions of each extracted sample in triplicate were prepared. The undiluted extracts were used as the highest concentration in each case, diluting it down to a 32-fold dilution as the lowest concentration. For the negative control, a portion of a random malt extract was boiled for 10 minutes at 80°C and subsequently centrifuged (20 minutes at 3000 ×*g*). Fifty microlitres of each diluted extract and control was then transferred in triplicate into a chilled 96-well microtiter plate. For the standard curve, a dilution of analytically pure LOX-1 from soybean (Sigma Aldrich, Missouri, USA) was tested against an excess linoleic acid (Sigma Aldrich, Missouri, USA). For the LOX-1 activity standard curves the soybean lipoxygenase was made up in triplicate doubling dilutions starting with 1000 units/mL to 31.25 units/mL.

The FOX reaction was initiated by adding 50 µL of a 0.70 mM linoleic acid solution to the extracts/standards in the microtiter plate, with exclusion of the controls, and incubated for 15 minutes at 37°C. After incubation 100 µL FOX reagent was added to each well, including that of the controls. The controls also received 50 µL of a 0.70 mM linoleic acid solution, which enabled the determination of any possible activity and background colour development in the

absence of substrate during incubation. The colour development in the reaction mixtures were determined 15 minutes after FOX addition at 620 nm using a BioRad microtiter plate reader.

Antiradical/antioxidant potential

AROP was measured using the AROP-FOX micro-assay as previously described in Chapter 4. To determine AROP activity, the reaction was initiated by adding 50 µL of a 0.5 mM hydrogen peroxide solution (Alpha Pharm, Allied drug Company, Durban, South Africa) to the extracts, with exclusion of the controls, which consisted of the sample solvent and incubated for 15 minutes at 37°C. After incubation 100 µL FOX reagent was added followed by 50 µL 50 mM Tris-HCl buffer, pH 7.4. The coloured reaction product was then determined 15 minutes after FOX addition on a BioRad microtiter plate reader at 620 nm.

For the standard curve, a dilution of pure L(+) ascorbic acid (Merck, Darmstadt, Germany) was tested against a constant concentration (0.5 mM) of hydrogen peroxide. The ascorbic acid was made up in doubling dilutions with 50 mM Tris-HCl buffer, pH 7.4 with the highest concentration being 40 mM. Fifty microlitre of each dilution was carried over to an ice cold micro-titre plate and each dilution was done in triplicate. The AROP-FOX assay was conducted as describe above.

BCA protein determination

A protein determination on each malt extract was done using a Pierce BCA protein determination kit (Thermo scientific, Rockford, Illinois, USA). The standard protein determination procedure outlined by the manufacturer was used in all protein concentration determinations. A standard protein concentration curve was generated by using a dilution range of bovine serum albumin (BSA) as reference.

LTP1 fingerprinting

A western blot was performed using LTP1 generated antibodies generously supplied by Prof. Evan Evans (School of Plant Science, University of Tasmania, Tasmania) to verify the presence of LTP1 in the different extracts. The proteins were transferred from the SDS-PAGE gel onto a nitrocellulose membrane (Pall Corporation, Pensacola, Finland) at 0.8 mA/cm^2 gel. The membrane with the transferred protein was incubated with 20 mL casein buffer (154 mM NaCl, 0.5 casein, 10 mM Tris-HCl and 0.02% thiomersal, pH 7.2) for 20 minutes under slight agitatation. This was followed by the incubation of the membrane with whole serum (1:5000) and further incubated for 60 minutes at room temperature. The membrane was then washed four times for 5 minutes with 20 mL PBS-Tween (0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO_4, 0.02% KH_2PO_4, 0.1% Tween 20, pH 7.2) after which it was placed in 20 mL casein buffer with 1: 10 000 goat anti-rabbit horse radish peroxidase-conjugate (BioRad, Parklands, South Africa) for another 60 minutes at room temperature. The membrane was then washed again four times for 5 minutes with 20 mL PBS-Tween and incubated for 30 minutes with Pierce ECL western blotting substrate (Thermo Scientific, Rockford, Illinois, USA) after which it was exposed for 15 minutes and developed.

The developed western blot results were scanned and converted to 16 bit grey scale and pixelated using UN-SCAN-IT *gel*^{TM}, version 6.1 software from Silk Scientific Corporation and a relative value was assigned to each LTP1-positive band‟s intensity for comparative studies (Fig. 1).

LTP1 and LOX-1 titration

The LOX-FOX assay (as previously described) was performed to monitor the effect of LTP1 on LOX-1 reactions. Pure soybean LOX-1 was diluted down from a starting concentration of 1000 units/mL to 125 units/mL at the lowest concentration. Fifty microlitres of each LOX-1

dilution was incubated in a microtiter plate with 10µL pure LTP1 (extracted as was described in Chapter 2) starting at the highest concentration of 5.0 mg/mL and diluted down as a doubling dilution series to 0.625 mg/mL. After a 15 minute incubation time, 50 µL of a 0.70 mM linoleic acid solution was added to all wells excluding the blanks. Controls were performed where LOX-1 was absent and LTP1 present and *vice versa*. Blank samples contained only the reagents‟ (LTP1 and LOX-1) solvents and substrate was only added after the FOX reagent had been added.

Data analysis

Data analysis was done using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, California USA) and STATISTICA, version 11 (StatSoft, Inc, 2012, Oklahoma, USA). The data analysed using GraphPad Prism was plotted using X-Y analysis, fitted to a linear regression curve (Fig. 2, 3 and 5) and a hyperbolic curve (Fig. 4). Two dimensional principle component analysis (PCA) was performed from a scatter plot using STATISTICA (Fig. 6).

Results and Discussion

In this study twenty eight malt samples were analysed as previously mentioned on the basis of LOX-1 and AROP content using the FOX assay as described in Chapters 3 and 4. The intact protein concentration in these samples were also analysed and FAN values were supplied by SABMiller (Caledon, South Africa) for each of the malts. SABMiller makes use of the ninhydrin method for determination of free amino nitrogen (FAN) as set out by the European Brewery Convention, Analytica-EBC (1998), FAN method 8.10, also described by Lie (1973). LTP1 was analysed for comparative purposes using western blotting and quantified by digitisation (Fig.1). Five variants were statistically analysed to detect possible trends between different malt characteristics. .

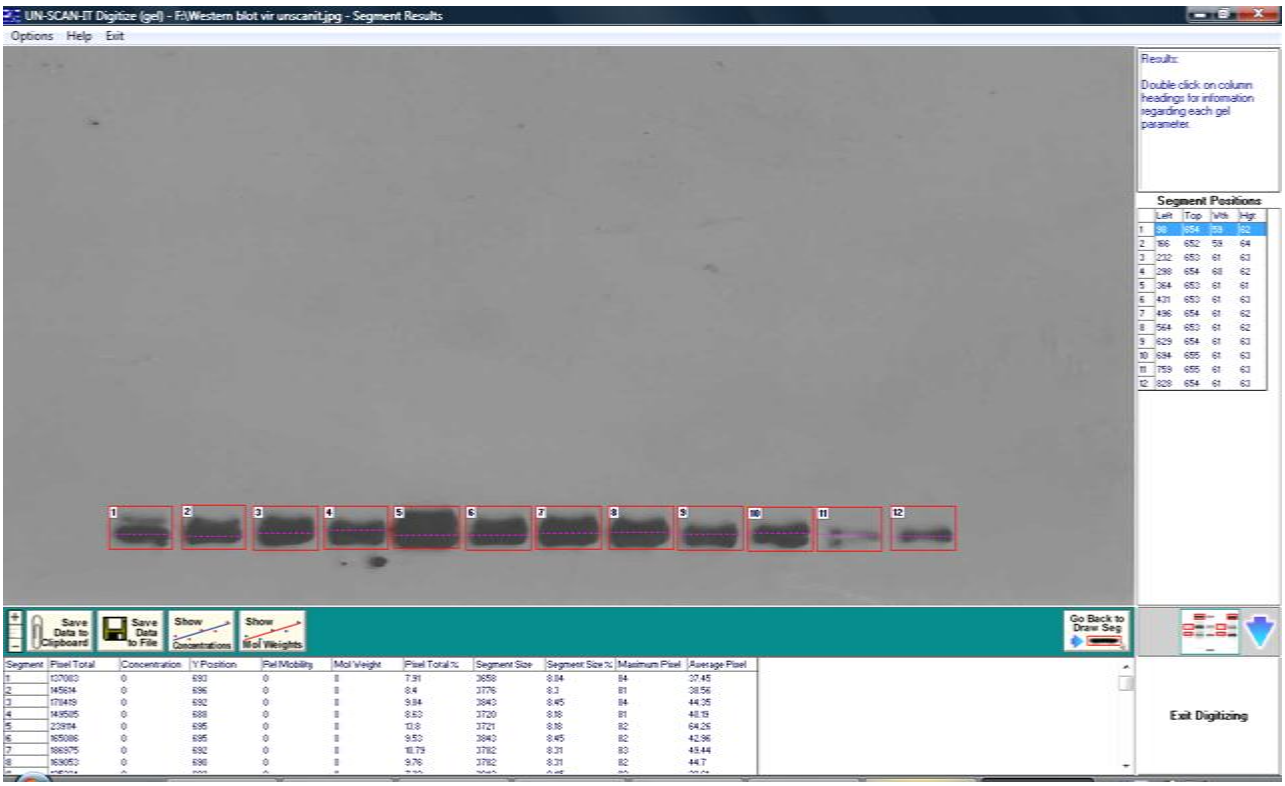

Fig. 1. An example of western blot results used for LTP1 quantification by densitometry. Scanned western blot results were analysed by selecting each band (red rectangles). UN-SCAN-IT *gel*™ (version 6.1 software from Silk Scientific Corporation) analyses convert each band into pixels and average pixels are calculated after a background subtraction is done.

Comparative values for the different variables, determined in the malt samples, are represented in Table 1, in addition with each sample‟s FAN value, as were supplied by SABMiller. Most of the intact protein levels were between 30 and 45 mg/g malt, with only malt 6 having a lower protein level. The FAN values were all between 150 and 215 mg/L wort. Most of the LOX activity levels were below 20000 units, with only six malts showing higher levels and three malts showing levels higher than 40000 units/g malt, corresponding to previously observed results (Douma *et al.*, 2003). Most of the AROP levels were above 1000 mmol/g malt with only five malts having very low levels and malt Be having very high levels. This observation is comparable to the results obtained by Samaras *et al.* (2005) when considering the total anti-oxidant concentration.

Table 1 Summary of the different parameters determined in malt extracts from a range of barley cultivars and seasons. Bracketed values indicates the standard error of the mean (SEM) of triplicate determinations.

Barley Cultivar code	Year	mg protein/ g malt	FAN mg/ L wort	LOX units/ g malt	mmol AROP/ g malt	LTP1 signal/ g malt
B	2008	34 (±1.8)	191	9897 (±852.7)	3259 (±418.8)	284
B	2010	36 (±2.2)	212	na	3234 (±534.8)	319
Be	2009	32 (±1.9)	181	12928 (±954.0)	9183 (±1315.0)	433
Cl	2009	34 (±0.9)	203	10133 (±872.9)	2611 (±225.1)	328
C	2009	36 (±1.7)	175	15860 (±1522.0)	1706 (±360.3)	415
Co	2010	38 (±2.2)	189	7411 (±958.5)	2939 (±623.7)	307
E	2009	35 (±2.4)	180	1099 (±59.9)	1715 (±81.7)	255
G	na	41 (±2.0)	180	42827 (±3744.0)	52 (±12.9)	409
H	2009	37 (±2.8)	205	9699 (±701.4)	1635 (±840.7)	322
He	2009	35 (±1.9)	181	10026 (±696.5)	801 (±347.9)	488
M	2009	40 (±2.1)	188	11569 (±32.4)	1595 (±36.2)	246
N	2009	36 (±3.2)	208	2973 (±304.3)	58 (±2.6)	205
Pe	na	32 (±1.4)	na	12333 (±125.0)	2551 (±862.3)	350
Pr1	na	42 (±2.8)	na	10246 (±387.3)	3140 (±451.9)	358
Pr2	na	31 (±0.7)	na	5428 (±524.4)	2409 (±229.7)	358
P	2009	33 (±2.9)	190	47688 (±358.9)	3393 (±357.4)	341
5	2009	26 (±2.5)	208	8620 (±358.7)	3507 (±798.0)	142
6	2010	34 (±1.6)	177	22170 (±649.4)	1788 (±480.8)	332
S	2009	38 (±2.0)	167	3200 (±362.1)	1013 (±151.7)	290
S	2010	37 (±1.7)	175	7536 (±595.1)	288 (±19.45)	332
Se	2009	44 (±3.2)	166	9712 (±56.1)	2802 (±796.7)	380
S-11	2009	30 (±1.8)	163	9945 (±147.5)	3741 (±103.9)	304
S-06	2009	36 (±1.7)	196	24293 (±141.3)	997 (±240.8)	313
S-4	2009	39 (±2.9)	195	66701 (±169.5)	1580 (±419.2)	355
S-4	2010	38 (±2.1)	178	7177 (±177.8)	2627 (±610.3)	307
S-5	2009	38 (±2.3)	206	22170 (±649.4)	3785 (±105.0)	330
Su	na	35 (±2.4)	na	20158 (±255.7)	1457 (±434.4)	281
T	2009	30 (±1.0)	155	10189 (±127.4)	3869 (±503.3)	358

na – not available

Although the exact concentration of LTP1 in each malt was not deducted, the results also indicated variation of LTP1 content, particularly intact/unmodified LTP1 and LTP1b in the malt library (Table 1). As FAN and intact protein concentration are both regarded as positive factors in fermentation the correlation between these two factors in our malt library were assessed. No definitive relationship between a malt‟s protein concentration and FAN content was observed, indicating that other nitrogen sources contribute to FAN, other than the intact protein and *visa*

versa (Fig. 2). The total intact protein as determined with the BCA method in the malts also did not show any direct correlation to the LTP1 or LOX-1 contents (results not shown). However, the BCA method was found to be sensitive to polyphenols (Siebert & Lynn, 2005) which could have skewed the correlations and would reflect in some artefact correlation between with AROP and the intact protein concentration. No such correlation was found indicating, that although the protein determination may have been influenced by polyphenols, it is assume a similar error was incorporated in this parameter.

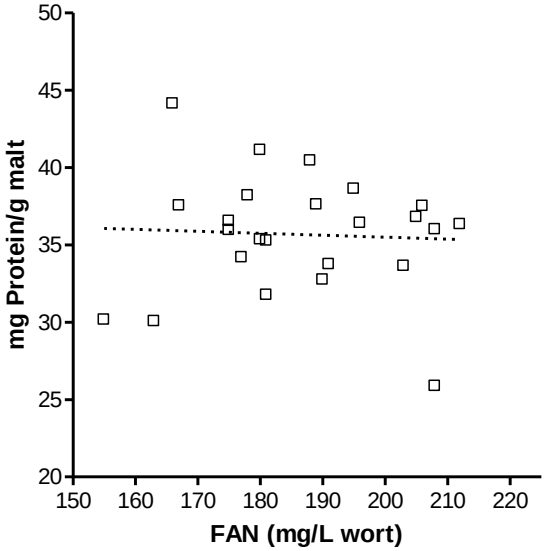

Fig. 2. Graphical representation of the relationship between FAN (mg/L wort) and protein concentration (mg/g malt) in different malts (n=28, linear fit $R^2 = 0.003$)

When the barley malt positive parameters AROP and FAN were normalised to the barley malt negative factor, LOX activity, a fair linear correlation ($R^2 = 0.57$) was observed (Fig. 3A). Also, a cluster of eight cultivars in the mid range with similar characteristics could be identified. Such a trend could be used in distinguishing between malts with possibly having good fermentation characteristics and/or longer shelf life potential and malts having lower fermentation abilities and shortened shelf lives.

As AROP is a flavour positive factor, and assuming that LTP is a flavour positive factor, the ratio of AROP/LOX was plotted against LTP1/LOX (Fig 3B). Normalisation of data to the variable denominator LOX incorporates its differential effect on AROP and LTP1. A relatively

good positive correlation (R^2= 0.66) was observed between AROP/LOX and LTP1/LOX. A cultivar with a high AROP/LOX ratio tended to have a high LTP1/LOX ratio. For this correlation a cluster of ten cultivars in the mid-range could be identified with similar characteristics. This assessment can be beneficial to the brewer since malts with high ratios of AROP/LOX and LTP1/LOX could prove to have a longer shelf-life and possibly better foaming characteristics.

