FACULTY OF ENGINEERING TECHNOLOGY
DEPARTMENT OF MICROBIAL AND MOLECULAR SYSTEMS (M^2S)
FOOD AND MICROBIAL TECHNOLOGY (CLMT)
Gebroeders De Smetstraat 1
B-9000 GHENT, BELGIUM
weronika.filipowska@kuleuven.be
iiw.kuleuven.be

WERONIKA ANNA FILIPOWSKA

DETERMINATION OF CRITICAL FACTORS IN MALT PRODUCTION RELATED TO THE FLAVOUR STABILITY OF FINAL BEER

INTERNATIONAL CENTRE FOR BREWING SCIENCE
SCHOOL OF BIOSCIENCES
Sutton Bonington Campus
Bioenergy & Brewing Science Building
Loughborough,LE12 5RD, UK

October 2021

KU LEUVEN

University of Nottingham
UK | CHINA | MALAYSIA

ARENBERG DOCTORAL SCHOOL
FACULTY OF ENGINEERING TECHNOLOGY

SCHOOL OF BIOSCIENCE
INTERNATIONAL CENTRE FOR BREWING SCIENCE

DETERMINATION OF CRITICAL FACTORS IN MALT PRODUCTION RELATED TO THE FLAVOUR STABILITY OF FINAL BEER

Supervisors:
Prof. Luc De Cooman
Prof. Chris Powell

Co-supervisors:
Prof. David Cook
Dr. Ing. Gert De Rouck
Prof. Guido Aerts

Weronika Anna Filipowska

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

October 2021

DETERMINATION OF CRITICAL FACTORS IN MALT PRODUCTION RELATED TO THE FLAVOUR STABILITY OF FINAL BEER

WERONIKA ANNA FILIPOWSKA

Supervisors:

Prof. Luc De Cooman (KU Leuven)
Prof. Chris Powell (University of Nottingham)

Co-supervisors:

Prof. David Cook (University of Nottingham)
Dr. Ing. Gert De Rouck (KU Leuven)
Prof. Guido Aerts (KU Leuven)

Members of the Examination Committee:

Prof. Frederik Desplentere (KU Leuven, Chair)
Prof. Ann Van Loey (KU Leuven)
Prof. Christophe Courtin (KU Leuven)
Prof. Stephen Lawrence (University of Nottingham)
Dr. Irina Bolat (Boortmalt)
Dr. Boris Gadzov (FlavorActiV)

Dissertation presented in partial fulfilment of the requirements for the degree of Doctor of Engineering Technology (PhD) from KU Leuven and Doctor of Philosophy from University of Nottingham.

October 2021

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

Uitgegeven in eigen beheer, Weronika Filipowska, Gent, Belgium.

ACKNOWLEDGEMENTS

Science and beer have a lot in common. Both are complex, intriguing, addictive, and taste best when being accompanied by others. During the past four years, I was fortunate to enjoy both science and beer together with many people whom I would like to thank for sharing with me their time, knowledge, and passion.

In the first place, I would like to thank the European Union community for financial support of science and encouragement for creating training networks such as the European Joint Doctorate in Food Science (EJDFoodSCI). I would like to express my gratitude to the EJDFoodSCI project coordinator prof. Aleksander Poreda and the whole consortium - KU Leuven, University of Nottingham, University of Agriculture in Kraków, University of Copenhagen, Ghent University, TU Berlin, VLB Berlin, FlavorActiV, Carlsberg, and Boortmalt - for providing an innovative doctoral training where knowledge and skills were gained via mobility, collaboration, and state-of-the-art research. Being part of this extraordinary 'educational experiment' brought me a multitude of valuable experiences. Additionally, I would like to thank my former supervisor, prof. Joanna Kawa-Rygielska, who encouraged me to apply for the EJD project and showed interest in the research progress throughout these years.

This thesis wouldn't be possible without the guidance of my Supervisors from KU Leuven - prof. Luc De Cooman, dr. ing. Gert De Rouck, prof. Guido Aerts - and from the University of Nottingham - prof. David Cook and prof. Chris Powell. Thank you for your expertise, for providing state-of-the-art facilities, and for your interest in the outcomes of my research work. Luc, I highly appreciate your persistent curiosity and eagerness to better understand not only beer-related matters but also the world around us, regardless of the circumstances. I have learned a lot from you. Thank you for your expert advice and for motivating me to give the best I could (or even more than that). Gert, your 'can-do' attitude was very helpful when approaching research challenges. Thank you for sharing with me your passion for beer and showing me the 'real' malting and brewing world during various conferences. David, thank you for your scientific input, understanding, and careful proofreading of this thesis manuscript. Guido and Chris, I am grateful for the interesting discussions we had during oral presentations. I am also thankful to dr. Barbara Jaskula-Goiris, Basiu, thank you for your time and effort to proofread every poster, publication, and finally this thesis manuscript.

Words of appreciation to my industrial mentors dr. Irina Bola and dr. Boris Gadzov. Irina, thank you for always being there when I really needed support and for your insightful advices on both professional and personal levels. Boris, I highly value your practical approach and the numerous suggestions you have given me whenever we had a chance to meet.

I would like to express my gratitude to the members of the Examination Committee for their commitment, constructive discussion during the preliminary defence and suggestions allowing further improvement of the manuscript.

An essential part of this technology-driven thesis was close collaboration with Boortmalt. I am thankful to dr. Irina Bolat for making this possible. I am very grateful to Geert Van D'Huynslager and his team for providing malt samples, for their support to the data collection, and for showing me how to perform numerous analysis during my industrial internship. Many thanks to Koen Hardy and Michiel Jorissen for their assistance with sample collection

throughout industrial-scale malting. I am also thankful to Dominique Geerts and the whole technical team, who always made time for the maintenance of micro-malting. To all Boortmalt team, thank you for inviting me to join your team for a couple of months so that I could learn the best practices from the 'Masters of malt'.

The beer (and science) would not taste the same without my 'young, ambitious, and brave' ESR's fellows. Jonas, Marcus, Celina, Magda, Tuur, and Maciej, I cannot imagine a better team. You were always there to pass a tisssue and to share joyful moments. Thank you for making this PhD journey an unforgettable experience.

In the last four years, I had a chance to form a part of the Enzyme Fermentation and Brewing Technology Lab (EFBT). I would like to thank Agata, Barbara, Brecht, Brenda, Celina, Filip, Florian, Gino, Ilse D.P., Ilse V.D.V., Jasper, Jeroen B., Jeroen V., Johanna, Jolien, Lode, Lore, Mati, Marjan, Nina, Paula, Peter, Tuur, Valerie, and Wouter, for your technical support, pleasant, collaborative way of working and for our chats over the lunch, cup of coffee, beer or…a broken fiber. Special thanks to Johanna, thank you for teaching me how to perform complicated analysis, your never-ending willingness to help, speaking polish, and being my friend. You are my Chimay Bleue. Agata, thank you for 'simply' being my angel and sharing our 'polish things'. You are my Rodenbach. Jeroen, I already miss our insightful conversations, discussions over the challenging problems and (to some extent) collection of malting samples. You are my non-alcoholic Jupiler (sorry…). Paula, thank you for teaching me how to handle UPLC and being ready to assist whenever I had a doubt, your Spanish temperament was always bringing a lot of entertainment. You are my Estrella. Maciej, thank you for believing in me and for a multitude of conversations over a multitude of beers. You are my Geuze Boon.

I also had a chance to come across amazing people at my partner institution the Bioenergy and Brewing Science Department, University of Nottingham. Alex, Carolina, Celina, Christina, Chris, Gabby, Imogen, Jo, Marcus, Neil, Steph thank you for integrating me into the team and sharing your stories over a pint (or two). A huge 'thank you' to Celina and Maximo for hosting me in the city of Robin Hood.

My sincerest gratitude goes to my family. Mamo, Tato dziękuję wam za wasze nieustanne wsparcie, rozbudzanie we mnie ciekawości świata i waszą wiarę w pomyślne zakończenie tego etapu. Zawsze mnie wysłuchiwaliście, wspieraliście radą, wykazywaliście zainteresowanie zarówno wynikami naukowymi jak i stanem ducha. Bardzo was kocham. Babciu, Dziadku, Braciszku, Emilko, Adamie dziękuję za wasze wsparcie, dobre słowo i za to że zawsze byliście (i jesteście) blisko mnie.

Muchas gracias a mi familia política por todo ánimo y apoyo que recibí durante los últimos años. Chus, gracias por invitarme a tú casa y tratarme como tú hija. Antón, no hay suficiente parabalas para expresar mi gratitud por todo lo que hiciste por mi (y por está tesis). Tú me enseñaste que 'no se dice, se hace'. Eres lo mejor que me ha dado la vida.

Last but not least, I would like to thank you – the reader – for showing interest in this work. Wishing you a pleasant read!

Weronika

SUMMARY

Flavour instability can be regarded as one of the most important quality problems faced by the brewing industry, as beer flavour starts to deteriorate almost instantly upon packaging. The loss of pleasant flavour attributes and appearance of off-flavours will impact palatability and, therefore, reduce drinkability of beer, which in turn may result in economic losses for the brewer. Efforts to prolong beer freshness may only be fully effective when the complex chemistry behind the phenomena of beer ageing is much better understood. Within this context, one of the current areas of active research is an investigation on the gradual appearance of sensory perceivable off-flavours during beer transport and storage, which, chemically, coincides with an increase in levels of a multitude of unwanted compounds. Among them particular volatiles, known as 'beer staling aldehydes'. This specific group of aldehydes is characterised by highly flavour-active compounds, perceived at low concentrations, such as Strecker degradation aldehydes (derived from amino acids), furfural (derived from pentose), and hexanal, as well as *trans*-2-nonenal (both derived from oxidation of unsaturated fatty acids). Two major mechanisms have been ascribed to the development of staling aldehydes during beer ageing in a package, namely *de novo* formation of aldehydes from a precursor compound (*e.g.* an amino acid) and release of aldehydes from a pre-formed bound-state adduct (*e.g.* cysteinylated aldehydes).

It is well-established that the quality of brewing raw materials (in particular malted barley, water, hops, and yeast), as well as the brewing process, affect final beer quality and also beer flavour deterioration. Among raw materials, malt, being the major source of extract, appears to play a pivotal role in beer flavour instability as it has been reported that particular malt quality parameters (such as Kolbach Index, free amino nitrogen content, levels of Strecker aldehydes) are connected to beer staling. Previously, this relationship has also been referred to as the 'beer staling potential of malt'. Interestingly, the same volatile compounds that are directly involved in beer staling, *i.e.* the above mentioned staling aldehydes, also develop during production of malt. However, unambiguous cause-effect relationships between particular malt constituents and particular compounds in beer (responsible for in-package ageing) have not been proven yet, demonstrating the huge complexity of beer flavour instability and, consequently, the requirement for more research regarding malt and the malting process in relation to this phenomenon.
Since during malting both free and bound-state aldehydes already develop, the central objective of this technology-driven PhD is to monitor the malting process in relation to this development and, in particular, to pinpoint critical moments during malting regarding generation of these unwanted compounds. In this regard, in-depth chemical-analytical evaluation of an industrial-scale malting process and industrial-scale pale lager malts in relation to the formation of (bound-state) aldehydes was performed. Next, targeted micro-malting aiming at malt of reduced beer staling potential and high brewing quality was assessed based on experimental design and numerical modelling.

Aiming at a successful assessment of the malting process, the conditions of sample preparation for GC-MS determination of staling aldehydes were optimised for various types of malting samples (*i.e.* green malt, partially kilned malt, finished pale lager malt). Next, the resulting protocol for determination of volatile, free aldehydes was applied together with an already available method for quantification of non-volatile cysteinylated aldehydes.

By applying this approach, we were able to achieve a truly integrated view on both volatile aldehydes and their non-volatile counterparts for malt samples of various origins throughout this whole doctoral study. Moreover, results obtained on (bound-state) aldehydes were assessed in relation to standard quality parameters of various pale lager malts in search for correlations among all of these variables and to improve our understanding of potential relationships that may be relevant to beer flavour instability.

Results obtained on industrial-scale samples demonstrated that the content of (cysteinylated) aldehydes in finished malt is clearly higher compared to its starting material, barley. During germination, strong increases in levels of the fatty acid oxidation aldehydes, *i.e.* hexanal and *trans*-2-nonenal, were found. Levels in Strecker aldehydes and furfural were, however, hardly affected at this stage. Next, when approaching the stage of drying at elevated temperature (in particular when arriving at a critical moisture content of approx. 6% - 9%), levels of most (cysteinylated) aldehydes showed a first dramatic increase, except for hexanal. Furthermore, levels of all (cysteinylated) aldehydes continued to increase rapidly during kilning-off, except for hexanal. Clearly, throughout this PhD, it was found that hexanal behaves differently compared to all other aldehydes, including the other fatty acid oxidation marker aldehyde, *trans*-2-nonenal.
Furthermore, a clear effect of process-associated physicochemical gradients (caused by pneumatic processing in relatively thick grain beds) on aldehyde formation in malting was demonstrated for the first time. Except for hexanal, the highest levels of free and bound-state aldehydes were found in samples derived from the bottom layer of the grain bed, which is most exposed to heat load during kilning. Accordingly, samples taken at the same time from the upper layer (exposed to significantly less heat load) showed the lowest levels of aldehydes.
Next to analysis of the industrial-scale malting process as a function of duration (*i.e.* stages of malting) and position of the grain in the bed (*i.e.* bottom, middle, top layer), emphasis was put on several, potentially critical malting variables. These results showed that both the degree of grain modification and kilning-off temperature impact – either indirectly (degree of modification) or directly (kilning-off temperature) – the generation of (cysteinylated) aldehydes during malting. Consequently, the degree of steeping and kilning-off temperature were selected as key variables to conduct additional, targeted micro-malting experiments aimed to investigate the potential feasibility of producing malt of reduced beer staling potential, combined with satisfying brewing quality. Modelling of individual responses, representing the measured aldehydes and malt quality parameters, as a function of the key variables, demonstrated that the steeping degree especially impacts levels of hexanal, whereas kilning-off temperature mainly affects levels of Strecker aldehydes, furfural, and *trans*-2-nonenal. Finally, numerical modelling of the selected malting variables (steeping degree, kilning-off temperature), suggested the feasibility of producing pale lager malt of superior overall quality on condition that adequate, mutual adjustment of both grain modification and kilning-off temperature is implemented.

SAMENVATTING

Smaakinstabiliteit kan worden beschouwd als één van de belangrijkste kwaliteitsproblemen in de brouwindustrie, omdat de biersmaak bijna direct achteruitgaat vanaf het verpakken. De aangename smaakeigenschappen verdwijnen en de aanwezigheid van onaangename componenten verminderen de drinkbaarheid van het bier, wat financiële gevolgen kan hebben voor de brouwer. Pogingen om de versheid van bier te verlengen zijn alleen effectief wanneer de complexe chemie achter bierveroudering beter begrepen wordt. Er wordt momenteel actief onderzoek gedaan naar de geleidelijke vorming van zintuiglijk waarneembare onaangename smaken tijdens biertransport en -bewaring, dewelke vanuit chemisch standpunt gepaard gaan met een toename van talrijke ongewenste componenten, waaronder de zogenaamde vluchtige 'bierverouderingsaldehyden'. Deze specifieke groep van aldehyden wordt gekenmerkt door sterk aroma-actieve verbindingen, die waar te nemen zijn bij lage concentraties, zoals de Strecker degradatie aldehyden (afkomstig van aminozuren), furfural (gevormd uit pentose), en hexanal, alsook *trans*-2-nonenal (beide gevormd door de oxidatie van onverzadigde vetzuren). Twee belangrijke mechanismes worden toegeschreven aan de ontwikkeling van verouderingsaldehyden tijdens bierveroudering in een verpakking, namelijk *de novo* vorming van aldehyden uit een precursor (bijvoorbeeld een aminozuur) en het vrijkomen van aldehyden uit een voorgevormd gebonden adduct (bijvoorbeeld cysteïne-gebonden aldehyden).

Het is welbekend dat zowel de kwaliteit van de brouwgrondstoffen (in het bijzonder gerstemout, water, hop en gist), als het brouwproces, een effect hebben op de finale bierkwaliteit en op de achteruitgang van de biersmaak. Mout, de belangrijkste bron van extract, lijkt een cruciale rol te spelen bij de instabiliteit van de biersmaak, aangezien bepaalde moutkwaliteitsvariabelen (zoals Kolbach Index, vrij aminostikstofgehalte, concentratie aan Strecker aldehyden) worden gelinkt aan bierveroudering. Tot dusver werd dit verband beschreven als het 'bierverouderingspotentieel' van mout. Interessant is dat dezelfde vluchtige componenten die een directe invloed hebben op de bierveroudering, namelijk de bovengenoemde verouderingsaldehyden, ook worden gevormd tijdens de productie van mout. Er is echter nog geen ondubbelzinnig oorzakelijk verband gelegd tussen bepaalde mout bestanddelen en bepaalde bestanddelen in bier (verantwoordelijk voor veroudering in verpakking), wat de complexiteit van biersmaakinstabiliteit en de nood aan verder onderzoek naar mout en het moutproces in relatie tot bierveroudering aantoont. Aangezien tijdens het mouten zowel vrije als gebonden aldehyden reeds worden gevormd, is het voornaamste doel van dit technologisch gedreven doctoraat het moutproces op te volgen met het oog op de ontwikkeling en in het bijzonder het vastleggen van het cruciale moment tijdens het mouten waarbij deze ongewenste bestanddelen zich ontwikkelen. Daarom werd een gedetailleerde chemisch-analytische evaluatie uitgevoerd van een geïndustrialiseerd moutproces en geïndustrialiseerd pilsmout met betrekking tot de vorming van (gebonden) aldehyden. Vervolgens werden gerichte micro-moutprocessen geëvalueerd op basis van experimentele ontwerpen en numerieke modellen gericht op mout met een verminderd bierverouderingspotentieel en hoge brouwkwaliteit.

Om het moutproces succesvol te evalueren werden de condities van de staalvoorbereiding voor GC-MS bepaling van verouderingsaldehyden geoptimaliseerd voor verschillende types moutstalen (namelijk groenmout, deels geëeste mout, afgewerkte pilsmout). Vervolgens

werd het bekomen protocol voor de bepaling van vluchtige, vrije aldehyden toegepast in combinatie met met een reeds bestaande methode voor de kwantitative bepaling van niet-vluchtige gecysteïnyleerde aldehyden. Door deze aanpak waren we in staat om, doorheen de volledige doctoraatstudie, een totaalbeeld te krijgen van zowel vluchtige aldehyden en hun niet-vluchtige tegenhangers voor moutstalen van verschillende oorsprongen. Ook werden de resultaten van (gebonden) aldehyden geëvalueerd met betrekking tot standaard kwaliteitsparameters van verschillende pilsmouten om zo correlaties tussen de verschillende variabelen te vinden en om meer inzicht te verwerven in potentiële verbanden die relevant kunnen zijn voor biersmaakinstabiliteit.

De resultaten van stalen op industriële schaal toonden aan dat het gehalte van (gecysteïnyleerde) aldehyden in het afgewerkte mout duidelijk hoger ligt dan in het startgrondstof, gerst. Tijdens het kiemen werden grote toenames aan vetzuuroxidatie aldehyden, zijnde hexanal en *trans*-2-nonenal, vastgesteld. De hoeveelheid Strecker aldehyden en furfural daarentegen veranderden nauwelijks in dit stadium. Vervolgens, tijdens het droogstadium op hoge temperaturen (in het bijzonder bij het kritische vochtgehalte tussen 6% - 9%) werd een eerste verhoogde toename vastgesteld van de meeste (gecysteïnyleerde) aldehyden, met uitzondering van hexanal. Bij het afeesten bleven alle (gecysteïnyleerde) aldehyden snel stijgen, met uitzondering van hexanal. In de bevindingen van het doctoraat viel op dat hexanal zich anders gedraagt dan alle andere aldehyden, zelfs het andere vetzuuroxidatie merker aldehyde, *trans*-2-nonenal. Verder werd ook voor de eerste keer een duidelijk gevolg aangetoond van procesgerelateerde fysicochemische gradiënten (veroorzaakt door de pneumatische verwerking in relatief dikke graanbedden) op aldehyde vorming tijdens het mouten. Met uitzondering van hexanal, werden de hoogste gehaltes aan vrije en gebonden aldehyden gevonden in stalen verkregen van de onderste laag van het graanbed, dat het meeste blootgesteld wordt aan de hitte tijdens het eesten. De stalen die tegelijkertijd werden genomen van de bovenste laag (blootgesteld aan beduidend minder hitte) toonden de laagste gehaltes aan aldehyden.
Naast de analyse van het industrieel moutproces in functie van tijd (namelijk de verschillende moutstadia) en positie van het graanbed (zijnde onderste, middenste, bovenste laag), werd ook de nadruk gelegd op verschillende, potentieel kritische moutvariabelen. Deze resultaten toonden aan dat zowel de graad van de graanmodificatie als de afeesttemperatuur een impact hebben, hetzij indirect (graad van modificatie) of direct (afeesttemperatuur) op de ontwikkeling van (gecysteïnyleerde) aldehyden tijdens het moutproces. Om die reden werd de mate van het weken en de afeesttemperatuur als belangrijke variabelen bevonden om aanvullend gerichte micro-mout experimenten uit te voeren met als doel te onderzoeken of het mogelijk is om mout te produceren met een verminderd bierverouderingspotentieel en dat in combinatie met een bevredigende brouwkwaliteit. Modelleren van individuele parameters, namelijk de geanalyseerde aldehyden en de mout kwaliteit parameters als belangrijke variabelen, heeft aangetoond dat het weekproces vooral een impact heeft op de hexanal gehaltes, terwijl de afeesttemperatuur vooral een invloed heeft op het gehalte aan Strecker aldehyden, furfural en *trans*-2-nonenal. Ten slotte, numerieke modellering van de geselecteerde mout variabelen (weekgehalte, afeesttemperateuur), suggereert de mogelijkheid tot het produceren van pilsmout van superieure kwaliteit op voorwaarde dat zowel graanmodificatie als afeesttemperatuur voldoende worden aangepast.

LIST OF ABBREVIATIONS AND SYMBOLS

(v/v)	volume for volume
(w/w)	weight for weight
2MB	2-methylbutanal
2MB-CYS	2-(sec-butyl)thiazolidine-4-carboxylic acid
2MB-d_{10}	2-methylbutanal-d_{10}
2MP	2-methylpropanal
2MP-CYS	2-isopropylthiazolidine-4-carboxylic acid
3,4-DDH	3,4-dideoxyhexosulose-3-ene
3,4-DDP	3,4-dideoxypentosulose-3-ene
3MB	3-methylbutanal
3MB-CYS	2-isobutylthiazolidine-4-carboxylic acid
5-HMF	5-hydroxymethylfurfural
9-LOOH	9-hydroperoxyoctadeca-10,12-dienoic acid
13-LOOH	13-hydroperoxyoctadeca-9,11-dienoic
a_w	water activity
C18:2	linoleic acid
C18:3	linolenic acid
CAS number	Chemical Abstracts Service number
CI	chemical ionisation
dm	dry matter
DMS	dimethyl sulphide
DNS	3,5-dinitrosalicylic acid
DP	diastatic power
DU	dextrinizing units
EBC	European Brewery Convention
FAN	free amino nitrogen
FID	flame ionization detector
FUR	furfural
FUR-CYS	2-(furan-2-yl)thiazolidine-4-carboxylic acid
GC	gas chromatography
GKV	germination kilning vessel
GM	germination
HEX	hexanal
HEX-CYS	2-pentylthiazolidine-4-carboxylic acid
HS	headspace
IS	internal standard
Ile	isoleucine
KI	Kolbach Index
Leu	leucine
LOD	limit of detection
LOQ	limit of quantification
LOX-1	lipoxygenase 1
LOX-2	lipoxygenase 2
M	molar

m/z	mass-to-charge ratio
Met	methionine
MET	methional
MET-CYS	2-(2-(methylthio)ethyl)thiazolidine-4-carboxylic acid
MS	mass spectrometry
mPas	Millipascal second
NCI	negative chemical ionisation
PC-1, PC-2	Principal Component 1 and 2
PCA	Principal Component Analysis
PDMS/DVB	polydimethylsiloxane/divinylbenzene
PFBHA	O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine
PFBO	hydrochloride pentafluorobenzyloxime
PFPD	pulsed flame photometric detector
Phe	phenylalanine
PHE	phenylacetaldehyde
PHE-CYS	2-benzylthiazolidine-4-carboxylic acid
PUG	partially unmodified grains
r	Pearson's correlation coefficient
r^2	coefficient of determination
ROS	reactive oxygen species
rmp	revolutions per minute
RSD	relative standard deviation
Rt	retention time
SIM	single ion monitoring
SMM	S-methylmethionine
SPME	solid phase microextraction
SD	standard deviation
T2N	*trans*-2-nonenal
TBI	thiobarbituric acid index
THFA	trihydroxy fatty acids
TP	total protein
TSP	total soluble protein
UPLC	ultra-performance liquid-chromatography
UV	ultraviolet
Val	valine
°WK	Windisch-Kolbach degrees

TABLE OF CONTENTS

CONTEXT AND AIMS OF THE STUDY

Characteristic flavour features of food products and beverages should remain pleasant and stable on storage. For the brewing industry, flavour instability represents a big challenge as beer flavour generally starts to deteriorate quite soon upon packaging - a phenomenon often described as 'beer staling'. This is especially true for pilsner type beers made from pale lager malt because fresh pilsners are relatively poor in aroma constituents, making their ability to suppress sensory perception of off-flavours appearing during storage very limited. Changes in the sensorial perception of beer upon storage are caused by losses of pleasant flavour attributes *(e.g.* a decrease in the intensity and quality of bitterness due to degradation of iso-α-acids[1]), and by the appearance of off-flavours (*e.g*. appearance of cooked potato and cardboard aromas, related to an increase in levels of the aldehydes methional and *trans*-2-nonenal, respectively[2]). These changes in beer flavour may have a considerable effect on palatability and, therefore, reduce drinkability, which in turn may result in economic losses for the brewer. Furthermore, beer flavour instability becomes more and more important when considering the increasing globalisation of the beer market and growing beer export figures (*e.g.* in 2019, approx. 20% of the total beer volume produced within the European Union was exported[3]). Especially exported beers may suffer from more rapid flavour deterioration since they are often exposed during transport and/or storage to elevated temperatures and vibrations, which have been reported to promote beer staling[4].

Previous research in the field of beer flavour chemistry has pointed to the important role of staling aldehydes in relation to beer ageing, as an increase in levels of staling aldehydes in packaged beer during transport and storage was found to coincide with the gradual appearance of sensory perceivable off-flavours[2,5,6]. Staling aldehydes are characterised as volatile, highly flavour-active compounds, *i.e.* they can be perceived at very low concentrations. The most representative staling aldehydes for beer ageing (also called 'marker aldehydes') are Strecker degradation aldehydes (derived from amino acids), furfural (derived from pentose), and hexanal, as well as *trans*-2-nonenal (both originating from oxidation of unsaturated fatty acids). In fresh beer, staling aldehydes are almost completely absent and, as indicated above, their appear gradually as a function of beer storage time. However, when looking into the beer preparation process, and even malt production, the above mentioned staling aldehydes appear to be present in considerable amounts in both malting and brewing samples[7,8]. Clearly, these observations point to the huge complexity regarding the origin(s) and appearance of staling aldehydes.

In respect of the brewing process, relatively high quantities of staling aldehydes have been reported at the onset of mashing[7,8]. During subsequent stages, their levels decline mainly due to evaporation during wort boiling[7,8] and enzymatic reduction of aldehydes by yeast during fermentation[8,9]. This explains why, in fresh beer, staling aldehydes are only present in trace amounts (mostly below their flavour threshold values). Nevertheless, during beer transport and storage, levels of staling aldehydes gradually increase[2,8] by mechanisms of formation that have not been fully resolved yet. Two major mechanisms have been proposed to explain the development of staling aldehydes during beer ageing in package, namely *de novo* formation of aldehydes from a precursor compound (*e.g.* formation of 3-methylbutanal from L-leucine[8]), and release of aldehydes from a non-volatile, pre-formed bound-state adduct (*e.g.* cysteinylated aldehydes[8]).

The same volatile compounds that are directly involved in beer flavour deterioration, *i.e.* staling aldehydes, also develop during the production of malt. Hypothetically, free aldehydes introduced with malt into the brewing process may bind to other molecules during wort production forming non-volatile bound-state adducts, which may be affected to a lesser extent by the brewing conditions, in contrast to their free, volatile counterparts. Consequently, aldehyde adducts might (partly) end up in finished, fresh beer, and dissociate again during storage, thereby giving rise to free, flavour-active aldehydes. In addition, malt comprises various compounds that may contribute to *de novo* formation of aldehydes. For instance, malt contains potential precursors (*e.g.* amino acids), intermediate reactants (*e.g*. α-dicarbonyls), and catalysts (*e.g.* lipoxygenase enzymes). Moreover, the presence of bound-state aldehydes has been demonstrated in malt (in the form of cysteinylated aldehydes)[8]. All of these compounds, *i.e.* free aldehydes, precursors, and bound-state forms, constitute to the so-called 'beer staling potential of malt'. It is suggested that the staling potential of malt presumably plays a key role in relation to final beer flavour (in)stability. However, an unequivocal link between particular compounds in malt and the development of staling aldehydes in ageing beer has not been demonstrated yet. Moreover, it is proposed that a more systemic approach through integrated research comprising malting, brewing, and beer ageing, should contribute to unravelling the possible relationship between the beer staling potential of malt and beer aging in package.

This technology-driven PhD focuses on the first major part of the production chain from barley to beer, *i.e.* malt preparation. More specifically, research aims at detailed monitoring of the malting process and at defining critical moments during malting, in relation to the formation of (bound-state) aldehydes.

Chapter 1 (*Literature review on the contribution of the malting process and finished malt to the formation of staling aldehydes*) aims at presenting an overview of the state-of-the-art regarding formation of staling aldehydes during different stages of malt production, and on the potential contribution of finished malt to beer flavour (in)stability.
The first experimental part of this PhD is focused on developing of a reliable, analytical methodology for determination of staling aldehydes in malting samples. Therefore, **Chapter 2** (*Method development for determination of aldehydes in malting samples*) deals with evaluation and optimisation of sample preparation, *i.e.* extraction of aldehydes from pale lager malts and other types of malting samples, followed by aldehyde quantification via HS-SPME-GC-MS.

The second experimental part of this work focuses on evaluation of the industrial-scale malting process in relation to the formation of (bound-state) aldehydes. **Chapter 3** (*Explorative monitoring of the evolution of free and cysteinylated aldehydes during malting)* aims at obtaining an overview of the evolution of (cysteinylated) aldehydes throughout industrial-scale pale malt production (from barley up to finished malt). Next, **Chapter 4** (*Evaluation of industrial-scale malting in relation to process-associated physicochemical gradients and the formation of staling aldehydes*) intends to pinpoint the most critical moment(s) during malting, regarding generation of (cysteinylated) aldehydes, and also to investigate as to which extent process-related, physicochemical gradients may contribute to this. In **Chapter 5** (*Evaluation of selected process variables of industrial-scale malting as a function of malt quality parameters and potential beer flavour (in)stability)*, research emphasis is put on investigation and evaluation of the influence of potentially critical malting variables, regarding levels of (bound-state) aldehydes in the finished malt.

In the final experimental part - **Chapter 6** (*Micro-malting in search of 'reduced beer staling potential – optimal brewing quality*') - micro-malting combined with modelling is performed to better understand potential relationships among important malt quality parameters/aldehydes and malting process variables that were selected, based on outcomes of chapter 5. Moreover, this study aims at investigation of the feasibility of preparing malt of reduced beer staling potential combined with high brewing quality.

Chapter 1

Literature review on the contribution of the malting process and finished malt to the formation of staling aldehydes

This chapter partly corresponds to:

Filipowska W., Jaskula-Goiris B., Ditrych M., Bustillo Trueba P., De Rouck G., Aerts G., Powell C., Cook D. & De Cooman L. (2021) On the contribution of malt quality and the malting process to the formation of beer staling aldehydes: a review. *Journal of the Institute of Brewing* 127(2) 107-126. doi:10.1002/jib.644

1.1. Contribution of free and bound aldehydes to beer flavour instability

1.1.1. Introduction

The microbiological, colloidal, foam, colour and flavour stability of beer are considered to be critical quality parameters influencing drinkability and brand acceptance by the consumer[10,11]. Unfortunately, various chemical reactions take place in the closed beer package, resulting in a change to the sensorial perception of beer over time, which starts almost instantly upon packaging. The most significant changes are the increase in negative off-flavours (*e.g.* cardboard associated with *trans*-2-nonenal) and the loss of pleasant flavour attributes (such as bitterness or estery character due to degradation of iso-α-acids and, *e.g.* acetate esters, respectively)[12–19]. Losses in esters also reduce their well-known masking effect, thereby leading to an even more pronounced perception of off-flavours[16,17,20]. Similarly, the synergistic effect caused by the sum of the intensities of beer ageing indicators allows to perceive off-flavours even when their concentrations found in beer do not exceed their individual flavour thresholds[16–18]. Exposure to high temperatures, light, vibrations during transport and/or contact with oxygen, as well as presence of certain transition metal ions, accelerate the rate of beer staling[4,21–24]. Unravelling the chemistry behind these changes and thus learning how to control the rate at which flavour change develops is the key to prolonging beer freshness.

Measures adopted to improve beer flavour stability are considered most effective when applied downstream and close to the packaged product[25]. However, the fact that staling precursors are developed upstream in the raw materials and brewhouse operations means that brewers with 'best in class' flavour stability control measures are currently looking to these upstream stages to better understand the source of major staling precursors. One area of active research is to focus on raw materials. Malt, as a major brewing ingredient, delivers to the brewing process various compounds, which can contribute to beer staling – *i.e.* amino acids, proteins, enzymes, reducing sugars, and staling aldehydes[6,7,26–33]. The content of these compounds in malt is influenced by factors such as barley variety and malting process, which also directly affect malt quality. Therefore, this introductory part of the thesis manuscript discusses current knowledge on the potential impact of malt quality and malting conditions on beer flavour deterioration with regard to staling potential of malt, in particular, staling aldehydes.

1.1.2. Free aldehydes

In the late 1960s, the search for potential beer staling markers pointed to aldehydes as a class of compounds of paramount importance, as their increase coincides with the appearance of off-flavours during beer ageing[5]. Moreover, free aldehydes show flavour-active properties and very low flavour thresholds, for example, *trans*-2-nonenal can be perceived at 0.03 µg/L, methional at 4.2 µg/L, and 2-methylbutanal at 45 µg/L when spiked individually to lager beer[16]. Further studies led to the identification of the most relevant aldehydes originating from various chemical pathways, which are indicators of lager beer staling – the so-called marker aldehydes[2]. The most frequently reported are hexanal, *trans*-2-nonenal, furfural, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, methional, and phenylacetaldehyde[2,6,9,17,34,35].

These aldehydes may arise through *de novo* formation and/or due to their release from a bound-state form. Various authors[17,36–47] have thoroughly discussed the possible reaction of *de novo* formation during malting and brewing. Marker aldehydes (see also section 'The most relevant formation pathways of marker aldehydes in relation to malt') can arise through the following mechanisms: (1) lipid and fatty acid oxidation (auto- and enzymatic oxidation); (2) Maillard reactions; (3) Strecker degradation (Strecker degradation of amino acids in a strict sense, Strecker-like reactions, direct Strecker aldehyde formation from Amadori compounds, direct oxidation of amino acids); (4) oxidative degradation of iso-α-acids; (5) aldol condensation of short-chain aldehydes; (6) oxidation of higher alcohols; (7) secondary oxidation of long-chain aldehydes, and (8) secretion by fermenting yeast.

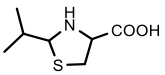
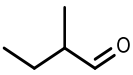
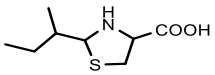
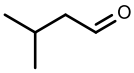
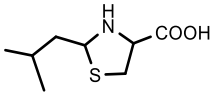
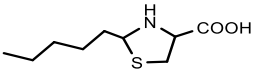
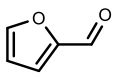
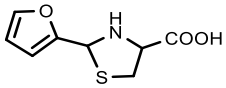
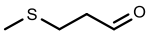
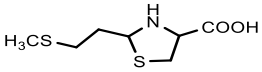
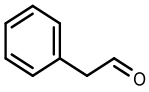
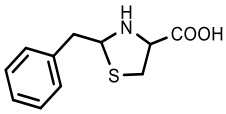
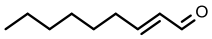
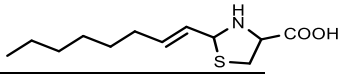
1.1.3. Bound-state aldehydes

Free aldehydes are prone to binding due to the high electronegativity of the double-bonded oxygen atom. Therefore, the electron-deficient carbonyl carbon is likely to be attacked by nucleophiles[48,49]. This may result in binding with other compounds, such as bisulphites[40,41], cysteine[50,51] or other amino acids[42]. During beer storage and under specific conditions inside the beer package (beer pH, storage temperature, vibrations during transport), those adducts may dissociate, releasing free aldehydes, thus causing an increase in off-flavours[17,37,52]. Unravelling the chemistry behind bound-state aldehydes is crucial in order to better understand beer staling.

The topic of aldehyde-adduct formation (the so-called binding) is very complex since numerous chemical pathways are possible. Beer staling aldehydes are of different origin, chemical structures and properties, and they may react with various nucleophiles at different rates and under different reaction conditions (such as pH value, temperature). The binding behaviour of selected beer staling aldehydes was thoroughly studied by Baert *et al.*[50,51]. The authors demonstrated that the nucleophilic addition of cysteine or bisulphite to the carbonyl group of an aldehyde is affected by the electrophilic character of an individual aldehyde, thus its structure (see Table 1-1). Accordingly, the electrophilicity of the carbon atom of the carbonyl group is influenced by the nature of the R group of the aldehyde (RCHO). In particular, the R group can be aliphatic (either saturated or unsaturated) or aromatic, and because of this electrophilic character of aldehydes will vary. Thus, aldehydes substituted with an aromatic R group (*e.g.* phenylacetaldehyde) are less prone to binding to nucleophilic compounds compared to saturated aliphatic aldehydes (*e.g.* 2-methylpropanal) because the conjugated system of the aromatic substituent is decreasing the electrophilicity of the carbon atom of the carbonyl group. For the same reason, α,β-unsaturated aliphatic aldehydes such as for instance *trans*-2-nonenal are less prone to binding nucleophilic compounds compared to saturated aliphatic aldehydes. Moreover, for saturated aliphatic aldehydes, the well-known inductive effect should be taken into account when comparing their reactiveness towards nucleophilic compounds. For instance, the inductive effect caused by the methyl group present at the 2-position of the R group, as is the case for 2-methylpropanal and 2-methylbutanal, reduces, to some extent, the readiness for binding nucleophiles[53]. Baert *et al.*[51] and Bustillo Trueba *et al.*[54] reported on the influence of beer (4.4), wort (5.2), and malt (6.0) pH on binding behaviour of aldehydes. A general trend of lower affinity to binding at lower pH, regardless of the nucleophile (cysteine or bisulphite) could be seen. This is because the more acidic pH (4.4) enhances protonation of the carbonyl

oxygen causing enolisation, which somewhat reduces the aldehyde readiness towards binding. Cysteine and bisulphite reactivity was hardly affected by the pH of the model solution (4.4 - 6.0), due to the relatively high pK_a values of the cysteine amino group (pK_a = 10.8) and the sulfhydryl group (pK_a = 8.3)[53].

Table 1-1. Molecular structure of the selected free aldehydes and their corresponding cysteinylated form, after[54,56].