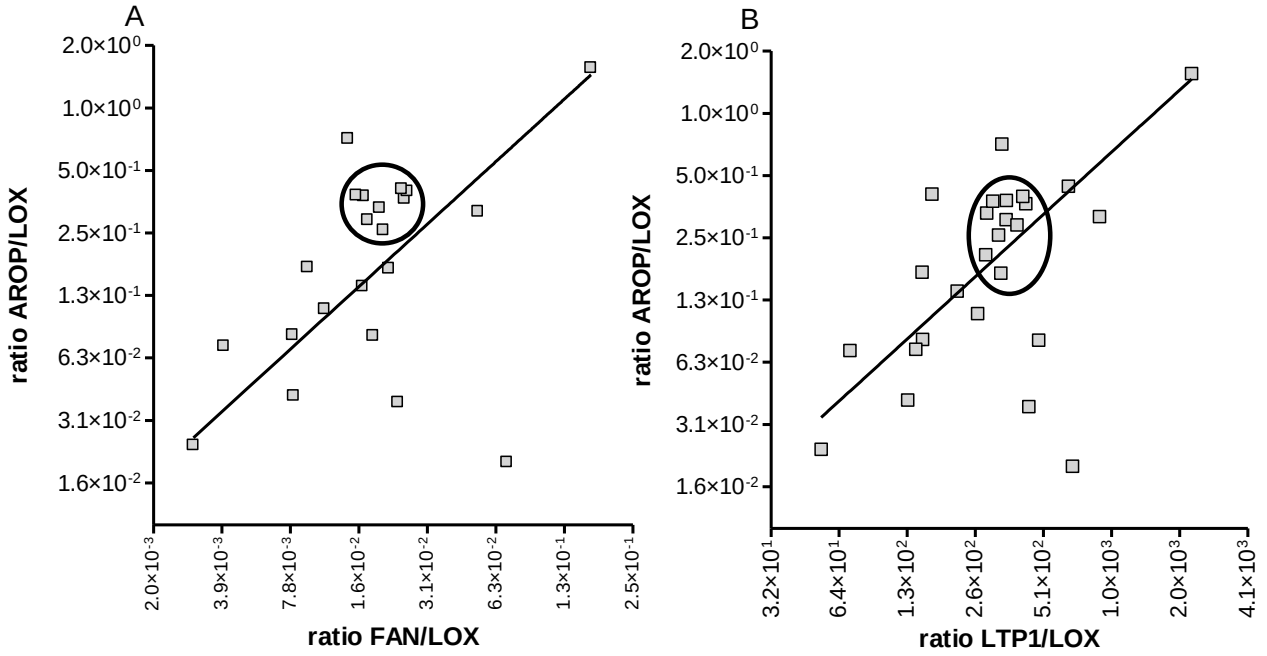

Fig. 3. Graphical representation of the relationships between the ratio **A:** AROP/LOX and FAN/LOX (n=23 and R^2=0.57) and relationship between the ratios **B:** AROP/LOX and LTP1/LOX (n=27 and R^2=0.66). The largest clusters of cultivars with similar characteristic are encircled in each graph. Axes of both graphs are given in $\log_2$ units to depict the spread of the cultivar parameter ratios.

It has been hypothesised that LTP1 is involved in the binding of certain LOX-pathway intermediates, i.e. 9- and 13- hydroxyoctadecadienoic acid (Bakan *et al.*, 2006) although it is still not clear if this binding is competitive and if it could enable LTP1 to inhibit LOX-1 reactions. On the other hand, AROP is again responsible for the scavenging of radicals essential to the LOX-pathway to form unwanted flavour compounds. Wu *et al.* (2011) showed that LTP1 also has antioxidant properties and this could explain why a relationship exists between AROP and LTP1 concentration when normalised by LOX content. A high LTP1/LOX ratio may thus be as much responsible for a stable flavour profile as the AROP/LOX ratio.

AROP was not found in our purified LTP1 samples with the AROP-FOX assay (results not shown). Therefore, in order to elucidate the relationship found in Fig. 3B, LOX-1 was titrated with LTP1 to assess whether LTP1 would influence the LOX-1 activity using our LOX-FOX micro-assay. According to Bakan *et al.* (2006) LTP1 can be bound to α-ketol 9-hydroxy-10-oxo-12*(Z)*-octadecenoic acid to form LTP1b. It has also been shown that this octadecenoic acid is a precursor for 2*(E)*-nonenal that is produced in barley as a breakdown product of linoleic acid *via* the LOX pathway, leading to the staling of beer (Kuroda *et al.*, 2002). We hypothesised that LTP1 could possibly inhibit the formation of 2*(E)*-nonenal by binding a pathway hydroxy lipid intermediate, probably α-ketol 9-hydroxy-10-oxo-12*(Z)*-octadecenoic acid. With the LOX-FOX assay where different concentrations of LOX-1 were incubated with different concentrations of LTP1 it was observed that LOX-1 activity is inhibited by LTP1 in a concentration dependant manner (Fig. 4). As a clear relationship exists between LTP1 and LOX-1, it does explain why a linear correlation was observed AROP/LOX versus LTP/LOX (Fig. 3B).

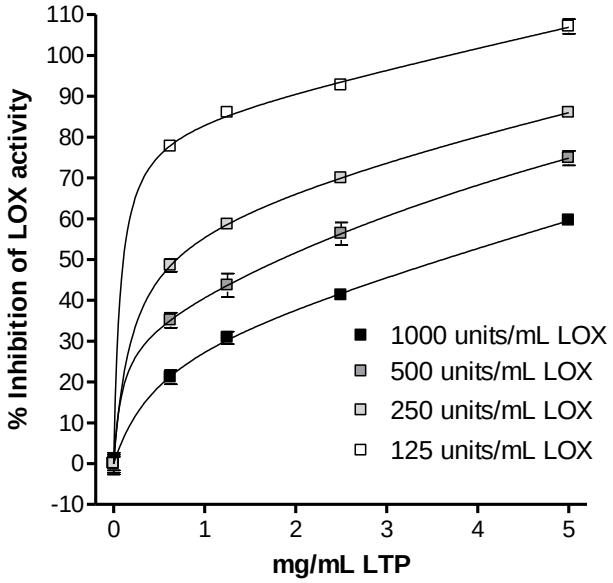

Fig. 4. Inhibition of LOX-1 activity by the different concentrations of LTP1 (hyperbolic trend lines fitted with R^2>0.999). Each data point is the mean of quadruplicate determinations with error bars representing the standard error of the mean (SEM).

Since LTP1 (modified and unmodified) is present at concentrations of 502-1144 µg/g (Evans & Hejgaard, 1999) and 350-700 µg/g (Moelskov Bech *et al.*, 1995) in malt and kilned

malt respectively, it is apparent that LTP1 is playing a much larger role in the prevention of flavour degradation than was originally thought. From this positive correlation between LOX and LTP1, the positive factors, FAN and LTP1, were again normalised with LOX as negative factor (Fig. 5). An excellent linear correlation (R^2=0.93) was found between FAN/LOX and LTP1/LOX. This highly significant linear general correlation between AROP/LOX and FAN/LOX may be beneficial to group and select malts/worts for blends with parameters beneficial for both good beer brewing character and flavour stability in the end product. This analysis also revealed close grouping of 14 cultivars in the mid-range of the trend. An R^2 of 0.15 for the linear fit between FAN and LTP1 indicated very little correlation without incorporation of the third negative parameter, LOX for normalisation of the two parameters.

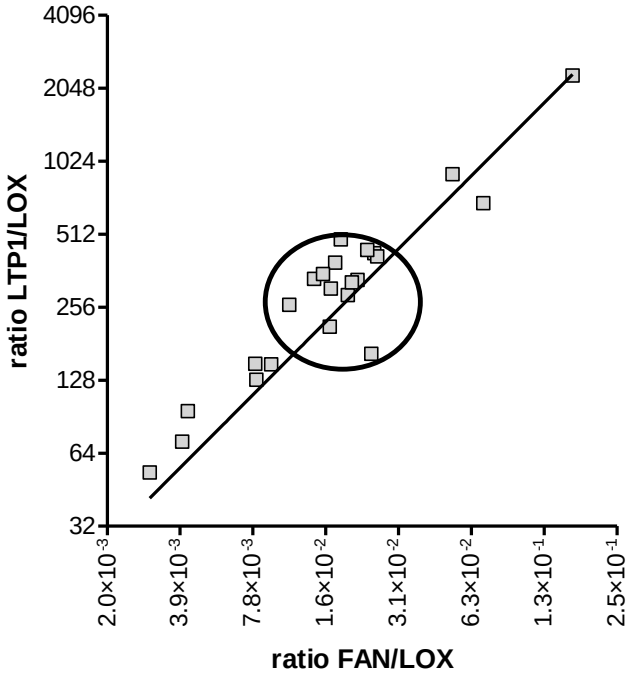

Fig. 5. Graphical representation of the relationships between the ratios LTP1/LOX and FAN/LOX (n=23 and R^2= 0.93). A grouping of 14 cultivars is encircled. Axes of graph are given in log 2 units to depict the spread of the cultivar parameter ratios.

Further analysis using principle component analysis (PCA) using protein concentration, AROP, LOX activity, LTP1 content and FAN concentration as principle components we also observed different groupings of the different barley malt cultivars on a two-dimentional platform

(Fig. 6). Scores of samples which are close to each are considered similar. A major grouping of 15 samples is found around the origin of the components, correlating with our previous results (refer to Figs. 3 and 5). From the two-dimensional PCA plot a minor grouping shows that malts G, Se, C and H seem to be more or less similar with regards to the components, while malts S-11 and T are regarded as similar. The malts of cultivars 5, N and Be were regarded in this PCA analyses as dissimilar to the rest.

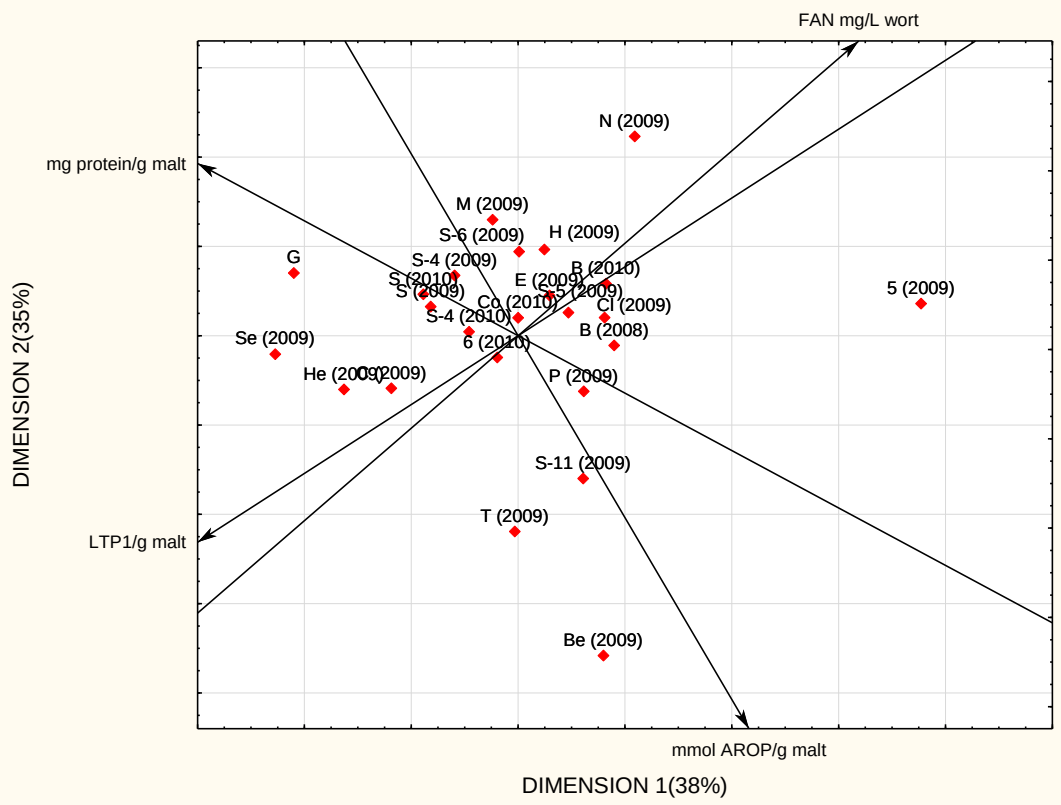

Fig. 6. Two dimensional PCA plot of the 28 barley malt samples with protein-, LTP1-, AROP-, LOX- and FAN concentration as principle components.

On the basis of the number of malt quality parameters analyses one can clearly distinguish between malts with a potentially higher tendency of producing a high quality end product with regards to flavour stability and foam. The relatively simple protocols followed in obtaining these results could benefit barley breeders, maltsters and brewers in screening for barley varieties specifically for the production of a high quality end product.

Conclusions

The ratio of AROP/LOX shows a fair linear trend with the ratio of FAN/LOX (Fig. 3A). Similarly, a fair to good linear trend was found with barley malt cultivars having a high AROP/LOX ratio also had a high LTP1/LOX ratio (Fig 3B). A correlation between LTP1 and LOX-1 was established in that LTP1 tend to be a potent inhibitor of LOX-1 activity and therefore the LOX-1 mediated pathway by possibly competing for one of the pathway‟s intermediates (Fig. 4). This explained the trend between AROP/LOX and LTP1/LOX. It also supported the excellent linear trend that was observed between FAN/LOX and LTP1/LOX (Fig. 6). The PCA analysis correlated with our findings that some barley malt cultivars share similar characteristics. From this study we suggest that barley malt cultivars with high FAN/LOX and high LTP1/LOX would be the best brewing malts for both good fermentation qualities and also good flavour and foam stability. This study also emphasises the complexity of malt character and that in order to choose the best malt for brewing LOX activity and LTP1 content must be determined in conjunction with FAN. This combined information on malts will enable brewers to better predict certain malt‟s brewing performance, assist in optimal blending of the malts and alleviate some of the fermentability and flavour stability issues in beer brewing.

References

Abernathy, D.G., Spedding, G. & Starcher, B. (2009). Analysis of protein and total usable nitrogen in beer and wine using a microwell ninhydrin assay. *Journal of the Institute of Brewing*, **115**, 122-127.

Agu, R.C. & Palmer, G.H. (2001). The effect of nitrogen level on the performance of malting baley varieties during germination. *Journal of the Institute of Brewing*, **107**, 93-98.

Bakan, B., Hamberg, M., Perrocheau, L., Maume, D., Rogniaux, H., Tranquet, O., Rondeau, C., Blein, J.P., Ponchet, M. & Marion, D. (2006). Specific adduction of plant lipid transfer protein by an allene oxide generated by 9-lipoxygenase and allene oxide synthase. *Journal of Biological Chemistry*, **281**, 38981-38988.

Bamforth, C.W. (1985). Cambridge prize lecture biochemical approaches to beer quality. *Journal of the Institute of Brewing*, **91**, 154-160.

Douma, A.C., Doderer, A., Cameron-Mills, V., Skadhauge, B., Bech, L.M., Schmitt, N., Heistek, J.C. & Van Mechelen, J.R. (2003). Low lipoxygenase 1 barley. World Intellectual Property Organization, Geneva, Switzerland, WIPO Patent No. 2002053721.

European Brewery Convention (1998). *Analytica-EBC.* Verlag Hans Carl: Nurnberg, Germany.

Evans, D.E. & Hejgaard, J. (1999). The impact of malt derived proteins on beer foam quality. Part I. The effect of germination and kilning on the level of protein Z4, protein Z7 and LTP1. *Journal of the Institute of Brewing*, **105**, 159-169.

Fox, G.P., Panozzo, J.F., Li, C.D., Lance, R.C.M., Inkerman, P.A. & Henry, R.J. (2003). Molecular basis of barley quality. *Australian Journal of Agricultural Research*, **54**, 1081-1101.