Free aldehyde	Molecular structure	Cysteinylated aldehyde	Molecular structure
2-methylpropanal		2-isopropylthiazolidine-4-carboxylic acid	
2-methylbutanal		2-(sec-butyl) thiazolidine-4-carboxylic acid	
3-methylbutanal		2-isobutylthiazolidine-4-carboxylic acid	
hexanal		2-pentylthiazolidine-4-carboxylic acid	
furfural		2-(furan-2-yl) thiazolidine-4-carboxylic acid	
methional		2-(2-(methylthio)ethyl) thiazolidine-4-carboxylic acid	
phenylacetaldehyde		2-benzylthiazolidine-4-carboxylic acid	
trans-2-nonenal		(E)-2-(oct-1-en-1-yl)thiazolidine-4-carboxylic acid	

Regarding the release of flavour-active free aldehydes, until now, indirect methods under extreme conditions were applied to measure the dissociation of bound-state aldehydes. In 1983 Baker *et al.*[40] presented the release of carbonyl compounds from their corresponding bisulphite adducts. In 1990 Drost *et al.*[55] introduced the concept of 'nonenal potential' as an indicator of the possible release of *trans*-2-nonenal from a bound-state form. In 2015 Baert *et al*.[51] used 4-vinylpyridine as an aldehyde 'releasing agent', for example, to demonstrate that the bound aldehydes are present in fresh beers. Only recently has development of analytical methodologies allowed a direct determination of cysteinylated aldehyde adducts in model solutions[54], and somewhat later, in malt, brewing and beer samples[33]. Bustillo Trueba *et al.*[54] conducted a detailed study investigating the chemical behaviour of cysteinylated aldehydes in model solutions. The results showed that the degradation rate of an adduct depends on the 2-substitution pattern (*i.e.* the nature of the R group) of the thiazolidine ring and on the pH value of the medium, *e.g.* at malt pH (6.0) decomposition of cysteine adduct was slower than at beer pH (4.4). Under the acidic

conditions, the nitrogen atom may be protonated, leading to destabilisation of the thiazolidine ring and ring opening.

pH-Sensitivity was also previously reported for bisulphite adducts, as Kaneda *et al.*[41] demonstrated that carbonyl compounds are present in a bound-state when SO_3^- is in the nucleophilic form (pH range 3 - 6). Conversely, imine adducts are more stable at higher pH (since with increase of pH rises Schiff base concentration), whereas a lower pH, similar to exposure to heat, promotes dissociation of the complex[42]. Therefore, in the pH of malt (6.0), bound-state aldehydes may be formed more easily than in beer and their stability may also be higher. An overview of possible interactions between saturated and unsaturated aldehydes and cysteine, an amine or bisulphite respectively, is shown in Figure 1-1.

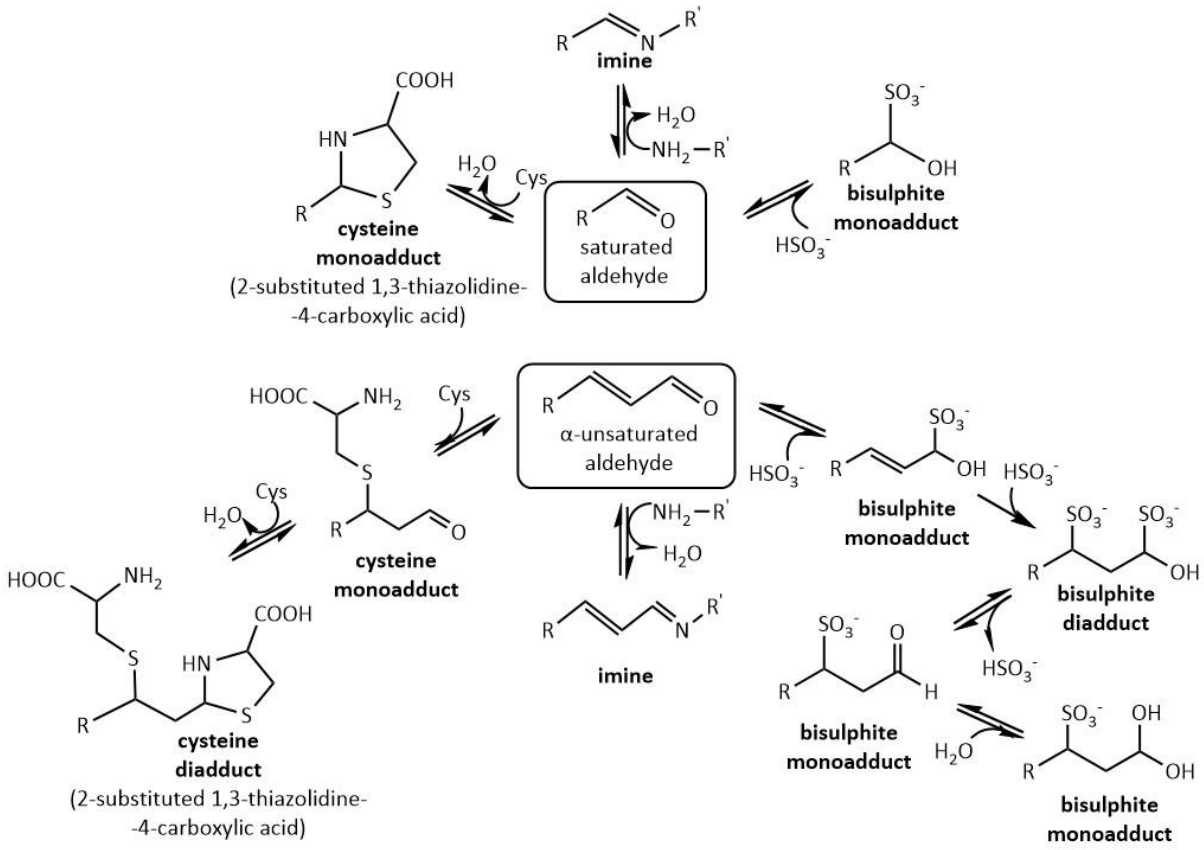


Figure 1-1. Overview of the possible interactions between saturated (*e.g.* methional) and α-unsaturated (*e.g. trans*-2-nonenal) aldehydes and cysteine, an amine or bisulphite, after Baert *et al.*[51].

1.1.4. The significance of free and bound-state aldehydes in beer ageing

The debate as to what extent bound-state forms may be responsible for beer staling is still ongoing. Regarding *de* novo formation, it has been suggested that aldehydes 'reappear' during beer ageing. Wietstock *et al.*[47] demonstrated that supplementation of fresh beer with leucine, isoleucine, and phenylalanine in the presence of oxygen leads to higher concentrations of the corresponding aldehydes (3-methylbutanal, 2-methylbutanal and phenylacetaldehyde, respectively) upon beer ageing for 30 weeks at 20°C. This indicates that *de novo* formation of Strecker aldehydes may indeed occur in a closed beer package and is enhanced by the presence of oxygen. Similar outcomes were obtained by Gibson *et al.*[57], who supplemented amino acids to fresh beer and observed an increase in Strecker aldehydes after forced ageing. Furthermore, Rangel-Aldao *et al.*[58] reported on the relevance of α-dicarbonyls (intermediate products of Maillard reactions) to aldehyde formation while storing beer at elevated temperatures (28°C). The authors determined lower levels of furfural and 5-hydroxymethyl furfural (HMF) in beers with the addition of an α-dicarbonyl trapping reagent. This is in agreement with Rakete *et al.*[46], who indicated that Maillard reactions resulting in the formation of *i.a.* furfural, occur to some extent during forced beer ageing (two weeks at 50°C) since intermediates necessary for the reaction are already present in beer.

On the other hand, it has been suggested that the conditions in a closed beer package do not promote *de novo* formation. For example, Lermusieau *et al.*[42] compared the content of *trans*-2-nonenal in oxygen-free and oxygen-receiving ageing beers. The results indicated that the increase in *trans*-2-nonenal over time is not caused by lipid oxidation in the beer package but it is due to the release of its free form from a bound-state. Moreover, Maillard reactions leading to *de* novo formation of *e.g.* furfural are favoured at conditions where pH is higher than typical beer pH values (for example, in malt)[48].

To date, free aldehydes originating from both potential pathways – *de novo* formation and release from a bound-state form – are considered to be contributors to beer flavour deterioration. Suda *et al.*[44] reported that approx. 85% of Strecker aldehydes determined in aged beers are derived from wort, whereas approx. 15% originate from *de novo* formation in packaged beer. Furthermore, both aldehydes free and bound-state, might be delivered to the brewing process with raw materials and/or could be formed during beer production[33]. Formation of imine adducts may occur during malting[59,60] and brewing[52], whereas bisulphite adducts might be formed during fermentation or downstream[40,55,61]. In summary, the above studies[33,40,44,52,55,59–61] points to the relevance of the malting and brewing process to the formation of bound-state aldehydes, as well as to malt as an essential source of beer staling compounds and their precursors.

1.1.5. The most relevant formation pathways of marker aldehydes in relation to malt

Malt provides aldehydes to the brewing process directly but also offers a variety of their precursors (Figure 1-2). Formation pathways of marker aldehydes are complex and consist of numerous steps, which strongly depend on the reaction conditions (*e.g.* pH, temperature, presence of substrates) and can lead to various intermediate and final products. This section focuses on particular reactions taking place in malt (or analogous conditions) and leading to the formation of marker aldehydes, namely: hexanal, *trans*-2-nonenal, furfural, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, methional, and phenylacetaldehyde.

These compounds represent the end products of some typical formation reactions, *e.g.* oxidation of unsaturated fatty acids, Maillard reactions and Strecker degradation of amino acids.

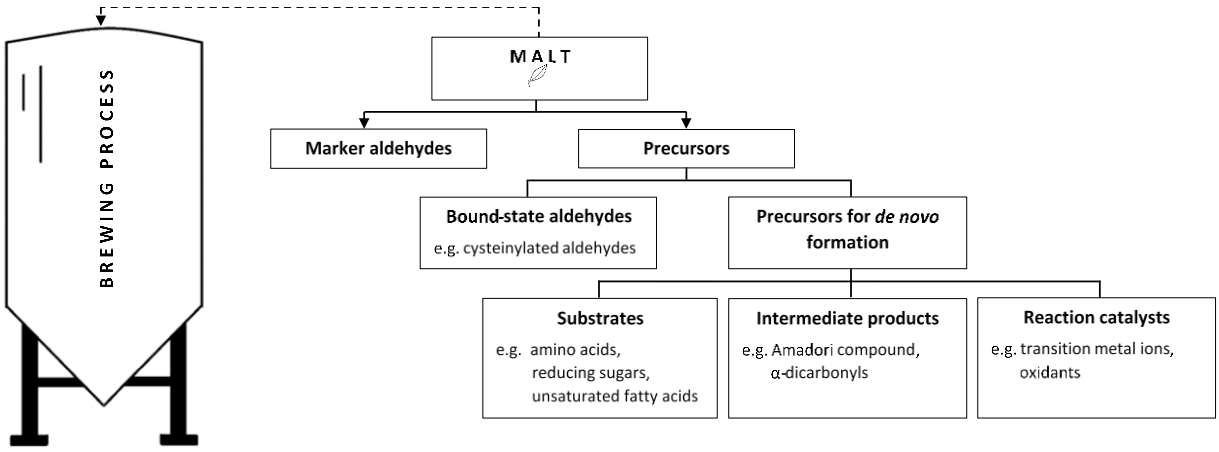


Figure 1-2. Compounds delivered with malt to the brewing process, which may potentially affect beer flavour (in)stability.

1.1.5.1. Hexanal and *trans*-2-nonenal

The main chemical pathways leading to the formation of hexanal and *trans*-2-nonenal are autoxidation and enzymatic oxidation of unsaturated fatty acids. In the enzymatic pathway (Figure 1-3), linoleic acid (C18:2) and linolenic acid (C18:3), representing up to 60 and 10% of the total fatty acids in malt, respectively[62], are released in the presence of water from triacylglycerols by lipase (pH optimum 6.8)[63]. The resulting free fatty acids are oxidised by lipoxygenase (LOX-1 and LOX-2, pH optimum 6.5)[64] to hydroperoxy fatty acids. LOX-1 yields 9-hydroperoxyoctadeca-10,12-dienoic acid (9-LOOH), whereas LOX-2 produces 13-hydroperoxyoctadeca-9,11-dienoic acid (13-LOOH). During malt kilning, most of the LOX activity is destroyed as both enzymes are heat-sensitive. However, LOX-1 is more heat-resistant than LOX-2, thus during mashing, the enzymatic formation of 9-LOOH may proceed at a higher rate[65]. Subsequently, 9- and 13-LOOH are subjected to enzymatic degradation to mono-, di-, and trihydroxy fatty acids followed by non-enzymatic breakdown resulting in carbonyl compounds. The pathway of 9-LOOH leads to *trans*-2-nonenal, whereas 13-LOOH yields hexanal[10]. Another possible oxidation of linoleic and linolenic acid esterified in triacylglycerol is by LOX-2, which also leads to the formation of carbonyl compounds[10,62].

Regarding autoxidation, in the cascade of reactions, unsaturated fatty acids can be oxidised by reactive oxygen species and via lipid peroxyl radicals into lipid hydroperoxides (9-LOOH and 13-LOOH) (Figure 1-4)[66]. Again, various compounds may be formed from these precursors in enzymatic and non-enzymatic reactions leading to hexanal and *trans*-2-nonenal. The rate of autoxidation is enhanced by high temperatures and presence of oxidants, *e.g.* transition metal ions (iron and copper)[17]. Malt, among other brewing raw materials, is rich in these ions, delivering up to 97.5% of iron and 94.3% of copper to the brewing process[67]. Another critical reaction from the perspective of malting is the secondary autoxidation of unsaturated aldehydes. For example, *trans*-2-nonenal can be autoxidised into shorter chain aldehydes, such as hexanal[68].

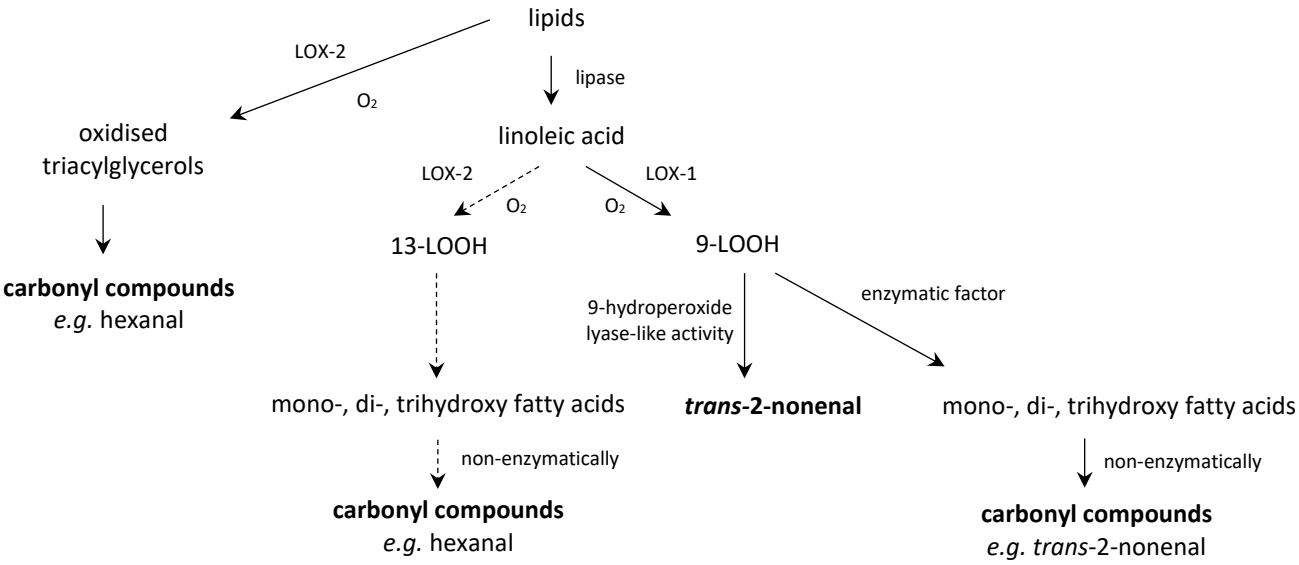


Figure 1-3. An overview of the enzymatic oxidation leading to the formation of hexanal and *trans*-2-nonenal, after Vanderhaegen *et al*.[10].

The continuous line refers to reactions of high rate, whereas the dashed line relates to pathways that proceed at a slower rate. LOX = lipoxygenase enzyme, 9-LOOH = 9-hydroperoxyoctadeca-10,12-dienoic acid, 13-LOOH = 13-hydroperoxyoctadeca-9,11-dienoic acid.

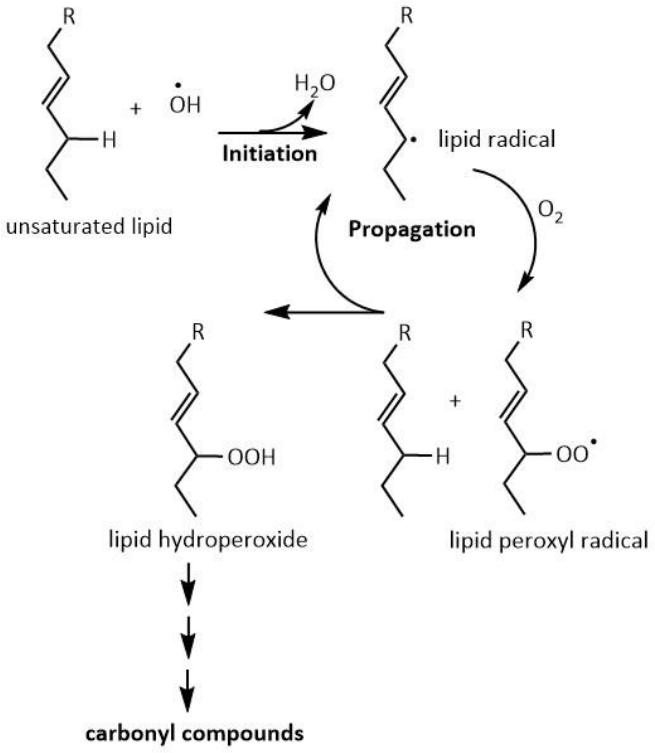


Figure 1-4. Overview of the oxidation cascade of unsaturated fatty acids, initiated by reactive oxygen species, after to Bamforth *et. al* [69].

1.1.5.2. Furfural

Furfural is one of the many products of Maillard reactions - a complex reaction chain initiated by an amine, amino acid, peptide or protein reacting with reducing sugar (Figure 1-5)[17,70]. The reaction is initiated by nucleophilic addition of an amino group to the reducing end of an open-chain of sugar, leading to N-glycosylamine (Schiff base) formation[71]. This intermediate undergoes Amadori rearrangement resulting in the formation of 1-amino-1-deoxyketose (Amadori compound), which undergoes enolisation and, depending on the pH, forms specific isomers. In the next stage, the amine is released and α-dicarbonyls are formed, *i.e.* 3-deoxyosone (pH<5), and 1- or 4-deoxyosone (pH>7). Upon dehydration of 3-deoxyosone followed by cyclisation of the intermediate α-dicarbonyl, and final dehydration, furfural is formed from pentose. The generated α-dicarbonyls can also act as a reactant in Strecker degradation of amino acids. The kinetics of Maillard reactions are strongly dependent on the nature and proportion of reactants, temperature, time, pH, and water activity[72–74]. For example, pH value affects the reactivity of amino group (pK_a values often around 9 or higher) and the proportion of open-chain to closed-chain forms of sugars (more aldose forms are present at higher pH). Also, a moderate water activity is required, allowing almost a subsequent addition and elimination of water molecule (see Figure 1-5). As Maillard reactions are mostly associated with the exposure of malt to high temperatures, these chemical pathways have been studied extensively with regard to dark speciality malts[75–77] and pale lager malts[78].

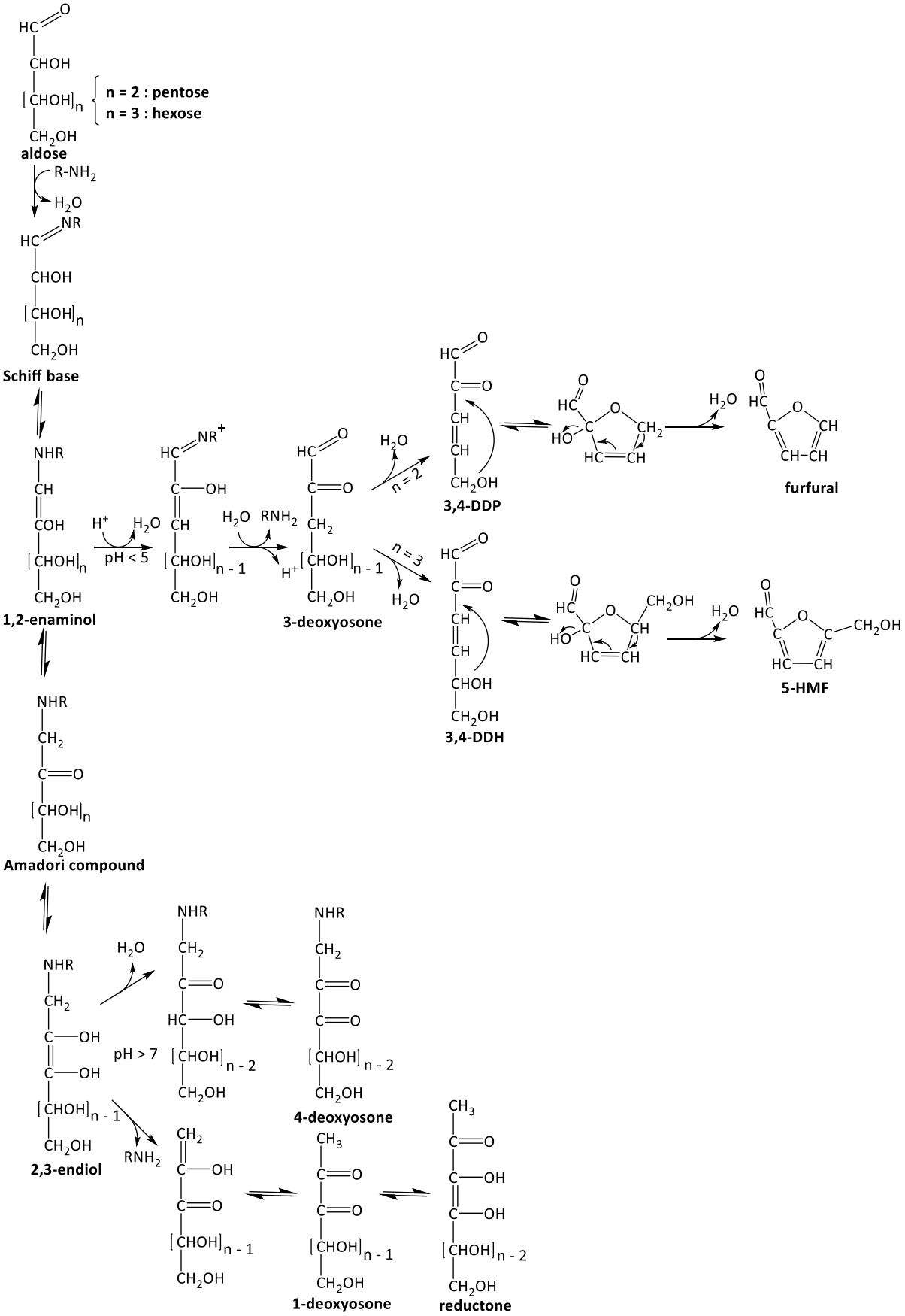


Figure 1-5. Overview of Maillard reactions leading to the formation of furfural, as described by Baert *et al.*[17].

3,4-DDP = 3,4-dideoxypentosulose-3-ene; 3,4-DDH = 3,4-dideoxyhexosulose-3-ene; 5-HMF = 5-hydroxymethylfurfural.

1.1.5.3. 2-Methylpropanal, 2-methylbutanal, 3-methylbutanal, methional, and phenylacetaldehyde

Strecker aldehydes may arise via several formation pathways. One of them is the Strecker degradation in a strict sense (Figure 1-6), which is a reaction between an amino acid and an α-dicarbonyl. In the case of beer staling marker aldehydes, the well-known amino acids act as precursors (valine is a precursor of 2-methylpropanal, isoleucine of 2-methylbutanal, leucine of 3-methylbutanal, methionine of methional, and phenylalanine of phenylacetaldehyde[17]), whereas a variety of α-dicarbonyls are derived among others from Maillard reactions[79]. Strecker degradation in a strict sense is initiated by nucleophilic addition of the unprotonated amino group to the carbonyl group resulting in the formation of a hemiaminal. Next, this unstable intermediate undergoes dehydration and subsequent irreversible decarboxylation forming an imine zwitterion. Water addition leads to an unstable amino alcohol, which breaks down into an α-ketoamine and an aldehyde[10,17,79,80].

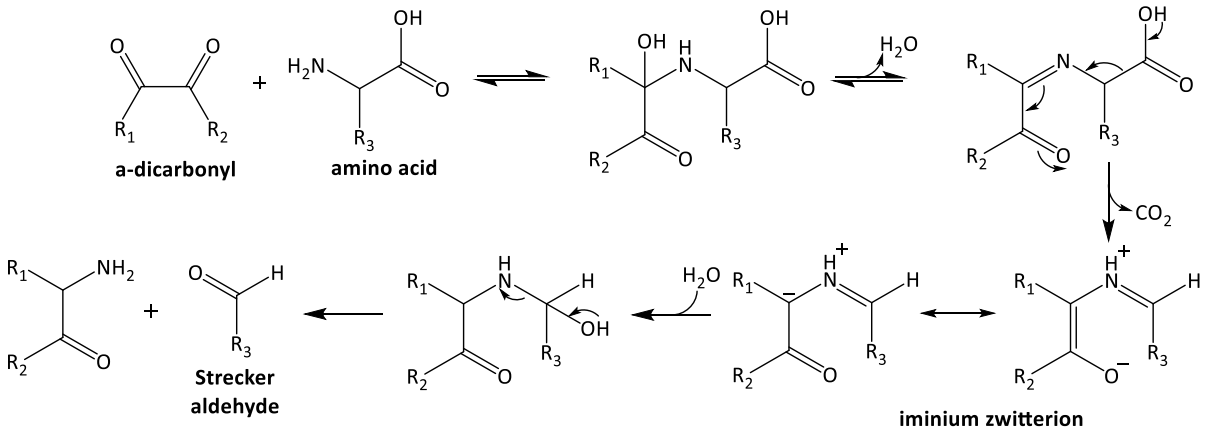


Figure 1-6. Strecker degradation in a strict sense - reaction of an α-dicarbonyl with an amino acid[17,66,81,82].

Alternatively, Strecker aldehydes may arise via Strecker-like reactions – reaction between an amino acid and an α,β-unsaturated carbonyl compound (*e.g*. *trans*-2-nonenal, furfural) (Figure 1-7)[80], or by direct oxidative degradation of amino acids (Figure 1-8)[83]. The latter was confirmed to occur in beer[47], however, further investigation is required with regard to barley, malt and model solutions of pH ≈ 6.

Another possibility is the reaction of Amadori compound (derived from Maillard reactions) with amino acid (Figure 1-9)[79]. This formation pathway is likely to take place in malt, even though it was studied only in model solutions (with and without the presence of transition metal ions)[82,85]. Firstly, compared to wort and beer, the pH of malt is somewhat more favourable to the formation of 1-amino-1-deoxyketoses (Amadori compounds) and, secondly, during germination, the proteolytic activity of malt increases, leading to the release of free amino acids from more complex structures. Therefore, in malting, conditions seem to be more favourable for the formation of substrates for this particular formation pathway of Strecker aldehydes.

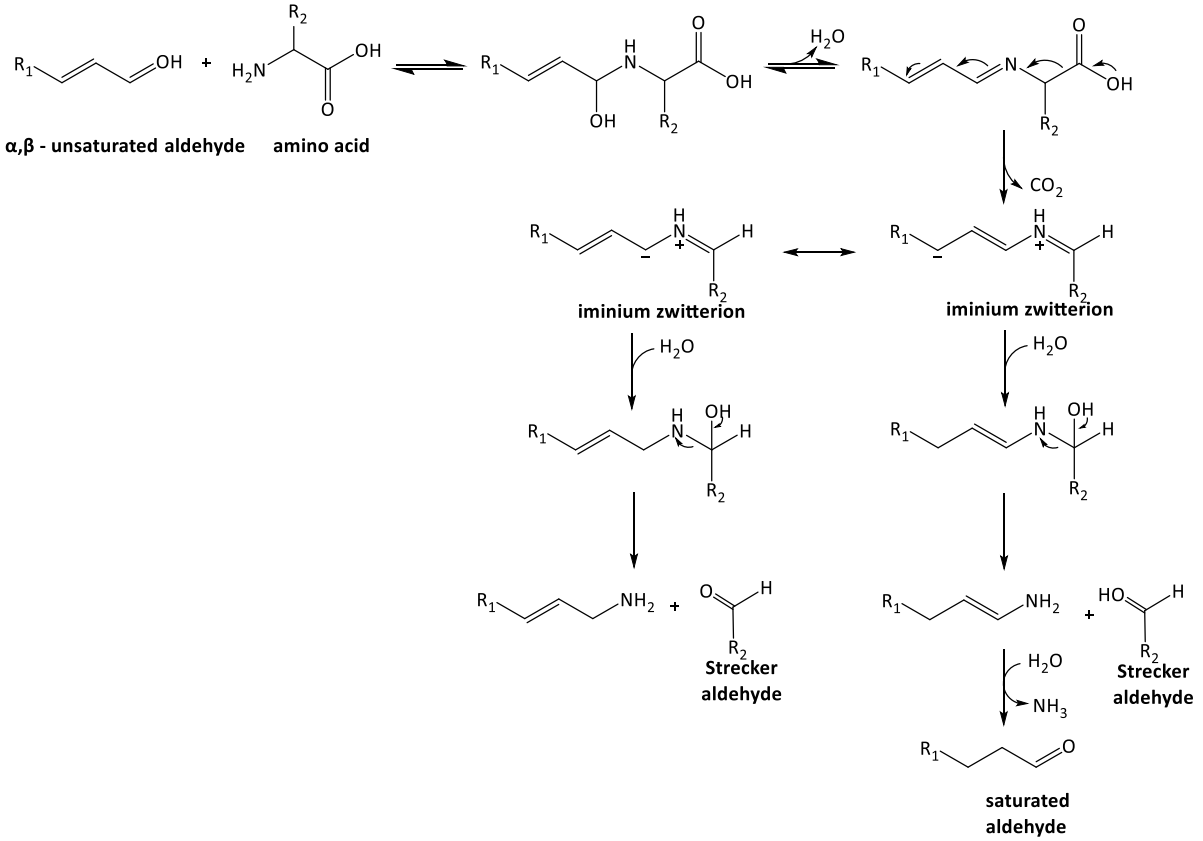


Figure 1-7. Strecker degradation between an α,β-unsaturated carbonyl compound and amino acid according to Rizzi *et al.*[80], after Baert *et al.* [17] and Bustillo Trueba[81].

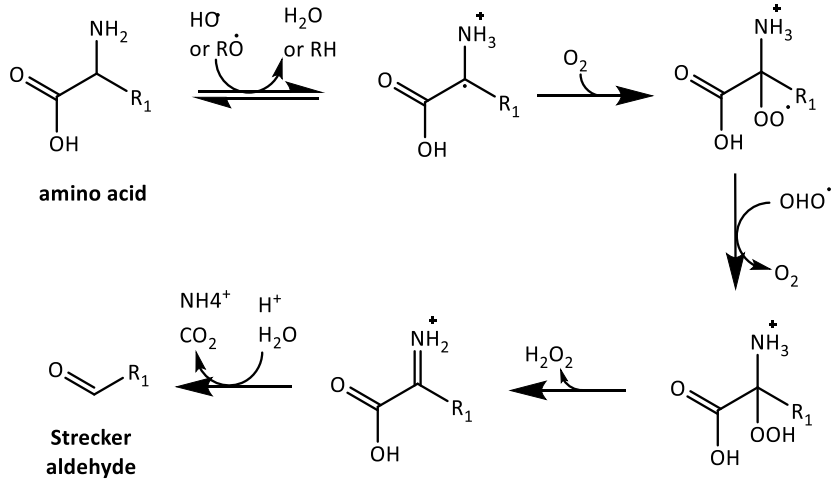


Figure 1-8. Overview of direct oxidative degradation of amino acids to Strecker aldehydes after Stadtman[84] and Wietstock et al.[47].

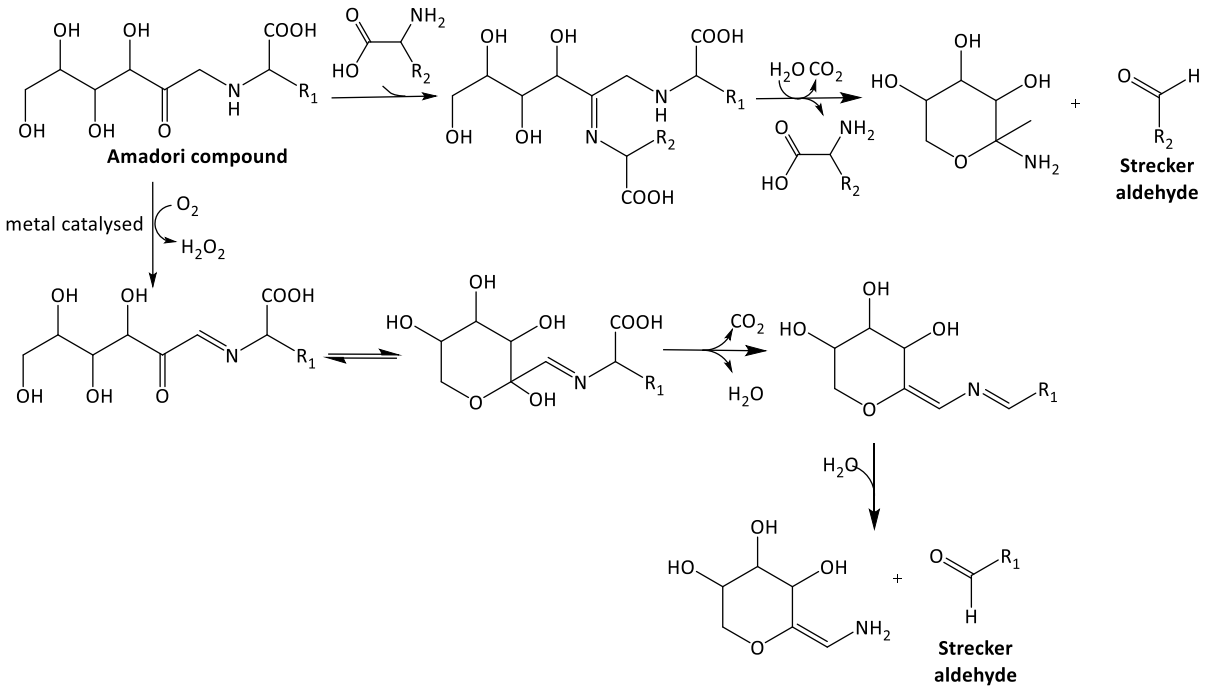


Figure 1-9. Overview of the formation of Strecker aldehydes from an Amadori compound and an amino acid or metal-ion catalysed reaction, according to Yaylayan[79], after Baert *et al.*[17] and Bustillo Trueba[81].

1.1.5.4. Important precursors, intermediates and catalysts for *de novo* formation of aldehydes

In the reactions of *de novo* formation of marker aldehydes, their precursors, intermediate products and catalysts play an essential role, the most relevant of which are presented in Table 1-2. The function of precursors, intermediates and some catalysts of marker aldehyde formation have been described in the above sections.

Catalysts play an important role in generation of staling aldehydes, especially from the perspective of oxidation reactions. In particular, important are transition metal ions as well as anti- and pro-oxidants. Transition metal ions such as iron, copper, and manganese are known to accelerate the rate of radical reactions, *i.e.* the so-called Fenton and Haber-Weiss reactions, which occur as follows[86]:

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$

$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + {}^{\bullet}O_2^- + 2H^+$

$Cu^{2+} + {}^{\bullet}O_2^- \rightarrow Cu^+ + O_2$

$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + OH^- + {}^{\bullet}OH$

The reaction chain starts with oxidation of Fe^{2+} by hydrogen peroxide to Fe^{3+}, resulting in the formation of hydroxyl radical ($^{\bullet}OH$) and hydroxyl anion (OH^-). Next, Fe^{3+} reacts with another molecule of hydrogen peroxide forming a superoxide radical ($^{\bullet}O_2^-$), two protons and reduced iron (Fe^{2+}). Then, the superoxide radical reacts with Cu^{2+} leading to oxygen and Cu^+.

Finally, the generated Cu^{+} reacts with hydrogen peroxide forming a hydroxyl radical and hydroxyl anion, and oxidising copper to its original Cu^{2+} form. Free radical species (in particular, hydroxyl radical) are exceptionally reactive, which can lead to non-enzymatic oxidation of lipids resulting in hexanal and *trans*-2-nonenal[17], as well as to direct oxidation of amino acids to Strecker aldehydes[83]. Malt, among other brewing raw materials, is rich in these ions, delivering up to 97.5% of iron and 94.3% of copper to the brewing process[67]. The extent of these reactions can be diminished by antioxidants, due to their reducing power, their radical-scavenging and metal-chelating properties, as well as by the so-called antioxidative enzymes such as peroxidase, catalase and superoxide dismutase[45,87–89]. Malt is a natural source of antioxidants *e.g.* flavan-3-ols, phenolic acids and ferulic acid, which are delivered to the brewing process[10,87]. The antioxidant properties of barley and malt are mostly associated with phenolic compounds such as phenolic acids, flavonoids, proanthocyanidins and tannins[90,91], as well as some of the Maillard reaction products (MRPs), in particular, melanoidins and reductones[92]. Phenolic compounds that are ultimately found in beer originate to a large extent from malt (*e.g.* pale malt delivers around 80% - 85% of polyphenols to beer, whereas dark speciality malt delivers approx. 95%), whereases hops deliver only a minor fraction of the total beer polyphenols[93–95]. Melanoidins and reductones (intermediates of Maillard reactions) are present in all types of malt, however, in particular in dark speciality malts as they are mostly formed during the roasting process due to intensive heating. As antioxidants, melanonids are superoxide scavengers and can interact with peroxide and hydroxyl radicals[96]. However, Hashimoto *et al.*[97] reported that these compounds also may catalyse the oxidation of higher alcohols to carbonyl compounds, thereby impairing beer flavour stability. Similarly, reductones act as radicals scavengers[92], but also they intensify Fenton and Haber-Weiss reactions by reduction of transition metal ions (*e.g.* Fe^{3+} to Fe^{2+})[98]. In accordance with these are the results by Hoff *et al*.[99], who reported higher iron content and higher radical intensities in dark worts compared to pale malt worts. Regarding the so-called anti-oxidative enzymes, barley contains superoxide dismutase, catalase, peroxidase, as well as ascorbate peroxidase[100–102]. However, peroxidase acts as an antioxidative enzyme by removing hydrogen peroxide from the system, it also oxidises barley phenolic compounds, thereby reducing the antioxidative potential of malt[103]. Other pro-oxidative enzyme actives are the well-known activities in lipid degradation, such as lipase, lipoxygenase and the hydroperoxide-reactive enzyme system (hydroperoxide lyase and hydroperoxide isomerase), as well as polyphenol oxidase, which catalyses oxidation of phenolic compounds[100,103,104]. Both the barley cultivar and malting process influence the above mentioned residual enzymatic activities found in the finished malt[87].

Table 1-2. Significant precursors and catalysts for the *de novo* formation pathways of selected beer staling aldehydess[17,36,44,46,47].

Aldehydes	Precursors	Catalyst
Fatty Acid Oxidation Products		
hexanal *trans*-2-nonenal	- lipids and unsaturated fatty acids - 9-LOOH, 13-LOOH - mono-, di- and trihydroxy fatty acids	- enzymes (*e.g.* lipase, lipoxygenase and hydroperoxide lyase) - oxygen - high temperatures - reactive oxygen species (ROS) (for autoxidation) - transition metal ions (Cu^{2+}, Fe^{2+}) (for autoxidation)
Maillard Reactions Products		
furfural	- amines, amino acids, peptides, proteins - reducing sugars (*e.g.* xylose) - α-dicarbonyls	- high temperature - high pH in the first stage of reaction, pH<5 for conversion of 1,2-enaminol
Strecker Degradation Products		
2-methylpropanal 2-methylbutanal 3-methylbutanal methional phenylacetaldehyde	- corresponding amino acids: valine, isoleucine, leucine, methionine, phenylalanine - α-dicarbonyls - α,β-unsaturated aldehyde - Amadori compounds	- high temperature - transition metal ions (Cu^{2+}, Fe^{2+}) - reactive oxygen species (ROS) - oxygen

1.2. Contribution of malt to beer flavour instability

To date, most research regarding beer staling has focused on the combined effects of brewing process and beer storage conditions on the chemistry of beer ageing. However, in 2004 Bamforth[43] stated: '(…) the scenario for malt in the context of flavour instability is so under-researched that it is impossible to be categorical either for or against its significance'. Since this time, the importance of malt quality in the context of beer flavour and flavour instability has been increasingly recognised[6,7,26,27,29,30,32,33,105–109]. Regarding beer staling aldehydes, according to De Clippeleer *et al.*[32], these compounds are primarily derived from malt to the brewing process, rather than from degradation of hop bitter acids, hence the latter seems to be of lesser importance. In support of this, Ditrych *et al.*[7] evaluated levels of staling aldehydes across the entire wort production process and reported the highest levels of staling aldehydes in mashing-in samples. Thus, malt can be seen as main brewing raw material delivering staling aldehydes to the brewing process.

Dong *et al.*[110] identified various flavour-active compounds in pale malt, among them marker aldehydes including 2-methylpropanal, 3-methylbutanal, 2-methylbutanal, hexanal, *trans*-2-nonenal and benzaldehyde. Determined quantities of marker aldehydes in pale malts are presented in Table 1-3[6,111–114]. The aldehyde profile of the different pale lager malt samples appeared to be quite similar; 3-methylbutanal was found in the highest concentration, followed by 2-methylpropanal and 2-methylbutanal, whereas *trans*-2-nonenal and methional were present in the lowest concentration. Variations in aldehyde content among these malts can be explained by different barley cultivars, crop years and malting

technologies (infrastructure and malting parameters), as well as different analytical approaches adopted (*e.g*. SIFT-MS, HS-SPME-GC-MS). Irrespective, similar compounds were identified in speciality malts, *i.e.* caramalt and dark malts, which in comparison to pale malts contained higher amounts of aldehydes[115–117]. For example, Yahya *et al.*[76] quantitatively determined around 9,000 µg/kg and 2,100 µg/kg of furfural in black malt and crystal malt, respectively, while Gibson *et al.*[57] reported on a 3-fold higher concentration of 2-methylpropanal and 2-methylbutanal in worts produced with dark malts in comparison to their pale malt derived counterparts. Moreover, Gastl *et al.*[26] by applying air recirculation during germination and a relatively long kilning cycle (36h) with kilning-off at 85°C, obtained malt, which lead to the aged beers of a high acceptance score. The authors suggested that the combination of a low Kolbach Index of malt and a low amount of Strecker aldehydes and hexanal in wort, as well as low heat load index in wort can result in enhanced beer flavour stability.

Table 1-3. Levels of marker, staling aldehydes determined in pale malts that varied in barley cultivars, and harvest year, malting technology[6,111–114] and their flavour description[9].

Aldehyde	Concentration range (µg/kg dry mass)	Flavour description
Fatty acid oxidation products		
hexanal	173 – 1,010[2] 495 – 1,123[3] 449 – 1,669[5]	bitter, winey
trans-2-nonenal	29 – 74[2] 17 - 46[3] 9 - 39[4] 210 - 580[5]	cardboard, papery
Strecker degradation products		
2-methylpropanal	612 – 2,311[2] 722 – 3,480[3] 1,128 – 3,469[5]	grainy, fruity
2-methylbutanal	467 – 1,119[2] 612 – 2,411[3] 980 – 3,279[5]	almond, malty
3-methylbutanal	1,741 – 2,585[1] 1,213 – 4,271[2] 3,053 – 4,215[3] 2,834 – 8,218[5]	malty, cherry, almond
methional	224 – 566[2] 383 – 1,014[3] 319 – 5,105[5]	cooked potatoes
phenylacetaldehyde	400 – 853[2] 198 – 1,014[3] 319 – 5,105[5]	flowery
Maillard reactions products		
furfural	185 – 477[1] 285 - 412[2] 89 - 651[3] 391 – 1,116[5]	caramel, bready

1 - GC-FID on the extract of water vapour distillation[112]; 2 - SIFT-MS on the ungrounded malt grains and HS-SPME-GC-MS on malt extract[111]; 3 - HS-SPME-GC-MS on malt extract[6]; 4 - HS-SPME-GC-FID on malt extract with the addition of NaCl[113]; 5 - HS-SPME-GC-MS on malt extract[114].

Regarding the relationship between final beer flavour stability and malt, Bustillo Trueba *et al.*[33] showed that malt is rich in bound-state aldehydes, namely cysteinylated aldehydes, which could contribute to the increase of free forms during beer ageing if these compounds 'survive' brewing process. Also, it has been reported that brewing with malt that is low in Kolbach Index[109] and low in free amino nitrogen[6,105], results in a lower rate of beer staling. Moreover, a high thermal heat load, in the form of Maillard intermediates reacting with thiobarbituric acid (TBI), measured in malt, in unboiled wort and fresh beer was found to be negatively related to the sensory score of beer freshness while examining forced-aged beers[26]. The authors concluded that a low TBI is a basis for better beer flavour stability. Furthermore, this parameter measured in malt correlates with boiled and unboiled Congress wort colour[26]. The above observations are in agreement with Furukawa Suárez *et al.*[27], who reported that addition of speciality malts (*e.g*. caramel malt), which are characterised by higher EBC colour and heat load values, increases the content of beer staling aldehydes in forced-aged beers. In addition to the above, antioxidant properties of malt appear to impact flavour stability as a high radical scavenging activity (positively correlated with phenolic content) contributes to prolonged beer freshness[29]. When evaluating the staling degree of forced-aged beers (forced aged for six days at 50°C, followed by one day at 0°C), which were brewed with or without a reduced content of phenolic compounds, Mikyška *et al*.[28] reported a higher stale flavour intensity in the beer variant with a diminished phenolic content. The authors interpreted it as malt polyphenols having a positive impact on the staling degree of forced-aged beers. Though the phenolic content was reduced by the addition of PVPP and the assumption was not tested on the purified malt polyphenols. As mentioned before, besides polyphenols, also Maillard reaction products (MRPs) are the well-known antioxidants present in malt. However, it has been demonstrated that brewing with the addition of dark speciality malts may result in a decreased oxidative stability of wort and beer, though dark malts are known for their high content of MRPs[27,118]. These outcomes are in line with other studies[98,119] reporting that MRPs or intermediates such as reductones with an enediol structure, may accelerate Fenton reaction, thereby yielding higher levels of reactive oxygen species (ROS) during beer ageing. Higher levels of ROS will in turn accelerate degradation of amino acids into Strecker aldehydes in darker beers[47,83]. Finally, when using electron spin resonance (ESR) spectroscopy, Kunz *et al.*[108], observed higher oxidative stability in beer brewed with 25%, 50%, 75%, and 90% raw barley, in comparison to a 100% malt reference beer. Thus, it appears that the use of unmalted barley, which is not exposed to heat load, compared to conventional malt, imparts prolonged beer flavour stability.

Therefore, malt can be seen as a key factor considering free aldehydes as such, as well as aldehyde precursors (*e.g.* bound-state aldehydes, amino acids, reducing sugars, *etc*.) and intermediate products (*e.g.* Schiff bases, α-dicarbonyls, *etc*.), since free aldehydes are largely removed during brewing[7] and fermentation process[120], in particular, aldehydes precursors and intermediate products may affect beer staling.

In addition to brewing trials, various barley breeding experiments were performed with the main focus on elimination of proanthocyanidins[121] and lipoxygenase activity (considering both LOX-1 and LOX-2 enzymes)[122–125], which are recognised as factors negatively influencing beer stability. Since the beer brewed with malted proanthocyanidin-free barley received a lower score in the sensory evaluation after one month of natural ageing than its proanthocyanidin control[121], it seems possible that the content of low molecular weight

polyphenols was insufficient to slow down oxidation reactions, resulting in an increase in off-flavours. A different approach – brewing with malted null-LOX barley – showed however, a positive influence on beer flavour stability. The latter was assessed as the *trans*-2-nonenal content and through sensory evaluation of forced-aged beers[122]. In another study conducted by Hirota *et al.*[123], low levels of *trans*-2-nonenal were determined in both forced-aged beers, when null-LOX malt was used as the main ingredient and when applied in combination with a LOX-normal pale malt. In the sensory evaluation conducted by well-trained panellists, null-LOX forced-aged beer obtained lower off-flavour and total staleness scores. More profound analysis, conducted by the same research group, allowed to compare malt quality parameters between LOX and null-LOX malts[125]. The authors reported no differences in general characteristics, except for LOX-activity (16.1 U/g vs 1.3 U/g). In forced-aged beers, *trans*-2-nonenal was detected in the range of 0.09 - 0.12 µg/L and 0.35 - 0.36 µg/L, when brewed with null-LOX and LOX-normal malt, respectively. In addition, Hirota *et al.*[125] suggested that the application of null-LOX malt would reduce the cost of energy required to inactivate LOX in the brewhouse. Hoki *et al.*[124] indicated that modifying the malting schedule (*e.g.* decreasing germination temperature, increasing kilning temperature and/or prolonging kilning time) could reduce LOX-activity already during malting, without the need of using null-LOX barley. Nevertheless, these adjustments would affect other malt quality parameters crucial for brewers, primarily malt colour. Therefore, the use of null-LOX barley may be more suitable to decrease *trans*-2-nonenal concentrations in malt and later on in aged beer. Additional brewing trials[124] with 74% of malted LOX-1-less barley variety 'Satuiku 2 go' and 26% adjuncts (starch, corn, rice) showed that fresh beer contained only 0.03 µg/L of *trans*-2-nonenal, whereas after 1 month at 30°C, aged beer and its control (beer brewed with LOX-normal malt) contained 0.11 µg/L and 0.16 µg/L, respectively. Considering 0.03 µg/L as an accepted flavour threshold value of *trans*-2-nonenal[16], brewing with LOX-1-less barley does not allow the concentration of this off-flavour to be maintained below sensory perception levels. Nevertheless, it does decrease the content of *trans*-2-nonenal to levels similar to those formed via the nonenzymatic reaction of autoxidation[17].

1.3. Evolution of marker aldehydes and their precursors during the malting process

The (bio)chemical composition of malt directly depends on grain to be malted[126,127] and the applied malting process[26,128,129]. In this thesis, particular attention is drawn to malted barley, as it is the primary cereal used for brewing. In brief, the malting process consists of grain sorting and cleaning, steeping, germination, and kilning. The main goals of malting are activation and formation of enzymes, partial degradation of endosperm matrix polymers (mainly proteins and β-glucans), improvement of grain friability and formation of colour as well as characteristic malt flavours. During malting, barley undergoes physical and chemical modifications when exposed to the following key factors for varying lengths of time: water, oxygen, and high temperature[128]. By adapting malting regime, quality parameters of pale malt can be modified, for example, lower germination temperatures tend to decrease soluble nitrogen[130]. Moreover, flavour, colour and reducing power of malt can be influenced by kilning regime or by roasting[131]. The latter allows production of speciality malts such as crystal malt, caramel malt and chocolate malt, with flavour profiles that are well defined[76,77,92,110,131–135].

The importance of the malting process in relation to beer flavour (in)stability is still poorly understood, even in the case of pale malts that represent the predominant grist material used across the brewing industry. Currently, available data on the influence of crucial malting parameters on the formation of free aldehydes and their precursors are summarised in Table 1-4, as well as in the following sections.

Table 1-4A. Overview of the influence of barley selection and malting on the formation of marker aldehydes and their precursors.

Malting stage	Influence		
	Positive	**Negative**	**Ambivalent**
Barley selection	- Selection of barley with low lipoxygenase potential or null-LOX barley[122–124]. - Selection of winter barley allows reduction of the levels of polyunsaturated fatty acids[151].	- Selection of barley with high nitrogen content increases pool of aldehyde precursors[6,105,109,155–158]. - Crop year, soil conditions and fertilisation may increase the nitrogen content[159,160] and levels of volatile compounds found in barley[111].	- Winter barley has a higher husk to endosperm ratio, which on the one hand results in a higher content of Maillard intermediates[144] and transition metal ions [67], however, on the other hand, it is rich in antioxidants[28,29,143,154].
Steeping	- Application of sonic waves improves the oxidative stability of wort by reduction of iron content in malt[148].	- Frequent aeration during the wet phase and multiple air rests lead to higher LOX-activity[64]. - Oxidation of steeping water with hydrogen peroxide or ozone results in an increased pool of aldehyde precursors[167].	- Long steeping time enhances steeping degree and thus, results in high grain modification. This may increase pool of aldehyde precursors but also increases content of antioxidants[152]. - Re-steeping of green malt in acidified solution increases the content of Maillard precursors but also decreases the quantity of Strecker aldehydes[168,169].
Germination	- Application of lower germination temperatures leads towards lower LOX-activity[64,65]. - Maintenance of low moisture content of green malt results in reduced levels of marker aldehydes present in malt[112]. - Grain asphyxiation or air recirculation during germination may suppress oxidation of unsaturated fatty acids and reduce LOX-activity[26,64]. - Acidification of green malt by lactic acid bacteria may indirectly reduce the content of aldehydes found in malt[179,180].	- Microbial contamination with *Fusarium* leads to higher levels of aldehydes found in malt[177,178]. - Excessive treatment of green malt with gibberellic acid may increase the pool of aldehyde precursors[181,182].	- High germination temperature leads to higher grain modification and an increased pool of aldehyde precursors[150,170]. However, it also results in a lower content of some staling aldehydes[112].

Table 1-4B. Overview of the influence of malting on the formation of marker aldehydes and their precursors.

Malting stage	Influence		
	Positive	**Negative**	**Ambivalent**
Kilning	- Moderate kilning off temperature (up to 80°C) increases the polyphenol content in the finished malt[188]. - Application of alternative drying methods may allow reduction of the heat load and grain inhomogeneity (difference between bottom, middle and top layer of a kiln bed)[191–193].	- High kilning temperature leads to a higher rate of Strecker aldehydes and furfural formation[77]. - High kilning-off temperature enhances radical formation[118]. - Inhomogeneity of malt caused by kilning grain in a thick layer (difference between bottom, middle and top layer of a kiln bed)[59]. - Application of sulphuring during kilning increases the content of soluble nitrogen[189]. - Higher kilning temperatures increase malt colour and TBI, as well as the content of Maillard reactions (e.g. reductones) and reactive oxygen species what may lead to increased levels of Strecker aldehydes[27,47,98,119].	- Exposure of malt to high kilning-off temperature for a long(er) time reduces LOX-activity[64,173,187]; however, the formation of Strecker aldehydes and furfural is intensified[77,124].
Storage	- Prolonged storage time decreases LOX-activity[186].	- Humid conditions increase levels of Strecker aldehydes and their precursors in malt[99,274].	

1.3.1. Barley

1.3.1.2. Structure of barley grain in relation to marker aldehydes and their precursors

Barley grain (*Hordeum vulgare, vulgare L.*) consists of approx. 65% - 68% of starch, 10% - 17% of protein, 3% - 9% of arabinoxylans, 4% - 9% of β-glucans, 2% - 3% of lipids, and 1.5% - 2.5% of minerals on a dry matter basis[136–138]. The moisture content of stored barley and malt is low (approx. 12% and <5% for barley and malt, respectively), which prevents extensive chemical transformations[128]. Moreover, barley and malt kernels consist of a multi-layered structure, including the husk, pericarp, testa, aleurone layer, embryo and endosperm (Figure 1-10). These are exposed during malting (albeit to differing extents) to water, air (oxygen boost), and heat, as well as to enzymatic, hormonal, and microbial activity. Therefore, the formation of marker aldehydes can differ between grain structures. According to Fox *et al.*[139] among all of the kernel structures, the husk, aleurone, scutellum and embryo are primarily associated with barley and malt quality.

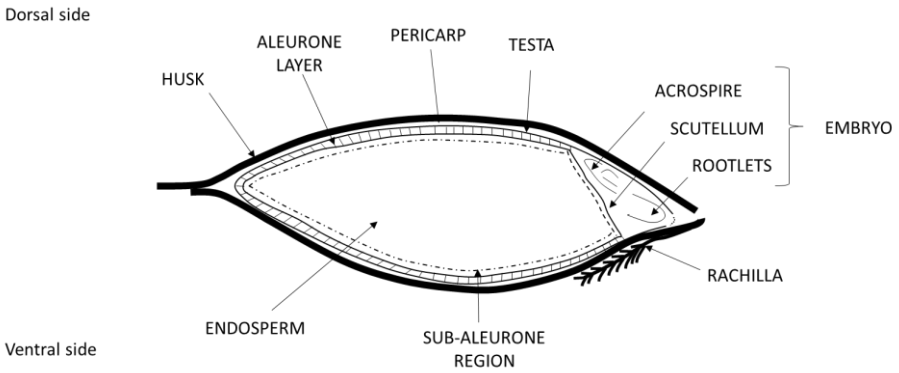


Figure 1-10. Depiction of barley grain structures, based on Briggs[140].

Husk, together with pericarp, accounts for 7% to 14% of grain dry matter basis, depending on the barley variety, grain size and growing environment[139,141]. From the perspective of beer flavour stability, organic radicals which catalyse various oxidation reactions leading to, for example, marker aldehydes are mainly found in the husk, rather than in the flour fraction of malt[118]. In addition, husk provides flavours that are often described as 'husky' or 'grainy'[142]. Lewis *et al.*[143] highlighted the importance of extracting malt polyphenols from the husk during the brewing process since these are responsible for a grainy taste, astringency and may also act as pro- or antioxidants. The latter may enhance or reduce the rate of marker aldehyde formation. Van Waesberghe *et al.*[144] suggested that beer flavour instability is significantly affected by husk components extracted during mashing and sparging, among them staling aldehydes, as well as their precursors and polyphenols. For example, beers brewed with malt containing husk showed higher TBI than beers brewed using a corresponding 'hulled' malt[144]. Though, the TBI is strongly correlated with the evolution of carbonyl compounds during beer ageing, 'de-husked beers' were more prone to staling, probably due to lack of husk polyphenols acting as antioxidants.

Another barley structure that may be important from the perspective of beer flavour instability is the aleurone layer, which makes up to 8% to 15% of the grain dry matter and contains proteins, lipids, glucan, and xylan[128,139,143]. Moreover, it is also rich in transition metal ions, such as iron, which may catalyse the formation of carbonyl compounds both in malt and wort[145]. During germination, the aleurone synthesises and secretes enzymes that play a

crucial role in the growth of the embryo and grain modification, including lipoxygenases, xylanase, peroxidases, polyphenol oxidases, dehydrogenases, esterases, phosphatases, phytases, proteases, lipases, β-glucanases, proteinases, peptidases, α-amylase, limit dextrinase, and α-glucosidase[139,143]. These enzymes catalyse degradation of polymers (*e.g*. proteins, starch) releasing low-molecular weight-compounds, which can act as precursors for marker aldehydes (*e.g.* free amino acids, initiating Strecker degradation or Maillard reactions). Moreover, some enzymes (*e.g*. LOX-1 and LOX-2) are directly involved in aldehyde formation pathway since they are implicated in the formation of lipid degradation aldehydes. Furthermore, the aleurone layer is rich in ferulic acid; a well-known antioxidant[146].

Barley endosperm accounts for 75% to 80% of a grain dry matter. The endosperm cell wall consists of β-glucans (approx. 75%) and arabinoxylans (approx. 20%)[139,146]. The remainder mostly consists of small and large starch granules embedded in a protein matrix[128,147]. Therefore, it can be seen to act as a reservoir for storage compounds and is an important structure from the perspective of the brewer since the extract yield depends on the overall starch content. However, the relationship between endosperm components and beer flavour instability is still not well established.

Embryo is a living tissue, which accounts for 3% to 5% of the grain dry matter[128]. It contains sugars, amino acids and lipids, which are mostly used to support the initial development of the embryo prior to breakdown of the endosperm reserves[139]. Embryo stimulates development of hormonal and enzymatic pathways and is the so-called starting point for grain transformation to a plant through the growth of the acrospire and rootlets[128]. The acrospire developed from the embryo during germination contains relatively high levels of lipoxygenase enzymes, soluble protein, free amino acids and S-methyl methionine (SMM), which are important from the perspective of beer staling, especially with regard to hexanal, *trans*-2-nonenal and Strecker aldehydes[148]. Brewing trials with the addition of 5% and 15% of acrospire material to the wort showed a significant increase in Strecker degradation aldehydes, hexanal and furfural measured in pitching wort, as well as a decline in the overall beer flavour stability, compared to the control brewing trails without the addition of the acrospire material[148].

1.3.1.2. The influence of barley variety and growing conditions

Herb *et al.*[149] stated that the barley variety and the growth environment are equally important contributors to beer flavour. However, not much is known about the direct influence of either of these parameters on beer flavour instability. It is well established that barley variety and growing conditions influence content of lipids, thus precursors of hexanal and *trans*-2-nonenal[150]. Spring barley contains higher levels of polyunsaturated fatty acids (including linoleic acid) than its winter counterpart[151]. Consequently, use of a winter barley variety might (to a certain extent) improve the quality of the final product (malt and/or beer), however, this still requires confirmation. Furthermore, the crop year, variety and application of fertiliser containing zinc can influence the content of antioxidants (mostly phenolic compounds)[152], which suppress aldehyde formation from radical reactions involving fatty acids, amino acids and higher alcohols[153]. Also, winter barley presents higher antioxidant activity than spring barley due to the thicker husk[154]. Various authors have stated that the high availability of nitrogen in the soil (mostly due to extensive fertilisation) increases the content of proteins in a barley grain[155–158], which may become a substrate pool for formation of aldehydes. Moreover, the nitrogen content in barley is related to various malt quality

parameters, for example, Kolbach Index, free amino nitrogen, soluble nitrogen, and colour[158]. Also, it is known that weather conditions during barley grain development in the field play an essential role, as drought leads to elevated levels of proteins. Fortunately, it can be compensated partially by the selection of suitable barley varieties and application of an optimised irrigation system[159,160]. Besides, barley variety and crop year influence levels of volatile compounds (among them staling aldehydes) present in barley and later in malt[111]. Svoboda *et al.*[113] detected *trans*-2-nonenal in the range from 0.28 µg/kg to 3.06 µg/kg of barley, when comparing 21 barley varieties. In addition, Cramer *et al.*[161] identified 3-methylbutanal, 2-methylbutanal, hexanal, 2-hexenal, 2-heptenal, and 2-nonenal as key odorants in barley. The highest concentration was detected for hexanal ranging from 46 µg/L up to 1,269 µg/L of barley extract, depending on the barley variety.

1.3.2. Steeping

The major aims of steeping are to increase grain water activity, as well as washing away dust and germination inhibitors, and improving friability by loosening of the grain structure[128]. Steeping is typically performed by periodic submersion of the kernels in water, according to the steeping regime (time, temperature, number of wet/dry phases, and water aeration). Various enzymes which catalyse reactions yielding aldehyde precursors (such as lipoxygenase and proteases) are activated when grain reaches a minimum of 32% of moisture content[128]. This can lead to an increase in the activity of transaminase, peptidase and protease, along with the rise in water content of the grain, resulting in a subsequent increase in amino acids[162]. Also, with an increase in the activity of saccharolytic enzymes, levels of carbohydrates such as maltotriose and sucrose decrease[163], while the concentrations of simple carbohydrates (fructose and glucose) increase concomitantly[164]. Furthermore, during steeping total polyphenol content decreases by around 6% - 7%[165], thus the pool of compounds, which may act as inhibitors of catalysts for *de novo* formation of aldehydes declines. The steeping regime also influences enzymatic activity during germination. For example longer steeping results in higher LOX-activity[107] and increased overall antioxidant activity of finished malt (the latter measured as the amount of free radicals, which can be eliminated by antioxidants present in the sample)[152]. Moreover, LOX-activity is also enhanced by applying aeration throughout the wet phase of steeping, and when the steeping schedule includes more than one air rest[64].

In order to improve the potential impact of steeping on beer flavour stability, Müller *et al.*[166] proposed implementation of vibrations at a frequency of 180 Hz - 200 Hz, creating sonic waves. This led to an iron reduction of approx. 30% in malt, thus improving oxidative beer flavour stability measured by electron spin resonance (ESR). An additional benefit of this treatment was improved grain washing, which enhanced water uptake and increased homogeneity of the finished malt. Ma *et al.*[167] reported that oxidation of steep water by hydrogen peroxide or ozone resulted in an increase in free amino nitrogen, β-glucan, and diastatic power. This could potentially improve the efficiency of malting as such, however, it would also deliver more aldehyde precursors to the brewing process. Mauch *et al.*[168,169] suggested that re-steeping of green malt under acid conditions, which in the original assumption should limit malting losses, could also increase the amount of beer staling markers present in fresh and aged beers. Such a rise was expected, especially in the case of furfural derived from Maillard reactions, as under acidic conditions the formation of its precursor (3-deoxyosone) is predominant over 1- and 4-deoxyosone formation[17].

In contrast, in aged beers brewed with acidified malt, the authors measured lower levels of oxygen indicators (2-methylbutanal, 3-methylbutanal), probably due to a pH effect, *i.e.* under acidic conditions, amino acids are protonated, and therefore less reactive towards Strecker degradation.

1.3.3. Germination

During germination, grain modification takes place. This is characterised by the breakdown of cell walls, proteins, lipids and starch as a consequence of enzyme formation and activation. The process is performed in humid and aerobic conditions at temperatures ranging from 16°C to 20°C for 4 to 5 days[128]. Germination rate and chemical changes are temperature dependent; when higher temperatures are applied, more intense grain modification is achieved, resulting in an elevated pool of staling-related compounds[150,170]. Analysis of 'non-conventional' malting, with germination at 'low' (12°C) or 'high' (18°C) temperatures and various moisture contents, showed the influence of these parameters on aldehyde formation[112]. Essentially, higher germination temperatures enhanced reduction of aldehydes to alcohols and resulted in malt with lower concentrations of 3-methylbutanal, furfural, and hexanal. Furthermore, lower moisture content in green malt resulted in reduced concentrations of these compounds, especially 3-methylbutanal[112].

Lipoxygenase enzymes LOX-1 and LOX-2 play an important role in the formation of precursors of some staling aldehydes[17,171]. LOX-activity increases approximately four-fold during germination and its rate depends mostly on the germination temperature and presence of oxygen[64]. Lower germination temperatures can lead to reduced LOX-activity in the finished malt[65]. Also, grain asphyxiation (reduction of oxygen content in the air passing through the grain bed) may have a similar effect[64]. In order to reduce LOX-activity in malt, Baxter[64] proposed acidification of steeping water or asphyxiation of grain during germination since both of the treatments resulted in a 3-fold decrease of LOX-activity. This is in line with outcomes presented by Gastl *et al.*[26], suggesting the use of recirculated air enriched in CO_2 during germination to suppress oxidation of unsaturated fatty acids. This system enabled production of malt with a lower concentration of hexanal. In the case when LOX-activity is high and oxygen is present in the system, enzymatic oxidation and auto-oxidation take place resulting in a decrease during germination and following it kilning, in the levels of triglycerides and a subsequent increase in free fatty acids, intermediates of *trans*-2-nonenal and hexanal, respectively[151,172,173]. This is most likely due to both LOX-activity and lipid metabolism of the grain. The highest concentrations of free fatty acids were found at the end of germination[174]. Similarly, because of intensive proteolysis during germination, the quantity of amino acids increases[175]. Frank *et al.*[164] observed a significant increase in amino acids, including valine (precursor of 2-methylpropanal). Similarly, the content of simple carbohydrates[163] and polyphenols[176] becomes elevated.

The volatile fraction of malt also can be influenced through microbial contamination of green malt by *Fusarium poae*[177] and *Fusarium graminearum*[178]. Chen *et al.*[178] reported a significant increase in the concentrations of 2-methylbutanal, pentanal, hexanal, and *trans*-2-nonenal in contaminated malts. The authors suggested that these compounds must have been emitted from the mycelium since the same aldehydes were detected in the sporulated mycelium itself. Therefore, contamination by *Fusarium* may directly deteriorate beer flavour and potentially its flavour stability. Introduction of lactic acid bacteria during germination may improve the biological stability of malt since it can prevent or reduce contamination

by *Fusarium* species[179]. It also affects aspects of brewing performance, such as filtration time, which can result in a decrease in total heat load, and ultimately a lower aldehyde content in beer[180].

From a technological perspective, addition of gibberellic acid is a common practice applied to enhance enzyme formation. This results in shorter germination times and more profound grain modification (lower β-glucan content, higher Kolbach Index, and higher friability of the finished malt)[128]. However, a careful dosage is essential, as overdosing will result in extensive rootlet formation, extract yield losses, as well as high sugar and soluble nitrogen levels. This can further lead to the development of abnormal colour of the final malt and might indirectly contribute to beer flavour instability as more aldehydes precursors are generated[181,182].

1.3.4. Kilning

During kilning, grain is dried gradually by a flow of warm air (from 55°C - 90°C for pale malts) in order to stop biochemical reactions, ensure product stability during storage, as well as to develop the desired colour and flavour characteristics. The kilning regime (processing time, temperature, humidity, and airflow) affects the physical and (bio)chemical properties of malt, *e.g.* moisture content, growth of the embryo, enzymatic activity and aroma composition[76,183]. In particular, aroma compounds (such as those conferring biscuit, toast, nutty, caramel flavours) and colour are highly impacted, leading to a broad range of commercially available malts such as Vienna or Crystal[184]. Kilning is also a critical step of the malting process regarding beer flavour instability, mostly due to the applied heat load and the decrease in moisture content, which accelerate the formation of marker compounds[26]. Regarding Strecker aldehydes, in the last stage of Strecker degradation, the presence of water is required for conversion of an iminium ion into an unstable amino alcohol[17]. Therefore, Strecker aldehydes (2-methylpropanal, 2-methylbutanal, 3-methylbutanal) are formed in humid conditions regardless of the temperature (tested range 105°C to 180°C), whereas in more dry conditions the formation occurs only at very high temperatures (above 130°C)[77]. As opposed to Strecker degradation aldehydes, the formation of furfural is also taking place under dry conditions[77], because it requires dehydratation of its direct precursor (3,4-dideoxypentosulose-3-ene). During kilning minor changes to the lipid content (and therefore formation of fatty acid oxidation products) are observed with a decrease in humidity. Grain modification terminates and the activity of lipases is significantly reduced[151,171]. In general, with an increase in drying temperature and a decrease in water activity inside the grain, the overall enzymatic activity gradually declines. For example, lipoxygenase activity in the finished pale malt reaches approx. 5% of the initial value[171]. Enzymatic inactivation takes place gradually as the conditions in the bottom, middle, and top layer of the kilning bed differ. This is caused by the introduction of warm air from the bottom and by the stationary position of the grain (*i.e*. no turning of the kernels) during the process. Hence, the bottom layer dries faster and enzyme inactivation occurs quicker than in the top layer[59]. Therefore, the moment of inactivation mostly depends on the position of a kernel in the grain bed and the particular properties of the enzyme under consideration[59,64,150,171,173,185,186]. As an example, Baxter[64] stated that lipoxygenase activity in green malt is relatively stable; however, a temperature increase up to 65°C reduces its activity by approx. 70% - 90% depending on the time of grain exposure to the high temperature. Kilning-off at 85°C - 90°C reduces this activity to 2%. Generally, higher

processing temperature and longer kilning times result in a significant reduction of LOX-activity in malt, which is positive from the perspective of beer flavour deterioration[173,187]. Nevertheless, increased heat load also affects other malt quality parameters such as aroma profile and, therefore could have an undesirable effect on beer flavour[124].

Kilning also influences antioxidants, which reduce the rate of oxidation reactions. The total polyphenol content increases during kilning (especially in the first phase of kilning) regardless of the tested barley variety[165,188]. A marked increase was identified for (+)-catechin and ferulic acid[165]. Inns *et al.*[188] showed that the content of ferulic acid increased until a temperature of 80°C was reached, then ferulic acid esterase was deactivated and, as a consequence, the enzymatic release of free phenols from their bound forms was suppressed. In general, high temperatures (as applied during kilning), enhance radical formation, which can also directly or indirectly affect beer flavour instability[118].

Some older malthouses with directly-fired kilns apply sulphuring during kilning in order to control the formation of carcinogenic nitrosamines[189]. This treatment however may increase the content of soluble nitrogen, leading to an increased pool of aldehyde precursors.

Huang *et al.*[133] aimed at optimisation of kilning regime as a function of selected indicators of beer quality and its flavour instability (LOX-activity, *trans*-2-nonenal, hexanal, methional, phenylacetaldehyde, and furfural content, heat load and the wort sensory score). The authors selected kilning-off temperature, kilning time and a withering time of 86.35°C, 3.19h and 14.00h, respectively. According to the authors, malting barley following the proposed regime can result in a high-quality pale malt with a low beer staling potential. A totally different approach is the application of alternative water removal methods such as freeze-drying, already widely used in the food industry for drying coffee, spices, meats, food ingredients and other high-value solid-phase food products[190]. Malt dried in this way, in comparison to its conventionally kilned counterpart, yields a higher amylolytic activity, higher extract yield, lower colour, as well as higher viscosity and turbidity of filtrated wort[191]. However, the effect of this treatment on staling aldehydes has not been reported to date. A further option is drying with electromagnetic waves, although a recent publication in this field focused on the effects related to enzyme survival and energy efficiency rather than on beer staling compounds[192]. Yet another proposed green malt drying technique is through vacuum oven drying[193]. This is designed for a continuous operation whereby the apparatus transports green malt through separate drying zones, which allows a more homogeneous malt to be produced. In order to facilitate moisture removal, the machine is equipped with a vacuum chamber to reduce vapour pressure. It would be interesting to investigate staling aldehyde formation during this process since two critical factors (temperature and the lack of homogeneity in conventional kilning caused by the thick bed) are obviously of reduced significance.

1.3.5. Storage

To the best of our knowledge, until today, studies regarding the evolution of beer staling aldehydes during storage in real, industrial conditions have not been carried out, however, some lab-scale experiments have been performed[99,186]. Hoff *et al.*[99] investigated the influence of storage time (up to 12 months), temperature (10°C and 20°C) and humidity (water activity of 0.231 and 0.432) on oxidative stability (measured as a radical content

in malt) and volatile profile (determined in sweet wort) of pilsner malt. The authors observed that the radical content measured in pilsner malt was positively correlated with water activity and that this parameter has increased when the sample was stored at higher temperatures. Regarding the volatile profile, during the first six months of storage, pilsner malt reminded uninfluenced by storage temperature and water activity. However, longer storage (12 months) at 20°C led to the loss of the content of some Strecker aldehydes, in particular 2-methylbutanal and 3-methylbutanal. On the contrary, the content of phenylacetaldehyde, the other Strecker aldehyde, increased over the storage time, regardless of the temperature that the sample was exposed to. In the other study conducted by Kaukovirta-Norja *et al.*[186], the authors investigated the influence of storage time (seven-months at 5°C) of pale malt on LOX-activity. The authors observed a 25% decline in LOX-activity after a period of seven months. The reduction occurred linearly as a function of time.

1.4. Conclusions

Despite extensive research on beer flavour instability, off-flavours appearing over time in a closed beer package remain a challenge for the brewing and malting industries. Malt delivers various compounds to the brewing process, many of which could potentially affect beer ageing. Among them are the so-called beer staling aldehydes, precursors for *de novo* formation of aldehydes and bound-state aldehydes. Marker aldehydes for beer flavour instability can arise in malt, wort and beer due to Maillard reactions, Strecker degradation, oxidation of amino acids, and (enzymatic) oxidation of unsaturated fatty acids. However, they may also be released from a bound-state, although it is acknowledged that the chemistry and the behaviour of bound-state aldehydes in a complex matrix such as malt require much more detailed investigation. It is well established that malt quality influences not only the brewing performance and the flavour of the final beer but is also crucial from the perspective of beer staling. For example, malt characterised by low Kolbach Index, heat load, colour, LOX-activity, Strecker aldehydes, transition metal ions content, and high antioxidant activity, can lead to more flavour stable beer. In particular, the malting regime plays a crucial role, as it determines the overall malt quality but also affects the formation of aldehydes, as well as their intermediates and precursors. Proper selection of barley variety, together with the adequate adjustment of steeping and germination conditions, allows control over grain modification and thus the reservoir of aldehydes precursors. These compounds may undergo aldehyde formation pathways during malting, but also during brewing and even in the final beer package. Kilning is the most critical stage of the malting process from the perspective of marker aldehydes, as high temperature enhances reaction rates, and the oxygen boost triggers the formation of radicals, which leads to intensification of autoxidation. This results in the rapid formation of beer staling compounds already present in malt. However, the influence of post-kilning malt-cooling is not well studied. Various proposals of the potential technological improvements have been suggested aimed at malt with a low beer staling potential. For example, the application of vibrations during steeping, microbiological management during germination or alternative drying techniques during kilning. It is anticipated that further investigations into malt properties and the handling of barley during malting will lead to a better understanding of the origins of the beer staling process and ultimately, may lead to enhanced beer flavour stability.

Chapter 2

Method development for determination of aldehydes in malting samples

This chapter partly corresponds to:

Filipowska W., Jaskula-Goiris B., Ditrych M., Schlich J., De Rouck G., Aerts G. & De Cooman L. (2021) Determination of optimal sample preparation for aldehyde extraction from pale malts and their quantification via headspace solid-phase microextraction followed by gas chromatography and mass spectrometry. *Journal of Chromatography A.* 1612:460647 doi:10.1016/j.chroma.2019.460647

2.1. Introduction

2.1.1. General introduction

Understanding the contribution of raw materials to the quality of a final product is crucial for the entire food industry. Malt, as the key ingredient used in beer production, delivers various compounds, which impact to brewing performance as well as flavour, visual properties and stability of the final product. The chemical composition of malt directly depends on the type of malted grain[30,126] and the malting process[128,129]. However, the influence of the latter on beer staling is insufficiently understood, likely due to the unavailability of an appropriate analytical methodology for quantification of marker aldehydes in malt. Therefore, this chapter describes determination of optimised sample preparation for extraction of aldehydes from pale malts, and evaluation of an analytical approach already available within the research group, allowing quantification of staling aldehydes via headspace solid-phase microextraction (HS-SPME) with on fibre derivatisation, followed by gas chromatography and mass spectrometry (GC-MS). Additionally, adjustment of the method was carried out, in order to enable analysis of samples coming from different stages of malting (*i.e.* green malt, partially kilned malt, and finished pale lager malt).

2.1.2. HS-SPME with on-fibre derivatisation, followed by GC-MS for quantitative determination of aldehydes

Headspace solid-phase microextraction (HS-SPME) is a commonly applied extraction technique, used to study composition of volatile fractions of beer[35,194], wort[7,195], and brewing raw materials, *e.g.* hops[196] and speciality malts[197]. Also, it has been applied on various cereals, such as wheat[198], rye[198], oat flakes[199], and barley[198]. HS-SPME is typically coupled with a separation and detection techniques such as gas chromatography and mass spectrometry (GC-MS), gas chromatography and flame ionization detector (GC-FID), or selected ion flow tube mass spectrometry (SIFT-MS)[26,55,110,111,133].

Regarding investigation of the volatile composition of pale lager malts, to date, various analytical methods have been applied, both for sample preparation and determination of volatile compounds. For example, malt-derived wort samples have been subjected to HS-SPME coupled with GC-MS (indirect determination)[26,133]. Another applied possibility is preparation of aqueous malt extract, which undergoes water-vapour distillation followed by solvent extraction prior to gas chromatography - flame ionisation detection (GC-FID)[112]. Alternatively, mixing malt slurry with sodium chloride solution followed by HS-SPME-GC-MS[110] or selected ion flow tube mass spectrometry (SIFT-MS)[111]. The above mentioned studies[26,110,112] mainly focused on a wide spectrum of malt volatile compounds, rather than on a selective quantification of a specific chemical group (*e.g.* carbonyl compounds), whereas the target of others[111,133] was, in particular, on aldehydes. The latter allowed to predict the concentration range at which staling aldehydes may be present in pale lager malts (for the comparison of quantified levels of aldehydes in pale lager malts via different analytical methods, see Chapter 1, Table 1-3).