Gorjanovic, S., Suznjevic, D., Beljanski, M., Ostojic, S., Gorjanovic, R., Vrvic, M. & Hranisavljevic, J. (2004). Effects of lipid-transfer protein from malting barley grain on brewers yeast fermentation. *Journal of the Institute of Brewing*, **110**, 297-302.

Guido, L.F., Curto, A.F., Boivin, P., Benismail, N., Gonçalves, C.R. & Barros, A.A. (2007). Correlation of malt quality parameters and beer flavor stability: Multivariate analysis. *Journal of Agricultural and Food Chemistry*, **55**, 728-733.

Hammond, J. & Smart, K. (2000). Yeast growth and nutrition.In: *Brewing yeast fermentation performance,* 1st ed. Katherine Smart, Blackwell Scientific, Oxford, pp.77-85.

Hirota, N., Kuroda, H., Takoi, K., Kaneko, T., Kaneda, H., Yoshida, I., Takashio, M., Ito, K. & Takeda, K. (2006). Brewing performance of malted lipoxygenase-1 null barley and effect on the flavor stability of beer. *Cereal Chemistry*, **83**, 250-254.

Hyde, W. & Brookes, P. (1978). Malt quality in relation to beer quality. *Journal of the Institute of Brewing*, **84**, 167-174.

Iimure, T., Nankaku, N., Hirota, N., Tiansu, Z., Hoki, T., Kihara, M., Hayashi, K., Ito, K. & Sato, K. (2010). Construction of a novel beer proteome map and its use in beer quality control. *Food Chemistry*, **118**, 566-574.

Jegou, S., Douliez, J.P., Molle, D., Boivin, P. & Marion, D. (2001). Evidence of the glycation and denaturation of LTP1 during the malting and brewing process. *Journal of Agricultural and Food Chemistry*, **49**, 4942-4949.

Jiang, X.L., Dong, X.Y., Yao, J., Dong, L.A., Chen, X., Li, M.D. & Zhao, C.X. (2011). Barley protein function as growth inhibitor in *Saccharomyces cerevisiae*. *Journal of Plant Biochemistry and Biotechnology*, **20**, 134-137.

Kader, J.C. (1996). Lipid-transfer proteins in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, **47**, 627-654.

Kuroda, H., Kobayashi, N., Kaneda, H., Watari, J. & Takashio, M. (2002). Characterization of factors that transform linoleic acid into di- and trihydroxyoctadecenoic acids in mash. *Journal of Bioscience and Bioengineering*, **93**, 73-77.

Lie, S. (1973). The EBC-ninhydrin method for determination of free alpha amino nitrogen. *Journal of the Institute of Brewing*, **79**, 37-41.

Linko, M., Haikara, A., Ritala, A. & Penttila, M. (1998). Recent advances in the malting and brewing industry. *Journal of Biotechnology*, **65**, 85-98.

Loiseau, J., Vu, B.L., Macherel, M.H. & Le Deunff, Y. (2001). Seed lipoxygenases: Occurrence and functions. *Seed Science Research*, **11**, 199-211.

Moelskov Bech, L., Vaag, P., Heinemann, B. & Breddam, K. (1995). Throughout the brewing process barley lipid transfer protein 1 (LTP1) is transformed into a more foam-promoting form. In: *Proceedings of Congress-European Brewery Convention*. Oxford University Press, Brussels, Vol 25, pp. 561-561.

Mundy, J. & Rogers, J. (1986). Selective expression of a probable amylase/protease inhibitor in barley aleurone cells: Comparison to the barley amylase/subtilisin inhibitor. *Planta*, **169**, 51-63.

Nelson, M.J. & Seitz, S.P. (1994). The structure and function of lipoxygenase. *Current Opinion in Structural Biology*, **4**, 878-884.

Nie, C., Wang, C., Zhou, G., Dou, F. & Huang, M. (2010). Effects of malting conditions on the amino acid compositions of final malt. *African Journal of Biotechnology*, **9**, 9018-9025.

Perrocheau, L., Bakan, B., Boivin, P. & Marion, D. (2006). Stability of barley and malt lipid transfer protein 1 (LTP1) towards heating and reducing agents: Relationships with the brewing process. *Journal of Agricultural and Food Chemistry*, **54**, 3108-3113.

Samaras, T.S., Camburn, P.A., Chandra, S.X., Gordon, M.H. & Ames, J.M. (2005). Antioxidant properties of kilned and roasted malts. *Journal of Agricultural and Food Chemistry*, **53**, 8068-8074.

Siebert, K.J. & Lynn, P. (2005). Comparison of methods for measuring protein in beer. *Journal of the American Society of Brewing Chemists*, **63**, 163-170.

Sorensen, S., Bech, L., Muldbjerg, M., Beenfeldt, T. & Breddam, K. (1993). Barley lipid transfer protein 1 is involved in beer foam formation. *Technical Quarterly*, **30**.

Van Nierop, S.N.E., Rautenbach, M., Axcell, B.C. & Cantrell, I.C. (2006). The impact of microorganisms on barley and malt quality - a review. *Journal of American Society of Brewing Chemists*, **64**, 69-78.

Vanderhaegen, B., Neven, H., Verachtert, H. & Derdelinckx, G. (2006). The chemistry of beer aging - a critical review. *Food Chemistry*, **95**, 357-381.

Wu, M.J., Clarke, F.M., Rogers, P.J., Young, P., Sales, N., O"Doherty, P.J. & Higgins, V.J. (2011). Identification of a protein with antioxidant activity that is important for the protection against beer ageing. *International Journal of Molecular Sciences*, **12**, 6089-6103.

Yano, M., Tsuda, H., Imai, T., Ogawa, Y. & Ohkochi, M. (2008). The effect of barley adjuancts on free amino nitrogen contents in wort. *Journal of the Institute of Brewing*, **114**, 230-238.

CHAPTER 6

Assessment of biochemically derived parameters in prediction of beer quality and shelf life

Introduction

Selecting barley varieties for the production of a high quality end product have proven a difficult task due to the complexity of the variables that need to be taken into account in brewing beer, as well as the costs involved. Many of the older barley screening methods for different traits may discriminate against superior quality varieties, due to the lack of sufficient biochemical insight. Screening methods include determination of moisture, free amino nitrogen (FAN), protein content, germinative properties, grain size, husk content, starch and fibre quality, water uptake properties, various enzyme determinations (β-glucanase, α-amylase), β-glucan content, grain hardness, hot- and cold water extract, Kolbach index, diastatic power, viscosity, fermentability and friability (Wainwright & Buckee, 1977; Fox *et al.*, 2003). According to Fox *et al,* 2003, the most important parameters are viscosity, diastic power, hot water extract, β-glucan content, fermentability, Kolbach index, FAN, α-amylase content, β-glucanse and friability. These barley malt quality parameters have definite limitations in predicting brewery performance (Fox *et al.*, 2003) and some difference of opinion exists over the use of such methods for the screening of barley, indicating that a need exists for better biochemical analysis. Specific barley quality traits include the analysis of grain protein content, storage protein, hordeins, non-storage proteins, protein Z, lipid transfer proteins (i.e. LTP1), barley carbohydrates, starch and non-starch polysaccharides (Fox *et al.*, 2003).

For the screening of malt and beer a new set of parameters were compiled to predict specific quality traits, such as beer foam and flavour stability and fermentability. These included

screening for FAN, protein content, LTP1, lipoxygenase (LOX) and anti-radical/oxidant potential (AROP).

Probably the most important aspects of beer quality are beer flavour (Lustig, 1999; Santos *et al.*, 2003) and foam stability (Bamforth, 1985; Evans & Bamforth, 2009) and these are also traits proving difficult to predict at an early stage. The shelf-life of beer is generally measured by flavour-, foam-, microbiological- colour- and colloidal stability (Vanderhaegen *et al.*, 2006). The major contributors toward compounds responsible for the stale flavour of aged beers are primarily as a consequence of oxidation of higher alcohols and unsaturated fatty acids, degradation of hop bitter acids, Strecker break-down of amino acids an aldol condensation (Malfliet *et al.*, 2008). Numerous methods are used to improve the stability of flavour in beer. The quality of the raw materials used in the production of beer, together with the brewing process and practices, are the main determinants of flavour stability (Lustig, 1999). The focus of this study will be on barley malt as raw material and how it could be used when selected correctly to enhance primarily the flavour stability and foam quality and secondly the colour of the product.

Parameters such as high natural AROP, minimal lipid degradation potential, high FAN (Lustig, 1999) and high LTP1 (Wu *et al.*, 2011) are seen as quality promoting attributes. Lipid degradation will be inhibited by the presence of anti-oxidants (Dvořáková *et al.*, 2008) combined with low LOX activity which is responsible for the conversion of linoleic acid to eventually form 2(*E*)-nonenal (Hirota *et al.*, 2006), one of the compounds responsible for the cardboard taint in aged beers. Various methods for the determination of 2(*E*)-nonenal exists to aid brewers in malt selection to improve flavour stability, but it requires highly specialised equipment. The biochemical background of this compound, which impacts its formation, is not taken into account (Kuroda *et al.*, 2005). Alternatively, the determination of AROP and LOX activity in malts can serve as predictor for the potential to form 2(*E*)-nonenal. In Chapter 4 the development

of a method for the determination of both LOX-1 and AROP, using an adapted ferrous oxidation-xylenol orange (FOX) micro-assay, is described. It was found that definite trends exist in certain malts and that LOX-1 and AROP may serve as predictors of flavour stability.

It has also recently been observed that LTP1, which plays a major role in the stability of beer foam (Evans & Bamforth, 2009), also acts as anti-oxidant (Wu *et al.*, 2011). In our previous research (Chapter 5) we found that LTP1 also inhibits the LOX activity in malt extracts, in particular LOX-1 activity, which makes its role in the prevention of flavour degradation more significant than was originally thought. This inhibition is probably due to LTP1 binding to a reactive oxylipin, identified by Bakan *et al.* (2006) to be 9(*S*), 10-epoxy, 12(*Z*)-octadecadienoic acid, to form LTP1b. This oxylipin is produced via the LOX pathway from linoleic acid and is further broken down to form 2(*E*)-nonenal (Kuroda *et al.*, 2005). For the determination of LTP1 in this study we performed a western blot, using specific LTP1 generated antibodies, for comparative reasons as was described in Chapters 2 and 5.

Typical flavour changes that occur during aging are the deterioration of bitterness, an intensifying sweet taste that coincides with caramel, burnt sugar and leather aromas (Vanderhaegen *et al.*, 2006) and special emphasis on the development of a cardboard/papery taste (Vanderhaegen *et al.*, 2006; Malfliet *et al.*, 2008). Another change includes an increase in ribes or blackcurrant favour (Vanderhaegen *et al.*, 2006).

The colour of beer can become darker upon prolonged storage due to a number of factors such as high storage temperatures, large bottle headspace and the presence of metal ions, melanoidins, diacetyls, aldehydes and hordeumin (Kuchel *et al.*, 2006). To evaluate beer colour over the 3 months the CIELAB colour space, as indorsed by Smedley (1995), was used. This method is a reproducible colour determinant. Colour is quantitatively estimated by the placement thereof in a three dimensional space. The sample is evaluated based on its placement on the different axes L*, a*, b* and C* (Smedley, 1995) (Fig. 1).

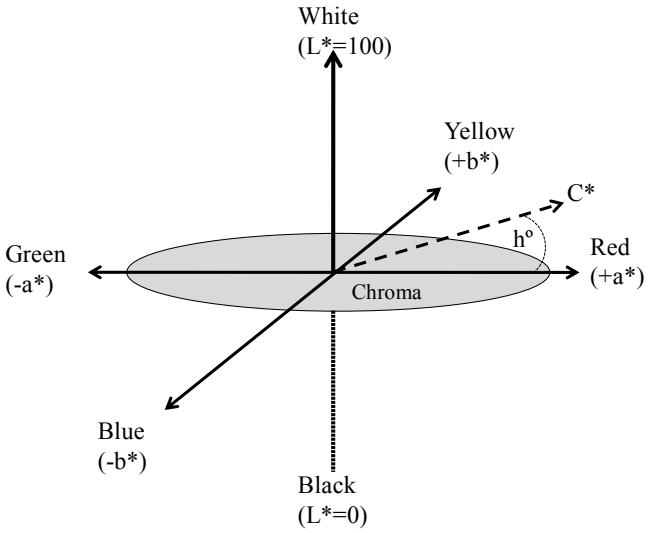

Fig 1. A representation of the CIELAB colour space. Figure adapted from the instrument manual (Corning, NY, USA).

During this study three malts for brewing trials were selected on the grounds of their FAN (as determined in their wort), LOX-1 activity, AROP and LTP1 content. The three selected biochemical parameters (LOX activity, AROP and LTP1 content) of the beers brewed with the different barley malt cultivars were determined and correlated with the original malt parameters. Beers were further assessed for foaming properties, colour and subjected to a sensory evaluation by a trained panel to determine whether the biochemical predictors can predict the quality of the fresh end product and after a three month storage period. First, LOX and AROP content of the malts and beer were determined using a FOX (ferrous oxidation-xylenol orange) as described previously (Chapters 3 and 4). It is expected that the beer with the highest AROP and lowest LOX content will have the better aging potential, while the same is true for the opposite. Second, a comparative LTP1 study was performed, to elucidate whether the LTP1 content impacted flavour and foam properties. It is expected that the beer with the higher LTP1 concentration would have the better aging potential and also possibly higher foam stability. Third, beer colour was monitored using the CIELAB method and finally beers were subjected to consumer and descriptive sensory analysis. The latter was used to determine which beer had the best ageing potential.

Materials and methods

Micro-brewery trials

Three different malts of the barley cultivars N, C and E was selected and generously donated by SABMiller Caledon, South Africa. Micro-brewery trials were conducted on the three different malts in four replicates each using the micro-brewery plant (Process Engineering, Stellenbosch University). For a single batch of „English pale ale", in short, 6 kg of milled barley malt was mixed with 15 L water, collected from the SABMiller"s Brewery at Newlands spring (South Africa), and mashed in a mashtun in consecutive steps of 30 minutes at 64˚C, 30 minutes at 68˚C and lastly 5 minutes at 75˚C. Lautering was done in a lautertun until the wort was clear and then transferred to the kettle. The spent grain was sparged (rinsed through) with an additional 20 L of water at 72˚C and also transferred to the kettle when clarity was obtained. The lautering step takes approximately one hour in total. The total volume of 35 L wort was brought to a boil at 100˚C for one hour and hops was added as per the following schedule; 30g T90 (IBU: 29.81) at the start of boil, 25g Cascade (IBU: 6.51) and 6g US4/78 (IBU: 2.96) 45 minutes later and lastly 20 g Glacier (IBU: 0.89), added 2 minutes before the end of boil. The boiled wort (reduced to 30 L) was subsequently cooled down to about 10˚C using an immersion chiller. Yeast (Safbrew, Beerlab, Belville, South Africa) was pitched at a rate of 12 g/L, fermented at 15˚C for 7 days and racked for 48 hours at 4˚C. Each 30 L batch was siphoned into two 10 L secondary fermentation vessels and 750 mL beer bottles along with 8.33 g/L priming sugar. One vessel for each batch was analysed immediately after secondary fermentation was complete and the second analysed three months later. The duration of secondary fermentation was 2 weeks. The trials were repeated twice to firstly have batches ready to age for three months and again three months later to have batches ready for evaluation as fresh beer.

Sensory analysis

Descriptive sensory analysis: Descriptive sensory analysis (DSA) was carried out on beer between 16 August and 18 November 2013. Two trials were conducted, the first consisting of three treatments of fresh beer (Beer 0), these beers were allowed to age for three months at room temperature (Beer 3) and included in the second trial, together with freshly brewed beer (Beer 0). The second trial therefore consisted of six treatments; three treatments aged beer (Beer 3) and three treatments fresh beer (Beer 0) (Table 1).