In our case, HS-SPME with on-fibre derivatisation, followed by GC-MS for quantification of staling aldehydes has been already available within the Enzyme, Fermentation and Brewing Technology (EFBT) research group, and has been applied previously on both beer model

solutions and beer samples[56,200] (see Figure 2-1). Therefore, this approach has been selected as the technique of choice for the quantitative determination of aldehydes in pale malts.

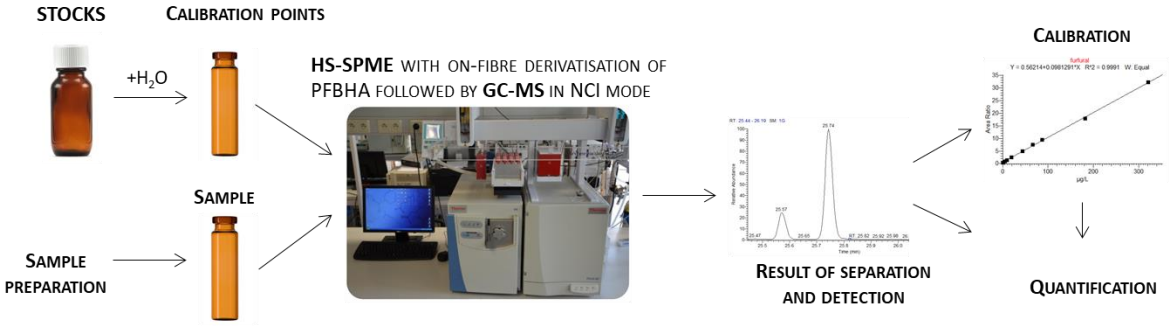


Figure 2-1. Overview of the analytical approach for determination of marker aldehydes as described by De Clippeleer[200] and Baert[56].

With respect to sample preparation, in the review on 'sample preparation techniques for determination of trace residues and contaminants in foods', Ridgway *et al.*[201] highlighted the importance of an adequate and uniform sample preparation technique in order to obtain trustful and repeatable results when analysing complex food matrixes (such as malt). Therefore, this chapter focuses on the evaluation and optimisation of the sample preparation technique followed by quantification of free aldehydes via HS-SPME-GC-MS.

2.2.2.1. HS-SPME with on-fibre derivatisation

The volatile fraction of malt is complex, consisting of *i.a.* carbonyl compounds, alcohols, acids, and furans[110]. Therefore, in order to enhance the method sensitivity and selectivity towards aldehydes, a derivatisation reagent (*o*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA)), which reacts with carbonyl compounds forming pentafluorobenzyloximes (PFBOs) (see Figure 2-2) at ambient temperature in aqueous solutions and at a wide pH range, was used[202]. An SPME fibre assembly (65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB)) was selected as the optimal fibre, since its coating is characterised by a high affinity to the PFBHA[35,203]. The SPME yield was assured by selecting an optimal temperature (30°C) and time (30 min) of the extraction process[35,200]. The combination of both parameters should lead to the highest possible SPME yield values with diminished *de novo* formation of artefacts. To ensure the robustness of the method, internal standard – consisting of stable isotope-labelled aldehydes – was spiked to all analysed samples. This approach minimises HS-SPME variations since aldehydes concentrations are obtained based on the peak area ratio, calculated as the ratio of the aldehyde peak area to the internal standard peak area.

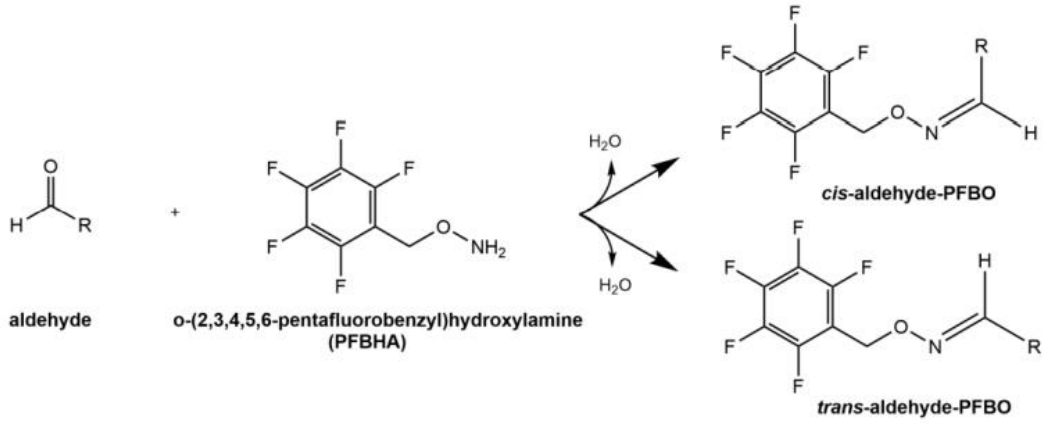


Figure 2-2. Derivatisation reaction of an aldehyde with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHA) forming pentafluorobenzyloximes (PFBOs), as described by De Clippeleer[200] and Baert[56].

2.2.2.2. GC-NCl-MS

Detection and quantification of the extracted aldehydes introduced to the GC column in the form of aldehyde-PFBHA-oxime, can be carried out in the electron impact ionisation (EI)[204] or chemical ionisation (CI) mode[205]. Comparison of the performance of both methods on a beer matrix (levels of aldehydes in beer are similar to the levels in extract derived from malt), demonstrated that EI is not sufficiently selective to identify all aldehydes of interest[200]. Obtained in EI mode, pentafluorobenzyl fragments were mostly analogous to the base peak (pentafluorobenzyl fragment at *m/z* 181), which is non-specific for the original compound. Additionally, this is a limiting factor for the use of isotope-labelled aldehydes as an internal standard since the chromatographic separation is not sufficient for specific detection of deuterated and their corresponding non-deuterated forms, which are coeluting. In contrast, application of chemical ionisation (CI) resulted in reduced fragmentation of the aldehyde-PFBHA-oxime ion. CI can be performed in a positive (PCI) or negative (NCI) mode. In CI primary electrons collide with reagent gas molecules (in this case methane) yielding ions and secondary electrons. Due to the further collision of these molecules, various positively and negatively charged molecular ions are formed. Positively charged ions have higher energy, thus their fragmentation continues, while the negative ions are being stabilised by a highly negative fluorine atom (from derivatization reagent). The proposed ion formation of the PFBOs via NCI technique is presented in Figure 2-3**.** As a consequence, in NCI more peaks related to the original aldehyde were present, which improved detection and also allowed to distinguish isotope-labelled aldehydes from their corresponding non-deuterated forms. Sensitivity and selectivity of the NCI method was further increased by performing the analyses in SIM scan mode, instead of full scan mode (*m/z* 50-350)[200]. Among the available fragments (see Table 2-1) the selected mass-to-charge ratios (*m/z*) were based on the previous work of Baert[56]: 247 (2-methylpropanal), 261 (2-methylbutanal and 3-methylbutanal), 270 (2-methylbutanal-d_{10}), 241 (furfural), 275 (hexanal), 279 (methional), 287 (benzaldehyde-d_6), 268 (phenylacetaldehyde), and 315 (*trans*-2-nonenal)[56]. Representative chromatograms for each compound obtained from malt extract sample spiked with an internal standard are presented in Figure 2-4.

Furthermore, the previously optimised parameters influencing analytical GC-MS performance have been selected for this study. An optimal reagent gas flow was found to be 1.5 mL/min, as the highest intensity of all analysed ions was measured[56,200]. An ion source temperature of 185°C was selected from the range 170-200°C, as the peak intensity of all measured aldehydes was the highest while conducting the analysis at this temperature[56].

Table 2-1. Overview of mass-to-charge ratios (*m/z*) of characteristic ions from pentafluorobenzyloximes (PFBOs) of selected beer staling aldehydes, obtained by negative chemical ionisation. Data obtained from Baert[56].

Aldehyde	2-methylpropanal	2-methylbutanal	2-methylbutanal-d_{10}	3-methylbutanal	hexanal	furfural	methional	benzaldehyde-d_6	phenylacetaldehyde	*trans*-2-nonenal
$[M]^{-\bullet}$	267	281	291	281	295	291	299	307	315	335
$[M\text{-}HF]^{-\bullet}$	247	261	270	261	275	271	279	287	295	315
$[M\text{-}HF\text{-}HCN]^{-\bullet}$	220	234	243	234	248	244	252	260	268	288
$[M\text{-}HF\text{-}NO]^{-}$	217	231	240	231	245	241	249	257	265	285

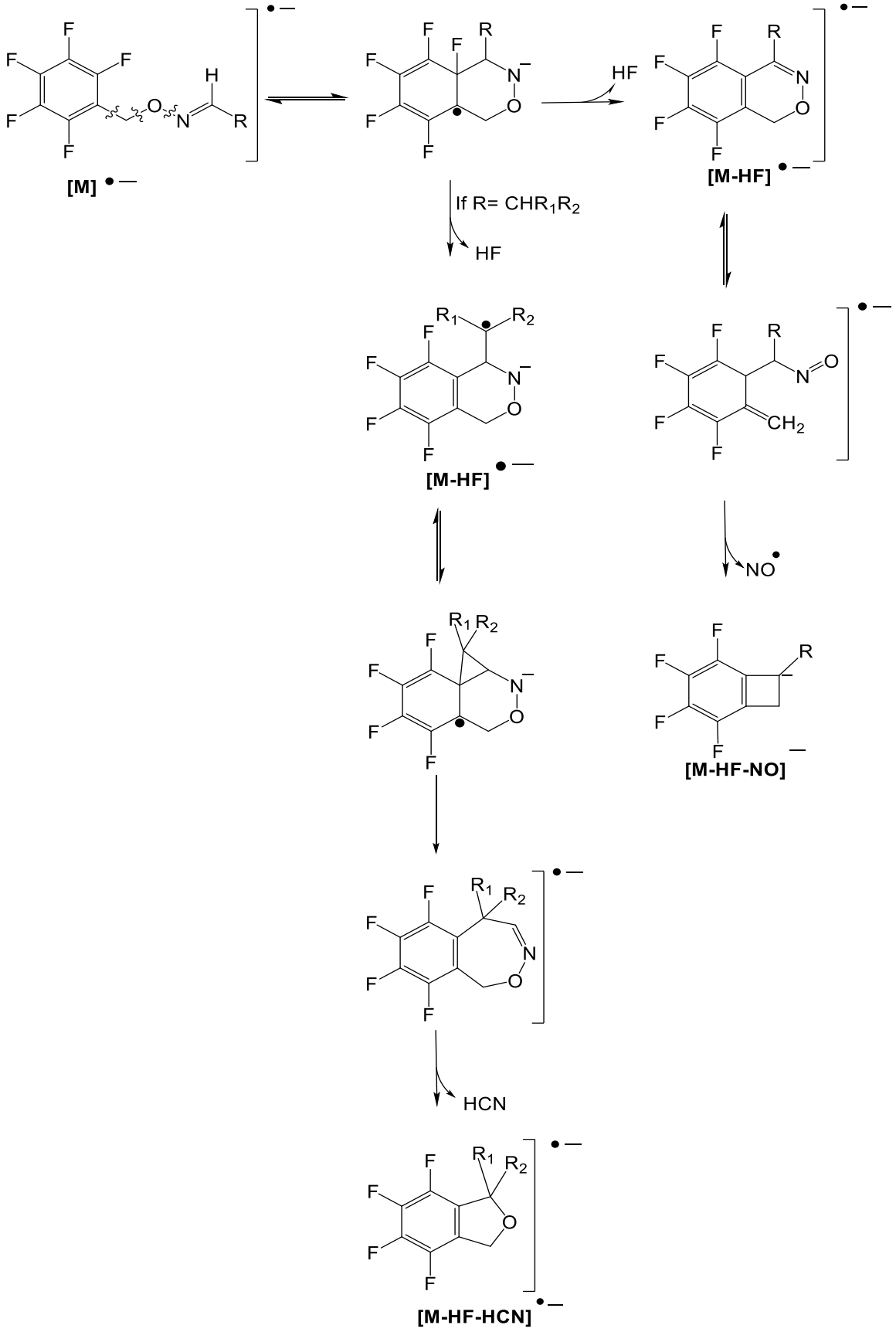


Figure 2-3. Proposed fragmentation positions by single-bond cleavage and ion formation of PFBOs, formed by reaction between the derivatisation reagent PFBHA and an aldehyde, with negative chemical ionisation (NCl), as described by De Clippeleer[200], Baert[56], and Bustillo Trueba[81].

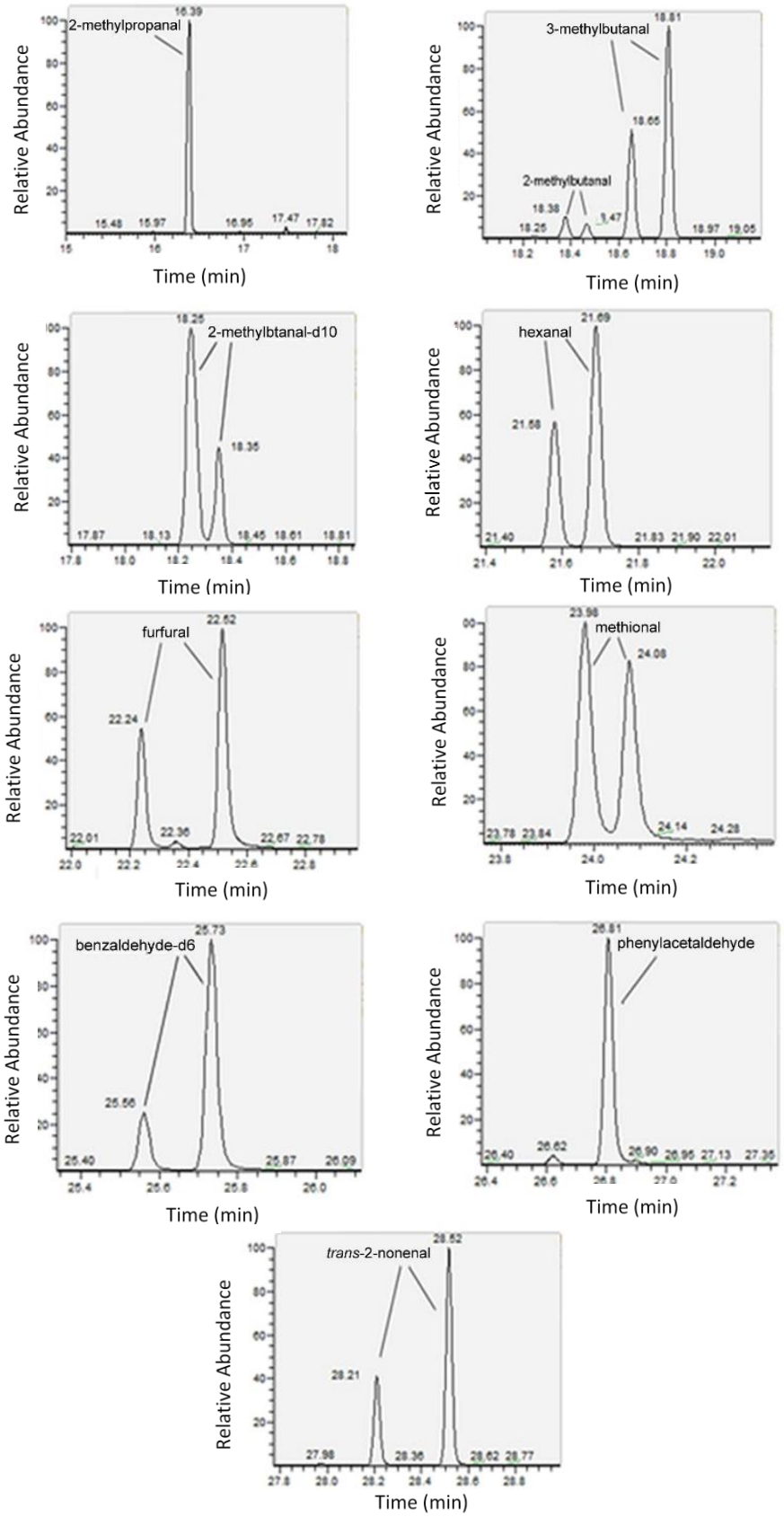


Figure 2-4. Representative GC–MS chromatograms of marker aldehydes present in malt extract samples, spiked with internal standard (2-methylbutanal-d_{10} and benzaldehyde-d_6); monitored in SIM mode.

2.2. Materials and methods

2.2.1. Chemicals

Determination of free aldehydes in malt. Selected marker aldehydes 2-methylpropanal (≥99%, CAS 78- 84-2), 2-methylbutanal (≥95%, CAS 96-17-3), 3-methylbutanal (≥97%, CAS 590-86-3), hexanal (≥98%, CAS 66-25-1), furfural (≥99%, CAS 98-01-1), phenylacetaldehyde (≥95%, CAS 122-78-1), methional (≥97%, CAS 3268-49-3), and *trans*-2-nonenal (≥95%, CAS 18829-56-6) were purchased from Sigma-Aldrich, USA. Internal standard contained a deuterated form of 2-methylbutanal (2-methylbutanal-d_{10}, upon request, MercaChem, The Netherlands) and benzaldehyde (benzaldehyde-d_6, CAS 17901-93-8, Sigma-Aldrich, USA. Aliquots of these aldehydes were spiked into absolute ethanol (≥99.5%, CAS 64-17-5, Merck KGaA, Germany) and stored at -20°C in amber glass vials. The derivatisation reagent o-(2,3,4,5,6-pentafluorobenzyl hydroxylamine hydrochloride)(PFBHA) (99%, CAS 57981-02-9) was purchased from Sigma-Aldrich USA. SPME fibres (65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB)) were obtained from Supelco, USA. Ultrapure type 1 grade (Milli-Q) water was used, which was obtained from Synergy 185 system from Millipore, France. All gasses (nitrogen, helium, and methane), as well as liquid nitrogen were purchased from Air Liquide, Belgium. Sodium chloride (CAS 7647-14-5) was purchased from Sigma Aldrich, USA.

Determination of cysteinylated aldehydes in malt. Cysteinylated aldehydes, 2-isopropylthiazolidine-4-carboxylic acid (2MP-CYS ≥99%, CAS 14347-75-2), 2-(sec-butyl)thiazolidine-4-carboxylic acid (2MB-CYS ≥99%, CAS 1214831-88-5), 2-isobutylthiazolidine-4-carboxylic acid (3MB-CYS ≥98%, CAS 215669-71-9), 2-(2-(methylthio)ethyl)thiazolidine-4-carboxylic acid (METH-CYS ≥99%, CAS 53943-83-2), 2-benzylthiazolidine-4-carboxylic acid (PHEN-CYS ≥94%, CAS 50739-30-5), 2-pentylthiazolidine-4-carboxylic acid (HEX-CYS ≥92%, CAS 69588-05-2), and 2-(furan-2-yl)thiazolidine-4-carboxylic acid (FUR-CYS ≥99%, CAS 72678-98-9) were synthesised according to Ershov's methodology [206] as described by Bustillo Trueba *et al.* [207]. Citric acid monohydrate (≥99.5%, CAS 5949-29-1), boric acid (≥99.5%, CAS 10043-35-3), tri-sodium phosphate dodecahydrate (≥98%, CAS 10101-89-0), dehydrated calcium chloride ($CaCl_2$ ≥95.0%, CAS 10043-52-4), (L)-lactic acid (≥90.0 %, CAS 50-21-5), and formic acid LC-MS grade (≥98%, CAS 64-18-6) were purchased from Merck, Germany. LC-MS grade acetonitrile (CAS 75-05-8) was acquired from Biosolve Chemie, France.

2.2.2. Instrumental conditions for HS-SPME with on-fibre carbonyl derivatisation, followed by GC-MS

The investigated aldehydes - 2-methylpropanal (2MP), 2-methylbutanal (2MB), 3-methylbutanal (3MB), methional (MET), phenylacetaldehyde (PHE), furfural (FUR), hexanal (HEX), and *trans*-2-nonenal (T2N) - were determined by HS-SPME with on-fibre carbonyl derivatisation, followed by GC-MS according to De Clippeleer[200] and Baert[56]. The procedure started with conditioning of the PDMS/DVB fibre, which was installed on the arm of a CombiPAL autosampler (CTC Analytics AG, Switzerland). The assigned vial was automatically transferred from the cooling tray at 5°C to the agitator at 30°C, where internal standard (20 µg/L of 2-methylbutanal-d_{10} and 2 µg/L of benzaldehyde-d_6) was added. Sample was then homogenised by shaking at 500 rpm for 2 min (cycles of 5 s shaking, 2 s rest).

Simultaneously, a PDMS/DVB fibre was exposed for 10 min to the headspace of the derivatisation reagent (1 g/L PFBHA aqueous solution) while being shaken at 250 rpm (cycles of 5 s shaking, 2 s rest). In the next stage, the fibre loaded with PFBHA was exposed for 30 min to the sample's headspace, whereby the reaction between the derivatisation reagent and carbonyl compounds was taking place, forming pentafluorobenzyl oxime (PFBO) derivatives. Sample was shaken 250 rpm (cycles of 5 s shaking, 2 s rest). Afterwards, carbonyl compounds in the form of PFBOs were thermally desorbed for 3 min at 250°C in the injector of a Focus GC Gas Chromatograph (Thermo Fisher Scientific Inc., USA). Liner volume was 0.5 mL. The inlet was set up to the split mode with a split flow of 10 mL/min and a split ratio of 12. Compounds were carried through the Rtx-1 Crossbond 100% dimethyl polysiloxane capillary column (40 m length, 0.18 mm i.d., 0.20 µm film thickness, Restek, USA) by the carrier gas (helium) at a flow rate of 0.8 mL/min. The GC oven temperature program started at 50°C for 2 min, followed by an increase of 6°C/min up to 250°C; in the last phase, the temperature was maintained for 5 min. The temperature of the transfer line between GC and mass spectrometer (MS) was held at 260°C. Detection was achieved using an ISQ^{TM} Single Quadrupole Mass Spectrometer (Thermo Fisher, USA) operating in negative chemical ionisation mode (NCI), with the ion source temperature at 185°C. Methane was used as the reagent gas, with a flow rate of 1.5 mL/min. The following parameters of MS were set: electron lens - 1.5 V; electron energy - 70 eV; emission current - 50 µA; detector gain 3.00×10^5. The detection and quantification were performed in Selected Ion Monitoring (SIM) mode, by choosing one characteristic ion with a negative charge per compound. The following *m/z* values were selected: 247 (2MP), 261 (2MB and 3MB), 270 (2MB-d_{10}), 241 (FUR), 275 (HEX), 279 (MET), 287 (benzaldehyde-d_6), 268 (PHE), 315 (T2N). XCalibur^{TM} (Thermo Electron Corporation, USA) was used for data processing.

2.2.3. Detection and quantification of aldehydes in aqueous model solutions via HS-SPME GC-MS - method validation

Authentic reference compounds of eight staling aldehydes were purchased from Sigma-Aldrich, USA (see section 2.2.1). First, eight individual standard stocks were prepared by transferring an aliquot of the authentic reference compound to ethanol. Accordingly, each stock contained a single aldehyde in the range of mg/L. Next, these individual standard stocks were used for preparation of seven calibration standards. The latter were obtained by mixing aliquots of the individual standard stocks and further diluting them in ethanol to the required concentration (µg/L range). The concentration range of each aldehyde was selected based on the expected aldehyde levels in pale malt extracts, according to the literature[111,133,208], and to the values obtained from our preliminary study. All stocks and calibration standards were stored at -20°C in 15 mL amber glass screw-capped bottles.

Further, the calibration samples were prepared by spiking 40 µL of the calibration standard to 10 mL of N_2-flushed Milli-Q water. The seven calibration samples were always freshly prepared in 20 mL amber glass GC-vials and were closed with magnetic bimetal crimp caps containing polytetrafluoroethylene (PTFE)/silicone septa (Interscience, Belgium). Concentration ranges obtained in the calibration samples after 250-fold dilution are presented in Table 2-2. Sample preparation was performed under oxygen-limited conditions in the anaerobic workstation (Whitley Anaerobic Workstation A100 model B, Don Whitley Scientific Ltd., UK).

Table 2-2. Concentration ranges of individual aldehydes in calibration samples obtained by 250-fold dilution of stock standard solution.

Compound	Concentration range in calibration samples (µg/L)
2-methylpropanal	2.07 – 59.83
2-methylbutanal	2.15 – 39.22
3-methylbutanal	5.24 – 57.20
hexanal	2.16 – 36.01
furfural	0.25 – 14.72
methional	0.21 – 14.64
phenylacetaldehyde	2.11 – 33.46
trans-2-nonenal	0.55 – 20.80

Calibration of all selected marker aldehydes was performed via external calibration, whereas matrix effects were controlled by spiking internal standard to the samples (IS; 20 µg/L of 2-methylbutanal-d_{10} and 2 µg/L of benzaldehyde-d_6). IS was added to all types of samples, including calibration samples, malt extracts and quality control samples. 2-Methylbutanal-d_{10} was used to quantify 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, and hexanal, whereas benzaldehyde-d_6 was applied for quantification of furfural, methional, phenylacetaldehyde, and *trans*-2-nonenal. Differences among internal standard peak areas did not exceed 7% (for both deuterated aldehydes) across different calibration points and malt extracts (data not shown). This indicates that the matrix has only a minor effect on volatilisation and subsequent quantification.

The method was evaluated in terms of linearity, goodness of fit, limit of detection (LOD), limit of quantification (LOQ), accuracy, and repeatability[209–211]. Linearity was determined by fitting the linear calibration curve y = ax + b, where peak area ratio (y) was plotted as a function of analyte concentration (x). Goodness of fit (R) was based on the square root of calculated coefficient of determination (R^2). LOD was determined as $3S_b/S$, where S_b is the standard error of the intercept of the vertical axis and S is the slope of the calibration curve. LOQ was calculated as $10S_b/S$. Accuracy was expressed as the mean value of the recovery (%) of 10 separate replicates, thus samples containing Milli-Q water and authentic reference compounds in concentrations expected in pale lager malts (14.54 µg/L of 2MP, 13.47 µg/L of 2MB, 30.71 µg/L of 3MB, 5.83 µg/L of HEX, 3.22 µg/L of FUR, 2.29 µg/L of MET, 12.79 µg/L of PHE, 3.35 µg/L of T2N). Repeatability was presented as the relative standard deviation (RSD in %) of 10 separate replicates analysed under the same conditions.

2.2.4. Investigation of extraction conditions for the analysis of aldehydes in malt

In all performed experiments, prior to HS-SPME-GC-MS analysis, malt extract was prepared in a standardised manner by mixing malt grist (fine milling applied by analytical mill A10, IKA, UK) with N_2-flushed Milli-Q water for a certain period of time at a set temperature. The mixture was prepared in a serum bottle closed with a sealed, aluminium crimp cap. Then, after 15 min of sedimentation, 10 mL of suspension were transferred to a 20 mL GC-vial. The vial was closed with sealed, aluminium crimp cap and the sample was further subjected to HS-SPME-GC-MS analysis. All samples, except for samples in the 'oxygen study' and

'extraction temperature study', were prepared under nitrogen gas in an anaerobic workstation. Five extraction parameters were investigated: sample amount, extraction time and temperature, ultrasonication time and extraction in oxygen-limited conditions. In order to study the influence of sample amount on extraction of aldehydes, 50 g of malt were freshly milled, out of which 0.5 g, 1.0 g, 2.0 g, 3.0 g, 5.0 g or 10.0 g were mixed with 100 mL of N_2-flushed Milli-Q water. The impact of extraction temperature was evaluated by adding previously heated Milli-Q water (20°C, 40°C, 50°C, 60°C or 70°C, respectively) to the milled malt. The contact stage was maintained at the desired temperature during the full extraction time. Before transferring extract to a GC-vial, the solution was immediately cooled down to approx. room temperature using an ice bath. The effect of stirring time on aldehyde extraction was examined by stirring the extract for 0 min, 5 min, 10 min, 15 min, 30 min, 45 min, 60 min or 90 min, respectively. The results were compared with ultrasonication for 0.5 min, 1 min, 2 min, 5 min or 15 min, respectively. Finally, the influence of oxygen-limited conditions on extraction of aldehydes was investigated by carrying out sample preparation under oxygen-limited conditions in an anaerobic workstation (120 ± 43 ppb of O_2 measured by c-TPO, Haffmans/Pentair, Germany) and under aerobic conditions, respectively.

2.2.5. Evaluation of the salting-out effect on SPME extraction of aldehydes

The salting-out effect was studied by adding 0.5 g, 1.0 g or 2.0 g of NaCl to corresponding GC-vials prior to transferring 10 mL of malt extract and subjecting the sample for HS-SPME-GC-MS analysis. Malt extract was prepared according to the optimised sample preparation parameters (including sample amount, extraction time and temperature, as well as oxygen-limited conditions).

2.2.6. Analysis of method intermediate precision of optimised malt extraction, combined with HS-SPME-GC-MS analysis

In order to assess the intermediate precision of the method, an extract of a pale lager malt was prepared according to the optimised conditions in this chapter (see results section 2.3.4) Next, 10 mL of the supernatant were transferred to 20 mL GC-vial and were subjected to HS-SPME-GC-MS analysis. The analysis was performed in triplicate. The above described procedure (including preparation of malt extract and determination of aldehydes) was repeated seven times, by two independent researchers, on the same equipment, over a period of approx. 2 months. From each series (one malt extract and three GC-MS measurements), a mean value was calculated. The intermediate precision was based on the relative standard deviation (RSD) from the mean values of the seven repetitions [209, 211].

2.2.7. Method application to various industrial pale malts

All malt samples were provided by Boortmalt Antwerp, Belgium.

The optimised extraction method followed by HS-SPME-GC-MS analysis was applied on six industrial-scale pale malts: A - six-row winter barley Etincel, produced according to the pale malt standard malting procedure; B - six-row winter barley Etincel, kilning-off at 95°C; C - two-row spring barley Passarel, with additional sulphuring during drying of green malt; D - two-row spring barley Passarel, produced according to the pale malt standard malting procedure; E - two-row spring barley Irina, with asphyxiation of green malt prior to drying;

F - two-row spring barley Irina, produced according to the pale malt standard malting procedure. The applied kilning-off temperature of the standard protocol was 85°C.

2.2.8. Determination of moisture content of malt

For all analysed malt samples, moisture content was determined according to the EBC 4.2 method in order to express the obtained aldehyde concentrations on a dry weight basis.

2.2.9. Determination of the content of cysteinylated aldehydes by UPLC-MS

The following cysteinylated aldehydes were determined in pale malt samples: 2-isopropylthiazolidine-4-carboxylic acid (2MP-CYS), 2-(sec-butyl)thiazolidine-4-carboxylic acid (2MB-CYS), 2-isobutylthiazolidine-4-carboxylic acid (3MB-CYS), 2-(2-(methylthio)ethyl)thiazolidine-4-carboxylic acid (MET-CYS), 2-benzylthiazolidine-4-carboxylic acid (PHE-CYS), 2-(furan-2-yl)thiazolidine-4-carboxylic acid (FUR-CYS), and 2-pentylthiazolidine-4-carboxylic acid (HEX-CYS). Determination was carried out by ultra-high-performance liquid chromatography (UPLC) coupled with mass spectrometry (MS) according to the protocol described by Bustillo Trueba *et al.*[33,207]. For the extraction of cysteinylated aldehydes from malt, 5 g of freshly and finely milled malt was mixed with 95 g of Milli-Q water. The solution was agitated at 200 rpm for 5 min at ambient temperature (Grant-Bio Multifuncional PSU20-I Orbital Shaker, UK). Then the mixture was centrifuged primarily at 9,000 rmp for 10 min at 10°C (Hettich Zentrifugen Universal 320R, Germany), and secondly at 11,000 rmp for 5 min at 10°C (Eppendorf centrifuge 5424R, Belgium). The supernatant was filtered through a SPARTAN HPLC Syringe filter (regenerated cellulose, 13 mm syringe filter, 0.2 µm pore size). An aliquot of 1 mL was transferred to a 2 mL amber LC vial closed with a screw cap with PTFE septum. Calibration curves were prepared by dissolving standards (synthesised by Paula Bustillo Trueba *et al.*[207]) in a solution of Carmody buffer (pH = 9) in a concentration range 1-1,000 µg/L. For each cysteinylated aldehyde, linear calibration fittings were obtained.

2.2.10. Method adaptation for malting samples of varying moisture content

Barley and samples coming from different stages of malting process such as green malt, partially kilned malt as well as finished malt, vary to a high extent in biochemical properties, water content, and friability. These affect milling properties of the material, resulting in *e.g.* formation of a heterogeneous paste while grinding green malt, which may lead to reduced availability of aldehydes for extraction. Therefore, in order to facilitate comparison of data obtained on different types of malting samples, a freeze-drying technique, also applied by other researchers on green malt and spent grains[110,177,212], was tested on three types of malting samples, containing high (43.1%), moderate (13.1%), and low moisture (4.7%) content, respectively. Samples were milled and freeze-dried for 2h, 4h, 6h, 8h or 10h, respectively. The water content (see 2.2.8), levels of free aldehydes (according to the optimised HS-SPME-GC-MS method) and levels of cysteinylated aldehydes (see 2.2.9) were determined. Milling and sample transfer operations were carried out at low temperature (approx. -20°C) to minimise the loss of compounds and to prevent the formation of artefacts.

Freshly collected samples were immediately frozen at -20°C. Further sample transport and storage were also conducted at this low temperature. The sample preparation procedure

began with 250 g of grain being submerged in liquid nitrogen. Next, frozen kernels were milled and the slurry was immediately transferred to six round-bottom flasks (each containing approx. 40 g of milled grain), closed with parafilm, and stored at -20°C. Then, the material was freeze-dried for 2h, 4h, 6h, 8h or 10h, respectively. After this time, it was milled for the second time. All malt powders were stored in amber glass, nitrogen flushed containers at -20°C.

2.2.11. Statistical analysis

Results are presented as mean values (n=3). For each individual aldehyde, quantified concentration values were expressed as the % of the maximum value obtained under particular conditions of the extraction. This allowed a more comprehensive comparison among the results obtained for all compounds since free aldehydes in malt are present in different concentration ranges. Statistical significance was analysed by the Student's t-test (comparing two variables) and by One-way ANOVA followed by Tukey's HSD post-hoc test (comparing more than 2 variables). In both cases, p-value ≤0.05 was considered statistically significant. Following software was used for the statistical analysis: Excel 365 (Microsoft, USA) and SPSS Statistics 26 (IBM, USA).

2.3. Results and discussion

2.3.1. Detection and quantification of aldehydes in aqueous model solutions via HS-SPME-GC-MS

Calibration was based on model solutions, where reference compounds were spiked at different concentrations into N_2-flushed Milli-Q water. For each marker aldehyde, a 7-point calibration curve was plotted and evaluated with respect to: linearity (slope, intercept, standard error of the slope and intercept, correlation coefficient), LOD, LOQ, precision, and accuracy (see 2.2.3). Calibration curves obtained for all carbonyls of interest were of linear character in the concentration ranges expected for pale malts, which was indicated by correlation coefficients values (≥ 0.9988) (see Table 2-3). LOD and LOQ values depended on the analysed compound and ranged from 0.28 µg/L to 0.99 µg/L and 0.92 µg/L to 3.31 µg/L, respectively. Precision, presented as the relative standard deviation (%) of 10 replicates (solution of water spiked with a known concentration of reference compounds in concentrations to be expected in pale malts), did not exceed 5.3%, demonstrating repeatability within the sample. Accuracy (trueness of the measurement based on 10 replicates) deviated by max. ± 5%.

Table 2-3. Validation of method for quantification of free aldehydes in model solution in concentration ranges expected in pale lager malts via HS-SPME-GC-MS.

Compound	Range	Linearity					Limit of detection	Limit of quantification	Repeatability	Accuracy
		Sensitivity	Intercept	Standard error of slope	Standard error of intercept	Correlation coefficient				
	(µg/L)	$(\mu g/L)^{-1}$					(µg/L)	(µg/L)	%	%
2MP	0.00 - 59.83	0.894	-0.297	0.006	0.150	0.9999	0.50	1.68	4.8	105
2MB	0.00 - 39.22	1.364	-0.523	0.013	0.237	0.9998	0.52	1.73	2.3	102
3MB	0.00 - 57.20	2.556	-0.419	0.028	0.847	0.9997	0.99	3.31	3.4	103
HEX	0.00 - 36.01	3.148	-0.943	0.024	0.378	0.9999	0.36	1.20	5.3	95
FUR	0.00 - 14.72	0.747	0.352	0.009	0.069	0.9995	0.28	0.92	3.4	103
MET	0.00 - 14.64	0.104	0.003	0.001	0.010	0.9994	0.30	0.99	5.2	95
PHE	0.00 - 33.46	0.040	0.006	0.001	0.006	0.9998	0.46	1.53	4.4	96
T2N	0.00 - 20.80	13.794	-6.102	0.298	2.805	0.9988	0.61	2.03	1.3	99

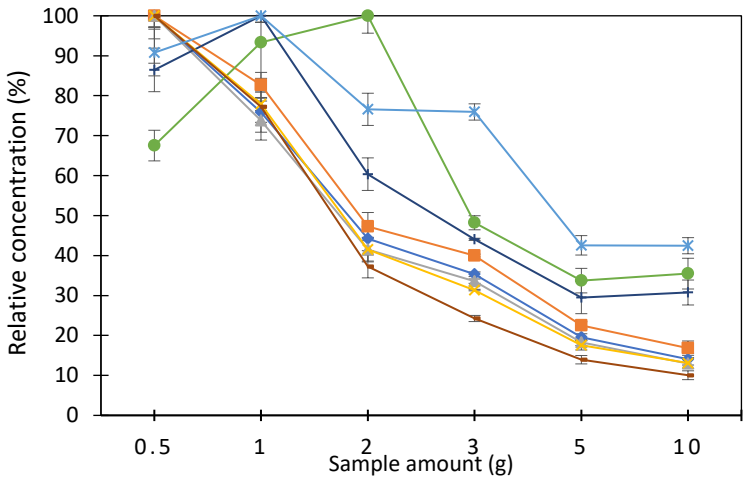
2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, T2N = *trans*-2-nonenal.

2.3.2. Investigation of extraction conditions for the analysis of aldehydes in malt

In order to optimise the sample preparation technique, we aimed at the highest possible extraction rate of selected marker aldehydes present in pale malt. Nevertheless, the extraction might be affected by the differences among aldehydes, such as their physico-chemical properties (*e.g.* volatility, polarity, *etc.*) and chemical structure (*e.g.* short-chain, aromatic ring, saturation, *etc.*). Therefore, in order to minimise the possibility of favouring extraction of any particular aldehyde during sample preparation, as well as to prevent *de novo* formation of aldehydes, every extraction parameter was investigated individually.

2.3.2.1. Sample amount

In order to achieve a high extraction yield and high sample homogeneity, the effect of the amount of malt taken for sample preparation was investigated. The content of free aldehydes was evaluated in samples containing 0.5 g to 10.0 g of milled pale malt in 100 mL of N_2-flushed Milli-Q water (see Section 2.2.4). For each determined aldehyde, as illustrated in Figure 2-5, a decrease in the efficiency of extraction was observed, when 2.0 g or more of milled malt was taken. Most of the investigated aldehydes (5 out of 8), reached the highest extraction rate at 0.5 g/100 mL. However, some compounds (phenylacetaldehyde, furfural and, in particular, methional) reached their maximum extraction concentrations when somewhat larger sample size was applied (1.0 g - 2.0 g). Aiming at high sample homogeneity and, thus representativeness, as well as maximal extraction of aldehydes, a consensus had to be reached regarding sample size selection. As presented in Figure 2-5, while applying 1.0 g of malt in 100 mL of water, the difference (spread) between the highest and the lowest point on the y-axis (relative concentration recalculated to malt dry mass) is the smallest. As a result, this sample size was selected for further optimisation of the extraction procedure.



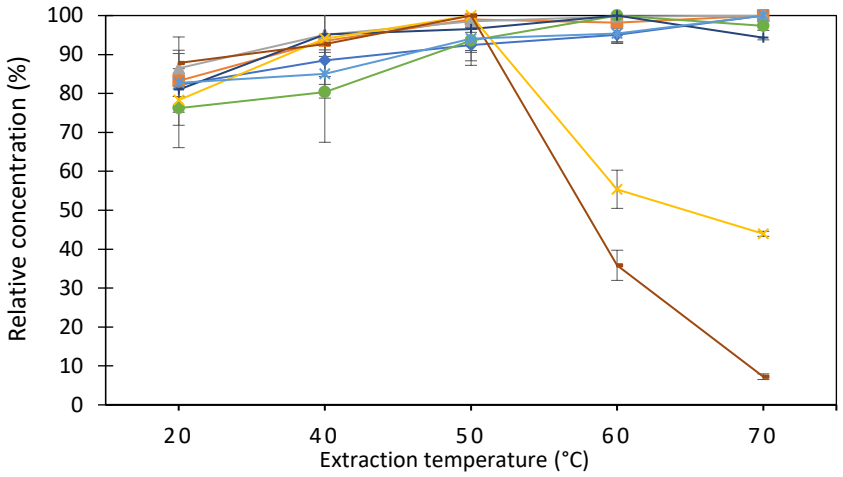
Aldehyde	0.5 g	1 g	2 g	3 g	5 g	10 g
2MP	a	b	c	d	e	e
2MB	a	b	c	d	e	e
3MB	a	b	c	c	d	d
MET	a	b	b	c	d	c d
PHE	a	b	c	d	e	e
HEX	a	b	c	d	e	e
T2N	a	b	c	d	e	e
FUR	a	b	c	c	d	d

Figure 2-5. Influence of sample amount on the extraction of free aldehydes from pale malt samples.

Expressed as % of the maximum concentration (concentration of the compound/concentration of the corresponding internal standard). Horizontal axis indicates the sample amount in grams. Statistical analysis of concentration of aldehydes for each compound is by HSD Tukey's, which distinguishes statistically different ($p \leq 0.05$) mean values (groups a, b, c, d, e).

2.3.2.2. Extraction temperature

In this section, extraction of aldehydes in temperature ranging from 20°C up to 70°C is evaluated (see Section 2.2.4). This experiment, in contrary to others, have been carried out in aerobic conditions due to technical reasons. In general, concentrations of individual marker aldehydes increased with an increase in extraction temperature, except for hexanal and *trans*-2-nonenal (see Figure 2-6). A considerable decrease at elevated temperatures (60°C and 70°C) in levels of these fatty acid oxidation aldehydes may be related to partial inactivation of catalysing the oxidation lipoxygenase enzymes[173], as well as to the lower oxygen content in warmer water (the experiment was carried out in aerobic conditions). Levels of all aldehydes reached maximum values at 50°C, however we suspect that this may be due to *de novo* formation of carbonyl compounds, in particular, furfural, hexanal, and *trans*-2-nonenal. For example, Maillard reactions, proceeds faster at higher temperatures (*e.g.* >50°C), enhancing formation of furfural[92]. Also, enzymatic oxidation of unsaturated fatty acids may be promoted at temperatures close to the conditions regarded as optimal for lipoxygenase enzymes (47°C, pH=6)[213]. The effect of temperature on the extraction of Strecker aldehydes was negligible; no significant difference was found while carrying out the extraction at 20°C and 40°C (group a), as well as at 50°C, 60°C and 70°C (group b and/or group c). Considering behaviour of all studied compounds, and in particular, because of relatively high extraction rates, 20°C was chosen as temperature for extraction of aldehydes from malt samples.



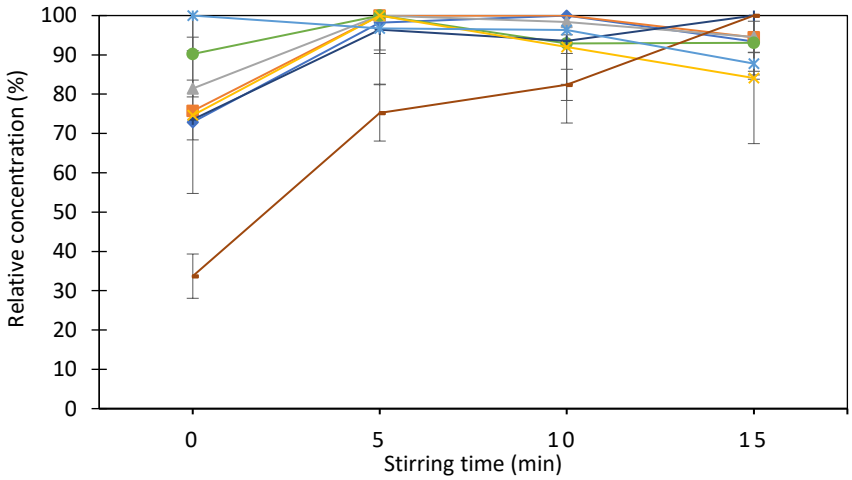
Aldehyde	20°C	40°C	50°C	60°C	70°C
2MP	a	a b	a b c	b c	c
2MB	a	a b	b	b	b
3MB	a	a b	b	b	b
MET	a	a b	a b	b	a b
PHE	a	b	b	b	b
HEX	a	a b	b	c	c
T2N	a	a	a	b	c
FUR	a	a b	a b c	b c	c

Figure 2-6. Effect of temperature on extraction of free aldehydes from pale malt samples.

Horizontal axis indicates extraction temperature in Celsius degrees (°C). Statistical evaluation of concentration of aldehydes for each compound is by HSD Tukey's, which distinguishes statistically different ($p \leq 0.05$) mean values (groups a, b, c).

2.3.2.3. Stirring time

The influence of stirring time (0 min - 90 min) (see also Section 2.2.4) on the extraction of free aldehydes from pale malt was investigated. Within the first 5 min of extraction, concentrations of all studied aldehydes reached their maximum value, except for *trans*-2-nonenal (see Figure 2-7). The highest measured value of *trans*-2-nonenal was determined after 15 min of stirring. Moreover, stirring for longer than 15 min had an insignificant effect on the extraction of all selected free aldehydes (data not shown). Therefore, aiming at the highest extraction of all studied aldehydes from malt matrix, 15 min was selected as the preferred stirring time.



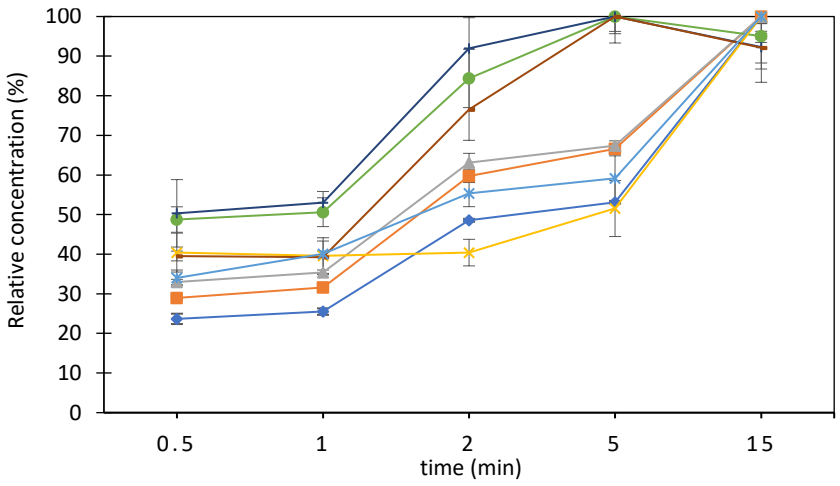
Aldehyde	0 min	5 min	10 min	15 min
2MP	a	b c	c	b
2MB	a	b	b	c
3MB	a	b	b	c
MET	a	a	a	a
PHE	a	b	b	b
HEX	a	a	a	a
T2N	a	b	b	c
FUR	a	a b	a b	b

Figure 2-7. Influence of stirring time on extraction of free aldehydes from pale malt samples.

Horizontal axis indicates the stirring time in minutes. Statistical evaluation of concentration of aldehydes for each compound is by HSD Tukey's, which distinguishes statistically different ($p \leq 0.05$) mean values (groups a, b, c).

2.3.2.4. Ultrasonication vs stirring

Ultrasonication is a well-established method for improved extraction of various components since the effect of acoustic cavitation facilitates release of compounds and accelerates mass transport between biological cells and solvent[214]. Therefore, different ultrasonication times (0.5 min - 15 min) (see 2.2.4) were tested on aldehyde extraction from malt. As presented in Figure 2-8, concentrations of all quantified aldehydes increased as a function of time. In comparison to the other evaluated aldehydes, extraction of phenylacetaldehyde, methional, and *trans*-2-nonenal seem to improve from the first moment of ultrasonication, to reach maximum after 5 min and remine at a high level after 15 min. Conversely, levels of 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, hexanal, and furfural increased significantly ($p \leq 0.05$) over the time of ultrasonication. Therefore, since the highest levels of all aldehydes were obtained after 15 min, this ultrasonication time was selected as preferred. In addition, when comparing ultrasonication for 15 min with vigorous stirring for 15 min (which previously was selected as an optimal extraction time (see Section 2.3.2.3)), stirring resulted in higher levels of 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, and methional. Consequently, stirring for 15 min was selected as a preferred mixing/extraction method.



Aldehyde	0.5 min	1 min	2 min	5 min	15 min
2MP	a	a	b	c	d
2MB	a	a	b	c	d
3MB	a	a	b	c	d
MET	a	a	b	c	b c
PHE	a	a	b	b	b
HEX	a	a	a	a	b
T2N	a	a	b	c	b c
FUR	a	a	b	b	c

Figure 2-8. Influence of ultrasonication time on extraction of free aldehydes from pale malt samples.

Horizontal axis indicates extraction time in minutes. Statistical evaluation of concentration of aldehydes for each compound is by HSD Tukey's, which distinguishes statistically different ($p \leq 0.05$) mean values (groups a, b, c, d).

2.3.2.5. Oxygen-limited conditions vs ambient conditions

Oxygen plays an important role in several chemical pathways resulting in formation of various aldehydes. For example, oxidative reactions may lead to the formation of hexanal and *trans*-2-nonenal (oxidation of unsaturated fatty acids)[17], as well as to generation of Strecker aldehydes *i.a.* phenylacetaldehyde, 2-methylbutanal, and 3-methylbutanal (via direct oxidation of amino acids)[83]. Therefore, in this section we have investigated to what extent ambient conditions could impact formation of aldehydes (artefacts) when extracting carbonyls from malt, compared to performing sample preparation under oxygen-limited conditions.

Results presented in Figure 2-9, show that sample preparation carried out under ambient conditions affected 5 out of 8 marker aldehydes. In particular, clearly higher levels of hexanal (approx. 3-fold) were measured in samples prepared under ambient conditions. This may be explained by *de novo* formation of hexanal during extraction. Moreover, at ambient conditions higher levels of furfural and methional were measured, whereas lower levels of phenylacetaldehyde and *trans*-2-nonenal. Regarding the latter, at this stage the explanation of these results is not straightforward. Nevertheless, based on the overall picture of all studied staling aldehydes, sample extraction under oxygen-limited conditions prevents/minimises formation of the so-called artefacts, thus it has been selected as an optimised extraction parameter.

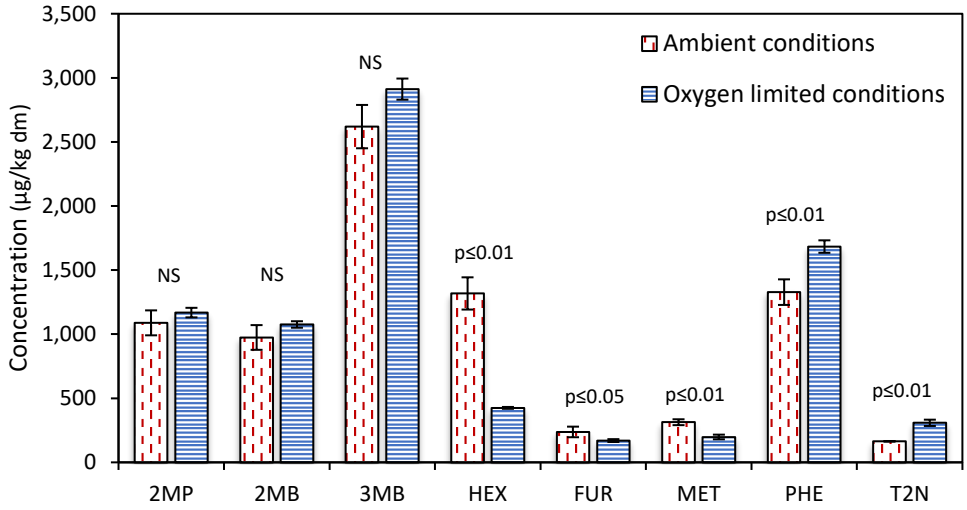


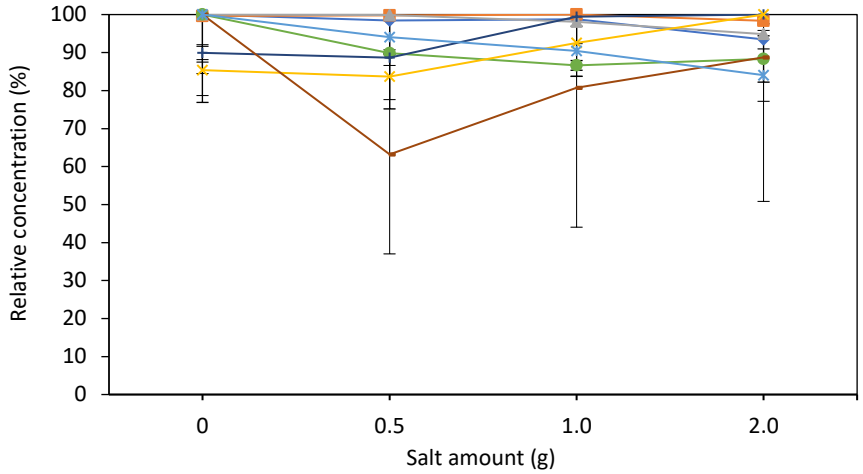
Figure 2-9. Influence of oxygen-limited vs ambient conditions during sample preparation on extraction of marker aldehydes from pale malt.

Statistical comparison is presented as the result of the Student's t-test with a confidence level of 0.95; NS – non-significant difference; x-axis – aldehydes; y-axis – concentration of aldehydes.

2.3.3. Evaluation of salting-out effect on SPME extraction of aldehydes

Addition of sodium chloride to sample prior to SPME analysis of volatile compounds is a commonly applied technique to increase extraction from headspace, as the ionic strength influences partition coefficient between gas phase and liquid phase[110,215]. Therefore, the effect of the addition of different amounts of NaCl to the GC-vial (0.5 g - 2.0 g of NaCl) with malt extract was tested (see Section 2.2.5).

The results show that the addition of NaCl do not have a major impact on levels of marker aldehydes, as there was no significant difference between analysed variants (group a) (see Figure 2-10). Furthermore, in the case of *trans*-2-nonenal, standard deviation between replicates exceeded 20%, when NaCl was added. As a result, addition of NaCl was rejected for the further experiments.



Aldehyde	0 g	0.5 g	1.0 g	2.0 g
2MP	a	a	a	a
2MB	a	a	a	a
3MB	a	a	a	a
MET	a	a	a	a
PHE	a	a	a	a
HEX	a	a	a	a
T2N	a	a	a	a
FUR	a	a	a	a

Figure 2-10. Influence of the salting-out effect on the extraction of free aldehydes from pale malt.

Horizontal axis indicates the amount of NaCl added to the GC-vial in grams. Statistical evaluation of concentration of aldehyde for each compound is by HSD Tukey's, which distinguishes statistically different ($p \leq 0.05$) mean values (group a).

2.3.4. Analysis of method intermediate precision

The aim was to estimate the method intermediate precision by applying the optimised sample preparation procedure (1.0 g of fine milled pale malt in 100 mL of N_2-flushed Milli-Q water, extracted for 15 min at 20°C, under oxygen-limited conditions) on a pale malt sample (for details see, Section 2.2.6). Intermediate precision measured as relative standard deviation (RSD) (n=7), for all compounds, have not exceed 20% (see Table 2-4). This is a satisfactory outcome, especially since malt during storage may undergo a variety of (bio)chemical transformations. Hence, the RSD values might be considered as a combined result of method variance, and changes in the volatile composition of the malt sample during malt storage.

Table 2-4. Evaluation of method intermediate precision.

Parameter	2MP	2MB	3MB	HEX	FUR	MET	PHE	T2N	SUM
Concentration (µg/kg dm)	1,508	1,330	3,177	641	317	222	1,243	290	8,828
RSD (%)	17	18	15	12	14	16	9	15	11

RSD – relative standard deviation of analysis repetition (n =7) on the same pale malt sample.

2.3.5. Method application on industrial-scale pale malts

As previously shown by other authors[7,8,32], malt may be considered as a primary source of staling aldehydes introduced with raw materials to the brewing process. Consequently, adequate quantitative determination of these compounds in malt is of primary importance in relation to beer quality.

In this part of the thesis, the optimised extraction procedure, followed by HS-SPME-GC-MS, was applied on various industrial-scale, pale malt samples (A-F), in order to compare their free aldehyde profiles. For details on the samples see section 2.2.10. The results, summarised in Figure 2-11, show statistically significant variations between different malt samples in relation to the levels of individual aldehydes. In general, the sum of free aldehydes varied among all samples from 8,064 µg/kg dm (sample A) to 23,515 µg/kg dm (sample B). Regarding individual aldehydes, 3-methylbutanal was always found in the highest concentrations (varying from 2,834 µg/kg dm to 8,218 µg/kg dm), while *trans*-2-nonenal in the lowest (from 210 µg/kg dm to 580 µg/kg dm). This is in accordance with the outcomes of De Clippeleer *et al*.[111], who also measured beer staling aldehydes in malt applying a similar analytical method (HS-SPME-GC-MS on a malt extract).
Strecker aldehydes were found in the subsequent ranges: 2-methylpropanal: 1,190-3,469 µg/kg dm, 2-methylbutanal: 980-2,834 µg/kg dm, 3-methylbutanal: 2,834-8,218 µg/kg dm, methional: 383-1,298 µg/kg dm, and phenylacetaldehyde 481-5,105 µg/kg dm, respectively. These ranges are similar to the ones reported by other authors, regardless of the applied analytical method[6,111–113]. Moreover, the analysis of Strecker aldehydes showed that, independent of a malt sample, there is a positive correlation among all Strecker aldehydes, *i.e.* the higher the concentration of 3-methylbutanal, the higher the concentration of the remaining Strecker aldehydes. Correlation coefficients (calculated based on the data of the six analysed in this section malts) of 3-methylbutanal concentration as a function of individual Strecker aldehydes were as follow: 0.99 for 2-methylpropanal, 0.99 for 2-methylbutanal, 0.84 for methional, and 0.93 for phenylacetaldehyde. Similarity in the behaviour of Strecker aldehydes may be explained by

analogous formation pathways and by related chemical nature of the involved compounds[17]. The highest content of 3-methylbutanal and, therefore, Strecker aldehydes was determined in sample B while the lowest in sample A (it is to be expected as sample B received the highest heat load being kilned-off at 95°C).

Furthermore, concentration of Maillard reaction product – furfural – ranges from 391 µg/kg dm to 1,116 µg/kg dm. In samples A, C, D, F the measured levels were similar, in contrast to malts B and E, when values were significantly higher ($p \leq 0.05$). This could be explained by the higher processing temperatures during malting of B (kilning-off at 95°C) and E (no cooling during grain asphyxiation) since higher heat load index was found to be related to the increased furfural levels[26].

Regarding fatty acid oxidation aldehydes, higher amounts of hexanal were found in samples A, C, D, F, whereas levels in malts B and especially E were significantly lower ($p \leq 0.05$). On the other hand, a significantly higher concentration ($p \leq 0.05$) of *trans*-2-nonenal was measured in malt B compared to other malts. A similar differential behaviour in testing pale malt samples between hexanal and *trans*-2-nonenl has been reported previously[6].

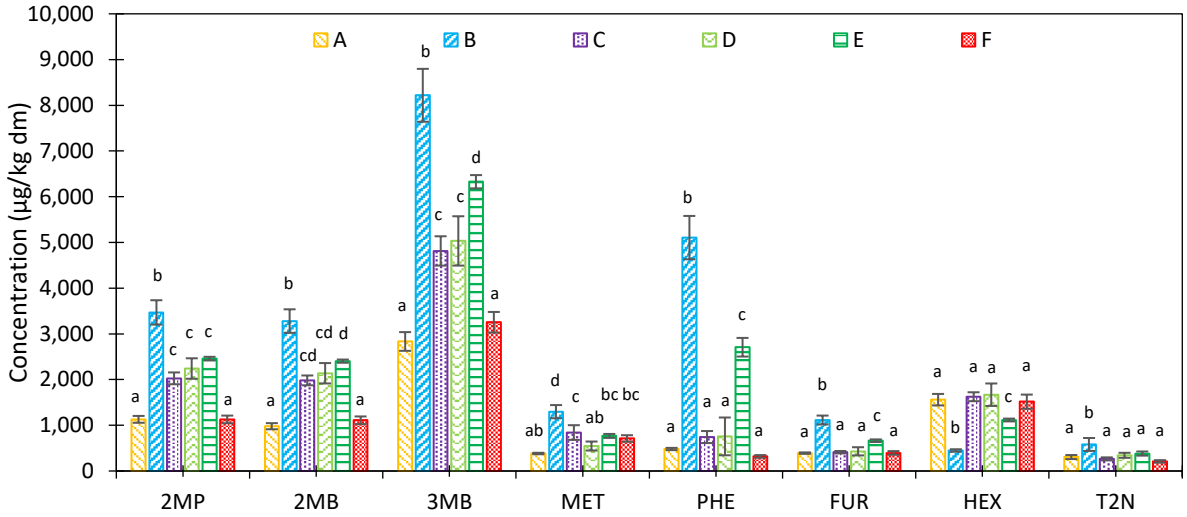


Figure 2-11. Levels of marker aldehydes (µg/kg dm) in various industrial scale, pale malts.

Statistical analysis of concentration of aldehydes for each compound is by HSD Tukey's, which distinguishes statistically different ($p \leq 0.05$) mean values (groups a, b, c, d). Malts: A - six-row winter barley Etincel, produced according to pale malt standard malting procedure; B - six-row winter barley Etincel, kilned-off at 95°C, C - two-row spring barley Passarel, with additional sulphuring during drying of green malt; D - two-row spring barley Passarel, produced according to pale malt standard malting procedure; E - two-row spring barley Irina, with asphyxiation of green malt prior to drying; F - two-row spring barley Irina, produced according to pale malt standard malting procedure. 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, T2N = *trans*-2-nonenal.

2.3.6. Method adaptation for samples of varying moisture content

Three types of samples coming from different stages of malt production (green malt, partially kilned malt, and finished malt) and varying in water content (43.1%, 13.1%, and 4.7%, respectively) were subjected, prior to malt extract preparation, to a 'pre-treatment step' - freeze-drying and subsequent milling. Each sample was freeze-dried for 0h, 2h, 4h, 6h, 8h or 10h to reduce its water content facilitating further grinding, which decreases particle size, and

as a consequence, should enhance extraction of compounds from malting samples. Determination of water content of freeze-dried samples, as well as measurements of free and cysteinylated aldehydes levels, were carried out.

Evolution of milling properties of each sample type was assessed by visual inspection with the focus on particle size (see Figures 2-12 to 2-14). Green malt, which originally contained approx. 43% of water was difficult to mill as it was creating an inhomogeneous paste containing large fragments of grain. Freeze-drying improved the milling properties (compare Figure 2-12-A and B) and resulted in a relatively homogeneous, fine powder (see Figure 2-12-C). Similarly, partially kilned malt, which originally contained approx. 13% of moisture, was inhomogeneous upon milling, as husk and endosperm were still distinguishable (see Figure 2-13-A). Freeze-drying, followed by milling enabled production of a relatively homogeneous powder (see Figure 2-13-B and C). In contrast, no improvement was observed in the case of finished malt (approx. 4.7%) as a result of freeze-drying (see Figure 2-14), since already the first milling of malt (see Figure 2-14A) resulted in a fine, homogeneous powder.

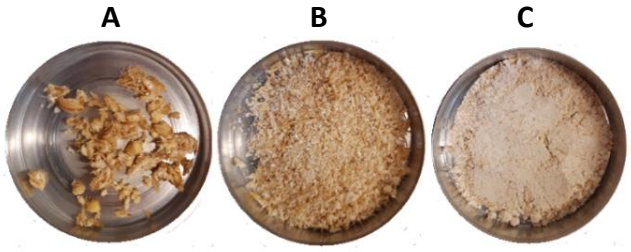


Figure 2-12. Visual analysis of freeze-dried and subsequently milled, green malt (original moisture content of approx. 43%) with focus on particle size.
Samples obtained after 0h (A), 6h (B) and 10h (C) of freeze-drying and subsequent milling.

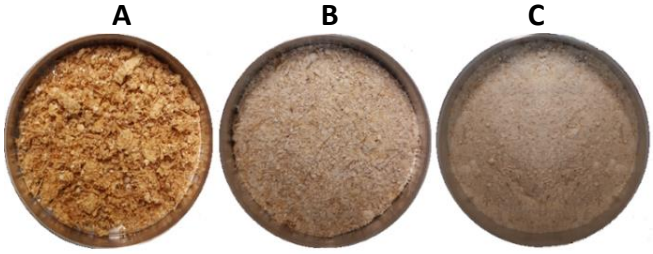


Figure 2-13. Visual analysis of freeze-dried and subsequently milled, partially kilned malt (original moisture content of approx. 13%) with focus on particle size.
Samples obtained after 0h (A), 6h (B) and 10h (C) of freeze-drying and subsequent milling.

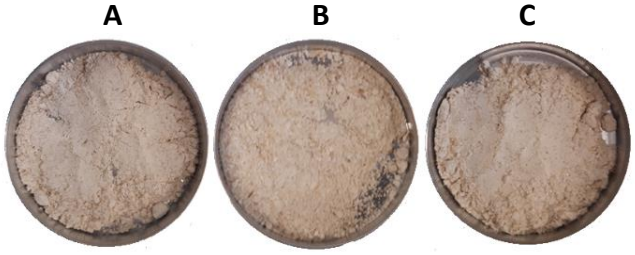


Figure 2-14. Visual analysis of freeze-dried and subsequently milled finished malt (original moisture content of 4.7%) with focus on particle size.
Samples after 0h (A), 6h (B) and 10h (C) of freeze-drying and subsequent milling.

Freeze-drying gradually decreased moisture content of all subjected samples (see Figure 2-15). During 10h of treatment, moisture of green malt decreased from 43.1% to 2.6%, while moisture content of partially kilned malt and finished malt decreased from 13.1% to 2.9%, and 4.7% to 1.7%, respectively.

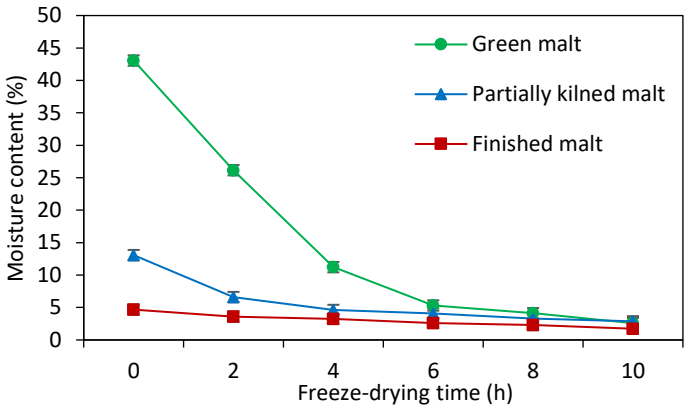


Figure 2-15. Decrease in moisture content during freeze-drying, determined in green malt, partially kilned malt and finished malt, respectively.

Free aldehydes were determined in all freeze-dried samples (see Figure 2-16). In the case of green malt, generally, the longer freeze-drying resulted in higher extraction of free aldehydes from the prepared malt powder, except for methional and furfural, which were not detected in any sample (<LOD). The highest values were obtained in the samples which were subjected to freeze-drying for 8h and 10h, and no significant differences between the values obtained for these two samples were noticed. The observed increase in the determined values of aldehydes is most likely due to the decrease in particle size upon milling (see Figure 2-12), thus increased surface area and a more efficient extraction during sample preparation for HS-SPME-GC-MS analysis. Moreover, the proposed pre-treatment (lyophilisation with subsequent milling) allowed detection of 2-methylbutanal, which was not detected in non-freeze-dried (0h) and shortly freeze-dried (2h - 4h) samples. The highest extraction of aldehydes in the shortest time of pre-treatment was achieved after 8h of green malt freeze-drying (4.1% of moisture content). Similar outcomes were obtained for partially kilned malt, for which initial moisture was at a moderate level (13.1%). Thus, again, methional and furfural were not detected (<LOD) in any sample, while the content of other extracted aldehydes increased up to 6h of freeze-drying. Therefore, freeze-drying of partially kilned malt for 6h, resulting in moisture content of approx. 4.1% was found to be optimal. In contrast, freeze-drying of finished malt showed no significant effect on the content of determined aldehydes (as mentioned before, the non-lyophilised finished malt sample already formed a homogeneous powder). The above results show that implementation of a pre-treatment step (submersion in liquid nitrogen, cold grinding, and freeze-drying with subsequent milling) prior to malt extract preparation for HS-SPME-GC-MS, allows determination of higher levels of free aldehydes in intermediate malting samples that considerably vary in moisture content. Samples should be freeze-dried until approx. 4% of moisture content, since further lyophilisation and milling do not further contribute to the extraction of aldehydes. Besides, due to the volatility of free aldehydes, the impact of freeze-drying on the levels or composition of these compounds cannot be disregarded. Nevertheless, the increased homogeneity of the sample and enhanced extraction are in favour of applying the proposed pre-treatment method.

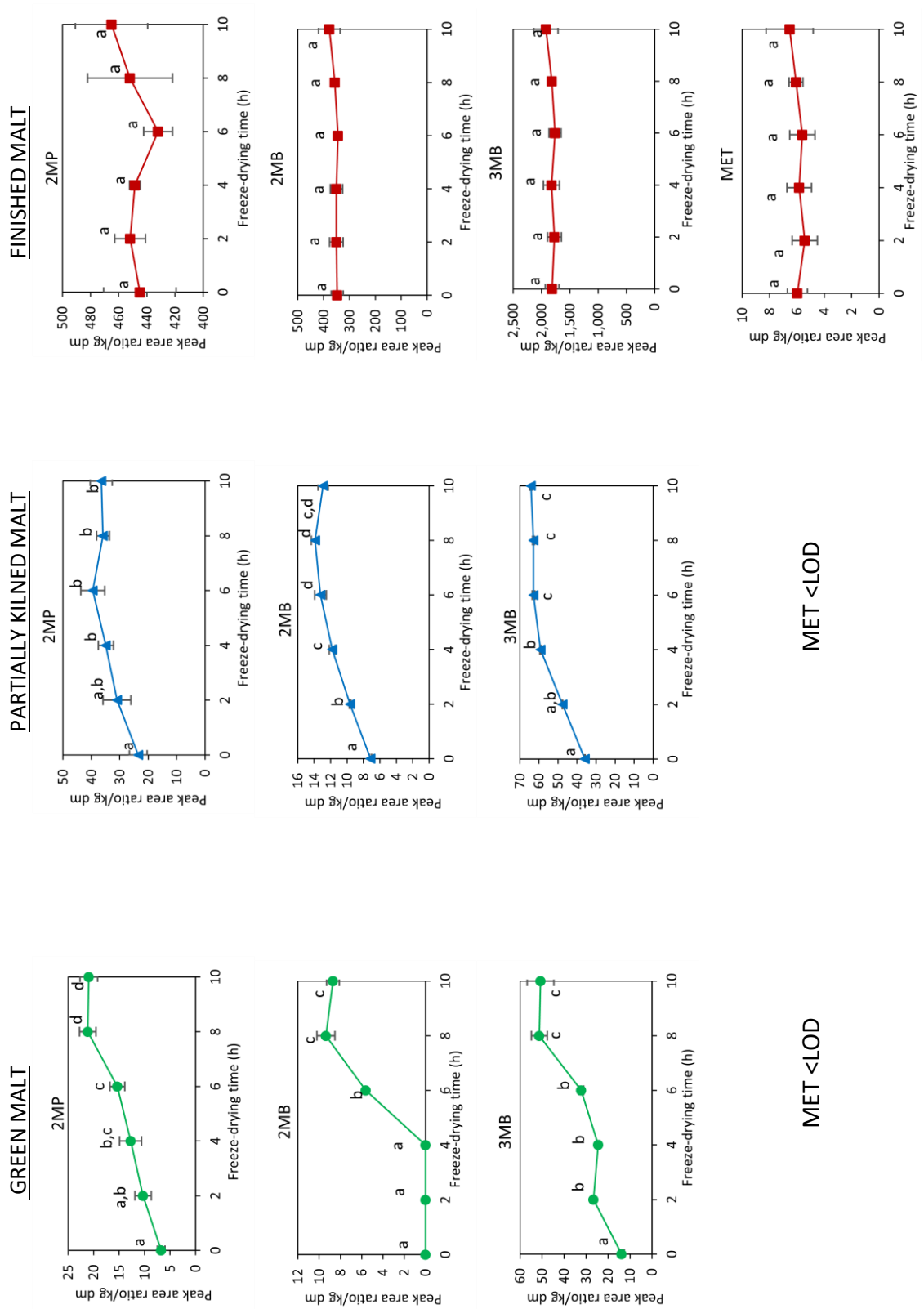


Figure 2-16A. Free aldehydes determined in green malt, partially kilned malt and finished malt as a function of freeze-drying time (h).

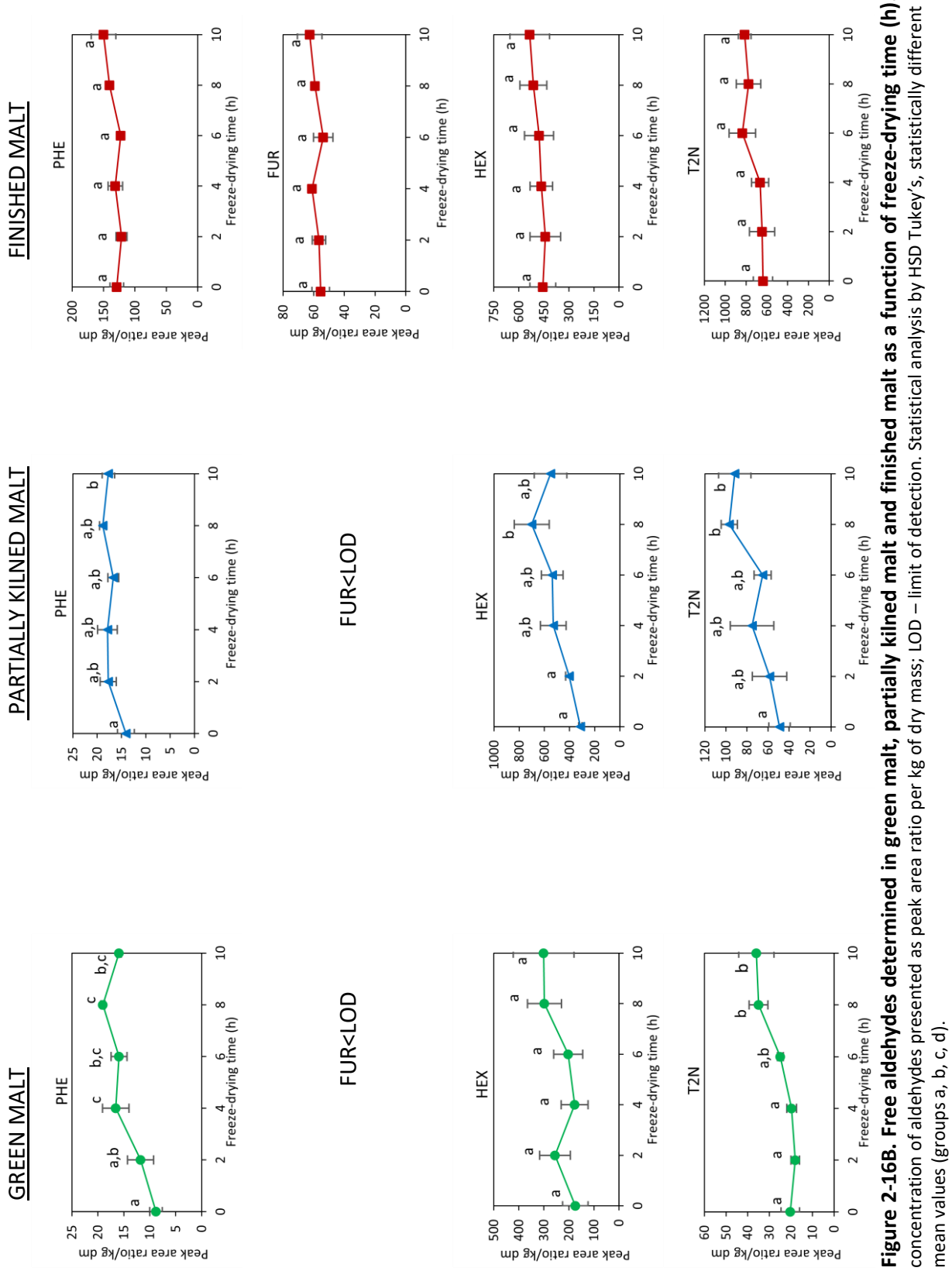


Figure 2-16B. Free aldehydes determined in green malt, partially kilned malt and finished malt as a function of freeze-drying time (h). y-axis - concentration of aldehydes presented as peak area ratio per kg of dry mass; LOD – limit of detection. Statistical analysis by HSD Tukey's, statistically different ($p \leq 0.05$) mean values (groups a, b, c, d).

Furthermore, cysteinylated aldehydes were determined in all freeze-dried samples (see Figure 2-17). In the case of green malt, cysteinylated aldehydes were below the limit of detection in all tested samples (0h - 10h freeze-drying treatment). Similar outcomes were obtained for the partially kilned malt, except cysteinylated hexanal, for which extraction increased till 6h of the pre-treatment. Again, this may be due to the decrease in particle size (see Figure 2-13), thus improved extraction. In the finished malt, six out of seven cysteinylated aldehydes were detected (all except cysteinylated furfural). Therefore, lyophilisation with subsequent milling did not significantly affect the determined content of these bound forms. In summary, lyophilisation up to a moisture content of approx. 4% and subsequent milling have been chosen as an optimal pre-treatment. Again, an effect of lyophilisation on the content and composition of cysteinylated aldehydes cannot be excluded. However, in contrast to volatile free aldehydes, bound forms are non-volatile, thus the effect of the pre-treatment should be of less significance.

Interestingly, data point to a significant increase in the levels of free aldehydes as a function of the malting process (see Figure 2-16), which may also explain why cysteinylated aldehydes were only quantifiable in finished malt samples.