Table 1. Coding of beer and different treatments evaluated using descriptive sensory analysis

Beer variety code	Fresh beer	Aged beer (3 Months)
C	C0	C3
E	E0	E3
N	N0	N3

The first trial conducted in August served as a training session and to compile a full set of descriptors. A trained panel, consisting of 10 panel members, was trained in accordance to the method as set out by Lawless & Heymann (2010). Judges were trained prior to the analysis in August and November in six training sessions of one hour each. The training session was also performed as described by Lawless & Heymann (2010) according to „Generic Descriptive Analysis". The judges were assessed for consistency. The sensory attribute, cardboard-like flavour, which is brought on by the presence of 2(*E*)-nonenal was emphasised for the judges, but no such particular flavour was found in any of the treatments. For the identification of this cardboard aroma, cardboard and the pure chemical 2(*E*)-nonenal (Sigma Aldrich, Missouri, USA) was used as reference. The pure chemical was diluted at very low concentrations to simulate the cardboard aroma. Even at extremely low concentrations (at 3.4 mg/L then diluted down to 3.4 µg/L) the panel did not associate the aroma with cardboard, but it was described as insect-like or plant-like. The dilutions were based on the observations by Guido *et al.,* (2005) which stated that 2(*E*)-nonenal can be detected at the low flavour threshold of 0.035 µg/L. The lack of sensory presence of this descriptor could be due to the fact that the descriptor might be

incorrectly referenced in some literature. We will thus use overall stale beer characteristics for shelf life estimation.

The six treatments of beer were analysed according to the sensory descriptors set out in Table 2.

Table 2. Summary of the 21 beer sensory descriptors analysed by the trained panel.

	Term		Reference standard	Scale
Aroma attributes	**Malty***		Malt flour (SABMiller, Caledon, SA) in water	0 = None 100 = Prominent malty aroma
	Hoppy*		Variety of hop cultivars	0 = None 100 = Prominent hoppy aroma
	Fruity*	**Tropical**	Tropical fruit juice (Dairybelle, Bloemfontein, SA)	0 = None 100 = Prominent fruity aroma
		Citrus	Orange and lemon peel	
		Pear	Apple & pear juice (Ceres, Ceres, SA)	
	Stale beer aroma		One year old Castle Lager (SABMiller)	0 = None 100 = Prominent stale aroma
	Smoky			0 = None 100 = Prominent smoky aroma
	Honey/sweet associated		Honey, stale beer	0 = None 100 = Extremely honey-like aroma
	Diacetyl/Butter/ Butterscotch		Butterscotch 1043727 (Sensient, Cape town, SA)	0 = None 100 = Prominent buttery aroma
	Sherry-like			0 = None 100 = Prominent sherry aroma
Palate attributes	**Fullness***			0 = None 100 = Prominent fullness
	Malty*			0 = None 100 = Prominent malty taste
	Hoppy*			0 = None 100 = Extremely hoppy taste
	Sweet			0 = None 100 = Prominent sweet taste
	Sour			0 = None 100 = Extremely sour taste
	Bitter*			0 = None 100 = Prominent bitter taste
	Bitterness quality (lingering)*			0 = None 100 = Prominent bitterness quality
	Fruity*			0 = None 100 = Prominent fruity taste
	Citrus*			0 = None 100 = Prominent citrus taste
	Smokey			0 = None 100 = Prominent smokey taste
	Fruity*			0 = None 100 = Prominent fruity taste

Generally regarded as ***fresh beer attributes**

Stale beer was used as a reference standard in combination with references for each individual aroma descriptor. All tastings were conducted in a sensory laboratory at the Department of Food Science, Stellenbosch University. The beer analysis was conducted within one hour after opening in a temperature (21˚C) and light controlled room.

Fifty millilitres of each sample, poured without head, was served in an ISO clear wine tasting glass at room temperature and labelled with a three-digit number code. The samples were served in a randomised order marked with a three-digit random code as generated by Compusense® Five data collection software (Version 5.4, Compusense Inc., Guelph, Ontario, Canada). Water, apple slices and plain crackers (Woolworths, South Africa) were served to each panel member to cleanse their palate between tasting the different treatments of beer. The panel used of a 100 mm unstructured line scale to mark the intensity of each of the attributes where a score of 0 indicated that the beer contained very low amounts or none of the attribute measured and a score of 100 indicated that the specific attribute was perceived to be high in intensity of the attribute measured.

The sensory data was analysed using analysis of variance (ANOVA) and SAS™ statistical software (Statistical Analysis System, Version, 9.2, 2006, SAS Institute Inc., CARY, NC, USA). The data was tested for normality using the Shapiro-Wilk test (Shapiro & Wilk, 1965) and outliers were removed before the final ANOVA. Student‟s t-least significant difference (LSD) were calculated at a 5% significance level and used to compare the means over the treatments. Pearson‟s correlation coefficient (Snedecor & Cochran, 1967) was used to verify any correlations between sensory attributes, proximate composition and physical characteristics. Principal Component Analysis and Discriminant analysis were used to test for attribute and treatment associations using XL STAT™ statistical software (Version 2011, Addinsoft, New York, USA). The data was subjected to Principle Component Analysis (PCA) and Discriminant

Analysis (DA) to elucidate whether relationships exist between sensory, physical and proximate data (Rencher & Christensen, 2012).

Consumer Analysis: Consumer analysis was conducted at the Department of Biochemistry on 16 August 2013. Consumers were recruited on the basis that they consume beer regularly (once a week or more). The three different fresh beer samples (month 0) were tested for degree of liking by a consumer panel using a nine-point hedonic scale where the three beer samples were rated on a scale from 1 to 9 according to Lawless and Haymann (2010): 9 = *Like extremely*; 8 = *Like very much*; 7 = *Like moderately*; 6 = *Like slightly*; 5 = *Neither like nor dislike*; 4 = *Dislike slightly*; 3 = *Dislike moderately*; 2 = *Dislike very much* and 1 = *Dislike extremely*. Judges were asked to indicate which term best describes their attitude towards each sample. A total of 74 judges were used to evaluate the beer samples.

Samples were presented to each judge in a randomised complete block design. Each judge received 70 mL of each sample served in an ISO clear wine tasting glass at 4˚C. All analyses were conducted within 3 hours after opening the beer in a temperature (21±1ºC) and light controlled room.

The sensory data for "the degree of liking for the beer samples" were analysed using analysis of variance (ANOVA). Student"s t-LSD,s (Least Significant Difference) were calculated at a 5% significance level and used to determine whether preference for a certain beer sample differed significantly between different age and gender groups.

Chemical analysis

LOX and AROP determination: Malt analyses were done as described in Chapter 3. Beer was centrifuged (5 minutes at 3000 x *g*) to completely clarify/degas the solution. Six doubling dilutions of each sample were prepared in triplicate. The undiluted extracts were used as the highest concentration in each case, diluting it down to a 32-fold dilution as the lowest

concentration. For the negative control, beer was boiled for 10 minutes at 80°C and subsequently centrifuged (20 minutes at 3000 ×*g*). Fifty microlitres of each diluted sample and control was then transferred in triplicate into a chilled 96-well microtiter plate.

The LOX activity was determined as previously described by the FOX-LOX micro-assay (Chapter 3). It should be mentioned that the LOX-FOX assay does not discriminate between LOX-1 and -2 activity, however it is believed that LOX-2 does not have a major contribution towards total LOX activity (Yang *et al.*, 1993) after kilning (as explained in Chapter 3). AROP was measured using the AROP-FOX micro-assay as previously described in Chapter 4.

LTP1 determination: A western blot was performed using LTP1 generated antibodies generously supplied by Prof Evan Evans (School of Plant Science, University of Tasmania, Tasmania) to verify the presence of LTP1 in the different beers. The LTP1 content of the beers was determined as described in Chapter 5.

Intact protein determination: A protein determination on each malt extract was done using a Pierce BCA protein determination kit (Thermo scientific, Rockford, Illinois, USA). The standard protein determination procedure outlined by the manufacturer was used in all protein concentration determinations. A standard protein concentration curve was generated by using a dilution range of bovine serum albumin (BSA) as reference.

FAN analyses: SABMiller makes use of the ninhydrin method for determination of free amino nitrogen (FAN) as set out by the European Brewery Convention, Analytica-EBC (1998), FAN method 8.10 and also described by Lie (1973).

Visual analysis

*CIE L*a*b* colour analysis:* CIE L*a*b* colour was measured using a calibrated color-guide glas b. 45/0, 20 mm (BYK-Gardner, Germany) by pipetting 10 mL of each degassed sample into a 60 X 15 mm tissue culture dish (Corning, NY, USA) and taking 3 separate

readings. The L*, a* and b* values were recorded in triplicate for each biological repeat from which the C* value was calculated as $C^*=\sqrt{(a^{*2}+b^{*2})}$. Fresh and aged beers were analysed at 0 and 3 months.

Foam quality tests: The foam stability of each of the different brewed batches was determined in triplicate. The method used was based on the principles of the sigma-value method described by the American Society of Brewing Chemists (ASBC), Methods of Analysis (2009). In short, 100 mL beer was dropped from a separation funnel with a 4.9 cm diameter from a height of 30 cm into a measuring cylinder with a similar diameter as the separation funnel. Time 0 is the moment the last liquid is drained from the separating funnel into the measuring cylinder and the volume of foam and liquid is recorded at this point and for each consecutive 10 second interval for up to 4 minutes (240 seconds). Each cultivar‟s (N, C, E) technical repeat, of which there were 4, was measured in triplicate.

Results and discussion

Malts and beers N, C and E were analysed according to their LTP1- and LOX content as well as for AROP and protein concentration. FAN values were supplied by SABMiller and the same beer samples were also evaluated based on their foam, colour and flavour stability.

Chemical analysis

LOX, AROP, LTP1 and BCA protein determination was done on malt and beer samples. FAN values were supplied by SABMiller (Table 3). As described in Chapter 5, AROP was classified as a flavour positive factor, LTP1 as both a flavour and foam positive factor (with sigma as the parameter in beer to evaluate foam) and FAN and protein concentration as fermentation positive factors, while LOX as a flavour negative factor.

Protein concentration did not differ significantly throughout malt samples and only showed significant decreases from malt to beer for malt samples N and C, but only slightly for E. Beer C had the highest FAN value, indicating that it would probably have better fermentability than beer N and E. Beer N and E had very similar FAN values (Table 3). During the brewery trial the fermentability of all the malts were found to be within the normal limits, with a similar decrease in specific gravity from the original gravity (OG) of 1.346 °P (69.8 Brix) to a final gravity (FG) of 1.024 °P (6.1 Brix).

LOX concentrations determined in the malt samples differed significantly at a 5% significance level between C, E and N with E having the highest concentration followed by N and lastly C. For the determination of LOX activity in the different beer samples, no significant difference was observed between beer N and E, although both of these differed significantly from beer C (being significantly less than the previous) at a 10% level of significance (Table 3). LOX-1 concentrations also significantly decreased (>75%) from malt to beer. This was expected due to it LOX-1 being denatured to a great extent which is shown to occur at about 65°C (Kobayashi *et al.*, 1993; Ludikhuyze *et al.*, 1998) during the brewing process. The apparent presence of LOX-1 activity in beer contradicts findings by Schwarz & Pyler (1984). Although the LOX-FOX assay is highly sensitive for LOX activity, with a limit of detection as low as 16 LOX activity units, LOX activity determined in beer could be artefact activity due to radicals, specifically as a consequence of the Mallard reaction during brewing. A more detailed explanation for the putative or artefact LOX activity is given in Chapter 3. However, if Lox activity or if radicasl were present as indicated by the FOX assay, this may have an influence on the shelf-life of the beer.

AROP did not differ significantly between malt samples at a 10% level of significance, but decreased significantly (>90%) in beer samples to 5 mmole/g malt (Table 3). This was a larger decrease than the 29% which was previously observed for *in vitro* antioxidant capacity

(Fantozzi *et al.*, 1998), but is very much dependant on the degree of malt modification and temperature (Samaras *et al.*, 2005) and will vary accordingly. However, when considering AROP/LOX the following trend was found for both the malts and their respective beers, namely C > N ≈ E. This indicated that Beer C could be protected best from staling.

Table 3. Parameter values of the different beers. Superscript alphabetical rank indicates a significant difference between C, E and N with P<0.1 for LOX (beer) and AROP (malt and beer), P<0.001 for LTP1 and P<0.05 for protein concentration (malt and beer) and LOX (malt). Bracketed values indicate the standard error of the mean (SEM). For the LTP1 (malt), LOX (malt), AROP (malt) and protein concentration (malts) estimation N=4 and for LOX (beer), AROP (beer) and Protein (beer) determination N=3×4

	N		E		C	
	Malt	**Beer**	**Malt**	**Beer**	**Malt**	**Beer**
LOX (Units/g malt)	3232^{b} (±10.87)	753.5^{d} (±40.38)	4518^{a} (±182.7)	819.5^{d} (±57.90)	2467^{c} (±162.2)	572^{e} (±48.32)
AROP (mmol)/g malt	868.3^{a} (±69.16)	5.0^{b} (±0.0005)	1178^{a} (±88.14)	5.0^{b} (±0.0005)	1118^{a} (±84.59)	5.0^{b} (±0.0005)
LTP1 signal/g malt	355.80	30.51^{b} (±0.09)	360.06	24.73^{c} (±0.71)	262.00	37.23^{a} (±0.60)
LTP/LOX	0.11	0.04	0.08	0.03	0.11	0.07
AROP/LOX	0.27	0.007	0.26	0.006	0.45	0.009
Protein mg/g malt	1.09^{a} (±0.03)	0.708^{b} (±0.01)	0.90^{ab} (±0.02)	0.728^{b} (±0.03)	1.09^{a} (±0.08)	0.790^{b} (±0.02)
FAN mg/L wort	171		170		179	

The unmodified LTP1 concentration decreased significantly from malt to beer for all samples. A reduction of 76-82% for LTP1 from malt to beer was observed. In an extensive review by Evans & Bamforth (2009) it was found that LTP1 decreased to 5-20%, depending on the severity of the boiling. It was also reported that a 97% reduction in LTP1 concentration occurred during wort boiling (Evans *et al.* (1999). This result may be due to the specificity of the LTP1 antibody (which was also used in this study) not recognising the modified LTP1 (Van Nierop *et al.*, 2002). This therefore lead to an under estimation of the total LTP1 concentration,

but still focussed on intact unmodified LTP1 and LTP1b. Ultra-performance ESMS analysis of the dried beer samples at 5 mg/mL failed to detect intact/unmodified LTP1 and LTP1b (results not shown), correlating with our western Blot analyses and that of Evans *et al.* (1999). For the estimation of LTP1 throughout the different repeats within a beer made from a specific cultivar a significant difference was found at a 1% level of significance. Beer C was significantly the highest, while beer N was significantly lower than beer E, the latter being the significant lowest (Table 3). If LTP1/LOX ratio was used for the malts quality assessment, the prediction for shelf life pertaining to beer quality would be as follows C ≈ N > E. From these analysis it is also predicted according to LTP1 content, LOX content and LTP1/LOX ratio that the prediction of shelf life pertaining to beer quality is similar to that in malts, but with a distinction between C and N namely: C > N ≥ E. This analysis again indicated that Beer C could have the best shelf life. However, a possible relationship between the loss of LTP1 and the LOX activity exists. For example malt E had both the highest LOX activity and LTP1 content, but ended with the lowest LTP1 content in the beer, as well as the greatest loss of LOX activity (82%). Conversely malt C had the lowest LOX activity and LTP1 content and ended with the highest LTP1 content in the beer. For a relationship between LTP1 and FAN it was observed that malt E had the lowest concentration of both LTP1 and FAN, while malt C had the highest concentration of both, making malt C the potentially better fermenter as well as having a potentially more stable beer foam.

Visual analysis

Colour analyses were done in triplicate on all beer samples. The CIELAB method was used as was endorsed by Smedley (1995) for its application in beer colour determination. Although some significant differences existed between the cultivars, no significant difference was observed within a cultivar over the three months (Table 4). No significant difference was observed between beer samples or within a sample over time at a 10% significance level. It was

expected that the colour should become more intense upon prolonged storage, which indicates that beer colour remained stable for three months and most probably will only start showing colour changes, indicating aging, over a longer period. Colour was also evaluated by a trained sensory panel (Table 6) and also no trend in the darkening of the aged beer was observed.

Table 4 Tabulated CIE L*a*b* colour measurements for the fresh and 3 months aged beer and Sigma value for foam measurement. Bracketed values indicates the standard error of the mean (SEM) of 4x3 replicates. Superscript alphabetical rank indicates a significant difference between C, E and N with P<0.1 for sigma value.

Beer		Colour				Foam
		L*	a*	b*	C*	Sigma Value (seconds)
N	Fresh	74.65 (±0.15)	-0.7308 (±0.04)	23.7 (±0.19)	6.777 (±0.03)	186.9^{a} (±4.669)
	Aged	74.06 (±0.31)	-0.7017 (±0.07)	22.84 (±0.29)	6.652 (±0.04)	
E	Fresh	74.65 (±0.16)	-0.525 (±0.03)	25.2 (±0.38)	7.023 (±0.05)	190.8^{a} (±2.445)
	Aged	74.59 (±0.12)	-0.445 (±0.05)	25.48 (±0.37)	7.073 (±0.05)	
C	Fresh	74.44 (±0.16)	-0.7683 (±0.06)	24.05 (±0.37)	6.821 (±0.06)	192.1^{a} (±2.194)
	Aged	75.02 (±0.26)	-0.5142 (±0.10)	24.82 (±0.30)	6.971 (±0.03)	

For foam analysis, each cultivar"s (N, E and C) quadruplicate technical repeats were measured in triplicate and sigma values are given in Table 4. No difference in foam stability was observed between the different beers, probably due to the inherent experimental error of the assay methodology (Table 4). However, the foam head of beer C, containing more protein and LTP1, could clearly visually be distinguished from the other two beers.

Consumer sensory analysis

A consumer analysis trial on the three fresh beers were conducted in order to assess the general preference for the three beers and to rule out any issue of brew bias. The consumer panel consisted of 74 members of whom 23 were female (31% of total group) and 51 were male (69% of total group) consumers. Nine and a half percent of panel members were below the age of 20, 60.8% were between the ages 20-29, 21.6% between ages 30-39, 4% between ages 40-49 and

4% were older than 50 years old. Eighty one percent of consumers were categorised as being regular consumers of beer i.e. consuming beer once every week or more, while 19% of panel members consume beer on a less regular basis.

According to Table 5, female consumers showed an equal degree of liking ($P>0.05$) for the three beers brewed with different malt varieties. The male consumers showed an equal degree of liking for Beer N and Beer E, with the degree of liking for Beer C significantly lower ($P\leq0.05$) compared to that of Beers N and E. Mean hedonic values for the total group show a similar pattern (N=74) to that of the male consumers (Fig. 2). From these results it was clear that there was no overt bias in the preference for the different beers with only a slight preference of beers N and E by male consumers.

Table 5 Overall consumer preference for the three different beer variants

Treatments	Mean hedonic value		
	Total group (N=74)	Female consumers (N=23)	Male consumers (N=51)
Beer N	6.7838[a]	6.1304[a]	7.0784[a]
Beer C	6.0811[b]	6.1739[a]	6.0392[b]
Beer E	7.000[a]	6.0870[a]	7.4118[a]
LSD (P=0.05)	**0.4334**	**0.9946**	**0.4275**

LSD = Least significant difference at the 5% level of significance
Figures with different superscript in the same column differ significantly at the 5% level of significance

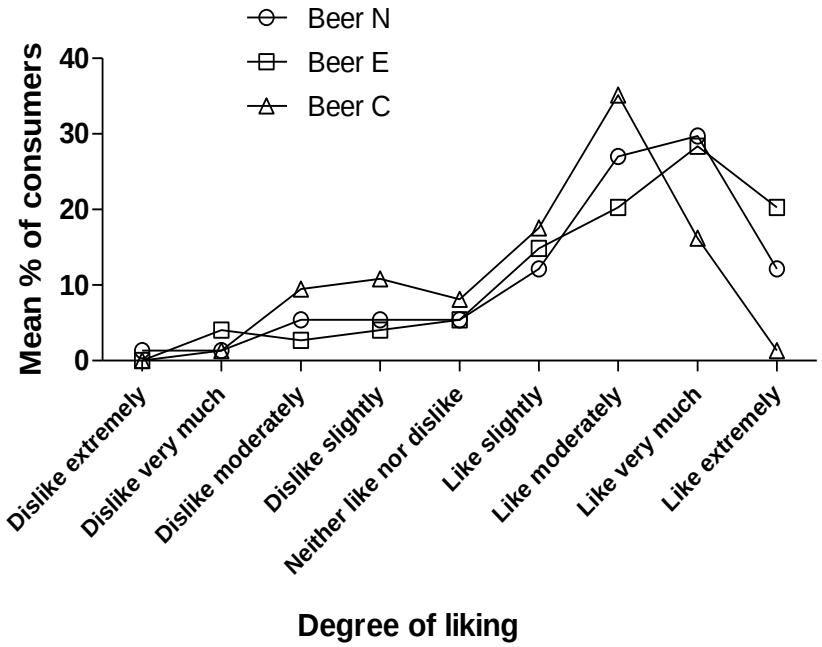

Fig. 2. The distribution of scores for the consumer analysis for the total group (N=74)

Descriptive sensory analysis

The sensory analysis of the beer at 0 and 3 months using descriptive sensory analysis and a trained panel enabled us to determine which beer had the better shelf life potential.

The current investigation focused on the change in overall flavour and palate attributes between beers over the three month storage period. The sensory attributes in each of the six treatments, three treatments of fresh beer denoted as C0; E0 and N0, and three treatments of aged beer denoted as C3, E3 and N3, are presented in Table 6. General fresh and stale beer attributes were initially compiled from work done by Malfliet *et al.* (2008), Angelino *et al.*(1999) and Meilgaard *et al.* (1979), but modified during training sessions as the panel saw fit.

It was found that each treatment of beer stored for three months (Beer C3, E3 and N3) showed a significant decline ($P \leq 0.05$) from the corresponding fresh beer treatments (Beer C0, E0 and N0) in fullness, bitterness, bitterness quality (lingering), malty-, hoppy and fruity flavour as well as malty-, hoppy and fruity aromas. Aged beer treatments showed significant ($P \leq 0.05$) increases in sweetness, sourness, stale beer aroma, honey or sweet associated aromas when compared to the corresponding fresh beer treatments (Beer C0, E0 and N0) (Table 6).

Standardised PCA plots, using the correlation matrix, are popular tools for the sensory analysis of samples to illustrate the relationship between individual samples as well as the relationship between the various attributes. In Fig. 3A the PCA plot for beer attributes is shown in relation and position to one another. A clear divide is observed between positive (fullness, fruity flavour/aroma, hoppy flavour/aroma, bitterness, bitterness quality, malty flavour/aroma, smoky flavour/aroma and all other fruit aromas/flavours) characteristics on the right and negative (sour taste, stale beer aroma, honey-like, sherry-like and to some extent diacetyl and butter associated aromas) sensory characteristics on the left. This plot can be generally used to determine which characteristics associated with which samples or which treatments are closely related to one another and also to predict which flavour and aroma attributes show correlation. However more detailed correlation matrixes are required to determine direct correlations between samples and attributes as can be seen in Table 7.

In Fig. 3A, most of the correlations observed in Table 7 appear on the PCA plot, but two outliers, namely colour and diacetyl are in need of further classification. No significant correlation could be established for colour or diacetyl with any of the other attributes (Table 6 and 7), making these independent attributes from the rest. The positioning of the quadruplicate repeats of the six treatments relative to each other is shown in the corresponding scores plot in Fig. 3B. Two distinct clusters are observed on axis 1, between aged (Beer 3) and fresh beer (Beer 0) indicated in solid lines. Fresh samples (Beer 0) associates with attributes found in the right quadrant, while aged beer (Beer 3) associates to attributes in the left quadrant. Within the fresh beer (Beer 0) cluster, three separate clusters were observed with beer E0 and N0 clustering close together, while beer C0 clustered together separately on another part of the scale (indicated with dashed lines). Within the aged beer (Beer 3), it seems that Beer N3 forms a separate cluster, while Beer C3 and Beer E3 cluster together (indicated in dashed lines).

Table 6 ANOVA Table for mean sensory scores (±SD) of six treatments of beer

	Treatment						LSD
Attribute	**C0** (fresh)	**C3** (aged)	**E0** (fresh)	**E3**(aged)	**N0** (fresh)	**N3**(aged)	**(P=0.05)**
Colour	6.25^{a}(±0.84)	5.15^{b} (±1.00)	3.25^{c} (±1.05)	5.12^{b} (±1.34)	3.70^{c} (±1.04)	3.62^{c} (±1.10)	**0.95**
Fullness	62.90^{a}(±8.44)	30.46^{c} (±15.03)	60.27^{ab} (±12.26)	30.67^{c} (±16.83)	57.51^{b} (±10.80)	30.06^{c} (±14.67)	**5.01**
Malty flavour	37.75^{ab}(±7.80)	24.53^{c} (±8.73)	38.56^{a} (±7.37)	21.30^{d} (±8.86)	35.32^{b} (±7.24)	23.63^{cd} (±8.92)	**3.11**
Hoppy flavour	35.20^{ab}(±11.82)	19.51^{c} (±10.61)	37.02^{a} (±12.44)	17.19^{c} (±11.23)	32.76^{b} (±11.27)	17.37^{c} (±9.92)	**3.16**
Sweet	9.98^{b}(±7.85)	14.80^{a} (±8.70)	10.28^{b} (±8.91)	13.88^{a} (±8.95)	10.53^{b} (±8.12)	13.51^{a} (±9.48)	**2.43**
Sour	2.00^{b}(±5.16)	4.52^{a} (±6.77)	0.50^{b} (±2.20)	5.84^{a} (±7.68)	1.76^{b} (±4.48)	4.28^{a} (±5.02)	**2.01**
Bitter	34.28^{a}(±5.93)	19.78^{c} (±8.30)	34.63^{a} (±6.14)	20.93^{c} (±7.99)	27.33^{b} (±7.84)	20.90^{c} (±9.19)	**3.97**
Bitterness quality	33.23^{a}(±8.43)	17.35^{c} (±9.12)	34.92^{a} (±7.98)	18.30^{c} (±10.34)	25.71^{b} (±8.23)	19.38^{c} (±9.87)	**5.65**
Citrus flavour	3.78^{a}(±5.84)	1.50^{b} (±3.61)	4.03^{a} (±5.94)	1.57^{b} (±3.64)	3.25^{ab} (±6.15)	1.60^{b} (±4.38)	**1.82**
Smokey flavour	18.67^{a}(±13.62)	3.51^{c} (±8.01)	13.55^{b} (±12.46)	4.00^{c} (±9.00)	14.37^{b} (±12.30)	5.51^{c} (±11.53)	**3.84**
Fruity flavour	8.00^{a}(±4.64)	6.00^{b} (±5.45)	8.26^{a} (±5.01)	4.75^{b} (±5.54)	8.00^{a} (±5.16)	5.03^{b} (±5.55)	**1.58**
Malty aroma	38.31^{a}(±7.08)	24.52^{b} (±8.05)	38.71^{a} (±7.75)	26.05^{b} (±10.27)	38.92^{a} (±9.29)	26.77^{b} (±9.39)	**2.66**
Hoppy aroma	32.80^{a}(±8.78)	20.76^{b} (±11.41)	34.35^{a} (±9.59)	18.26^{b} (±9.84)	35.91^{a} (±10.79)	19.68^{b} (±9.76)	**3.39**
Fruity aroma	25.20^{a}(±8.74)	16.40^{b} (±8.48)	27.44^{a} (±9.55)	17.50^{b} (±8.10)	28.56^{a} (±8.93)	18.28^{b} (±9.33)	**3.58**
Tropical aroma	13.08^{a}(±6.89)	7.77^{b} (±7.35)	15.93^{a} (±8.91)	9.30^{b} (±8.27)	16.42^{a} (±8.43)	8.50^{b} (±8.02)	**3.48**
Citrus aroma	3.33^{ab}(±4.77)	2.00^{bc} (±4.05)	3.51^{a} (±4.84)	1.26^{c} (±3.34)	2.83^{ab} (±4.58)	1.26^{c} (±3.34)	**1.38**
Apple/pear aroma	13.43^{a}(±13.43)	7.64^{b} (±10.05)	7.85^{a} (±14.31)	8.75^{b} (±9.55)	7.85^{a} (±13.73)	7.40^{b} (±7.88)	**3.16**
Stale beer aroma	6.16^{c}(±15.65)	34.57^{ab} (±16.43)	7.00^{c} (±17.27)	31.88^{b} (±15.58)	6.00^{c} (±13.92)	38.38^{a} (±15.14)	**5.30**
Smoky aroma	17.73^{a}(±13.26)	4.75^{c} (±9.33)	14.76^{ab} (±12.29)	4.75^{c} (±10.12)	13.60^{b} (±12.44)	5.65^{c} (±11.18)	**3.83**
Sweet /honey aroma	4.21^{b}(±10.03)	22.92^{a} (±12.43)	6.03^{b} (±11.70)	24.93^{a} (±11.69)	5.01^{b} (±10.12)	28.45^{a} (±10.15)	**6.20**
Diacetyl/Butterscotch	3.33^{a}(±5.77)	4.34^{a} (±6.99)	3.76^{a} (±6.66)	5.67^{a} (±8.00)	5.13^{a} (±7.12)	5.91^{a} (±8.18)	**3.20**
Sherry-like aroma	0.00^{c}(±0.00)	4.12^{b} (±8.20)	0.02^{c} (±0.11)	4.37^{b} (±7.19)	0.00^{c} (±0.00)	5.52^{a} (±8.89)	**0.97**

C, E, N = barley malt cultivar code; 0, 3 = 0 & 3 month aged beer; LSD = least significant difference at a 5% level of significance; values in brackets indicates the SD (standard deviation)

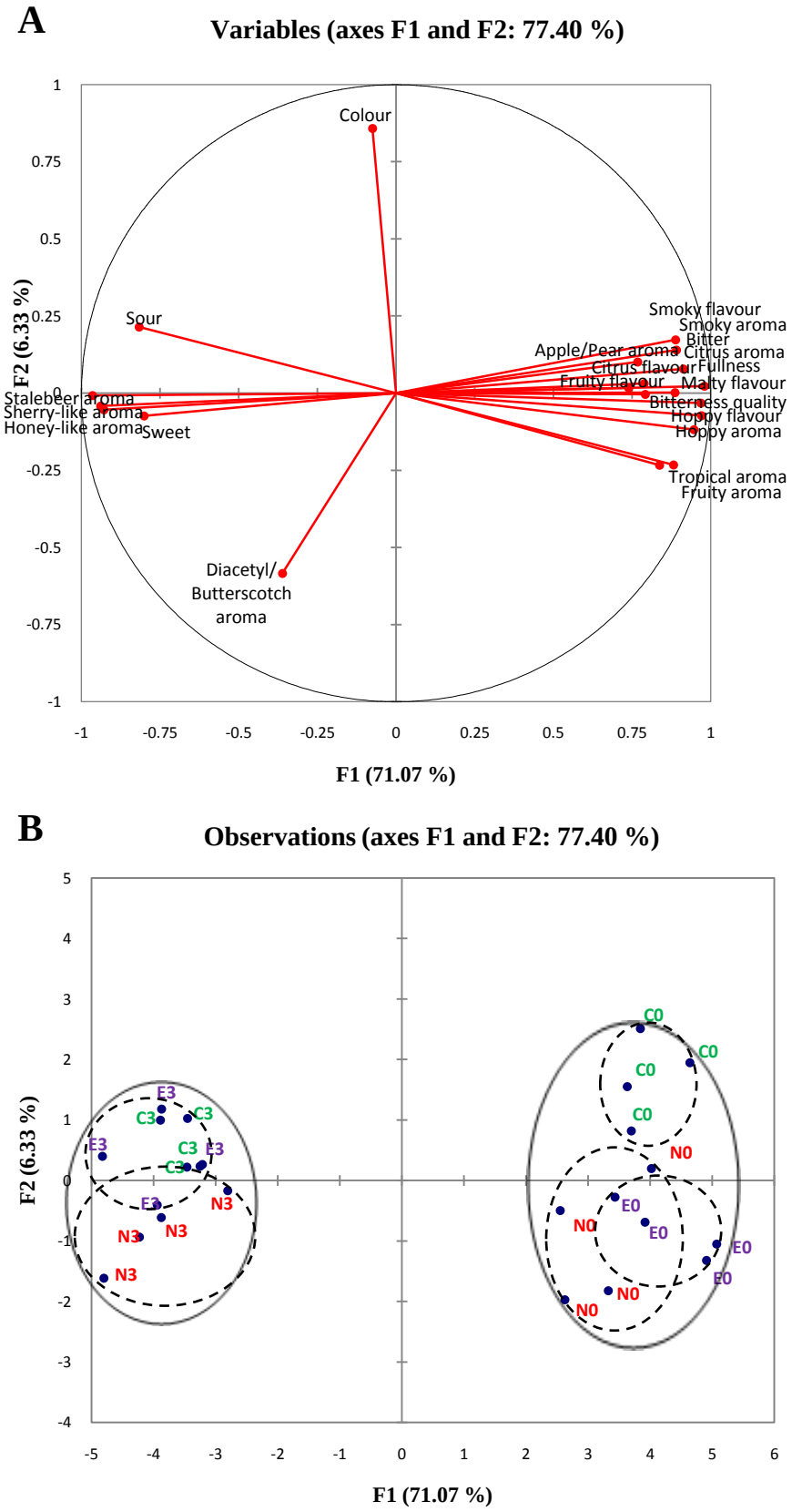

Fig. 3. PCA loading (**A**) and scores plot (**B**) illustrating the positioning of the 22 beer attributes and the 4 repeats of the 6 beer treatments respectively. N, E and C refer to a treatment and 0 and 3 refers to fresh and aged beers respectively.