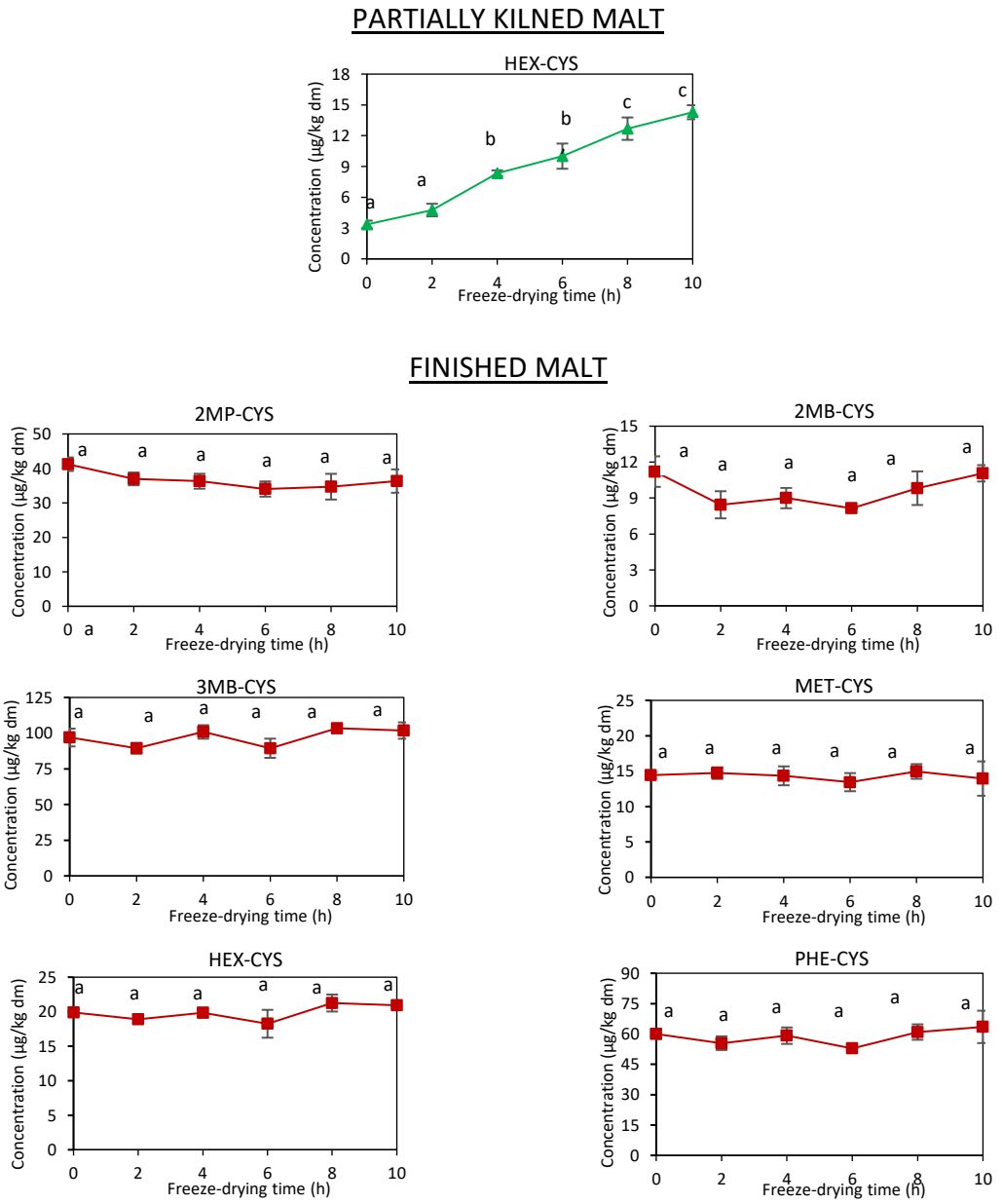


Figure 2-17. Cysteinylated aldehydes (µg/kg dm) determined in partially kilned malt and finished malt as a function of freeze-drying time (h).

Statistical analysis of levels of bound-state aldehydes as a function of freeze-drying time, for each individual compound by HSD Tukey's, distinguishes statistically different ($p \leq 0.05$) mean values (groups a, b, c).

2.4. Conclusions

Validation of HS-SPME-GC-MS method, for determination of staling aldehydes in pale malt using reference compounds, resulted in: very good linearity (R≥0.9988), low LOD (0.28 µg/L to 0.99 µg/L) and low LOQ (0.92 µg/L to 3.31 µg/L), satisfactory repeatability (1.3% to 5.3%) and accuracy (±5%). Furthermore, the effect of extraction conditions on the levels of free aldehydes determined in pale malt samples was evaluated regarding: sample amount, extraction time, extraction temperature, ultrasonication time, and oxygen concentration during extract preparation. The results showed that sample amount, time, temperature, oxygen concentration, and ultrasonication affect extraction, and thereby, determined concentration of marker aldehydes. While comparing different sample amounts, in the range from 0.5 g to 10.0 g of milled pale malt in 100 mL of N_2-flushed Milli-Q water, 1.0 g of fine milled malt was selected due to the high sample homogeneity and higher aldehyde extraction yield. Among extractions carried out at temperatures ranging from 20°C to 70°C, 20°C was chosen for further extraction optimisation in order to prevent the formation of artefacts. Various extraction times (0 min - 90 min) were studied, resulting in selection of 15 min, as any longer extraction had no significant effect on the measured aldehydes concentrations. The outcomes of the extraction time experiment were compared with ultrasonication for 0.5 min up to 15 min, which was found to significantly ($p \leq 0.05$) decrease the quantified values of: 2-methylpropanal, 2-methylbutanal, 3-methylbutanal and methional. Eventually, sample preparation under ambient and oxygen-limited conditions was compared. The results indicated potential *de novo* formation of aldehydes during malt extraction under ambient conditions. In conclusion, the optimisation of sample preparation resulted in the selection of oxygen-limited conditions, at which 1.0 g of milled pale malt in 100 mL of N_2-flushed Milli-Q water is stirred for 15 min at 20°C. Moreover, addition of NaCl (0.0 g - 2.0 g) prior to HS-SPME was disregarded since no positive effect of the addition of sodium chloride was observed. Furthermore, intermediate precision for the optimised sample preparation did not exceed 20% for any of the compounds studied. Consequently, the optimised method was applied on various industrial-scale pale malts, showing statistically significant differences in the concentrations of free aldehydes amongst the analysed malts. This indicates that the proposed method is suitable to perform a qualitative and quantitative study on the determination of staling aldehydes in pale malts. The methodology allows assessing the amounts of staling aldehydes that might be introduced to the brewing process. Moreover, the method was adjusted to analyse samples coming from different stages of the malting process, thus varying in moisture content. The proposed pre-treatment method (freeze-drying until 4% of moisture content, followed by milling) positively affected extraction of free and cysteinylated aldehydes. Even though possible losses and/or changes in aldehyde composition cannot be excluded, the increased homogeneity of sample and enhanced extraction are in favour for applying the lyophilisation pre-treatment method on the intermediate malting samples

Chapter 3

Explorative monitoring of the evolution of free and cysteinylated aldehydes during malting

3.1. Introduction

It is well-established that malt being one of the major brewing raw materials contributes to the performance of brewing process (*e.g*. by impacting filtration time) and impacts the quality of final product (*e.g.* by determining beer colour, mouthfeel, and foam stability). Moreover, malt may also contribute to beer flavour (in)stability, as it delivers to the brewing process various compounds with the risk to compromise beer staling, among them, compounds directly related to the appearance of off-flavours during beer storage, thus free, staling aldehydes[6,7,26,111–114].

When monitoring the evolution of free aldehydes throughout brewing process, the highest levels were found at the onset of mashing, indicating that malt may be the major source of staling compounds[7,8]. Further, during wort boiling[7,8] and fermentation[8,120,216] a dramatic decrease in levels of aldehydes has been reported, leading to very low concentrations in fresh beer. However, during storage levels of staling aldehydes gradually increase (likewise off-flavours)[2,8]. Consequently, the potential importance of staling aldehydes delivered with malt to the brewing process cannot be disregarded. More specifically, it has been hypothesised[56,81] that free aldehydes coming from malt may bind to other compounds during mashing (and possibly already during malting itself), thus changing their physicochemical properties (*i.e.* aldehydes become non-volatile and (probably) non-accessible to yeast), which may result in 'aldehyde survival' throughout the brewing stages, and, finally, their presence in a closed beer package, in the form of one or more type(s) of bound-state aldehyde adducts. Next, during beer transport and storage, bound form(s) may dissociate, thereby releasing free, volatile, and flavour-active aldehydes. The reaction could be enhanced by external factors, such as vibrations and/or increased temperature during transport[4,22], as well as conditions within the beer package, hence continually changing chemical equilibria within the beer matrix.

Furthermore, free aldehydes delivered with malt to the brewing process may reflect aldehyde precursors (*e.g.* amino acids), intermediate reactants (*e.g*. α-dicarbonyls), and catalysts (*e.g*. lipoxygenase enzymes), which contribute to *de novo* formation of aldehydes via, for example, Strecker degradation, Maillard reaction, oxidation of unsaturated fatty acids, and radical reactions[17]. Previous studies on the evolution of amino acids[217], 3-deoxyglucosone[78] as well as lipolytic and oxidative changes[151,171] during the malting process, indicated that the most pronounced changes occur during late germination and kilning. In addition, upon analysing volatile compounds during malting process (among them free aldehydes), Dong *et al.*[110] found higher levels of most of measured volatiles in kilned malt, in comparison to germinating barley and barley samples.

Based on the above literature, we hypothesise that the malting process is involved in beer flavour (in)stability, and, in particular, that the finished product of malting, *i.e.* malt, is a key factor in relation to beer flavour (in)stability, especially regarding development of staling aldehydes during beer transport/storage. However, scrupulous monitoring of the malting process in relation to the generation of staling aldehydes has not been carried out before. Therefore, the major objective of chapter 3 is to obtain an overview of the evolution of staling aldehydes during malting. More specifically, the first experimental part of chapter 3, aimed at explorative investigation of the evolution of aldehydes and their cysteinylated counterparts throughout pale malt production (from barley till finished malt). To this end, methodology for

quantification of free aldehydes (HS-SPME-GC-MS) and cysteinylated aldehydes (UPLC-MS) was applied on dedicated samples collected throughout the malting process, with the focus on kilning since this stage has been suggested by others to play an important role in aldehyde formation[26,59,110,133].

In addition, aiming at more reliable determination of the potential significance of the malting process in relation to aldehyde formation, in the second part of chapter 3, levels of free and cysteinylated aldehydes were monitored in key process samples, comprising barley, germinating barley and finished pale lager malt, derived from three different, independent industrial-scale malting batches.

3.2. Materials and Methods

3.2.1. Chemicals

Detailed information on the chemicals used for determination of free and cysteinylated aldehydes may be found in chapter 2, section 2.2.1.

Determination of TBI. Thiobarbituric acid (≥98%, CAS 504-17-6) was purchased from Merck KGaA, Germany, acetic acid (99%, CAS 64-19-7) from Sigma-Aldrich, USA.

Determination of amino acids. Amino acids were determined with the use of AccQ•Tag Derivatisation Kit (Waters, USA). Carrez I and Carrez II were purchased from Sigma-Aldrich, USA.

Determination of malt quality parameters. Carboxymethylcellulose (CMC) (CAS 9004-32-4), potassium phosphate monobasic (≥99%, CAS 7778-77-0), sodium phosphate dibasic dodecahydrate (≥99%, CAS 10039-32-4), potassium iodate (≥99.9%, CAS 7758-05-6), glycine (CAS 56-40-6), glucose (CAS 492-62-6), dinitro salicylic acid (CAS 206-156-8), phenol (CAS 108-95-2), and sodium sulphite (≥98%, CAS 7757-83-7), were purchased from Sigma-Aldrich, USA. Ethylenediaminetetraacetic acid (EDTA) (CAS 25102-12-9), ammonium iron citrate (CAS 1185-57-5), ninhydrin (CAS 485-47-2), (D)-fructose (CAS 57-48-7), hydrochloric acid (≥98%, CAS 7647-01-0), and citric acid monohydrate (≥99.5%, CAS-5949-29-1) were acquired from Merck, Germany.

3.2.2. Malts

Boortmalt Antwerp, Belgium provided all biological samples analysed in this chapter. Samples were collected according to the internal standard protocol for sample collection.

In section 3.3.1. of 'Results and Discussion', detailed sampling was performed at different time points of the industrial-scale standard malting process for pale malt production (batch G), with the focus on kilning. Samples were collected at the onset of the process (barley; six-row winter barley variety Etincel), after the second day (GM2), third and a half day (GM3.5) and fourth and a half day (GM4.5) of germination, and every two hours during kilning, starting from 10 hours of green malt drying (K10) and finishing at 24 hours (K24). Moreover, malt with rootlets (MR) and finished malt without rootlets (M) were collected.

In section 3.3.2. of 'Results and Discussion', six-row winter barley variety Etincel was subjected to three independent industrial-scale malting procedures for pale malt production resulting in three different malt batches H, I, and J, respectively. All malts were produced in the same malting facility of Boortmalt. Samples of barley, germinating barley (3.5 days of germination), and finished pale malt were analysed.

3.2.3. Determination of free aldehydes in malting samples

Quantitative determination of free aldehydes in samples from industrial-scale malting (barley, germinating barley, malt) was performed according to the optimised in chapter 2 procedure. Sample preparation consisted of 'pre-treatment step', thus freeze-drying samples to 4% of moisture content and subsequent milling. It was followed by preparation of extract by adding 99 mL of N_2-flushed Milli-Q water to 1.0 g of finely milled pale malt (experiments in section 3.3.2) or 1.0 g of freeze-dried powder (experiments in section 3.3.1). The extraction

was performed in 100 mL serum bottles closed with crimp caps to minimise contact with air and protected with aluminium foil from light. The mixture was stirred at 250 rpm for 15 min at 20°C. Next, malt particles were allowed to sediment for 15 min. Further, 10 mL of supernatant were transferred to 20 mL amber glass GC-vial and closed with a crimp cap. All operations were carried out in oxygen-limited conditions (in anaerobic workstation). Next, samples were subjected to HS-SPME-GC-MS analysis, which was carried out as described in chapter 2, section 2.2.2.

3.2.4. Determination of cysteinylated aldehydes in malting samples

Quantitative determination of cysteinylated aldehydes in samples from industrial-scale malting (barley, germinating barley, malt) was performed according to the optimised in chapter 2 procedure. In the study comparing levels of cysteinylated aldehydes in various malting samples (results section 3.3.1), all samples were freeze-dried to approx. 4% of moisture content prior to extract preparation. In experiments involving only finished malts, no pre-treatment (freeze-drying) was applied. Sample preparation and determination of cysteinylated aldehydes was performed as described in chapter 2, section 2.2.9.

3.2.5 Determination of TBI in malt

Determination of the thiobarbituric acid index (TBI) was performed following the adapted method of Coghe *et al*.[218], which itself is based on the method described by Thalacker and Brikenstock[219]. The colour reagent was prepared by dissolving 288 mg of thiobarbituric acid in 100 mL of 90% acetic acid. Malt extract was obtained by mixing (at ambient temperature) of 10 g finely milled malt with 100 mL of Milli-Q water for 30 min, followed by centrifugation at 9,000 rpm (5 min). Next, 2.5mL of supernatant were diluted up to 10 mL with Milli-Q water, and the colour reagent was added (5 mL). The mixture was incubated at 70°C for 70 min, followed by cooling on ice. Blank sample was prepared by diluting 2.5 mL of supernatant with 7.5 mL of Milli-Q water, and subsequent mixing with 5 mL of 90% acetic acid (no further heat treatment of blank sample). The absorption was measured at λ=448 nm. Subsequently, the TBI value was calculated as follows: TBI = ($A_{448} – A_{blank}$) x 40 (index for 10 g malt).

3.2.6. Determination of amino acids in malting samples

Amino acids in samples from maltings (barley, germinating barley, malt) were determined with the use of an Ultra Performance Liquid Chromatography (UPLC) separation system (Waters, USA) and PDA detector. Six amino acids were determined specifically, including valine, isoleucine, leucine, methionine, phenylalanine, and cysteine/cystine, respectively. Sample preparation consisted of mixing (at ambient temperature) 1 g of a finely milled biological material (either freeze-dried barley, freeze-dried germinating barley, or malt) with 10 mL of HCl (0.1*M*) for 30 min, followed by centrifugation for 5 min at 9,000 rpm. Then, in order to remove proteins, to 1 mL of supernatant were added 20 µL of Carrez I reagent (106 g potassium ferrocyanide trihydrate in 1L Milli-Q water) and 20 µl Carrez II reagent (220 g zinc acetate dihydrate and 30 mL of pure acetic acid made-up to a final volume of 1L with Milli-Q water). The sample was mixed and again centrifuged for 5 min at 9,000 rpm. Sample derivatisation was carried out manually with the application of the AccQ•Tag Derivatisation Kit and the accompanying procedure (Waters, USA). The UPLC system was equipped with an AccQ•Tag Ultra column (2.1 i.d. x 100 mm; Waters, USA). Column temperature was set at 60°C. Gradient elution, according to the prescribed AccQ•Tag Ultra method (Waters, USA),

was applied. Flow rate was 0.7 mL/min and total running time 9.5 min. Data processing was done using Empower 2 Software (Waters, USA).

3.2.7. Determination of malt quality parameters

Malt quality was assessed according to the European Brewery Convention methods (Analysis committee of the EBC, 2018). Directly on malt itself were measured: moisture content (EBC 4.2), homogeneity and partly unmodified grains (EBC 4.14), friability (EBC 4.15) and total nitrogen content (TN) (EBC 4.17, near-infrared (NIR) method). Diastatic Power (DP; EBC 4.12.1) and α-amylase activity (EBC 4.13) were determined in cold malt extract (5 g of milled malt mixed with 100 mL of 5% sodium chloride solution) by an automated continuous flow analyser (Skalar, The Netherlands). Congress wort, prepared according to EBC method 4.5.1, was used to determine the following parameters: extract yield (EBC 4.5.1), colour (EBC 4.7.1; spectrophotometric method), viscosity (EBC 4.8), total soluble nitrogen (TSN; expressed as g/100 g of malt) (EBC 4.9.2), free amino nitrogen (FAN) (EBC 4.10), wort pH (EBC 8.17) and β-glucan content (EBC 4.16). Total protein (TP; expressed as % dm) was calculated as TN x 6.25, whereas total soluble protein (TSP) was obtained as TSN x 6.25. Kolbach Index (KI, expressed in %) was calculated from the following formula KI = TSN/TN x 100. The following equipment was used for: mashing bath MBR25-V1a (Custom Laboratory Products, UK), AntonPaar DMA 5000 (Anton Paar GmbH, Austria), Spectrometer Thermo Scientific Evolution 60 S UV-Vis, Thermo Scientific drying oven (Thermo Fisher Scientific, USA), pH meter 7110 WTW (InoLab, France), FOSS WineScan Auto and Foss Infratec 1241 Analyzer (Foss, Denmark), and Skalar San++ System (Skalar, The Netherlands).

3.2.8. Statistical analysis

Results are presented as mean values and standard deviations (calculated from three analytical replicates). Statistical comparison between mean values was carried out by one-way ANOVA. To identify differences between multiple groups of samples, one-way ANOVA was followed by a post-hoc HSD Tukey's test. Statistical correlations amongst data were calculated via Pearson's correlation coefficients (r). Following software was used for statistical analysis: Excel 365 (Microsoft, USA) and SPSS Statistics 26 (IBM, USA).

3.3. Results and Discussion

3.3.1. Explorative monitoring of free and cysteinylated aldehydes through the industrial-scale malting

The major aim of this study is to monitor the industrial-scale malting process in order to obtain an overview of the evolution of free and cysteinylated aldehydes by applying the analytical methodology described in Chapter 2. Next, based on the data obtained, it will be investigated whether it is feasible to identify critical steps influencing the generation of these compounds. As far as we know, detailed profiling of free and cysteinylated aldehydes throughout industrial-scale malting has not been carried out before. Following samples were collected during the malting process and were analysed subsequently: barley, germinating barley (samples collected after 2, 3.5, 4.5 days of germination), dried green malt samples (*i.e*. samples collected every two hours after 10h up to 24h of green malt kilning), malt with rootlets and deculmed, finished malt. Information on temperature of incoming air and grain moisture content of the samples was provided by the malting company.

Quantitative data on free and cysteinylated aldehydes are presented in Table 3-1 and Table 3-2, respectively. Generally, an increase in the content of free and cysteinylated aldehydes is observed throughout the malting process, from barley up to finished malt. Among free aldehydes, the fatty acid oxidation products hexanal and *trans*-2-nonenal were quantifiable in all samples, except for the barley sample. However, Strecker aldehydes (2MP, 2MB, 3MB, MET, PHE) and the Maillard reaction product furfural were only quantifiable in dried green malt samples collected after 22h and 24h of drying (indicated as K22 and K24 in Table 3-1), as well as in malt with rootles (MR) and in finished malt.

Regarding cysteinylated aldehydes, except for the furfural adduct, all of these compounds were quantifiable in the malt samples with or without rootlets (see Table 3-2).

In the following sections, the evolution of free and bound-state aldehydes will be discussed, according to their pathways of formation (Strecker degradation of amino acids, Maillard reaction, and fatty acid oxidation, respectively). The evolution of free and bound-state aldehydes during malting (as will be further shown and discussed in Figures 3-1, 3-2, 3-3) is presented in terms of relative concentrations, *i.e.* for each compound, concentrations are shown as a percentage of the maximum value measured for that compound (as µg/kg dm) during the malting process.

Table 3-1. Quantification of free staling aldehydes in samples from different stages of the industrial-scale malting process (batch G).

Free aldehydes (µg/kg dm)	2MP	2MB	3MB	MET	PHE	FUR	HEX	T2N
Barley	<LOD (<14)	<LOD (<17)	<LOD (<49)	<LOD (<9)	<LOD (<70)	<LOQ (<35)	<LOD (<21)	<LOQ (<18)
GM2	<LOQ (<47)	<LOD (<17)	<LOD (<49)	<LOQ (<30)	<LOQ (<234)	<LOQ (<35)	597 ± 45	106 ± 29
GM3.5	<LOQ (<47)	<LOD (<17)	<LOD (<49)	<LOQ (<30)	<LOQ (<234)	<LOQ (<35)	624 ± 60	197 ± 62
GM4.5	<LOQ (<47)	<LOD (<17)	<LOD (<49)	<LOQ (<30)	<LOQ (<234)	<LOQ (<35)	436 ± 72	61 ± 6
K10	<LOQ (<47)	<LOD (<17)	<LOD (<49)	<LOQ (<30)	<LOQ (<234)	<LOQ (<35)	391 ± 39	143 ± 38
K12	<LOQ (<47)	<LOD (<17)	<LOD (<49)	<LOQ (<30)	<LOQ (<234)	<LOQ (<35)	397 ± 16	232 ± 28
K14	<LOQ (<47)	<LOD (<17)	<LOD (<49)	<LOQ (<30)	<LOQ (<234)	<LOQ (<35)	505 ± 41	267 ± 40
K16	<LOQ (<47)	<LOD (<17)	<LOD (<49)	<LOQ (<30)	<LOQ (<234)	<LOQ (<35)	445 ± 9	257 ± 119
K18	<LOQ (<47)	<LOD (<17)	<LOD (<49)	<LOQ (<30)	<LOQ (<234)	<LOQ (<35)	517 ± 59	327 ± 83
K20	<LOQ (<47)	<LOQ (<56)	<LOQ (<49)	<LOQ (<30)	<LOQ (<234)	<LOQ (<35)	590 ± 50	205 ± 92
K22	323 ± 12	335 ± 14	783 ± 45	51 ± 16	260 ± 22	222 ± 21	205 ± 31	268 ± 24
K24	347 ± 13	372 ± 2	836 ± 21	49 ± 14	284 ± 5	195 ± 17	114 ± 21	723 ± 190
MR	920 ± 4	1,010 ± 34	2,160 ± 76	158 ± 6	983 ± 74	497 ± 52	244 ± 25	457 ± 90
Malt	810 ± 6	851 ± 37	1,929 ± 83	112 ± 25	874 ± 65	586 ± 50	281 ± 25	453 ± 53

Measured aldehydes: 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, and T2N = *trans*-2-nonenal. Analysed samples: barley; GM = germinating barley (germinated for 2, 3.5, 4.5 days, respectively); K = samples taken at kilning, after 10 up to 24h of kilning; MR = malt with rootlets; malt = finished malt (without rootlets). Results are expressed as mean values (n=3) ± standard deviation. LOD = limit of detection; LOQ = limit of quantification. In brackets, estimated values of LOD and LOQ in µg/kg dm.

Table 3-2. Quantification of cysteinylated aldehydes in samples coming from different stages of the industrial-scale malting process (batch G).

Cysteinylated aldehydes (µg/kg dm)	2MP-CYS	2MB-CYS	3MB-CYS	MET-CYS	PHE-CYS	FUR-CYS	HEX-CYS
Barley	<LOD (<22)	<LOD (<10)	<LOD (<10)	<LOD (<16)	<LOD (<12)	<LOD (<22)	<LOD (<8)
GM2	<LOD (<22)	<LOD (<10)	<LOD (<10)	<LOD (<16)	<LOD (<12)	<LOD (<22)	<LOD (<8)
GM3.5	<LOD (<22)	<LOD (<10)	<LOD (<10)	<LOD (<16)	<LOD (<12)	<LOD (<22)	<LOQ (<18)
GM4.5	<LOD (<22)	<LOD (<10)	<LOD (<10)	<LOD (<16)	<LOD (<12)	<LOD (<22)	<LOQ (<18)
K10	<LOD (<22)	<LOD (<10)	<LOD (<10)	<LOD (<16)	<LOD (<12)	<LOD (<22)	<LOQ (<18)
K12	<LOD (<22)	<LOD (<10)	<LOD (<10)	<LOD (<16)	<LOD (<12)	<LOD (<22)	<LOQ (<18)
K14	<LOD (<22)	<LOD (<10)	<LOD (<10)	<LOD (<16)	<LOD (<12)	<LOD (<22)	<LOQ (<18)
K16	<LOD (<22)	<LOD (<10)	<LOD (<10)	<LOD (<16)	<LOD (<12)	<LOD (<22)	<LOQ (<18)
K18	<LOD (<22)	<LOD (<10)	<LOD (<10)	<LOD (<16)	<LOD (<12)	<LOD (<22)	<LOQ (<18)
K20	<LOD (<22)	<LOD (<10)	<LOQ (<24)	<LOD (<16)	<LOD (<12)	<LOD (<22)	<LOQ (<18)
K22	<LOD (<22)	<LOD (<10)	108 ± 6	<LOD (<16)	50 ± 2	<LOD (<22)	<LOQ (<18)
K24	<LOQ (<72)	<LOD (<10)	148 ± 5	<LOQ (<30)	73 ± 3	<LOD (<22)	<LOQ (<18)
MR	112 ± 2	30 ± 1	330 ± 18	34 ± 3	138 ± 4	<LOQ (<74)	21 ± 2
Malt	132 ± 2	36 ± 2	398 ± 14	42 ± 3	183 ± 3	<LOQ (<74)	39 ± 1

Measured cysteinylated aldehydes: 2MP-CYS = cysteinylated 2-methylpropanal, 2MB-CYS = cysteinylated 2-methylbutanal, 3MB-CYS = cysteinylated 3-methylbutanal, MET-CYS = cysteinylated methional, PHE-CYS = cysteinylated phenylacetaldehyde, FUR-CYS = cysteinylated furfural, and HEX-CYS = cysteinylated hexanal (cysteinylated *trans*-2-nonenal is not presented as the reference compound was not available). Analysed samples: barley; GM = germinating barley (germinated for 2, 3.5, 4.5 days, respectively); K = samples taken at kilning, after 10 up to 24h of kilning; MR = malt with rootlets; malt = finished malt (without rootlets). Results are expressed as mean values (n=3) ± standard deviation. LOD = limit of detection; LOQ = limit of quantification. In brackets, estimated values of LOD and LOQ in µg/kg dm.

3.3.1.1. Strecker aldehydes

The evolution of analysed Strecker aldehydes (2-methylpropanal, 2-methylbutanal, 3-methylbutanal, methional, and phenylacetaldehyde) during this particular industrial-scale malting process to obtain pale lager malt, is depicted in Figure 3-1A, whereas measurements on corresponding cysteinylated Strecker aldehydes are shown in Figure 3-1B. Only major changes in levels of free and bound-state aldehydes will be discussed below, because, in this particular experiment, all analysed samples were derived from a single industrial-scale batch, and information on potential batch-wise variation was not available at this stage of the PhD.

Among individual free Strecker aldehydes, a similar behaviour was observed during the entire malting process (see Figure 3-1A). This can be ascribed to the similar formation mechanism(s) of these compounds, for instance, Strecker degradation by reaction of an amino acid with an α-dicarbonyl intermediate, or direct oxidation of amino acids[17] (for more detailed information, see Chapter 1, section 1.1.5). Small amounts of Strecker aldehydes (below LOD and/or LOQ) were found in barley, as well as samples collected during germination, and during the first hours of kilning. This is not surprising, as Strecker aldehydes are considered to be side products of Maillard reactions, which are limited at low operating temperatures, like temperatures applied at these particular stages of malting[66,128]. A first considerable increase in levels of measured Strecker aldehydes (>LOQ) was observed after 20h of kilning, thus at the stage when the inlet air temperature was raised from 76°C to 78°C, and grain moisture dropped simultaneously from approx. 30% to 4.5%. In accordance with this observation, it is known from literature that Maillard reactions and maximum browning in food products in general, mainly occur at a water activity of approx. 0.6-0.7[92,220]. Furthermore, according to Barreiro *et al.* [221] and Laitila *et al.* [222], a water activity of 0.7 of malt corresponds to water content of approx. 14%, which fits within the above observed critical interval of water reduction of malt from 30% to 4.5%. Next to the above observed first increase in Strecker aldehydes, the most pronounced increase in levels of Strecker aldehydes (*i.e.* relative increase of approx. 60% - 70%) was however noticed during the cooling stage of malting. At the beginning of this stage, hot grain is exposed to a boost of fresh, oxygen-rich air. Thus, it can be envisaged that at this stage of the malting process, excessive levels of oxygen will provoke direct oxidation of amino acids and, consequently, may give rise to the corresponding free aldehydes[47,84]. Deculming (removal of rootlets) slightly decreased the content of Strecker aldehydes (5-30%), mainly affecting methional. This suggests that methional is present in rootlets and that adequate deculming can reduce its content in the finished malt.

During the malting process, cysteinylated Strecker aldehydes evolved similarly to their free counterparts (see Figure 3-1B), due to the existence of chemical equilibria between free and cysteinylated forms, as previously highlighted by Bustillo Trueba *et al.*[8].

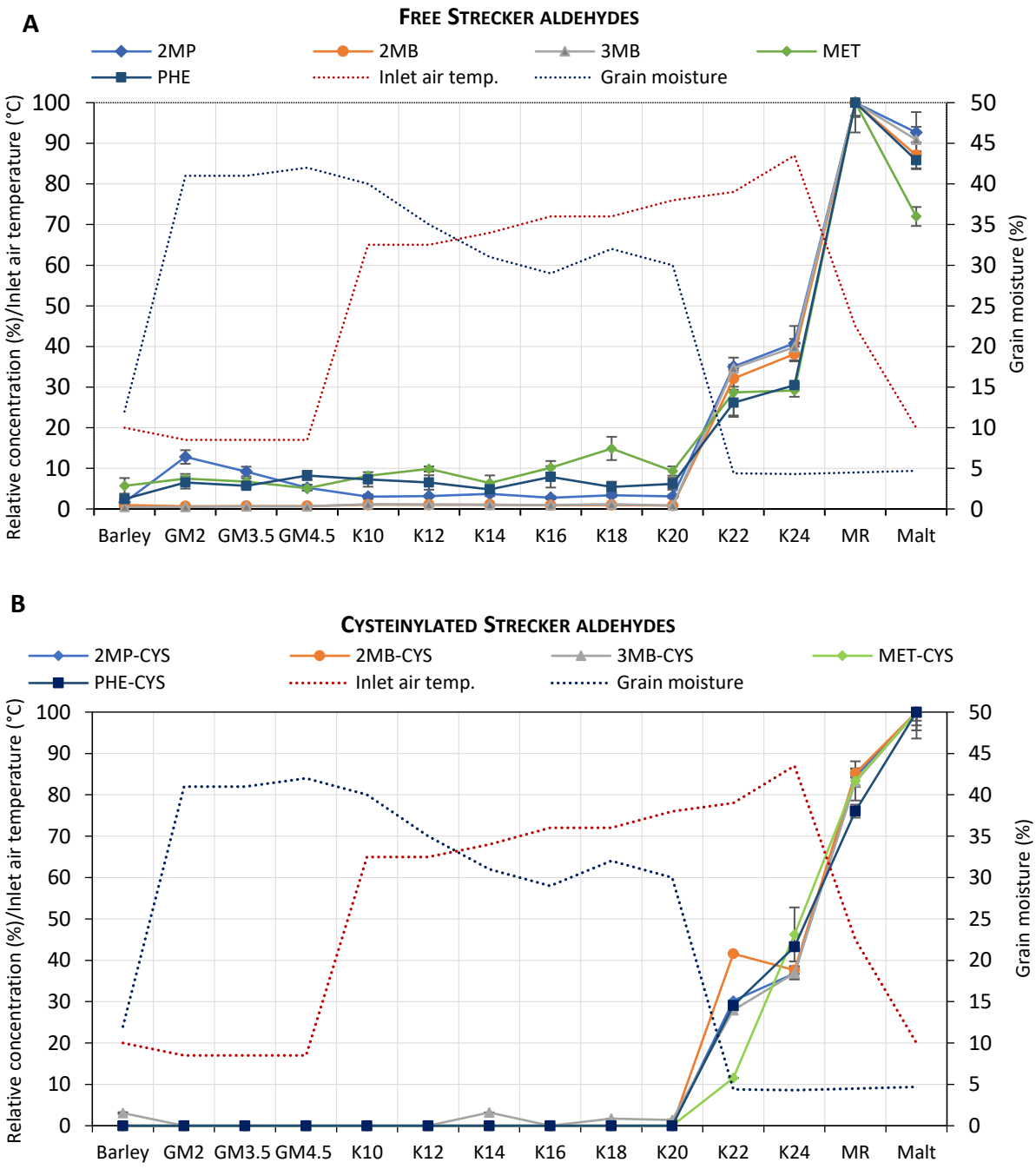


Figure 3-1. Evolution of free (A) and cysteinylated (B) Strecker aldehydes during industrial-scale preparation of pale lager malt.

Results on the evolution of aldehydes are expressed as a % of the maximum value measured for a compound during the malting process. Measured aldehydes: 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, and T2N = *trans*-2-nonenal, and their cysteinylated counterparts, namely: 2MP-CYS = cysteinylated 2-methylpropanal, 2MB-CYS = cysteinylated 2-methylbutanal, 3MB-CYS = cysteinylated 3-methylbutanal, MET-CYS = cysteinylated methional, PHE-CYS = cysteinylated phenylacetaldehyde, FUR-CYS = cysteinylated furfural, and HEX-CYS = cysteinylated hexanal (cysteinylated *trans*-2-nonenal is not presented as the reference compound was not available). Analysed samples: barley; GM = germinating barley (germinated for 2, 3.5, 4.5 days, respectively); K = samples taken at kilning, after 10 up to 24h of kilning; MR = malt with rootlets; malt = finished malt (without rootlets). Results are expressed as mean values (n=3), error bars = standard deviation.

3.3.1.2. Furfural - Maillard reaction product

The evolution of free and cysteinylated furfural during the monitored industrial-scale malting process is presented in Figure 3-2.

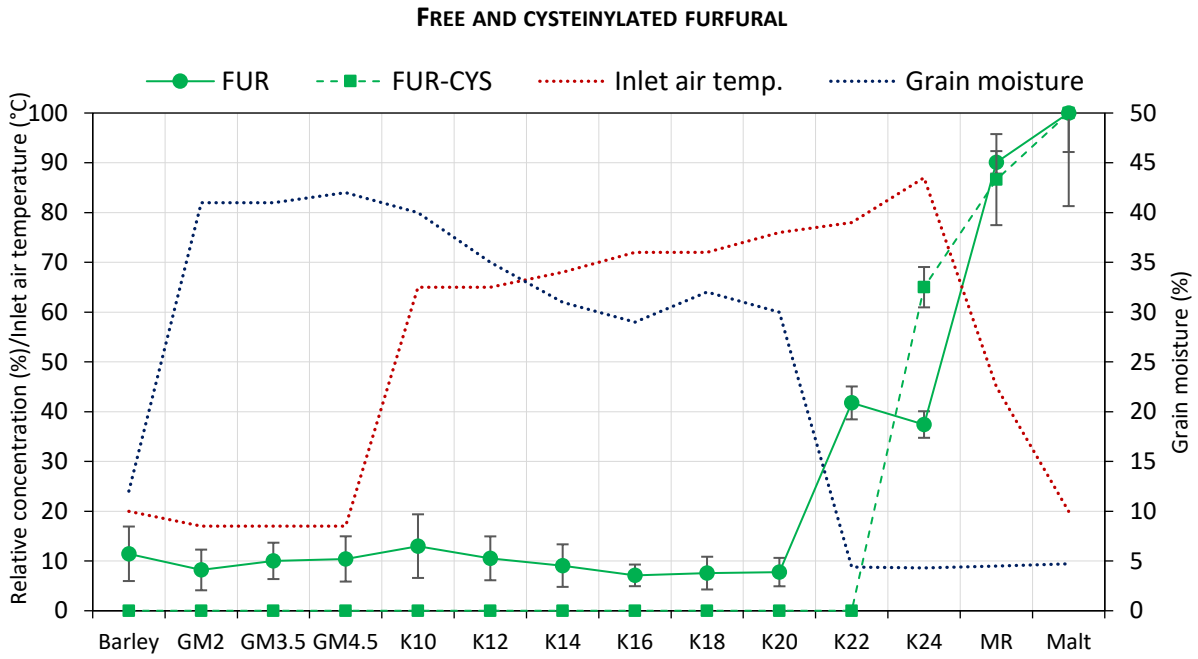


Figure 3-2 Evolution of free and cysteinylated furfural, during industrial-scale preparation of pale lager malt.

Results on the evolution of aldehydes are expressed as a % of the maximum value measured for a compound during the malting process. Measured aldehydes: FUR = furfural and FUR-CYS = cysteinylated furfural. Analysed samples: barley; GM = germinating barley (germinated for 2, 3.5, 4.5 days, respectively); K = samples taken at kilning, after 10 up to 24h of kilning; MR = malt with rootlets; malt = finished malt (without rootlets). Results are expressed as mean values (n=3), error bars = standard deviation.

The evolution of furfural levels as a function of malting was found to be similar to the evolution of Strecker aldehydes, which is explainable from a chemical point of view since both types of aldehydes are clearly connected to the Maillard reaction[66] (see also Chapter 1, section 1.1.5.1). Apparently, from barley up to 20h of kilning, only low levels of furfural (<LOQ) were found (see Table 3-1). However, between 20h and 22h of kilning *(i.e.* when the air inlet temperature was increased form 76°C to 78°C and a simultaneous decrease in moisture content from approx. 30% to 4.5% was observed), a first marked increase in furfural was found, similar to the previously observed increase in Strecker aldehydes. Next, and again similar to the behaviour of Strecker aldehydes, a second, pronounced increase in furfural was observed during cooling (relative increase of approx. 50%). Thus, in accordance with Strecker aldehydes, the appearance of furfural seems to be most strongly connected to the particular decline in grain moisture content (starting from approx. 30% of moisture), and to the presence of high levels of oxygen.

Regarding cysteinylated furfural, its measured concentration was always below the limit of quantification (see Table 3-2 and Figure 3-2), although significant levels of free furfural were found at the latest stages of kilning and in the final malt. As reported before[81], (slightly) acidic conditions such as in malt, are not favourable for the formation of the furfural-cysteine adduct, because of the relatively low electrophilic character of the aldehyde functional group of furfural (due to the aromatic nature of the R group of furfural).

3.3.1.3. Fatty acids oxidation products

The evolution of levels of hexanal and *trans*-2-nonenal during the monitored industrial-scale malting process is presented in Figure 3-3A, while the evolution of cysteinylated hexanal is shown in Figure 3-3B (cysteinylated *trans*-2-nonenal is not presented as the reference compound was not available).