Looking closer at the relationship and correlation of attributes towards one another (Table 7) it was found that fullness, as perceived on the palate, positively correlates to bitterness, bitterness quality (lingering), malty-, hoppy-, fruity-, citrus- and smoky flavours as well as hoppy- and fruity (overall, apple/pear, tropical and citrus)- smoky aromas (R^2>0.698). Fullness, however, correlates negatively with sweet-, sour-, stale beer-, honey-like and sherry-like flavours (R^2<-0.761). Overall fruity aroma correlates positively with all other fruit aromas (R^2>0.761). Another positive beer sensory attribute, malty flavour, correlates well with bitter, bitterness quality (lingering), hoppy-, fruity-, citrus- and smoky flavour as well as with hoppy and fruity aromas (R^2>0.751). Hoppy aroma again correlates well with bitterness, bitterness quality (lingering), fruity and smoky flavour and aromas (R^2>0.701). Both malty and hoppy flavour correlates negatively with sweet, sour, stale beer, honey and sherry-like aromas (R^2<-0.716). Stale beer aroma correlates positively with sweet, sour, stale beer, honey and sherry-like aromas (R^2>0.773). These correlations are in excellent agreement with literature (Clapperton *et al.*, 1976; Malfliet *et al.*, 2008), as well as in accordance with Meilgaard *et al.*"s flavour wheel (Meilgaard, 1982).

Variation within the group at month 0 only existed for colour, fullness, bitterness, bitterness quality (lingering), malty and hoppy flavours and smoky aroma and flavour (Fig. 4A), while all other attributes, as given in Table 5, showed no significant difference between fresh beer samples. Beer C0 scored significantly higher for fullness than Beer N0, while Beer E0 did not differ significantly from either N0 or C0. Beer C0 and E0 were significantly higher in bitterness than N0 and the same was true for bitterness quality (lingering). Beer E0 scored significantly higher in malty flavour than N0, while treatment C0 did not differ significantly in malty flavour when compared to N0 and E0.

Table 7. The correlation (R^2) matrix of aroma, flavour and colour attributes

Variables	Colour	Fullness	Sweet	Sour	Bitter	Bitterness Quality	Malty flavour	Hoppy flavour	Fruity flavour	Citrus flavour	Smoky flavour	Hoppy aroma	Fruity aroma	Tropical aroma	Citrus aroma	Apple/ pear aroma	Smoky aroma	Stale beer aroma	Honey-like aroma	Diacetyl/ butter-scotch aroma	Sherry-like aroma
Colour	**1.00**	-0.01	0.09	0.26	-0.01	-0.09	-0.07	-0.11	-0.04	-0.07	0.07	-0.14	-0.19	-0.18	0.01	-0.07	0.02	0.02	0.00	-0.19	0.04
Fullness	-0.01	**1.00**	-0.76	-0.76	0.92	0.89	0.97	0.97	0.75	0.76	0.88	0.93	0.85	0.81	0.70	0.77	0.88	-0.95	-0.91	-0.30	-0.91
Sweet	0.09	-0.76	**1.00**	0.74	-0.77	-0.76	-0.74	-0.75	-0.54	-0.61	-0.74	-0.69	-0.59	-0.55	-0.60	-0.54	-0.80	0.77	0.70	0.35	0.75
Sour	0.26	-0.76	0.74	**1.00**	-0.73	-0.78	-0.84	-0.83	-0.58	-0.55	-0.66	-0.79	-0.70	-0.66	-0.59	-0.51	-0.69	0.78	0.70	0.26	0.74
Bitter	-0.01	0.92	-0.77	-0.73	**1.00**	0.96	0.91	0.90	0.72	0.75	0.86	0.78	0.70	0.68	0.66	0.68	0.87	-0.83	-0.76	-0.28	-0.81
Bitterness quality	-0.09	0.89	-0.76	-0.78	0.96	**1.00**	0.89	0.90	0.69	0.72	0.78	0.76	0.68	0.66	0.63	0.65	0.82	-0.80	-0.72	-0.29	-0.75
Malty flavour	-0.07	0.97	-0.74	-0.84	0.91	0.89	**1.00**	0.96	0.75	0.76	0.87	0.91	0.83	0.79	0.68	0.75	0.86	-0.92	-0.88	-0.29	-0.88
Hoppy flavour	-0.11	0.97	-0.75	-0.83	0.90	0.90	0.96	**1.00**	0.77	0.70	0.83	0.91	0.83	0.79	0.74	0.72	0.87	-0.94	-0.91	-0.25	-0.92
Fruity flavour	-0.04	0.75	-0.54	-0.58	0.72	0.69	0.75	0.77	**1.00**	0.47	0.70	0.76	0.71	0.67	0.61	0.53	0.74	-0.76	-0.77	-0.26	-0.72

Citrus flavour	-0.07	0.76	-0.61	-0.55	0.75	0.72	0.76	0.70	0.47	**1.00**	0.62	0.66	0.56	0.61	0.51	0.63	0.59	-0.68	-0.60	-0.27	-0.60
Smoky flavour	0.07	0.88	-0.74	-0.66	0.86	0.78	0.87	0.83	0.70	0.62	**1.00**	0.80	0.73	0.65	0.65	0.66	0.93	-0.82	-0.80	-0.33	-0.84
Hoppy aroma	-0.14	0.93	-0.69	-0.79	0.78	0.76	0.91	0.91	0.76	0.66	0.80	**1.00**	0.92	0.88	0.68	0.79	0.79	-0.94	-0.90	-0.32	-0.90
Fruity aroma	-0.19	0.85	-0.59	-0.70	0.70	0.68	0.83	0.83	0.71	0.56	0.73	0.92	**1.00**	0.91	0.73	0.76	0.70	-0.88	-0.85	-0.19	-0.83
Tropical aroma	-0.18	0.81	-0.55	-0.66	0.68	0.66	0.79	0.79	0.67	0.61	0.65	0.88	0.91	**1.00**	0.66	0.68	0.60	-0.85	-0.81	-0.21	-0.77
Citrus aroma	0.01	0.70	-0.60	-0.59	0.66	0.63	0.68	0.74	0.61	0.51	0.65	0.68	0.73	0.66	**1.00**	0.65	0.62	-0.70	-0.73	-0.37	-0.72
Apple/ pear aroma	-0.07	0.77	-0.54	-0.51	0.68	0.65	0.75	0.72	0.53	0.63	0.66	0.79	0.76	0.68	0.65	**1.00**	0.60	-0.75	-0.73	-0.39	-0.75
Smokey aroma	0.02	0.88	-0.80	-0.69	0.87	0.82	0.86	0.87	0.74	0.59	0.93	0.79	0.70	0.60	0.62	0.60	**1.00**	-0.83	-0.81	-0.30	-0.85
Stale beer aroma	0.02	-0.95	0.77	0.78	-0.83	-0.80	-0.92	-0.94	-0.76	-0.68	-0.82	-0.94	-0.88	-0.85	-0.70	-0.75	-0.83	**1.00**	0.96	0.34	0.94
Honey-like aroma	0.00	-0.91	0.70	0.70	-0.76	-0.72	-0.88	-0.91	-0.77	-0.60	-0.80	-0.90	-0.85	-0.81	-0.73	-0.73	-0.81	0.96	**1.00**	0.36	0.95
Diacetyl/ butterscotch aroma	-0.19	-0.30	0.35	0.26	-0.28	-0.29	-0.29	-0.25	-0.26	-0.27	-0.33	-0.32	-0.19	-0.21	-0.37	-0.39	-0.30	0.34	0.36	**1.00**	0.36
Sherry-like aroma	0.04	-0.91	0.75	0.74	-0.81	-0.75	-0.88	-0.92	-0.72	-0.60	-0.84	-0.90	-0.83	-0.77	-0.72	-0.75	-0.85	0.94	0.95	0.36	**1.00**

Results for hoppy flavour show the same pattern as that of malty flavour. Treatment E0 was rated the highest for hoppy flavour, significantly higher ($P \leq 0.05$) compared to N0. No significant difference ($P > 0.05$) was found when comparing mean values for hoppy flavour of treatment C0 with N0 or E0. Variation within the group at month 3 only existed for stale beer, malty flavour, colour and sherry-like aroma (Fig. 4B) while all other attributes showed no significant difference between samples. Beer N3 had a significantly more stale beer aroma ($P \leq 0.05$) than Beer E3, while no significant difference in stale beer aroma was found when comparing mean values for Beer C3 to both Beer N3 and E3. According to Fig. 4B, Beer N3 was rated the highest for sherry-like aroma, significantly higher ($P \leq 0.05$) compared to Beer E3 and C3. No significant difference ($P > 0.05$) in sherry-like aroma was found between the latter two samples.

Discriminant Analysis was performed on the data collected to determine which attributes are responsible for discrimination between samples. As can be seen from Fig. 5 each treatment can be classified as an individual entity.

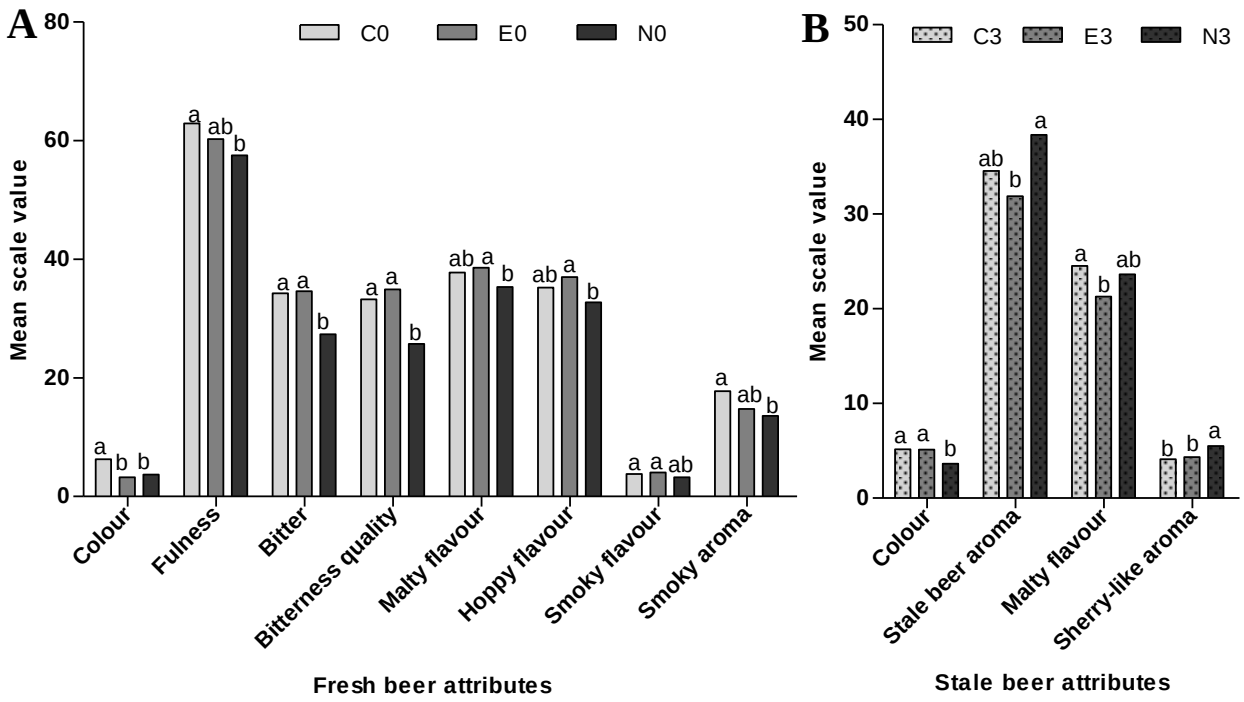

Fig. 4. Statistical mean of attributes intensities within fresh beer (Beer 0) (**A**) and aged beer (Beer 3) **(B)**. Only those attributes which are significantly different within a group (0 or 3) are shown. All excluded attributes did not show significant difference within the 0 or 3 beer age groups. Bars with different alphabetical letters are significantly different from each other ($P \leq 0.05$).

According to Fig. 5A 99.89% of variations between samples are explained. C0, E0 and N0 associate with the sensory attributes on the right side of P1 and it seems that E0 and N0 are more similar, while C0 associate more with traits in the top right quadrant than the previous two treatments. For the aged beers (3 month old, coded 3) we found that C3, E3 and N3 associate with the sensory attributes in the left quadrant. Beer C3 and N3 share more similar traits and E3 less similar traits than C3 and N3 (Fig. 5B). Arrows (Fig. 5B) indicate the shift in dominant sensory attributes from 0 to 3 months.

From Figs. 4 and 5B it seems that Beers C0 and E0 were the fuller bodied beers than Beer N0. After a three month period of aging a shift in sensory attributes occurred for all three fresh beers. All aged beers (N3, E3 and C3) were similar with regards to fullness, hoppy-, smoky- and fruity flavour, sweet, sour, bitter, bitterness quality (lingering), malty-, hoppy-, fruity-, tropical fruit-, citrus-, apple/pear-, smoky, sweet associated/honey-like-, and diacetyl/butterscotch aromas (Table 4). N3 had some of the lower scores for malty flavour, citrus aroma and one of the highest scores for stale beer and sherry-like aromas (Table 5). E3 and C3 did not show large mean differences in attributes, however, C3 still had a significantly higher malty aroma than N3 (Fig. 4 and 5B) when only investigating the attributes where the three aged beer treatments showed significant differences. C3 and E3 were similar with regards to stale beer and sherry-like aromas.

With regards to all descriptive sensory information so far discussed, it seems that Beer N showed the least potential for ageing, while it is difficult to establish large differences in ageing potential between Beers E and C. In summary, these results indicated that the quality of the aged beer can be rated as follows C ≈ E > N, correlating partially with our prediction from the malts and beer analysis that C has the best shelf-life quality parameters.

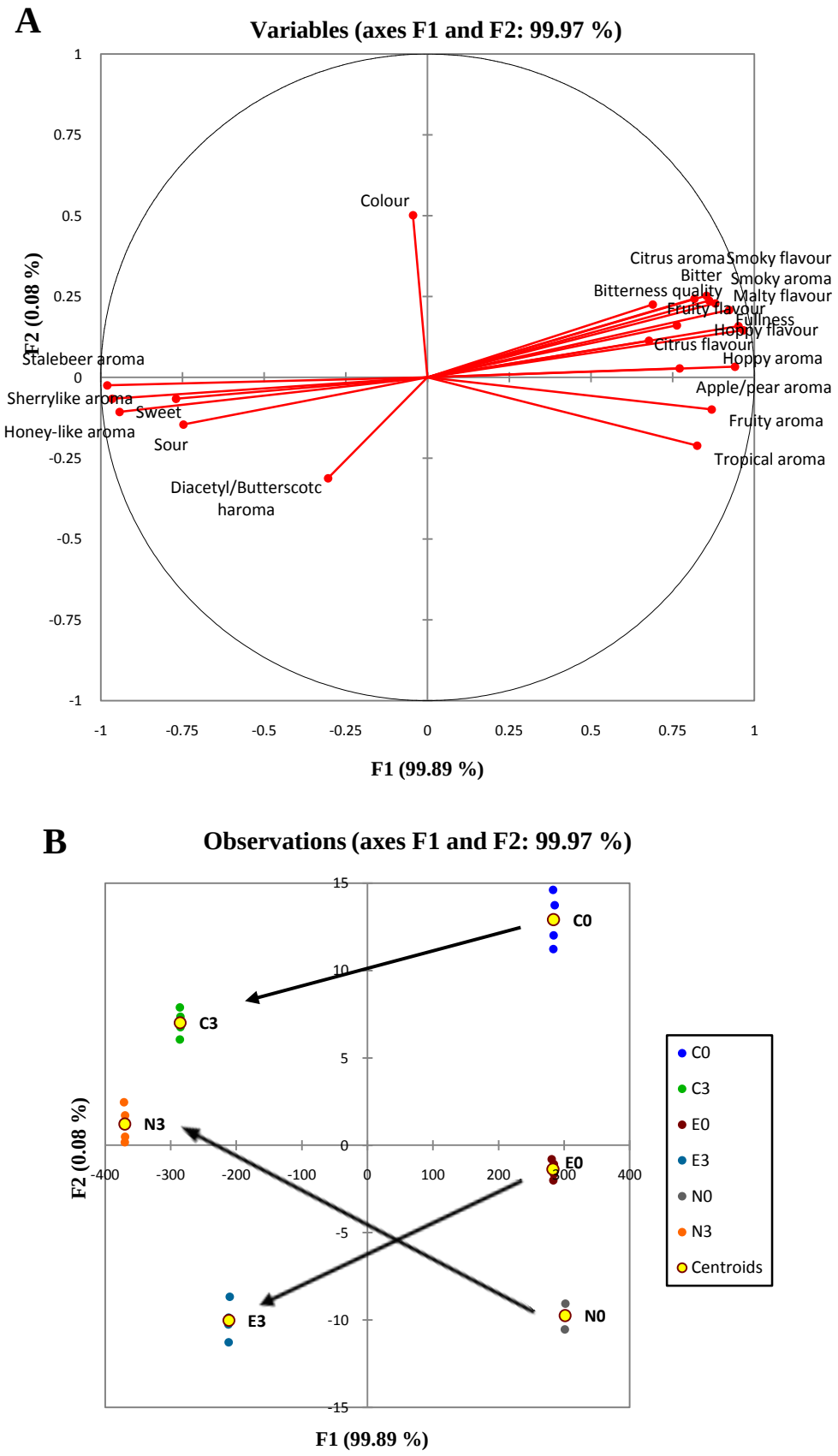

Fig 5. Discriminant analysis **A)** loading and **B)** scores plot illustrating the positioning of the 22 beer attributes and the means (centroids) of the 6 beer treatments respectively. N, E and C refer to a treatment and 0 and 3 refers to fresh and aged beers respectively. Arrows indicate how treatments aged and shifted with regards to dominant attributes.

Conclusions

A fair to good correlation when comparing the sensory results of the different beers and their potential for ageing with what was found with the prior screening of the barley malt was found. We predicted that beer brewed from Malt C would have the better aging potential followed by Malt N and then Malt E using the ratios of AROP/LOX and LTP/LOX. However, it was observed that Beer C ≥ E > N in terms of the sensory quality of aged beer, with C having a slightly better quality than E. Beer E and Beer C generally showed similar results when beer samples were subjected to sensory analysis of the aged beer, while aged beer N were rated more stale. The only large difference between C3 and E3 was for „malty flavour" for which C3 scored the highest, possibly indicating that C3 showed slightly better potential.

Malt E which was predicted to have the least stable flavour, which was not the case, based on the sensory results. This could be ascribed to the fact that LOX-1 did not decrease from malt to beer as would have been expected. Instead, Malt E, which had the highest LOX-1 content ended up with the lowest LOX-1 in the beer product, which might be the reason for it being more stable than was initially expected. Furthermore, consumers generally, and male consumers in particular, liked Beer E (and Beer N) more than they did Beer C, although the female consumers liked/disliked all beers equally. Using the sensory analysis we also observed that the trained panel found it difficult to identify the cardboard taint in the beer that is usually associated with the presence of 2(*E*)-nonenal. This and the consumer"s preference for beer E could further explain the discrepancy between our biochemical prediction of aged beer quality and the sensory analysis. The chemical analysis of the beer, however, correlated with the sensory analysis results. Beer C, having the more stable flavour, showed highest beer-LTP1, beer-protein and FAN and lowest beer-LOX-1. Beer N, showing the least stable flavour showed high beer-LOX-1 concentration, low beer-protein and FAN concentration and moderate beer-LTP1 concentration.

Although many other factors play a role in the stability of flavour in beers, the parameters we selected for barley malt screening did select the malt, namely Malt C leading to most flavour stable beer, but could not discriminate between Malt E and Malt N. However, in order to fully assess the prediction of beer quality from our parameters a much larger sample set would be needed. This prediction method is also limited to current knowledge of how LTP1 and LOX-1 interact and perform during brewing procedures, since we found that a possible relationship could exist between the loss of LTP1 and LOX activity. For example malt E had both the highest LOX activity and LTP1 content, but ended with the lowest LTP1 content in the beer, as well as the greatest loss of LOX activity (82%). Conversely malt C had the lowest LOX activity and LTP1 content and ended with the highest LTP1 content in the beer. Therefore it is possible that LOX can lead to some loss of LTP and *visa versa* which could lead to quality issues. A possible explanation if this is in fact the case could be that LTP1 could be binding to intermediates from the LOX-1 pathway to form LTP1 analogues or complexes not detected by means of western-blot assay where LTP1 specific antibodies are used.

From this study it is clear that LTP1, LOX and AROP does play crucial role in beer quality and could be used as three of the parameters to predict aged beer quality. These results could greatly benefit breeders, maltsters and/or brewers with barley selection or screening for analysis, blending and brewing purposes. The biochemical parameters evaluated was fairly representative of the end product quality in terms of flavour stability and foam. All analysis procedures were fairly simple to compile and perform and could therefore be performed in less specialised laboratories or where a large number of samples need to be screened on a regular basis. Future research regarding the relationship between LOX-1, other LOX enzymes, LTP1 and other LTPs and their role during the brewing process and beer aging, as well as larger sample sets for more stringent statistical evaluation would enable an even more accurate quality prediction.

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References

American Society of Brewing Chemists (2009). ASBC, methods of analysis. In: American Society of Brewing Chemists. Pilot Knob, Minnesota,

Angelino, S., Kolkman, J., Van Gemert, L., Vogels, J., Van Lonkhuijsen, H. & Douma, A. (1999). Flavour stability of pilsener beer. In: P*roceedings of Congress-European Brewery Convention*, pp. 103-112.

Bakan, B., Hamberg, M., Perrocheau, L., Maume, D., Rogniaux, H., Tranquet, O., Rondeau, C., Blein, J.P., Ponchet, M. & Marion, D. (2006). Specific adduction of plant lipid transfer protein by an allene oxide generated by 9-lipoxygenase and allene oxide synthase. *Journal of Biological Chemistry*, **281**, 38981-38988.

Bamforth, C.W. (1985). The foaming properties of beer. *Journal of the Institute of Brewing*, **91**, 370-383.

Clapperton, J., Dalgliesh, C. & Meilgaard, M. (1976). Progress towards an international system of beer flavour terminology. *Journal of the Institute of Brewing*, **82**, 7-13.

Dvořáková, M., Guido, L.F., Dostálek, P., Skulilová, Z., Moreira, M.M. & Barros, A.A. (2008). Antioxidant properties of free, soluble ester and insoluble-bound phenolic compounds in different barley varieties and corresponding malts. *Journal of the Institute of Brewing*, **114**, 27-33.

European Brewery Convention (1998). *Analytica-EBC.* Verlag Hans Carl: Nurnberg, Germany,

Evans, D.E. & Bamforth, C.W. (2009). *Beer foam: Achieving a suitable head.* In: Handbook of alcoholic beverages: Beer, a quality perspective, C. W. Bamforth, Russel, I., & Stewart, G. G. Ed, Elsevier: Burlington, MA. pp 1-60.

Evans, D.E., Sheehan, M.C. & Stewart, D.C. (1999). The impact of malt derived proteins on beer foam quality. Part II: The influence of malt foam-positive proteins and non-starch polysaccharides on beer foam quality. *Journal of the Institute of Brewing*, **105**, 171-177.

Fantozzi, P., Montanari, L., Mancini, F., Gasbarrini, A., Addolorato, G., Simoncini, M., Nardini, M., Ghiselli, A. & Scaccini, C. (1998). *In vitro* antioxidant capacity from wort to beer. *LWT-Food Science and Technology*, **31**, 221-227.

Fox, G.P., Panozzo, J.F., Li, C.D., Lance, R.C.M., Inkerman, P.A. & Henry, R.J. (2003). Molecular basis of barley quality. *Australian Journal of Agricultural Research*, **54**, 1081-1101.

Guido, L.F., Boivin, P., Benismail, N., Goncalves, C.R. & Barros, A.A. (2005). An early development of the nonenal potential in the malting process. *European Food Research and Technology*, **220**, 200-206.

Hirota, N., Kuroda, H., Takoi, K., Kaneko, T., Kaneda, H., Yoshida, I., Takashio, M., Ito, K. & Takeda, K. (2006). Brewing performance of malted lipoxygenase-1 null barley and effect on the flavor stability of beer. *Cereal Chemistry*, **83**, 250-254.

Kobayashi, N., Kaneda, H., Kano, Y. & Koshino, S. (1993). The production of linoleic and linolenic acid hydroperoxides during mashing. *Journal of Fermentation and Bioengineering*, **76**, 371-375.

Kuchel, L., Brody, A.L. & Wicker, L. (2006). Oxygen and its reactions in beer. *Packaging Technology and Science*, **19**, 25-32.

Kuroda, H., Kojma, H., Kaneda, H. & Takashio, M. (2005). Characterization of 9-fatty acid hydroperoxide lyase-like activity in germinationg barley seeds that transform 9(S)-hydroperoxy-10(E), 12(Z)-oktadecadienoic acid into 2(E)-nonenal. *Bioscience, Biotechnology, and Biochemistry*, **69**, 1661-1668.

Lawless, H.T. & Heymann, H. (2010). *Sensory evaluation of food: Principles and practices*. Springer, New York, pp. 79-99

Lie, S. (1973). The EBC-ninhydrin method for determination of free alpha amino nitrogen. *Journal of the Institute of Brewing*, **79**, 37-41.

Ludikhuyze, L., Indrawati, Van den Broeck, I., Weemaes, C. & Hendrickx, M. (1998). Effect of combined pressure and temperature on soybean lipoxygenase. 1. Influence of extrinsic and intrinsic factors on isobaric-isothermal inactivation kinetics. *Journal of Agricultural and Food Chemistry*, **46**, 4074-4080.

Lustig, S. (1999). Analytical tools and practical options to improve flavour stability. *Ferment*, **12**, 25-29.

Malfliet, S., Opstaele, F., Clippeleer, J., Syryn, E., Goiris, K., Cooman, L. & Aerts, G. (2008). Flavour instability of pale lager beers: Determination of analytical markers in relation to sensory ageing. *Journal of the Institute of Brewing*, **114**, 180-192.

Meilgaard, M., Dalgliesh, C. & Clapperton, J. (1979). Beer flavour terminology. *Journal of the Institute of Brewing*, **85**, 38-42.

Meilgaard, M.C. (1982). Prediction of flavor differences between beers from their chemical composition. *Journal of Agricultural and Food Chemistry*, **30**, 1009-1017.

Rencher, A.C. & Christensen, W.F. (2012). *Methods of multivariate analysis.* John Wiley & Sons, Hoboken, NJ, USA, pp.1-5

Samaras, T.S., Camburn, P.A., Chandra, S.X., Gordon, M.H. & Ames, J.M. (2005). Antioxidant properties of kilned and roasted malts. *Journal of Agricultural and Food Chemistry*, **53**, 8068-8074.

Santos, J., Carneiro, J., Guido, L., Almeida, P., Rodrigues, J. & Barros, A. (2003). Determination of E-2-nonenal by high-performance liquid chromatography with UV detection: Assay for the evaluation of beer ageing. *Journal of Chromatography A*, **985**, 395-402.

Shapiro, S.S. & Wilk, M.B. (1965). An analysis of variance test for normality (complete samples). *Biometrika*, **52**, 591-611.

Smedley, S. (1995). Discrimination between beers with small colour differences using the CIELAB colour space. *Journal of the Institute of Brewing*, **101**, 195-201.

Snedecor, G.W. & Cochran, W.G. (1967). Statistical methods. Ed. 6. Iowa State University Press, Ames.

Van Nierop, S.N.E., Evans, D.E., Axcell, B.C. & Cantrell, I.C. (2002). Studies on beer foam proteins in a commercial brewing process. In: *The institute & Guild of Brewing*, Asia Pacific, pp. 1-6.

Vanderhaegen, B., Neven, H., Verachtert, H. & Derdelinckx, G. (2006). The chemistry of beer aging - a critical review. *Food Chemistry*, **95**, 357-381.

Wainwright, T. & Buckee, G. (1977). Barley and malt analysis—a review. *Journal of the Institute of Brewing*, **83**, 325-347.

Wu, M.J., Clarke, F.M., Rogers, P.J., Young, P., Sales, N., O'Doherty, P.J. & Higgins, V.J. (2011). Identification of a protein with antioxidant activity that is important for the protection against beer ageing. *International Journal of Molecular Sciences*, **12**, 6089-6103.

Yang, G.S., Schwarz, P.B. & Vick, B.A. (1993). Purification and characterization of lipoxygenase isoenzymes in germinating barley. *Cereal Chemistry*, **70**, 589-595.

CHAPTER 7

Conclusion and future studies

Introduction

This study focussed on determining parameters in malts to predict two of the major beer quality determining factors namely, foam- and flavour stability. Addressing these specific issues were motivated by research done by Van Nierop (2005), whom investigated an industry problem of beer foam, which is still today a major challenge for brewers to overcome. More recently, however, the issue of beer shelf life was addressed, as this a major issue in the brewing industry, requiring further research. Both foam and flavour related issues led to the investigation of a number of quality determining factors and it was discovered that the proteins responsible for foam and flavour, had in fact much more in common than was originally thought.

Summary of work

One of the major problems in beer quality is the issue of stable foam (Bamforth, 1985). Numerous factors contribute to the formation of beer foam, but one protein, namely lipid transfer protein 1 (LTP1) has caught the attention of the industry and researchers. Not only is this protein one of the major beer stabilising proteins (Evans & Bamforth, 2009), but is also theorised to play a role in fermentation (van Nierop *et al.*, 2008) and has recently been identified as having anti-oxidant properties (Wu *et al.*, 2011) and thus has the potential of contributing to flavour stability. To fully understand this protein and whether it plays a role in beer foam, -flavour and fermentation, both LTP1 and LTP1b (LTP1 in association with a covalently bound lipid adduct) were purified and characterised both in terms of their tolerance to temperature and pH fluctuations and inhibitory effects towards two strain of *Saccharomyces cereviseae* (Chapter 2). A simplified five step protocol for the purification of LTP1 and LTP1b was successfully

developed with respective purified yields of 88 mg and 72 mg/100g barley. Both proteins were extremely tolerant to high temperatures reaching >90°C and remained relatively intact upon cooling to 25°C, as was investigated using circular dichroism (CD). It was also found that these proteins are more stable in a neutral to a more basic environment. With regards to their inhibitory effect on yeast, it was observed that only LTP1 showed antiyeast activity with IC_{50}'s of 15-23 µM. LTP is found in beer in concentrations of <35 µg/mL (Van Nierop *et al.*, 2004; Evans & Bamforth, 2009), therefore LTP1 antiyeast activity may lead to yeast inhibition and stuck fermentations. However, LTP1b was found to have little or no antiyeast activity which is probably due to the hydrophobic cavity being occupied by the already bound lipid moiety leading to weak interaction with lipid membranes. It would be of great benefit to have the modified form of LTP1 rather than LTP1 itself, since LTP1b would still be foam stabilising or possibly even more so than LTP1 (Wijesinha-Bettoni *et al.*, 2007), but would not have a detrimental effect on fermentation. In support of this it was found that LTP1 was converted into LTP1b during the 20 hour malt extraction and analysis of wort showed that the major fraction of LTP consists of LTP1b, glycosylated LTP1 and glycosylated LTP1b. It is therefore concluded that LTP1b and possibly glycocylated LTPs will not be a major inhibitory factor in fermentation, correlating with the observation by Gorjanovic *et al.* (2004) that LTP is inert in a brewing setup.