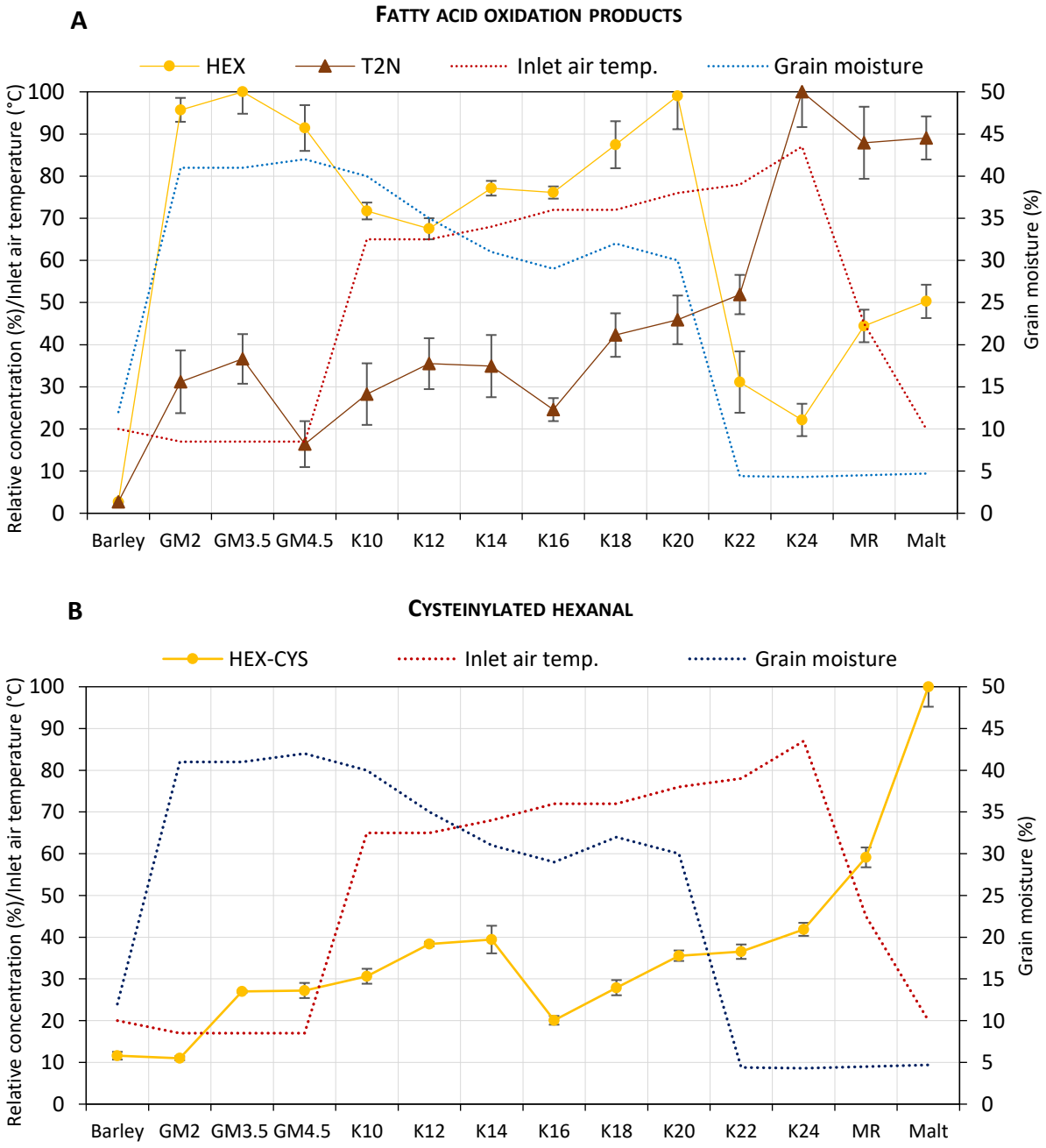


Figure 3-3. Evolution of hexanal and *trans*-2-nonenal (A) as well as cysteinylated hexanal (B) during industrial-scale preparation of pale lager malt.

Results on the evolution of aldehydes are expressed as a % of the maximum value measured for a compound during the malting process. Measured aldehydes: HEX = hexanal, T2N = *trans*-2-nonenal, HEX-CYS = cysteinylated hexanal. Analysed samples: barley; GM = germinating barley (germinated for 2, 3.5, 4.5 days, respectively); K = samples taken at kilning, after 10 up to 24h of kilning; MR = malt with rootlets; malt = finished malt (without rootlets). Results are expressed as mean values (n=3), error bars = standard deviation.

Clearly, hexanal and *trans*-2-nonenal evolve differently during industrial-scale malting, compared to Strecker aldehydes and furfural. This is due to the different formation mechanism, *i.e.* fatty acid oxidation (see Chapter 1 section 1.1.5.1).

In barley, the content of *trans*-2-nonenal appears to be <LOQ (see Table 3-1). A first significant increase in the level of this aldehyde is observed during the first two days of germination (106 µg/kg dm; see also Figure 3-3A). Although, at this stage (onset of germination), autoxidation cannot be excluded, enzymatic oxidation of unsaturated fatty acids is proposed as the prominent mechanism leading to *trans*-2-nonenal, due to LOX-activity, presumably LOX-1 activity[64]. A second marked increase in *trans*-2-nonenal (up to 723 µg/kg dm) is noticed within the time interval of 22h up to 24h of kilning (*i.e.* when the air inlet temperature was increased from 78°C to 87°C). Because of the high operating temperature and the low moisture content in the grain, it can be assumed that at this stage LOX-activity is suppressed and that rather autoxidation instead of enzymatic oxidation is the leading mechanism resulting in formation of *trans*-2-nonenal[64,118].

Regarding hexanal, in barley, its content was <LOD (see Table 3-1). As also noticed for *trans*-2-nonenal, in the case of hexanal, a first significant increase already occurs during the first two days of germination (597 µg/kg dm; see also Figure 3-3A). Analogous to *trans*-2-nonenal, this may be ascribed to enzymatic LOX-activity, presumably LOX-2 activity in the case of formation of hexanal. Next, between 20h and 22h of kilning (*i.e.* when the air inlet temperature is increased from 76°C to 78°C and a simultaneous decrease in moisture took place from approx. 30% to 4.5%), a pronounced decrease in hexanal was observed. Under these malting conditions, enzymatic formation of hexanal may be limited since it has been reported previously that about 70% - 90% of LOX-2 activity is already destroyed at a relatively low temperature of 65°C[64]. Moreover, at this stage of kilning, the efficient removal of water should facilitate evaporation of the relatively volatile hexanal. Finally, during the cooling stage of malt, again, some increase in hexanal content was found, likely caused by autoxidation at the onset of cooling, because of the still high temperature within the grain, in combination with the supply of high levels of oxygen through the use of fresh air.

As demonstrated by the experimental data, the evolution of hexanal and *trans*-2-nonenal as a function of the malting process shows similarities as well as differences, even though both aldehydes are derived from oxidation of unsaturated fatty acids. Observed differences may be related to the specific properties of lipoxygenase iso-enzymes (LOX-1 and LOX-2, leading to *trans*-2-nonenal and hexanal, respectively) and differences in the volatility of the aldehydes. Regarding samples collected during the stage of germination, the higher content of hexanal in comparison to *trans*-2-nonenal (see Table 3-1), can be ascribed to higher levels of LOX-2, as it is known to represent about 75% of total LOX-activity in germinated barley[223]. Also, LOX-2 shows higher affinity towards fatty acids esterified in triacylglycerols[224]. On the other hand, the different effect of kilning-off at high temperature on levels of hexanal and *trans*-2-nonenal, may (at least in part) be explained by the higher volatility of hexanal, compared to *trans*-2-nonenal[225].

Regarding the evolution of cysteinylated hexanal during the monitored malting process (see Figure 3-3B), a first increase in its content occurred after the second day of germination, when high levels of free hexanal were already present (see Figure 3-3A), and, therefore, together with free cysteine (as a result of proteolysis), available for adduct formation. A second pronounced increase in cysteinylated hexanal is observed during cooling of the malt

(relative increase of approx. 20%) when the levels of free hexanal also increased. Finally, also after deculming of malt, a relatively strong increase in the level of adduct of approx. 40% is noticed.

3.3.2. Explorative study on different pale malt industrial-scale maltings

The results obtained in the previous experimental section 3.3.1. were derived from a single batch of industrial malting. In this part of chapter 3, the major aim is to investigate key samples derived from several batches of industrial malting, to verify whether major findings obtained in section 3.3.1. remain applicable or not when investigating other malting batches. To this end, starting from the barley variety Etincel, samples were obtained from three different industrial-scale trials (batch H, I, and J), which were performed in the same malting facility. Levels of free aldehydes, cysteinylated aldehydes, and several amino acids, were determined in barley, germinating barley (collected at 3.5 days of germination), and finished pale malt.

In addition, data on standard quality parameters of the malt samples H, I, and J, provided by the maltings, are compared to measured levels of amino acids and aldehydes, in order to unravel potential relationships among the different sets of data.

3.3.2.1. Comparison of levels of aldehydes and selected amino acids in barley, germinating barley and finished pale malt, derived from three different industrial-scale malt productions

When looking at the overall evolution of free and cysteinylated aldehydes during malting, as expressed by the evolution of relative concentrations shown in Figure 3-4, similar data were obtained for the different batches batch H, I, and J. Clearly, except for hexanal, aldehydes were present at highest levels in finished malt, as previously observed in section 3.3.1.

More specifically, in barley, all investigated free and cysteinylated aldehydes were <LOQ. In germinating barley, detected Strecker aldehydes and furfural are also <LOQ, whereas levels of the fatty acid oxidation products (HEX and T2N) constitute approx. 100% of the relative concentration and approx. 35% of the relative concentration, respectively. In finished pale malt, levels of all aldehydes (except for HEX) were the highest (thus represented in Figure 3-4 as 100% of the relative concentration). These observations are in accordance with Dong *et al.*[110], who also reported higher levels of Strecker aldehydes in malt in comparison to germinating barley and green malt, in addition to higher levels of HEX determined in green malt, compared to kilned malt. Regarding bound-state aldehydes, in germinating barley, only HEX-CYS was found, reaching approx. 20% - 40% of its relative concentration. In finished pale malt, levels of cysteinylated aldehydes are at maximum, *i.e.* 100% of the relative concentration, except for FUR-CYS, which is <LOD (similar findings were obtained in section 3.3.1).

In summary, data obtained on the three independent industrial-scale malting batches, confirm our findings presented in section 3.3.1. Hence, it can be generalised that levels of free and cysteinylated aldehydes increase during malting process, reaching the highest values in finished pale malt (except for HEX, which already reaches its maximum in the beginning of germination).

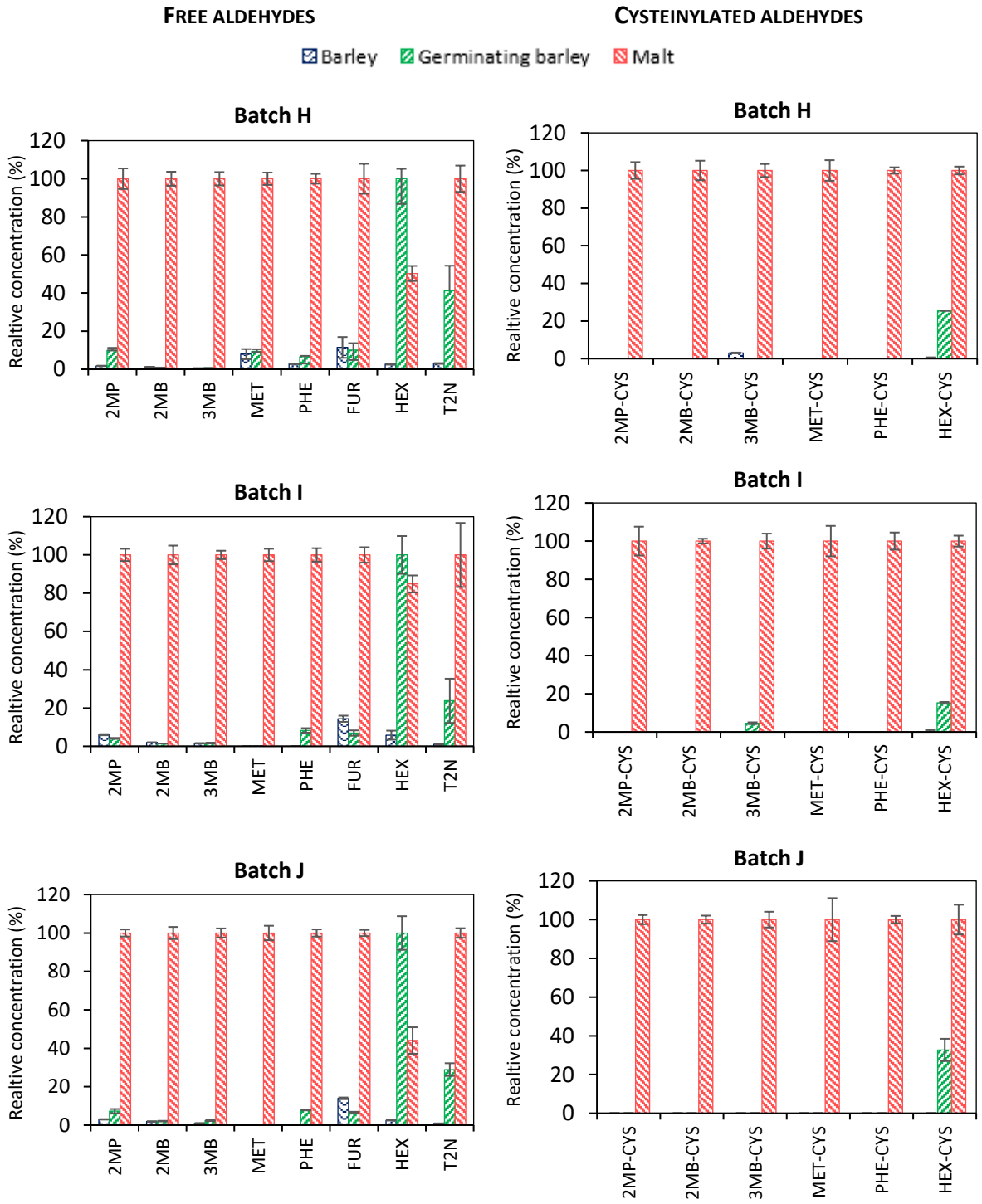


Figure 3-4. Free aldehydes (left side) and cysteinylated aldehydes (right side) determined in barley, germinating barley (3.5 days of germination) and finished malt, derived from three independent industrial-scale productions (malting batches H, I, J).

Results on the evolution of aldehydes are expressed as a % of the maximum value measured for a compound during the malting process. Measured aldehydes: 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB =3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, and T2N = *trans*-2-nonenal, and their cysteinylated counterparts, namely: 2MP-CYS = cysteinylated 2-methylpropanal, 2MB-CYS = cysteinylated 2-methylbutanal, 3MB-CYS = cysteinylated 3-methylbutanal, MET-CYS = cysteinylated methional, PHE-CYS = cysteinylated phenylacetaldehyde, FUR-CYS = cysteinylated furfural, and HEX-CYS = cysteinylated hexanal (cysteinylated *trans*-2-nonenal is not presented as the reference compound was not available). FUR-CYS was <LOD in all samples. Results are expressed as mean values (n=3), error bars = standard deviation.

Besides levels of aldehydes, levels of amino acids, some of which are potential precursors of Strecker aldehydes (valine, leucine, isoleucine, methionine, and phenylalanine, corresponding to 2MP, 2MB, 3MB, MET, and PHE, respectively), increase during the malting process in all three tested batches (see Figure 3-5). This is clearly the effect of proteolysis taking place during germination and at the beginning of green malt drying[128].

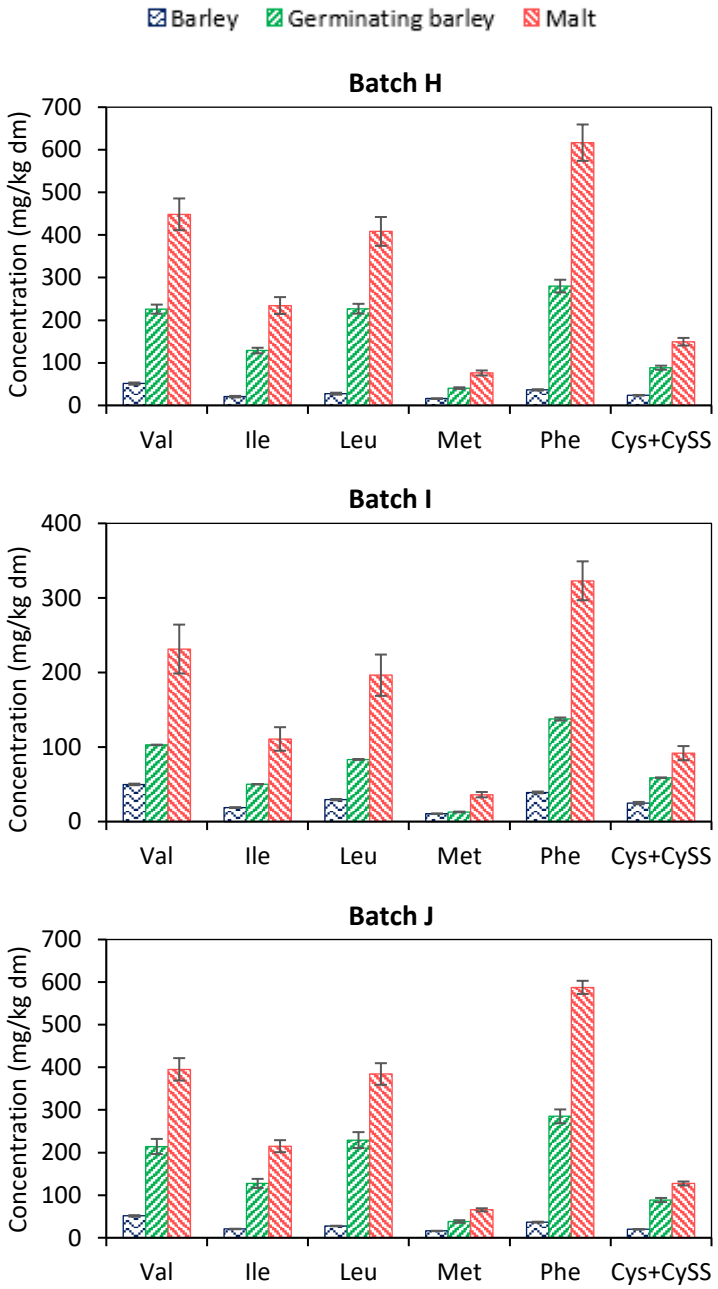


Figure 3-5. Levels of amino acids (mg/kg dm) determined in barley, germinating barley (3.5 days of germination) and finished pale malt, derived from three independent industrial-scale productions (malting batches H, I, J).

Measured amino acids: Val = valine, Ile = isoleucine, Leu = leucine, Met = methionine, Phe = phenylalanine, Cys+CySS = cysteine/cystine. Results are expressed as mean values (n=3), error bars = standard deviation.

Next to amino acids as potential precursors of Strecker aldehydes, also levels of cysteine/cystine were monitored during malting, and, in an agreement with the above, levels of cysteine/cystine also increase during malting. This allows for better understanding of the formation of cysteinylated aldehydes. For instance, in germinating barley, high levels of HEX (see Figure 3-4, right side) and moderate levels of cysteine (see Figure 3-5) lead to an increase in the content of HEX-CYS (see Figure 3-4, left side). Likewise**,** in finished malt, high levels of Strecker aldehydes and high levels of cysteine result in relatively high concentrations of cysteinylated Strecker aldehydes. All of these findings are in agreement with results obtained in section 3.3.1.

3.3.2.2. Comprehensive analysis of standard quality parameters, amino acids, and aldehydes in pale malts

Industrial maltings verify the quality of their product (*i.e.* malt) by assessing standard malt quality parameters, which inform on the physicochemical and biochemical properties of malt. Data on standard quality parameters of malts derived from batch H, I, and J are summarised in Table 3-3. Below is given a brief explanation of their significance, together with commonly recommended values for high-quality pale malt.

Standard malt quality parameters comprise: moisture content (g of water present in 100 g of malt, expressed as a %; typically 3% - 5%), extract yield (g of soluble material obtained from 100 g of malt recalculated to dry matter, expressed as % dm; typically >77% dm), malt colour (2.0 EBC - 4.5 EBC), and thiobarbituric acid index (TBI; a widely recognised indicator of applied heat load[226] and carbonyl compounds[227]). Furthermore, cytolytic modification is evaluated by friability and homogeneity of malt, along with β-glucan content and viscosity of Congress wort. Friability indicates the percentage of easily disintegrating kernels when pressed with a roller system within a friabilimeter. This parameter should exceed 80% to secure proper milling in the brewhouse[128,129]. Homogeneity expresses the percentage of uniformly modified grains. Typically, reported values are ≥95%, indicating that less than 5% of kernels can be either unmodified (reported as 'partly unmodified grains', PUG) and/or dead (reported as 'whole grains', WG)[228]. Further, β-glucan content informs on the quantity of residual (1,3)(1,4)-β-D-glucan fraction present in Congress wort, while viscosity (physicochemically) reflects the content of β-glucans, pentosans and proteins. Too high values of both of these parameters (>200 mg/L and >1.6 mPas, respectively) can lead to wort run-off problems, *i.e.* prolonged filtration times[129,229]. Proteolytic modification of malt is assessed via total soluble protein (TSP), Kolbach Index (KI) and free amino nitrogen (FAN). TSP is the percentage of proteins, peptides and amino acids that are solubilised in wort during mashing. Kolbach Index informs on total soluble protein in Congress wort, as a percentage of total protein (TP) measured in malt. FAN is determined by a colorimetric method with ninhydrin, which forms a colour complex with amino acids, ammonia and the terminal nitrogen groups of peptides and proteins. Insufficient proteolytic modification of pale malt (FAN <160 mg/L, KI <35%, TSN <3.7%) may lead to incomplete fermentation, whereas the excessive modification (FAN >250 mg/L, KI >47%, TSN >4.9%) may impair beer foam, result in a higher beer colour, and, possibly, in the appearance of beer off-flavours[129,140,228]. Amylolytic activity of malt is evaluated based on diastatic power (DP) and α-amylase activity. DP expressed in Windisch-Kolbach degrees (°WK; the amount of maltose formed by 100 g of malt in 30 min at 20°C), indicates the total activity of malt amylolytic enzymes (such as α-amylase, β-amylase, limit dextrinase, and α-glucosidase), hydrolysing starch to low-molecular-weight

sugars. High values (>220°WK) are required when brewing with a substantial addition of adjuncts[230]. Further, α-amylase activity, catalysing the hydrolysis of α-1,4-glycosidic linkages in starch, should exceed 30 dextrinising units (DU; the α-amylase activity that will dextrinize soluble starch in the presence of an excess of β-amylase at the rate of 1g/h at 20°C)[184,230].

Moreover, malt quality parameters may provide information on potential beer flavour deterioration during storage. For example, malt with a low Kolbach Index[109,208] and a low free amino nitrogen[6,105,208,231] was found to result in beers of enhanced flavour stability, whereas a high malt colour (pointing to excessive heat load), may lead to higher staling scores of aged beers[26,27].

As shown in Table 3-3, measurements of standard quality parameters of malts derived from batch H, I, and J were within the commonly accepted ranges for a high-quality pale malt. Only TP and TSP slightly exceeded the limits, which may be related to the barley variety and/or to the crop year[155]. In general, results obtained on batch H and J were quite similar, while batch I clearly differed. The latter is characterised by lower proteolytic modification (lower levels of TSP, KI, and FAN), as well as lower DP, α-amylase activity, and colour. Cytolytic modification assessed by β-glucan content, viscosity, and friability, was similar among the three tested batches. TBI was the parameter that differed mostly among the three different batches (the highest TBI value was determined in batch H, followed by batch J, and batch I).

Table 3-3. Malt quality parameters determined in finished pale malts, derived from three independent industrial-scale productions (malting batches H, I, J).

Malt quality parameter	Batch H			Batch I			Batch J			Recommended values for high-quality pale malt
	Mean		SD	Mean		SD	Mean		SD	
Moisture (%)	4.7 [a]	±	0.15	4.7 [a]	±	0.15	4.4 [a]	±	0.15	3-5 [1,2,4,6,7]
Extract yield (% dm)	79.8 [a]	±	0.4	78.4 [b]	±	0.4	79.2 [ab]	±	0.4	77-83 [1,2,3,5] > 80 [4,6,7]
Colour (EBC)	4.4 [a]	±	0.4	2.4 [b]	±	0.4	3.8 [a]	±	0.4	2-4.5 [1,2,4,5,6,7]
TBI (for 10 g dm)	22.9 [a]	±	0.1	11.1 [b]	±	0.2	18.0 [c]	±	0.1	-
Friability (%)	83.0 [a]	±	1.7	82.0 [a]	±	1.7	88.4 [b]	±	1.7	> 80 [1,4,5,6,7] > 85 [3]
Homogeneity (%)	95.2 [a]	±	0.5	95.4 [a]	±	0.5	97.8 [b]	±	0.5	> 95 [4,5]
Partly unmodified grain (%)	4.8 [a]	±	0.5	4.6 [a]	±	0.5	2.2 [b]	±	0.5	< 5 [4,5]
Whole grain (%)	2.0 [a]	±	0.4	2.5 [a]	±	0.4	1.6 [a]	±	0.4	< 3 [3,6]
β-Glucan (mg/L)	113 [a b]	±	20	136 [b]	±	20	84 [a]	±	20	< 250 [1,2]
Viscosity (mPas)	1.55 [a]	±	0.02	1.57 [a]	±	0.02	1.52 [a]	±	0.02	< 1.6 [1,2,4,5,7]
Total protein (% dm)	11.5 [a]	±	0.3	11.9 [a]	±	0.3	11.8 [a]	±	0.3	9.5-11.0 [3,4,7] < 10 [2,5]
Total soluble protein (% dm)	5.31 [a]	±	0.16	4.75 [b]	±	0.16	5.44 [a]	±	0.16	3.8-4.8 [2,3,6,7] 3.7-4.4 [5]
Kolbach index (%)	46.2 [a]	±	1.4	39.9 [b]	±	1.4	46.1 [a]	±	1.4	35-41[2] 38-44 [3,5] 37-45 [1,4,6,7]
Free amino nitrogen (mg/L)	194 [a]	±	9	157 [b]	±	9	202 [a]	±	9	160-250 [1,2,3,4,5,6,7]
Diastatic power (°WK)	390 [a]	±	25	280 [b]	±	25	360 [a]	±	25	> 220 [2,3,4,5,6,7]
α-Amylase activity (DU)	50 [a]	±	5.5	18 [b]	±	5.5	46 [a]	±	5.5	> 30 [1,4] > 40 [3,5]
pH	5.74 [a]	±	0.05	5.72 [a]	±	0.05	5.72 [a]	±	0.05	5.7-6.3 [1,7]

Results are presented as mean values ± standard deviation (SD). Statistical comparisons between the batches a, b, and c, by post-hoc HSD Tukey's test to distinguish among statistically significant different (p ≤0.05) groups (a, b, c). Sources from literature and/or companies: 1 – Briggs *et al.*[247]; 2 Kunze[129]; 3 – Eβlinger[230]; 4 – 'Rourk[228]; 5 – Davie[184]; 6 – corporative webpage Dingemans NV; 7 – corporative webpage Castle malting

Quantitative determinations of amino acids, free aldehydes, and cysteinylated aldehydes in malts derived from batch H, I, and J, are presented in Table 3-4**.** Higher levels of amino acids were found in batch H and J, in comparison to batch I. Among amino acids, which are potential precursors of Strecker degradation aldehydes, phenylalanine (Phe) was present in the highest concentration, followed by leucine (Leu) and valine (Val), isoleucine (Ile), and methionine (Met). Other authors[217,232] reported on a slightly different profile of these particular amino acids measured in different pale malts, *i.e.* Leu as major amino acid, followed by Phe, Val, Ile, and Met. Regarding aldehydes, the highest sum of free aldehydes was determined in batch H (6,007 µg/kg dm), followed by batch J (4,854 µg/kg dm), and batch I (3,507 µg/kg dm). In all samples, 3MB was present in the highest concentration, followed by PHE, 2MP, and 2MB. A similar profile of free aldehydes in pale malts has been reported by others[6,8]. Clearly, the above measured sequence in levels of volatile free aldehydes reflects the profile of non-volatile amino acids available in the literature [217,232]. In our study, as can be derived from Table 3-5, a strong, positive relationship (r ≥0.8) between individual amino acids and their corresponding, individual free Strecker degradation aldehyde was always found.

For bound-state aldehydes, the highest sum of cysteinylated aldehydes was determined in batch H (852 µg/kg dm), while comparable levels were found in batch I and J (461 µg/kg dm and 471 µg/kg dm, respectively). Cysteinylated aldehydes followed a similar pattern to their corresponding free forms, thus 3MB-CYS was present in the highest concentration, followed by PHE-CYS, 2MP-CYS and 2MB-CYS. Moreover, strong relationships (r ≥0.8) between individual, free Strecker aldehydes and their corresponding cysteinylated forms were found (see Table 3-5).

When comparing determined levels of each individual, free aldehyde with standard malt quality analysis, except for T2N, always very strong correlations (r ≥0.9) were found with colour, TBI, and extract yield (see Table 3-6). Furthermore, except for HEX and T2N, strong correlations (r ≥0.7) were found between individual, free aldehydes and TSP, KI, FAN, DP, and α-amylase activity (see Table 3-6). A positive relationship between FAN and the content of beer staling aldehydes measured in malt, has been reported previously[208]. In conclusion, it can be suggested that the levels of aldehydes determined in malt, in particular Strecker degradation aldehydes and furfural, appear to be connected to particular standard quality parameters of malt. Potential relationships with fatty acid oxidation aldehydes were less pronounced, although levels in hexanal correlated well with extract yield (r=0.80), malt colour (r=0.75), and TBI (r=0.82).

Table 3-4. Quantitative determination of amino acids, free aldehydes and cysteinylated aldehydes obtained for finished pale malts, derived from three independent industrial-scale productions (malting batches H, I, J).

Analytical parameter	Batch H			Batch I			Batch J		
	Mean		SD	Mean		SD	Mean		SD
Amino acids (mg/kg dm)									
Val*	449 [a]	±	37	231 [b]	±	33	395 [a]	±	26
Ile*	234 [a]	±	20	111 [b]	±	16	215 [a]	±	14
Leu*	408 [a]	±	34	196 [b]	±	28	384 [a]	±	25
Met*	76 [a]	±	6	36 [b]	±	4	66 [a]	±	4
Phe*	617 [a]	±	43	323 [b]	±	26	588 [a]	±	15
Cyss+Cys	149 [a]	±	9	92 [b]	±	10	127 [a]	±	5
Sum of free aldehydes (µg/kg dm)	6,007			3,507			4,854		
2MP	871 [a]	±	6	505 [b]	±	17	694 [c]	±	12
2MB	820 [a]	±	37	398 [b]	±	7	649 [c]	±	20
3MB	1,959 [a]	±	83	1,057 [b]	±	23	1,541 [c]	±	36
MET	122 [a]	±	25	78 [b]	±	3	108 [ab]	±	4
PHE	884 [a]	±	65	470 [b]	±	17	611 [c]	±	12
FUR	596 [a]	±	50	312 [b]	±	14	476 [c]	±	8
HEX	291 [a]	±	25	154 [b]	±	8	160 [b]	±	8
T2N	463 [a]	±	53	533 [a]	±	85	615 [a]	±	134
Sum of cysteinylated aldehydes (µg/kg dm)	852			461			471		
2MP-CYS	133 [a]	±	6	76 [b]	±	6	84 [b]	±	2
2MB-CYS	42 [a]	±	2	28 [b]	±	0	37 [a]	±	1
3MB-CYS	398 [a]	±	14	194 [b]	±	8	210 [b]	±	8
MET-CYS	36 [a]	±	3	25 [b]	±	2	31 [b]	±	3
PHE-CYS	182 [a]	±	3	84 [b]	±	4	90 [b]	±	2
FUR-CYS	22 [a]	±	0	19 [a]	±	3		<LOD	
HEX-CYS	39 [a]	±	1	35 [b]	±	1	19 [c]	±	1

Amino acids indicated with *, namely Val = valine, Ile = isoleucine, Leu = leucine, Met = methionine and Phe = phenylalanine are potential precursors of the corresponding Strecker degradation aldehydes: 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, respectively. Cyss+Cys = cystine/cysteine. Other compounds: FUR = furfural, HEX = hexanal, T2N = *trans*-2-nonenal, 2MP-CYS = cysteinylated 2-methylpropanal, 2MB-CYS = cysteinylated 2-methylbutanal, 3MB-CYS = cysteinylated 3-methylbutanal, MET-CYS = cysteinylated methional, PHE-CYS = cysteinylated phenylacetaldehyde, FUR-CYS = cysteinylated furfural, and HEX-CYS = cysteinylated hexanal (cysteinylated *trans*-2-nonenal is not presented as the reference compound was not available). LOD = limit of detection. Results are presented as mean values ± standard deviation (SD). Statistical comparisons between the batches I, J, and H, by post-hoc HSD Tukey's test to distinguish among statistically significant different ($p \leq 0.05$) groups (a, b, c).

Table 3-5. Pearson's correlation coefficients calculated for amino acids as a function of their corresponding Strecker aldehyde, as well as free Strecker aldehydes as a function of their corresponding cysteinylated aldehyde.

Compound 1	Compound 2	Correlation coefficient
valine	2-methylpropanal	0.96
isoleucine	2-methylbutanal	0.93
leucine	3-methylbutanal	0.92
methionine	methionine	0.91
phenylalanine	phenylacetaldehyde	0.82
2-methylpropanal	cysteinylated 2-methylpropanal	0.87
2-methylbutanal	cysteinylated 2-methylbutanal	0.83
3-methylbutanal	cysteinylated 3-methylbutanal	0.88
methionine	cysteinylated methionine	0.87
phenylacetaldehyde	cysteinylated phenylacetaldehyde	0.95

Degree of freedom (df) = 7, p-vale always <0.01.

Table 3-6. Pearson's correlation coefficients calculated for free aldehydes as a function of malt quality parameters.

Aldehyde/ Quality parameter	2MP	2MB	3MB	MET	PHE	FUR	HEX	T2N
Extract	0.90	0.91	0.91	0.92	0.93	0.92	0.80	-
Moisture content	-	-	-	-	-	-	-	-
Colour	0.95	0.94	0.95	0.92	0.96	0.92	0.75	-
TBI	1.00	0.99	0.99	0.84	0.98	0.98	0.82	-
Friability	-	-	-	-	-	-	-	0.78
Homogeneity	-	-	-	-	-	-	-	0.78
Total protein	-	-	-	-	-	-	-	0.85
Total soluble protein	0.79	0.75	0.76	0.83	0.73	0.80	-	-
Kolbach Index	0.88	0.86	0.86	0.89	0.76	0.89	-	-
Free amino nitrogen	0.81	0.77	0.78	0.82	0.74	0.81	-	-
Diastatic power	0.93	0.92	0.93	0.92	0.87	0.95	0.73	-
α-Amylase activity	0.93	0.91	0.92	0.90	0.82	0.94	-	-
β-Glucan content	-	-	-	-	-	-	-	-
Viscosity	-	-	-	-	-	-	-	-
pH	-	-	-	-	-	-	-	-

'-' – correlation coefficient not presented as p-value >0.05 (not significant), when correlation coefficient presented p-vale ≤0.05; degree of freedom (df) = 7. Aldehydes: 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, and T2N = *trans*-2-nonenal.

3.4. Conclusions

The study carried out in chapter 3 presents the first detailed investigation on the evolution of both free and cysteinylated aldehydes throughout the industrial-scale malting process. In addition, possible relationships between free aldehydes, cysteinylated aldehydes, and standard quality parameters of malt were analysed.

Exploratory monitoring of levels of free and cysteinylated aldehydes throughout industrial-scale malting, demonstrated that the content of both free and cysteinylated aldehydes, clearly increases from barley to finished malt. More specifically, the initial stage of germination points to enzymatic fatty acid oxidation as reflected by the formation of hexanal and *trans*-2-nonenal, whereas kilning (in particular, the stage of green malt drying when moisture declines from approx. 30% to 4.5%) appears to be critical for the formation of (cysteinylated) Strecker aldehydes, as well as for generation of furfural. Moreover, at this particular stage, a pronounce decrease in levels of hexanal were observed. A second pronounced increase in levels of *trans*-2-nonenal was observed during kilning-off. Finally, at the very end of malting, *i.e.* cooling of malt, increased levels of Strecker aldehydes, furfural and cysteinylated aldehydes were noticed in this study.

Comparison between levels of aldehydes in barley, germinating barley and finished pale malt, derived from three different malting batches, confirmed the increase in levels of aldehydes during the malting process, as observed in the single batch experiment, described in the previous paragraph. Thus, the highest values of free and cysteinylated aldehydes were again found in finished pale malt, except for hexanal, which already reached its maximum at the beginning of germination.

Finally, from comprehensive analysis of standard quality parameters, amino acids, and aldehydes in pale malts, it can be concluded that a particular set of malt quality parameters (thiobarbituric acid index (TBI), colour, total soluble protein (TSP), Kolbach Index (KI), free amino nitrogen (FAN), diastatic power (DP), and α-amylase activity) seem to be associated with the development of free aldehydes, especially Strecker degradation aldehydes. Moreover, strong positive correlations ($r \geq 0.8$) were always found between individual amino acids and their corresponding, individual free Strecker aldehydes. Also, a positive relationship ($r \geq 0.8$) between levels of individual, free Strecker aldehydes and levels of their corresponding cysteinylated forms was found, in accordance with generally known thermodynamics of chemical equilibrium.

Chapter 4

Evaluation of industrial-scale malting in relation to process-associated physicochemical gradients and the formation of staling aldehydes

4.1. Introduction

Based on the state-of-the-art of literature (for an overview, see Table 1-4, chapter 1, and Filipowska *et al.*[233]), it is generally accepted that both germination and kilning represent critical stages in malting, in relation to the development of staling aldehydes. Furthermore, our explorative study on the evolution of (cysteinylated) aldehydes during industrial-scale malting (chapter 3), provided additional support to this concept. In chapter 3, it was found that the initial stage of germination is critical to the formation of hexanal and *trans*-2-nonenal, while a particular stage of green malt drying (reduction in moisture from approx. 30% to 4.5%) resulted in higher levels of (cysteinylated) Strecker aldehydes and furfural. In addition, cooling of malt at the very end of malting also contributed to increased levels in aldehydes (except for free hexanal). These observations on the evolution of aldehydes throughout a single, industrial-scale, pale malt production process, provided promising, new insights, which will be re-analysed and further evaluated in this part of the PhD. Consequently, the first objective of chapter 4 is to more reliably specify the most critical point(s) regarding generation of free and cysteinylated aldehydes during malting. To this end, a comprehensive analysis on the levels of both free and cysteinylated aldehydes is performed on dedicated samples collected throughout two independent, industrial-scale pale malt productions, with the focus on germination, kilning, and cooling.

From the technological point of view, commonly applied pneumatic malting techniques lead to inevitable inhomogeneity among kernels positioned at different heights of a thick grain bed layer. The resulting gradients become especially pronounced during kilning when dry, warm air is supplied from the floor level of the kiln and turning is omitted[128,129]. Consequently, kilning conditions (in particular, conditions of temperature and moisture) may highly differ between kernels positioned in the upper and the lower layers of a grain bed, thereby affecting finished malt quality[143,234,235]. For example, higher Kolbach Index[235] was determined in malts derived from the upper layer. Moreover, a lower α-amylase activity[234] and LOX-activity[59] were measured in malts collected from the bottom layer. Since some of these parameters (*e.g.* Kolbach Index) were found to be related to aldehydes (see chapter 3), we hypothesise that the formation of staling aldehydes in different grain bed layers might also be affected. Therefore, the second aim of this study is to investigate as to which extent currently inherent, process-related gradients might contribute to differential formation of aldehydes. Thus, levels of (cysteinylated) aldehydes will be determined in dedicated samples collected from the bottom, middle, and top layers of the grain bed during industrial-scale kilning, and the results obtained will be evaluated in relation to the progress of the drying process and the applied head load.

4.2. Materials and methods

4.2.1. Chemicals

Detailed information on the chemicals used for determination of free and cysteinylated aldehydes may be found in chapter 2, section 2.2.1. Chemicals required for determination of TBI and standard malt quality parameters are specified in chapter 3, section 3.2.1.

4.2.2. Malting process

Industrial-scale operations were performed at the malting facility of Boortmalt Antwerp, Belgium. Six-row winter barley variety Etincel was subjected to two independent industrial-scale malting procedures for pale malt production, resulting in the malting batches K and L. The malting regime comprised 34h of steeping, 96h of germination with periodic turning, and approx. 22h - 23h of kilning. According to the information provided by the malting company, steeping degrees were similar for both batches, as well as moisture contents measured at the end of germination. Furthermore, total kilning times for batches K and L were 22h 31 min and 21h 32 min, respectively. The one hour difference in kilning time results from the kilning cycle being controlled by the conditions during the real-time performance of kilning, rather than by a pre-set time-temperature program. Consequently, kilning time of batch K was prolonged for 30 min at the stage of 72°C and 20 min at the stage of 85°C. At the end of malting max. 50 min of cooling (95% of fan speed) was applied.