The lipid moiety bound to LTP1 to form LTP1b was identified to be α-ketol 9-hydroxy-10-oxo-12*(Z)*-octadecenoic acid (Bakan *et al.*, 2006), which is also an intermediate in the LOX-1 pathway to later form 2(*E*)-nonenal, the compound associated with a cardboard-like stale flavour in beer. The question arose to whether LTP1 might be able to inhibit the formation of 2(*E*)-nonenal by competing for the intermediate required by LOX-1 for its formation. During the trials it was found that this was indeed so (Chapter 5). It was observed that LTP inhibited LOX-1 activity due to LTP1 possibly binding the necessary substrate resulting in the formation of LTP1b and could therefore have an even greater contribution towards flavour stability than was originally thought. Although Wu *et al.* (2011) found LTP1 to have antioxidant properties, we did

not come to the same conclusion using our FOX-AROP assay. Keeping the possible roles of LTP1 towards beer quality in mind it is clear to see why this protein has become of such great interest to researchers and why the need for further classification exists.

Another large concern for the brewing industry is the deterioration of flavour in the final packaged product over time (Takashio & Shinotsuka, 1998). This phenomenon can be ascribed to various chemical reactions, but one of the more significant reactions is the formation of a 2(*E*)-nonenal which gives stored beer a characteristic "cardboard taint" (Drost *et al.*, 1990). 2(*E*)-nonenal is formed via the lypoxygenase (LOX)-1 pathway from linoleic acid (Kuroda *et al.*, 2002; Kuroda *et al.*, 2003). LOX is therefore considered a malt negative factor, but it is known that the absence of oxygen or presence of anti-radical/oxidants can inhibit the oxidative breakdown of products such as linoleic acid to form unwanted flavour components (Vanderhaegen *et al.*, 2006). It would be of great benefit for the maltster/brewer to be able to blend malts on the basis of their predicted, potential shelf life, according to a malt's LOX concentration and anti-radical/oxidant potential (AROP). A need for a high-throughput, reliable and robust assay for the determination of both LOX-1 and AROP was identified that can be easily adapted for an on-site laboratory and requiring little specialised equipment. A 96-well plate micro-assay was therefore developed by adapting a ferrous oxidation-xylenol orange (FOX) assay for the determination of LOX (Chapter 3) and AROP (Chapter 4). Both assays for the determination of LOX and AROP gave highly repeatable results. Each assay was compared to other respective methods used for LOX and AROP determination. The LOX-FOX micro-assay gave highly repeatable LOX activity data and compared well with a widely used 234 nm spectrophotometric LOX assay, while the AROP-FOX micro-assay compared favourably to the cupric ion reducing antioxidant capacity (CUPRAC) assay. Both assays were successfully applicable to barley malts, worts and brewery extracts revealing a great variation in LOX and AROP between barley malts, harvest seasons and kiln sampling positions. According to the LOX-FOX assay, LOX activity was maintained during the brewery process. However, the LOX-

FOX assay is dependent on Fe^{2+} oxidation to Fe^{3+}, the assay can be influenced by radicals giving a false high LOX activity. Maillard reaction taking place during the mashing could lead to radicals activity (McMurrough *et al.*, 1983; Woffenden *et al.*, 2002; Samaras *et al.*, 2005) and artefact LOX, but the Maillard product on the other hand can act as anti-oxidants suppressing LOX activity (Drost *et al.*, 1990; Kobayashi *et al.*, 1993). Extreme care was taken to limit oxidation by N_2 flushing of reagents and Maillard radicals could already be quenched in cooled samples by antioxidant action. Therefore it is hypothesised that LOX activity may be present or that artefact LOX may be due to radicals, which both could lead to problems during mashing producing off-flavour compounds and precursors that would compromise the end product. As expected the LOX activity, however, decreased greatly (>99%) during the brewing process to form wort.

Using the medium throughput assays, as well as established assays, selected biochemical markers in 28 different barley malt cultivars over different harvest seasons were analysed to elucidate whether and why trends appear between factors seemingly unrelated (Chapter 5). The variables that were examined represented both malt positive and negative qualities. LOX concentration is seen as detrimental to flavour stability and is thus regarded a flavour negative factor, while AROP is seen as a flavour positive factor. Free amino nitrogen (FAN) and protein content represents fermentation positive factors and was also included in sample screening. LTP1, recently proven to have antioxidant characteristics (Wu *et al.*, 2011) and also now shown to inhibit LOX-1 activity is regarded as both foam and flavour positive. An excellent linear correlation ($R^2 = 0.93$) was found between FAN/LOX (fermentation positive factor, normalised with a flavour negative factor) and LTP1/LOX (flavour positive – normalised with a flavour negative factor). This trend could make it possible for brewers to select a malt variety with either high FAN/LOX and LTP1/LOX or low FAN/LOX and LTP1/LOX or blending it accordingly. A malt variety with high FAN/LOX and LTP1/LOX is predicted to have good fermentability and beer ageing potential. This trend was further substantiated by our novel observation that LTP1

significantly inhibits LOX-1 reactions. More linear trends were observed for the ratios AROP/LOX and FAN/LOX (R^2=0.57) as well as for AROP/LOX and LTP/LOX (R^2= 0.66) with distinct malt groupings, some predicted with better brewing characteristics than other. Malt character is extremely complex and when selecting a malt variety, one should consider multiple quality determining variables.

In order to determine whether the analysis of biochemical markers, elucidated in Chapter 5, will have any relation to sensory aging, we ran micro-brewery trials with three different malts which were chosen on the grounds of their biochemical parameters (Chapter 6). The malts and beers (fresh and aged for three months) were analysed for LTP1-, protein and LOX concentration, as well as for AROP and FAN content. Predictions on ageing potential for the beers brewed were based on their malt analysis, since this will be where screening will take place in a brewery setup. The fresh and three month aged beers were analysed using descriptive sensory analysis and we found that our predictions using the chemical analysis of the beer correlated well with what was found when using a trained panel to evaluate the beer. The malt with the highest LOX, lowest LTP1, AROP, FAN and protein concentration was expected to have a shorter shelf life and less stable beer foam and *vice versa*. This was true for the malt predicted to be the most flavour stable, but the biochemical predictions could not discriminate between the other two malts and sensory results ranked the lowest quality rated malt second. This was most probably due to the large decrease in LOX-1 and LTP1 concentration from malt to beer in this malt. It is possible that LOX-1 could be leading to a loss of LTP1 and *vice versa*, which could lead to quality issues. It was, however, clear that LTP1 and LOX play a crucial role in beer quality and could serve as tools to predict aging potential. Further investigation is needed to determine the relationship of LTP1 and LOX-1 during the brewing process and how they influence each other. Flavour is also a very complex, multi-facetted field of study and the quantification of certain compounds may not always represent what the consumer tastes due to the differences between compounds' flavour thresholds and the fact that some flavours may

mask others. In this study it was observed that a playoff of fullness in fresh beer samples *versus* stale beer flavour in aged samples. In other words, a fresh beer associating with a fuller flavour is also associated with a less stale beer flavour in aged beers, which could also explain the slight inconsistency between chemical and sensory results. However, in order to fully assess the prediction of beer quality from our parameters a much larger sample set would be needed.

Future research and closing remarks

Shelf life and foam stability of beer have since long been a concern for the brewing industry. Numerous methods are being applied to enhance both of these quality aspects. During this study a relationship between these two qualities are drawn, which was absent until recently when (Wu *et al.*, 2011) identified the main foam promoting protein, LTP1 as having anti-oxidant properties. This relationship was strengthened by establishing that LTP1 is able to inhibit LOX-1 activity to some extent. LTP1 have been the focus of many studies regarding its positive effect on foam stability as well as its more negative effect on fermentation. An excess of LTP1 would therefore hold more advantages than disadvantages, due to it showing very little antiyeast activity after it had been modified during the brewing process, it foaming properties and putative protection in terms of flavour.

It has proven difficult for brew house laboratories to screen efficiently for biochemical markers which would impact the end product quality. The major determinant of implementing such systems is the viability of these methods and whether screening for certain markers or the presence thereof would actually represent end product quality. The current research, however, showed that determining LOX-1, AROP, LTP1, FAN and protein content of malts would give a relatively good prediction on malt performance regarding beer foam and flavour stability. Methods for such screening need to be adapted for brew house laboratory, where little specialised equipment is required and samples can be accurately screened in bulk. The adapted

FOX assay as described during this research can therefore easily be implemented in such a setup for the medium-throughput screening of LOX-1 and AROP almost simultaneously. We made use of western blot assays for the analysis of LTP1 in various samples, which is not a complicated procedure, but is limited, by its utilisation of specialised equipment and difficulty to upscale sample size. An ELISA based assay, as described by Evans & Hejgaard (1999), would therefore be recommended for robust total LTP1 determination in future analyses. Such an ELISA assay can be adapted by using specific antibodies, as in this study, to determine unmodified/intact LTP1 and LTP1b, and an antibody preparation with broad LTP specificity to determine total LTP1 concentration. Another area in need of investigation is an assay-based method for the prediction of fullness of the end product, since there is a possible link between beer fullness (also described as the full bodied mouth feel of beer (Clapperton, 1974)) and stale-beer flavour. It is possible that the fullness of a beer and all those attributes associating with fullness may be masking some of the stale beer flavour attributes. If such an assay can be implemented in combination with the FOX assays for LOX and AROP described during this research and an LTP1 ELISA for LTP1 determination, a more accurate prediction could be made for malts and their end-product quality. Beer fullness can be ascribed to various aspects and would be a great challenge to predict at an early stage. Amino acid and protein quality of a malt determine how well and to what extent it is utilised during fermentation and would therefore greatly impact beer flavour and fullness. Therefore, more detailed and focused analyses of protein and FAN content of malt and the elucidation of its impact on end product quality linked to our quality parameters could improve the beer quality prediction from malts using biochemical parameters.

Many methods have been applied in the past for possible prediction of beer flavour through the analysis of barley malt in order to improve blending procedures for optimal product quality. It is of great importance to include the screening for a wide range of markers, to ensure quality on more than one quality aspect. These markers should then also be a good representation of the end product.. It can be can stated that the screening for the biochemical markers discussed

during this research proved to be a good representation of the end product quality as determined using sensory analysis i.e. flavour stability as well as foam stability. Methods used here are distinguished from others by its potential to be easily modified for a brewery laboratory environment and therefore facilitate the ease of regular screening procedures. Further research is however required on the interaction between LTP1 and LOX-1, since it our research highlighted that these proteins have an effect on each other, which could in turn influence beer quality.

References

Bakan, B., Hamberg, M., Perrocheau, L., Maume, D., Rogniaux, H., Tranquet, O., Rondeau, C., Blein, J.P., Ponchet, M. & Marion, D. (2006). Specific adduction of plant lipid transfer protein by an allene oxide generated by 9-lipoxygenase and allene oxide synthase. *Journal of Biological Chemistry*, **281**, 38981-38988.

Bamforth, C.W. (1985). The foaming properties of beer. *Journal of the Institute of Brewing*, **91**, 370-383.

Clapperton, J. (1974). Profile analysis and flavour discrimination. *Journal of the Institute of Brewing*, **80**, 164-173.

Drost, W., B., Berg, V.d., R., Freijee, M., F.J., Velde, V.d., G., E., Hollemans & M. (1990). *Flavor stability.* St. Paul, MN, ETATS-UNIS: American Society of Brewing Chemists. pp. 8.

Evans, D.E. & Bamforth, C.W. (2009). *Beer foam: Achieving a suitable head.* In: Handbook of alcoholic beverages: Beer, a quality perspective, C. W. Bamforth, Russel, I., & Stewart, G. G. Ed, Elsevier: Burlington, MA, pp 1-60.

Evans, D.E. & Hejgaard, J. (1999). The impact of malt derived proteins on beer foam quality. Part i. The effect of germination and kilning on the level of protein Z4, protein Z7 and LTP1. *Journal of the Institute of Brewing*, **105**, 159-169

Gorjanovic, S., Suznjevic, D., Beljanski, M., Ostojic, S., Gorjanovic, R., Vrvic, M. & Hranisavljevic, J. (2004). Effects of lipid-transfer protein from malting barley grain on brewers yeast fermentation. *Journal of the Institute of Brewing*, **110**, 297-302.

Kobayashi, N., Kaneda, H., Kano, Y. & Koshino, S. (1993). The production of linoleic and linolenic acid hydroperoxides during mashing. *Journal of Fermentation and Bioengineering*, **76**, 371-375.

Kuroda, H., Furusho, S., Maeba, H. & Takashio, M. (2003). Characterization of factors involved in the production of 2(E)-nonenal during mashing. *Bioscience, Biotechnology, and Biochemistry*, **67**, 691-697.

Kuroda, H., Kobayashi, N., Kaneda, H., Watari, J. & Takashio, M. (2002). Characterization of factors that transform linoleic acid into di- and trihydroxyoctadeconoic acids in mash *Journal of Bioscience and Bioengineering*, **93**, 73-77.

McMurrough, I., Hennigan, G.P. & Loughrey, M.J. (1983). Contents of simple, polymeric and complexed flavanols in worts and beers and their relationship to haze formation. *Journal of the Institute of Brewing*, **89**, 15-23.

Samaras, T.S., Camburn, P.A., Chandra, S.X., Gordon, M.H. & Ames, J.M. (2005). Antioxidant properties of kilned and roasted malts. *Journal of Agricultural and Food Chemistry*, **53**, 8068-8074.

Takashio, M. & Shinotsuka, K. (1998). Preventive production of beer against oxidation - recent advances in brewing technology. *Food Science and Technology International Tokyo*, **4**, 169-177.

Van Nierop, S.N.E. (2005). Investigation of malt factors that influence beer production and quality. PhD Department Biochemistry, University of Stellenbosch, South Africa

van Nierop, S.N.E., Axcell, B.C., Cantrell, I.C. & Rautenbach, M. (2008). Optimised quantification of the antiyeast activity of different barley malts towards a lager brewing yeast strain. *Food Microbiology*, **25**, 895-901.

Van Nierop, S.N.E., Evans, D.E., Axcell, B.C., Cantrell, I.C. & Rautenbach, M. (2004). Impact of different wort boiling temperatures on the beer foam stabilizing properties of lipid transfer protein 1. *Journal of Agricultural and Food Chemistry*, **52**, 3120-3129.

Vanderhaegen, B., Neven, H., Verachtert, H. & Derdelinckx, G. (2006). The chemistry of beer aging - a critical review. *Food Chemistry*, **95**, 357-381.

Wijesinha-Bettoni, R., Gao, C., Jenkins, J.A., Mackie, A.R., Wilde, P.J., Mills, E. & Smith, L.J. (2007). Post-translational modification of barley LTP1b: The lipid adduct lies in the hydrophobic cavity and alters the protein dynamics. *FEBS Letters*, **581**, 4557-4561.

Woffenden, H.M., Ames, J.M., Chandra, S., Anese, M. & Nicoli, M.C. (2002). Effect of kilning on the antioxidant and pro-oxidant activities of pale malts. *Journal of Agricultural and Food Chemistry*, **50**, 4925-4933.

Wu, M.J., Clarke, F.M., Rogers, P.J., Young, P., Sales, N., O'Doherty, P.J. & Higgins, V.J. (2011). Identification of a protein with antioxidant activity that is important for the protection against beer ageing. *International Journal of Molecular Sciences*, **12**, 6089-6103.