Sampling: at onset of the process (barley); after every day of germination, starting with onset of germination (GM0) and finishing after the fourth day of germination (GM4); during kilning, every 1-2h between 12-18h of green malt drying (K12-K18), and every 0.5-1h between 18.5h (K18.5h) up to the end of kilning; during cooling every 10-15 min staring from the onset of cooling (C0) up to 50 min (C50), and at the end of the process (finished pale malt without rootlets).

Sampling during germination was performed according to the internal standard protocol for sample collection. Samples during kilning were collected from three different layers of the grain bed, *i.e*. bottom layer (5 cm), middle layer (56 cm) and top layer (117 cm), measured from the floor level of a germination-kilning vessel. Samples during cooling were collected from the top layer.

4.2.3. Determination of free aldehydes in samples from industrial-scale malting

Quantitative determination of free aldehydes in samples from industrial-scale malting was performed according to the optimised in chapter 2 procedure – description of sample preparation is presented in chapter 3, section 3.2.3, while instrumental conditions in chapter 2, section 2.2.2. In this study, concentrations are presented as ‘peak area ratio/kg dm’. Term ‘peak area ratio’ refers to ratio between area of a peak of an individual aldehyde and area of a peak of the corresponding internal standard. The obtained value is corrected for the dilution factor from sample preparation (x100) and moisture content of an analysed sample.

4.2.4. Determination of cysteinylated aldehydes in samples from industrial-scale malting

Quantitative determination of cysteinylated aldehydes in various samples from industrial-scale malting was performed on freeze-dried powders of either barley, germinating barley, green malt or finished malt samples, adapted to a moisture content of approx. 4% (for more details on the procedure of freeze-drying, see chapter 2, section 2.2.10). Determination of cysteinylated aldehydes was further carried out as described in chapter 2, section 2.2.9. In this study, concentrations are presented as 'peak area ratio/kg dm'. Term 'peak area ratio' refers to ratio between peak area of an individual cysteinylated aldehyde and area of the same compound determined in the 'quality control' sample. The 'quality control' is prepared in the same way as calibration sample (see chapter 2, section 2.2.9), and it is analysed immediately after each malt extract sample. The 'peak area ratio' is further corrected for the dilution factor (x20) and moisture content of an analysed sample.

4.2.5. Determination of TBI in samples from industrial-scale malting

Determination of the thiobarbituric acid index (TBI) was performed on 2 g of freeze-dried powders of either barley, germinating barley, green malt or finished malt samples, adapted to a moisture content of approx. 4% (for more details on the procedure of freeze-drying, see chapter 2, section 2.2.10). TBI was measured following the adapted method of Coghe *et al*.[218], which itself is based on the method described by Thalacker and Brikenstock[219] (for more details, see chapter 3, section 3.2.5.).

4.2.6. Determination of moisture content in samples from industrial-scale malting

Moisture content was assessed according to the European Brewery Convention methods (EBC Analytica, 2018) – EBC method 3.2 was applied for barley, while EBC method 4.2 was used for germinating barley, green malt, and finished malt samples.

4.2.7. Determination of standard quality parameters of finished malt

Standard quality parameters of finished malt were assessed according to the European Brewery Convention methods (EBC Analytica, 2018) as described in chapter 3, section 3.2.7.

4.2.8. Statistical analysis

Statistically significant differences were analysed by one-way ANOVA (p-value ≤0.05 was selected for statistical significance). To identify differences between multiple groups of samples, one-way ANOVA was followed by a post-hoc HSD Tukey's test (applied software: SPSS Statistics 26 by IBM, USA). Statistical correlations amongst data were calculated via Pearson's correlation coefficients (r) (applied software: SPSS Statistics 26 IBM, USA).

4.3. Results and discussion

4.3.1. Evaluation of quality parameters of pale lager malts derived from two industrial-scale malt productions

This section will discuss the basic quality parameters of pale lager malts, derived from two independent, industrial-scale malting processes (malting batch K and malting batch L), carried out at the same malting facility. Significant differences between the quality of both malts are found (see Table 4-1). Malt L clearly shows a higher degree of proteolytic modification (higher levels of total soluble protein, Kolbach Index, and free amino nitrogen), as well as higher enzymatic potential (higher diastatic power and α-amylase activity). The difference in cytolytic modification is limited, however, it can be regarded as significant, since a lower β-glucan content and a higher viscosity were determined in Congress wort derived from malt L. The higher viscosity may be related to a higher percentage of partly unmodified grains. The latter includes the count of whole grains, which have been reported to increase wort viscosity[236]. In addition, no statistically significant difference was found in colour between the two malts, whereas the thiobarbituric acid index (TBI) in malt L was significantly higher than in malt K (even though the total kilning time of batch K was longer). The apparent contradiction between colour and TBI is due to the fact that malt L is more strongly modified, which is associated with more formation of carbonyl compounds (during the later kilning stage), and, therefore, with a higher TBI value. Thus, it appears that, at least for pale lager malts, measured TBI values do not necessarily directly reflect the amount of heat load applied during kilning. Accordingly, regarding the content of free aldehydes, malt L in comparison to malt K, contains approx. 2.5 times more free aldehydes. In particular, much higher levels of Strecker aldehydes (3.1-fold up to 4.4-fold, depending on the nature of the aldehyde) were determined in malt L. Based on the data on proteolytic modification of the malts and levels of Strecker aldehydes, it is hypothesised that higher proteolytic modification may lead to higher levels of Strecker aldehydes in finished malt, since higher amounts of suitable precursors (amino acids[217]) and reactants (α-dicarbonyls[78]) for Strecker degradation have been reported in more modified malts. Next to more Strecker aldehydes, 4-fold higher levels of furfural are present in malt L, which is also reflected by the higher TBI value. Furthermore, malt L contains approx. two times the amount of hexanal and *trans*-2-nonenal, when compared to malt K. The content of cysteinylated aldehydes was approx. 2.2 times higher in malt L, in comparison to malt K.

Comparison of free and cysteinylated aldehydes shows that levels of individual free aldehydes are always higher than levels of their cysteinylated counterparts. Furthermore, 3MB appears to be the major free aldehyde, followed by PHE, 2MP, and 2MB. Likewise, 3MB-CYS was found in the highest concentration in all samples, followed by PHE-CYS, 2MP-CYS and 2MB-CYS.

In summary, measurements on standard malt quality parameters and levels of (cysteinylated) aldehydes differ significantly for malt K and malt L. Therefore, in the following sections, the malting batches K and L will be discussed separately.

Table 4-1. Comparison of standard quality parameters and levels of free and cysteinylated aldehydes, determined in finished malts derived from the industrial-scale maltings K and L.

Malting process/ Analytical parameter	Malting K			Malting L			Statistical significance
	Mean		SD	Mean		SD	
Moisture (%)	4.4	±	0.2	4.3	±	0.2	
Extract yield (% dm)	79.7	±	0.4	81.5	±	0.4	
Colour (EBC)	2.7	±	0.4	3.3	±	0.4	
TBI (for 2 g dm)	7.66	±	0.11	13.38	±	0.14	*
Friability (%)	77.8	±	1.7	78.4	±	1.7	
Homogeneity (%)	93.2	±	0.5	91.8	±	0.5	*
Partly unmodified grains (%)	6.8	±	0.5	8.2	±	0.5	*
β-Glucan (mg/L)	306	±	20	259	±	20	*
Viscosity (mPas)	1.56	±	0.02	1.61	±	0.02	*
Total protein (% dm)	10.6	±	0.3	10.5	±	0.3	
Total soluble protein (% dm)	3.8	±	0.2	4.5	±	0.2	*
Kolbach index (%)	35.7	±	1.4	42.5	±	1.4	*
Free amino nitrogen (mg/L)	122	±	9	173	±	9	*
Diastatic power (°WK)	379	±	25	483	±	25	*
α-Amylase activity (DU)	44.0	±	5.5	60.0	±	5.5	*
pH	5.99	±	0.05	5.91	±	0.05	
Sum of free aldehydes (µg/kg dm)	2,997			7,666			
2MP	375	±	16	1,270	±	234	*
2MB	370	±	25	1,252	±	263	*
3MB	655	±	45	2,033	±	138	*
MET	71	±	11	312	±	46	*
PHE	380	±	39	1,493	±	362	*
FUR	176	±	16	705	±	135	*
HEX	191	±	29	358	±	42	*
T2N	124	±	15	244	±	43	*
Sum of cysteinylated aldehydes (µg/kg dm)	548			1,216			
2MP-CYS	92	±	6	334	±	4	*
2MB-CYS	36	±	3	100	±	1	*
3MB-CYS	218	±	20	477	±	21	*
MET-CYS	27	±	2	65	±	3	*
PHE-CYS	110	±	7	209	±	3	*
FUR-CYS	<LOD			<LOD			
HEX-CYS	65	±	4	31	±	1	*

Compounds: 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, and T2N = *trans*-2-nonenal, 2MP-CYS = cysteinylated 2-methylpropanal, 2MB-CYS = cysteinylated 2-methylbutanal, 3MB-CYS = cysteinylated 3-methylbutanal, MET-CYS = cysteinylated methional, PHE-CYS = cysteinylated phenylacetaldehyde, FUR-CYS = cysteinylated furfural, and HEX-CYS = cysteinylated hexanal (cysteinylated *trans*-2-nonenal is not presented as the reference compound was not available). Results are presented as mean values (n=2 or 3, depending on the measured analytical parameter) ± standard deviation (SD). Statistical comparison between batches by Student's t-test; * = statistically significant (p-value ≤ 0.05).

4.3.2. Monitoring of free and cysteinylated aldehydes throughout industrial-scale malting

A comprehensive analysis of the levels of (cysteinylated) aldehydes was performed on dedicated samples collected throughout two independent, industrial-scale pale malt productions, with the focus on germination, kilning, and cooling.

Quantitative data on free and cysteinylated aldehydes determined in samples collected throughout malting process K and L are presented in Appendix A and Appendix B, respectively. Since several data appear to be <LOQ, in this and the following sections, the evolution of free and cysteinylated aldehydes during malting will include only measured values above LOD (however, not necessarily above LOQ), and will be presented therefore as 'peak area ratio/kg dm', as a function of malting time. As a consequence, potential differences among samples can be presented in a clear, reliable way (for more information on calculation of 'peak area ratio/kg dm' see section 4.2.3., of 'Materials and Methods').

The evolution of the sum of (cysteinylated) aldehydes throughout industrial-scale pale malt production (batches K and L) is depicted in Figure 4-1. The overall pattern appears to be similar in both malting batches, even though considerably higher levels of (cysteinylated) aldehydes were determined in samples derived from malting L. Clearly, in both batches, the content of (cysteinylated) aldehydes increases from barley to finished malt. During germination, a limited increase in total aldehydes is observed, while during kilning (in particular during the last hours of this stage), the increase is much more pronounced. In both batches K and L, samples collected from the bottom, middle, and top layer of the kiln, respectively, showed differences. Clearly, the lowest amount of aldehydes was always found in the top layers (differences in levels of (cysteinylated) aldehydes, caused by process-related gradients will be discussed in detail in section 4.3.3.). Finally, regarding these malting batches K and L, cooling only shows a minor effect on (cysteinylated) aldehydes.

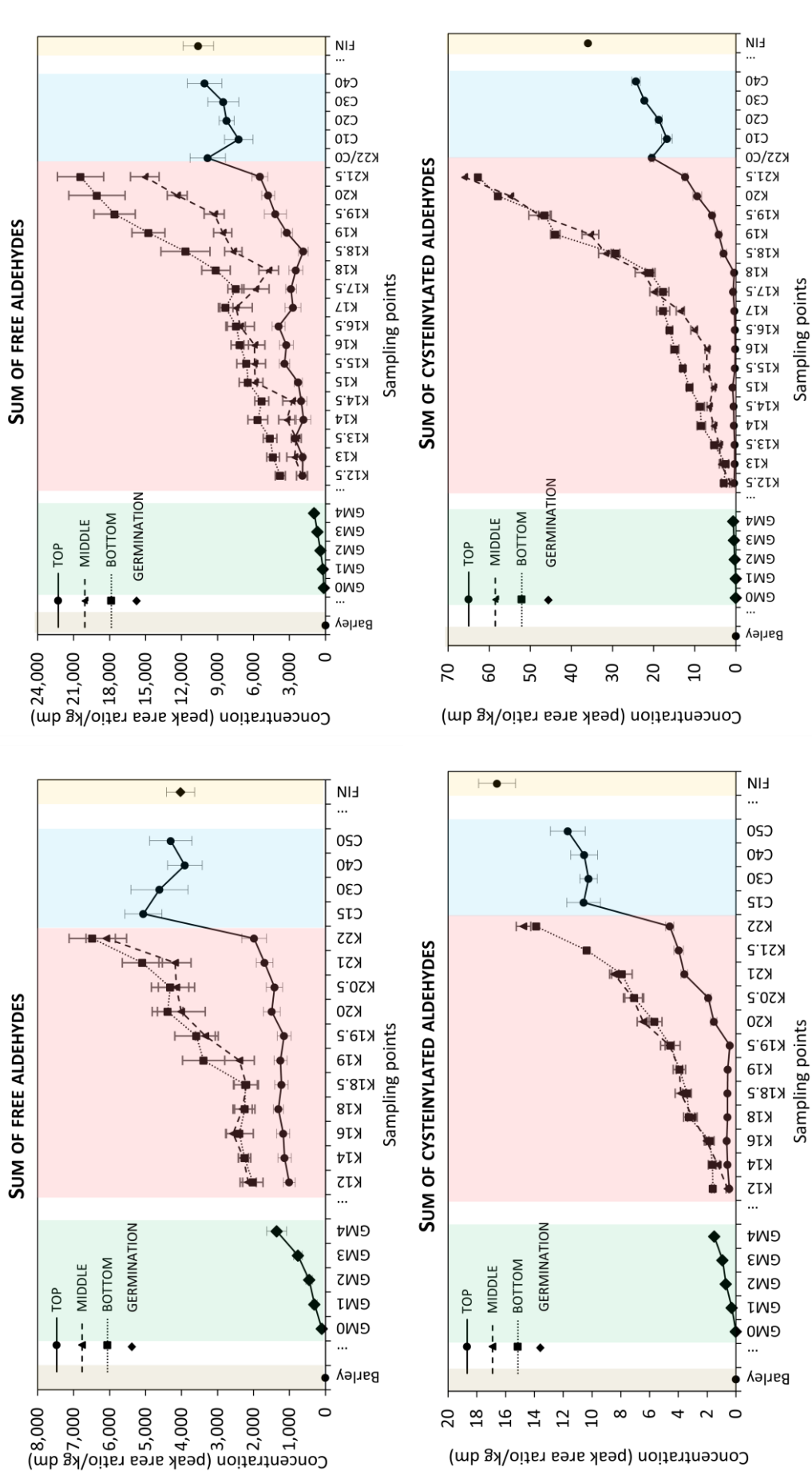


Figure 4-1. Evolution of the sum of free aldehydes and the sum of cysteinylated aldehydes during industrial-scale malting, resulting in batch K (left side) and batch L (right side).

Analysed samples: barley; GM = germinating barley (GM0 = onset of germination; GM1-GM4 = germinated for 1, 2, 3, 4 days, respectively); K = samples taken at kilning, after 12h (K12) up to 22h (K22) of kilning; C = samples taken at cooling, after 0 min (C0) up to 50 min (C50); FIN = finished malt (without rootlets). Sampling during germination is indicated by diamonds (◆); during kilning, samples were collected from TOP (•), MIDDLE (▲) and BOTTOM (■) grain bed layer, while during cooling, samples were taken only from the TOP layer (•). Results are expressed as mean values (n=3), error bars = standard deviation.

The evolution of individual free aldehydes as a function of the duration of industrial-scale malting (batches K and L) is depicted in Figure 4-2. Measurements on the corresponding cysteinylated aldehydes are shown in Figure 4-3. Only major changes in levels of free and bound-state aldehydes will be discussed, because each time point is represented by a single, biological sample, and because representative sampling from industrial-scale productions of tons of malt is anyway highly complicated.

The results obtained for individual (cysteinylated) aldehydes resemble the evolution described for the sum of (cysteinylated) aldehydes. Generally, evolution patterns are similar for malting K and malting L, and they differ among aldehydes originating from different chemical pathways. On the one hand, Strecker aldehydes all show a similar behaviour during the malting process, likewise furfural, as both types of aldehydes are connected to the Maillard reaction[233]. However, the evolution of the fatty acid oxidation products, hexanal and *trans*-2-nonenal, is clearly different from the other aldehydes (for instance, more pronounced increases during germination are noticed, because of LOX-activity). When comparing hexanal and *trans*-2-nonenal, a differential behaviour can be observed, which can be ascribed to specific properties of the lipoxygenase enzymes involved in their formation (LOX-1 leading to *trans*-2-nonenal and LOX-2 to hexanal)[65,223], as well as to differences in volatility of these aldehydes[225]. In general, cysteinylated aldehydes evolved similarly to their free counterparts.
All of the above observations made for the malting batches K and L are largely in accordance with our previous findings on malting batch G (see chapter 3).

Regarding particular stages of malting, in barely, all investigated free and cysteinylated aldehydes were <LOD. During germination, a first increase in levels of (cysteinylated) hexanal, *trans*-2-nonenal, and 2-methylpropanal was observed. As mentioned before, the increase in levels of fatty acid oxidation products during germination is well known from literature[64,110,112] and also in accordance with our previous findings (see chapter 3). Furthermore, an increase in levels of 2-methylpropanal during germination has also been reported by Dong *et al.*[110]. During kilning, levels of all individual (cysteinylated) aldehydes significantly increase (except for hexanal). The observed increases remain moderate until approx. 18 h of kilning (for both batches K and L), while in the later stage, levels in individual aldehydes increase much faster, in particular in the bottom layer of the kiln. This is due to more exposure to heat, which is known to accelerate the formation of, in particular, furfural and Strecker aldehydess[77,110,133].
Regarding malt cooling, no statistically significant differences among the various samples collected as a function of cooling time, are found (see Table 4-2). In chapter 3, however, cooling appeared to have a pivotal impact on aldehyde formation. Therefore, more detailed investigations regarding the stage of malt cooling are required.

In summary, the results obtained from monitoring of two independent, industrial-scale, pale lager malt production processes, indicate that germination and, in particular kilning should be regarded as critical stages of malt production in relation to the formation of aldehydes. In addition, these findings largely confirm our results obtained in chapter 3. Moreover, it is shown for the first time that process-related gradients appear to impact formation of (cysteinylated) aldehydes during kilning. In the following section, statistical analysis is performed in order to determine the potential significance of the observed differences in levels of aldehydes between the grain bed layers of the kiln.

Table 4-2. Statistical comparison between levels of free and cysteinylated aldehydes in samples collected during the cooling stage of the malting batches K and L.

Cooling time (min)/	**Cooling K**				**Cooling L**				
Compound	**15**	**30**	**40**	**50**	**0**	**10**	**20**	**30**	**40**
2MP / 2MP-CYS	a / I	a / I	b / I,II	a,b / II	ab / I	a / I	ab / I	ab / II	b / II
2MB / 2MB-CYS	a / I	a / I,II	a / I,II	a / II	ab / I	a / II	ab / III	ab / IV	b / IV
3MB / 3MB-CYS	a / I	a / I,II	a / II	a / II	a / I	a / II	a / II	a / III	a / III
MET / MET-CYS	a / I	a,b / I,II	b / II	a,b / I,II	ab / I	a / II	ab / II	ab / I	b / I
PHE / PHE-CYS	a / I	a / I,II	a / I,II	a / II	ab / I	a / II	ab / I	ab / III	b / IV
FUR / FUR-CYS	a / -	a / -	a / -	a / -	ab / -	c / -	ac / -	a / -	b / -
HEX / HEX-CYS	a / I	a / I,II	a / II	a / I,II	ab / I	ab / II	ab / II	a / I,II	b / I,II
T2N	a	a	a	a	a	b	b	b	b

Compounds: 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, and T2N = *trans*-2-nonenal, 2MP-CYS = cysteinylated 2-methylpropanal, 2MB-CYS = cysteinylated 2-methylbutanal, 3MB-CYS = cysteinylated 3-methylbutanal, MET-CYS = cysteinylated methional, PHE-CYS = cysteinylated phenylacetaldehyde, FUR-CYS = cysteinylated furfural, and HEX-CYS = cysteinylated hexanal (cysteinylated *trans*-2-nonenal is not presented as the reference compound was not available). FUR-CYS<LOD. Statistical comparisons between malt samples collected during cooling is performed by post-hoc HSD Tukey's test to distinguish among significant different groups ($p \leq 0.05$) (groups for free aldehydes: a, b, c; groups for cysteinylated aldehydes I, II, III, IV).

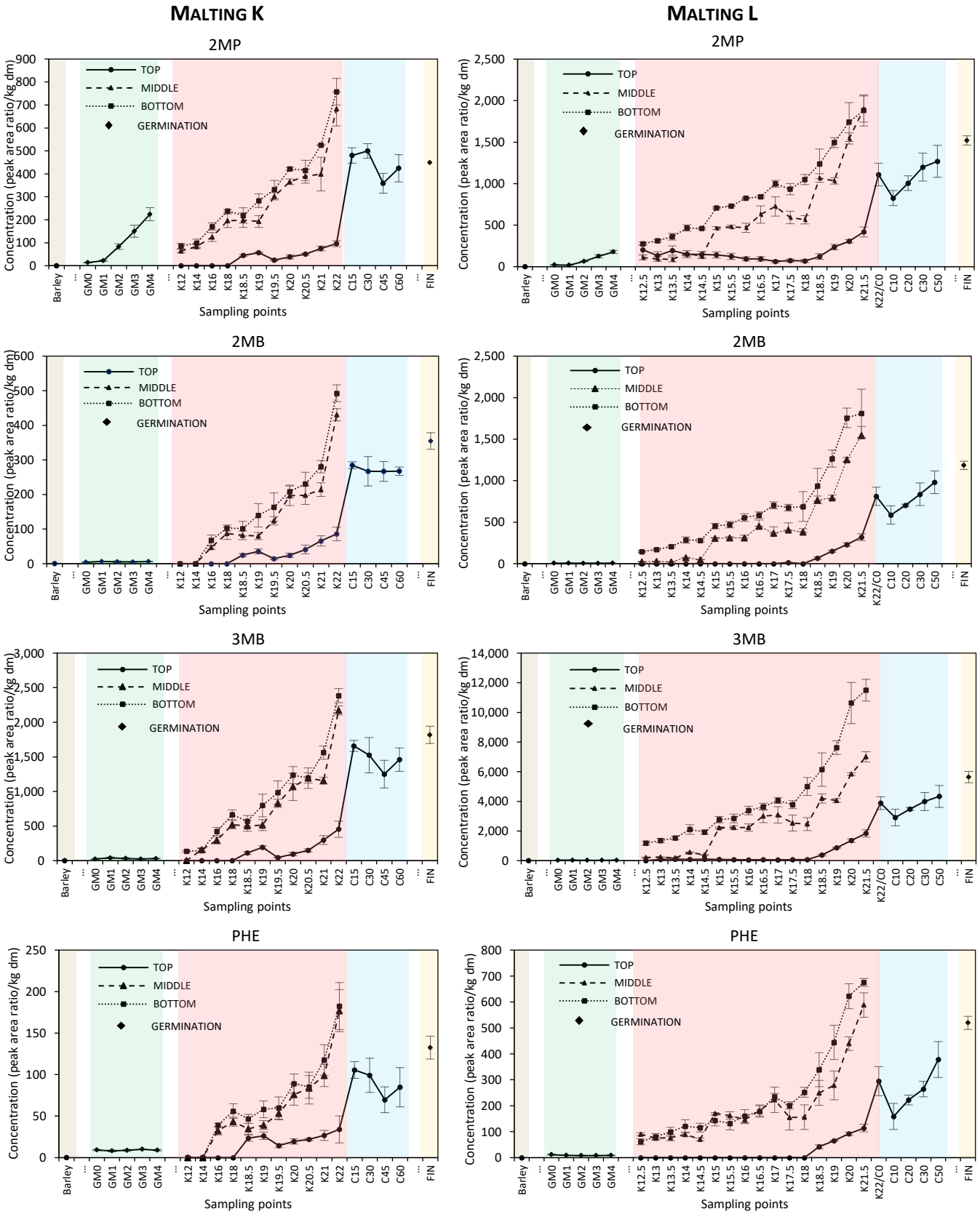


Figure 4-2A. Evolution of individual free aldehydes during industrial-scale malting, resulting in batch K (left side) and batch L (right side).

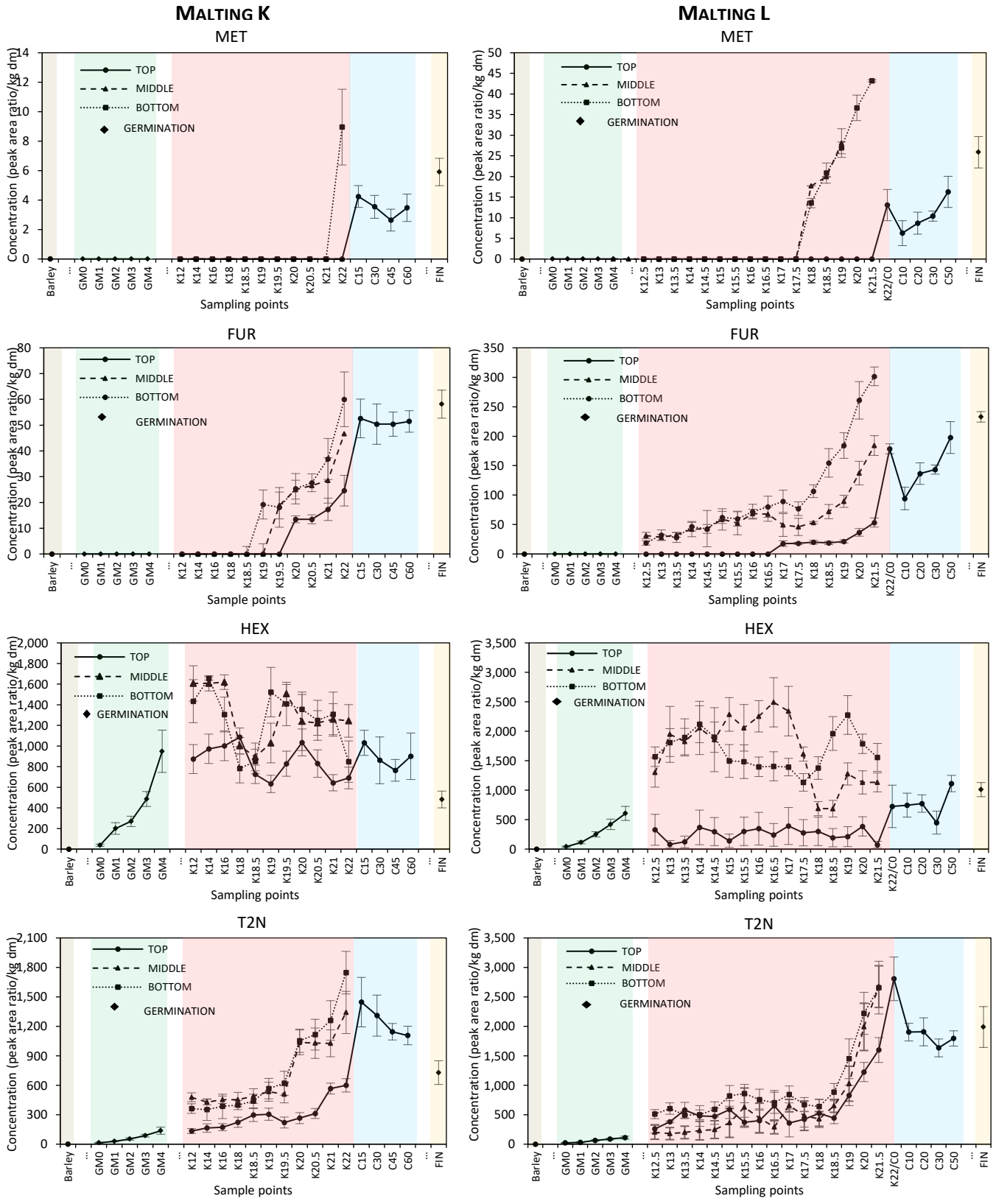


Figure 4-2B. Evolution of individual free aldehydes during industrial-scale malting, resulting in batch K (left side) and batch L (right side).

Analysed samples: barley; GM = germinating barley (GM0 = onset of germination; GM1-GM4 = germinated for 1, 2, 3, 4 days, respectively); K = samples taken at kilning, after 12h up to 22h of kilning; C = samples taken at cooling, after 0 min up to 50 min; FIN = finished malt (without rootlets). Sampling during germination is indicated by diamonds (◆); during kilning, samples were collected from TOP (•), MIDDLE (▲) and BOTTOM (■) grain bed layer, while during cooling, samples were taken only from the TOP layer (•). Compounds: 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, and T2N = *trans*-2-nonenal. Results are expressed as mean values (n=3), error bars = standard deviation.

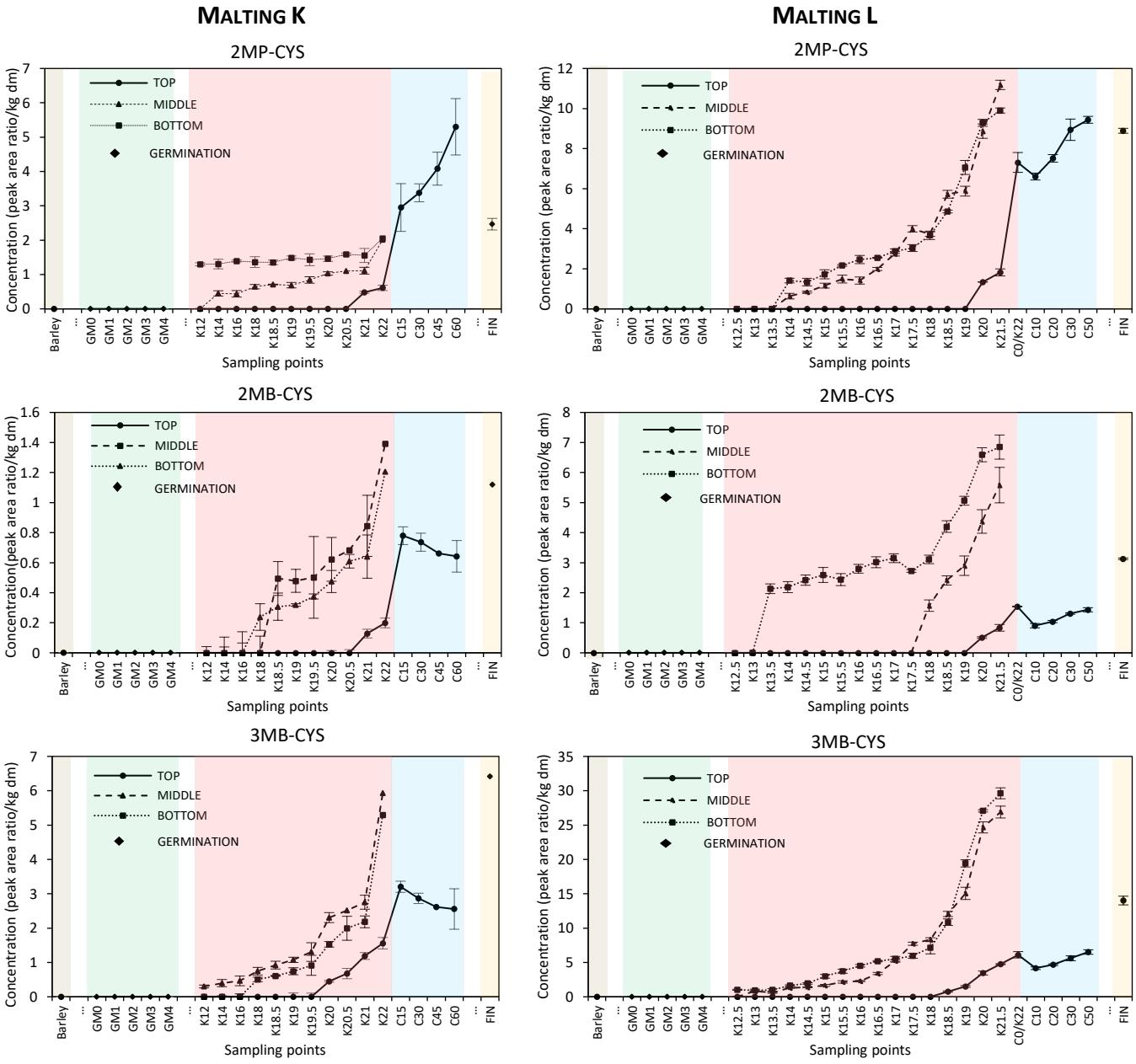


Figure 4-3A. Evolution of individual cysteinylated aldehydes during industrial-scale malting, resulting in batch K (left side) and batch L (right side).

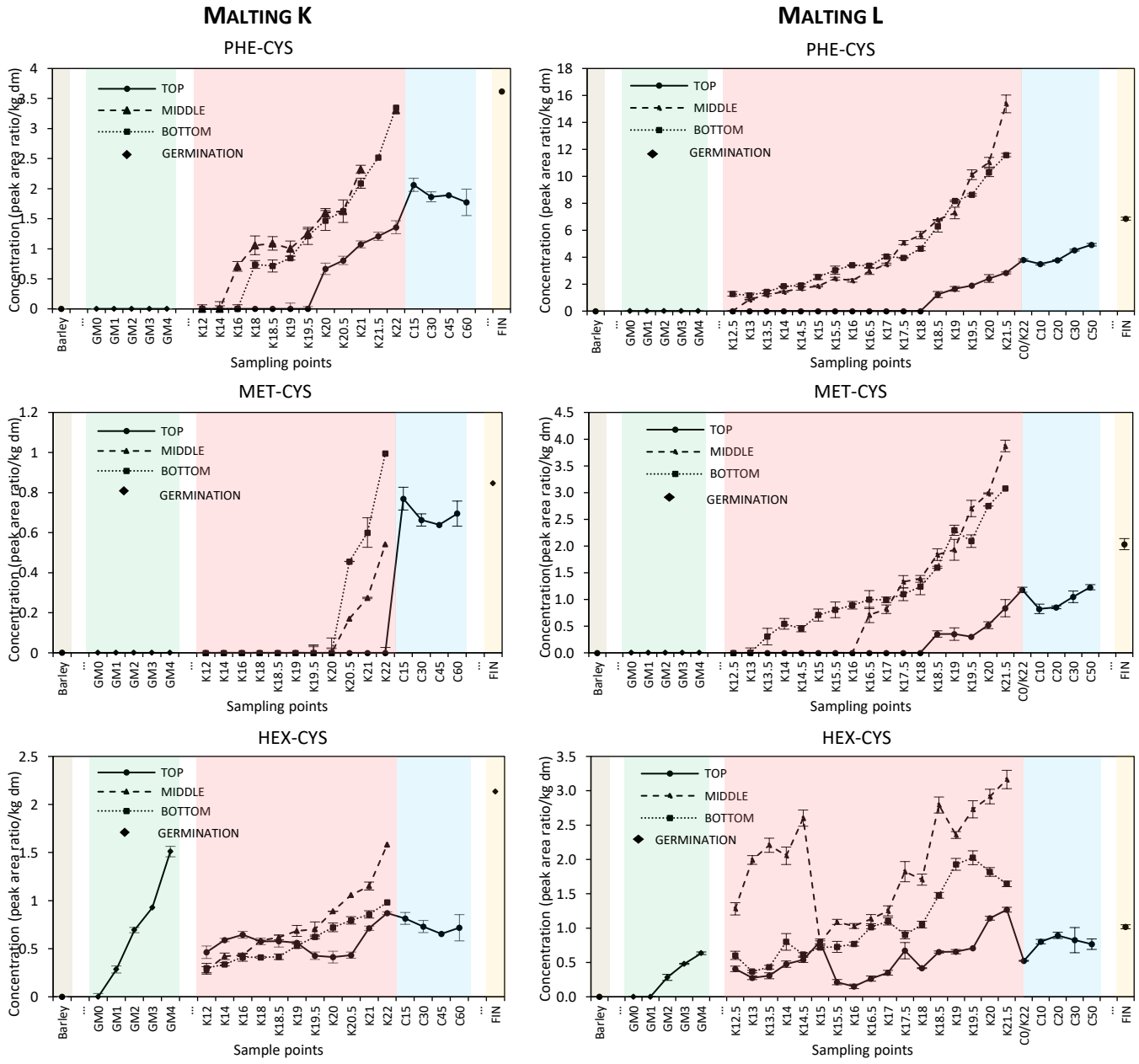


Figure 4-3B. Evolution of individual cysteinylated aldehydes during industrial-scale malting, resulting in batch K (left side) and batch L (right side).

Analysed samples: barley; GM = germinating barley (GM0 = onset of germination; GM1-GM4 = germinated for 1, 2, 3, 4 days, respectively); K = samples taken at kilning, after 12h up to 22h of kilning; C = samples taken at cooling, after 0 min up to 50 min; FIN = finished malt (without rootlets). Sampling during germination is indicated by diamonds (◆); during kilning, samples were collected from TOP (•), MIDDLE (▲) and BOTTOM (■) grain bed layer, while during cooling, samples were taken only from the TOP layer (•). Compounds: 2MP-CYS = cysteinylated 2-methylpropanal, 2MB-CYS = cysteinylated 2-methylbutanal, 3MB-CYS = cysteinylated 3-methylbutanal, MET CYS = cysteinylated methional, PHE-CYS = cysteinylated phenylacetaldehyde, FUR-CYS = cysteinylated furfural, and HEX-CYS = cysteinylated hexanal (cysteinylated *trans*-2-nonenal is not presented as the reference compound was not available). Results are expressed as mean values (n=3), error bars = standard deviation.

4.3.3. Evaluation of process associated gradients in relation to the formation of staling aldehydes

The results presented in the previous part 4.3.2., strongly suggest that process-related gradients, caused by pneumatic processing in a relatively thick grain bed, and thereby provoking physicochemical differences between the various layers, may also impact formation of (cysteinylated) aldehydes. Since these gradients arise mainly during kilning, we will focus the analysis on this stage of malting. The objective is to statistically evaluate in the malting batches K and L, variations among levels of aldehydes determined in various samples derived from the bottom, middle and top layer of the kiln, as a function of kilning time.

For all (cysteinylated) Strecker aldehydes, similar results were obtained (see Appendix C and D for free and cysteinylated Strecker aldehydes, respectively). Therefore, as an example, only the results obtained on 3-methylbutanal (3MB) and cysteinylated 3-methylbutanal (3MB-CYS) are presented here. Generally, statistically significant differences in levels of 3MB were found when comparing the three grain bed layers (see Figure 4-4). Kernels positioned in the bottom layer, and, because of that, directly exposed to dry, warm air, always showed the highest levels of 3MB. On the contrary, the lowest levels of this aldehyde were found in kernels derived from the top layer (exposed to relatively more humid and cooler air). In batch K, levels of 3MB determined in the bottom and middle layer were quite comparable throughout kilning, whereas in batch L, a clear difference between all layers can be observed, especially after longer kilning times. At the end of kilning, approx. 5 times (batch K) and 6 times (batch L) higher levels of 3MB were determined in the bottom layer compared to the top layer (for quantitative data on 3MB and other free aldehydes, see Appendix A).
Regarding 3MB-CYS (see Figure 4-4), statistically significant differences between kernels positioned in the bottom and top layer are also observed in both batches K and L. Differences in the evolution of 3MB between the bottom and middle layer in both batches K and L can be seen, but they are less pronounced. At the end of kilning, approx. 3 times (batch K) and 6 times (batch L) higher levels of 3MB-CYS were determined in samples derived from the bottom layer versus the upper layer (for quantitative data on 3MB-CYS and other cysteinylated aldehydes, see Appendix B).

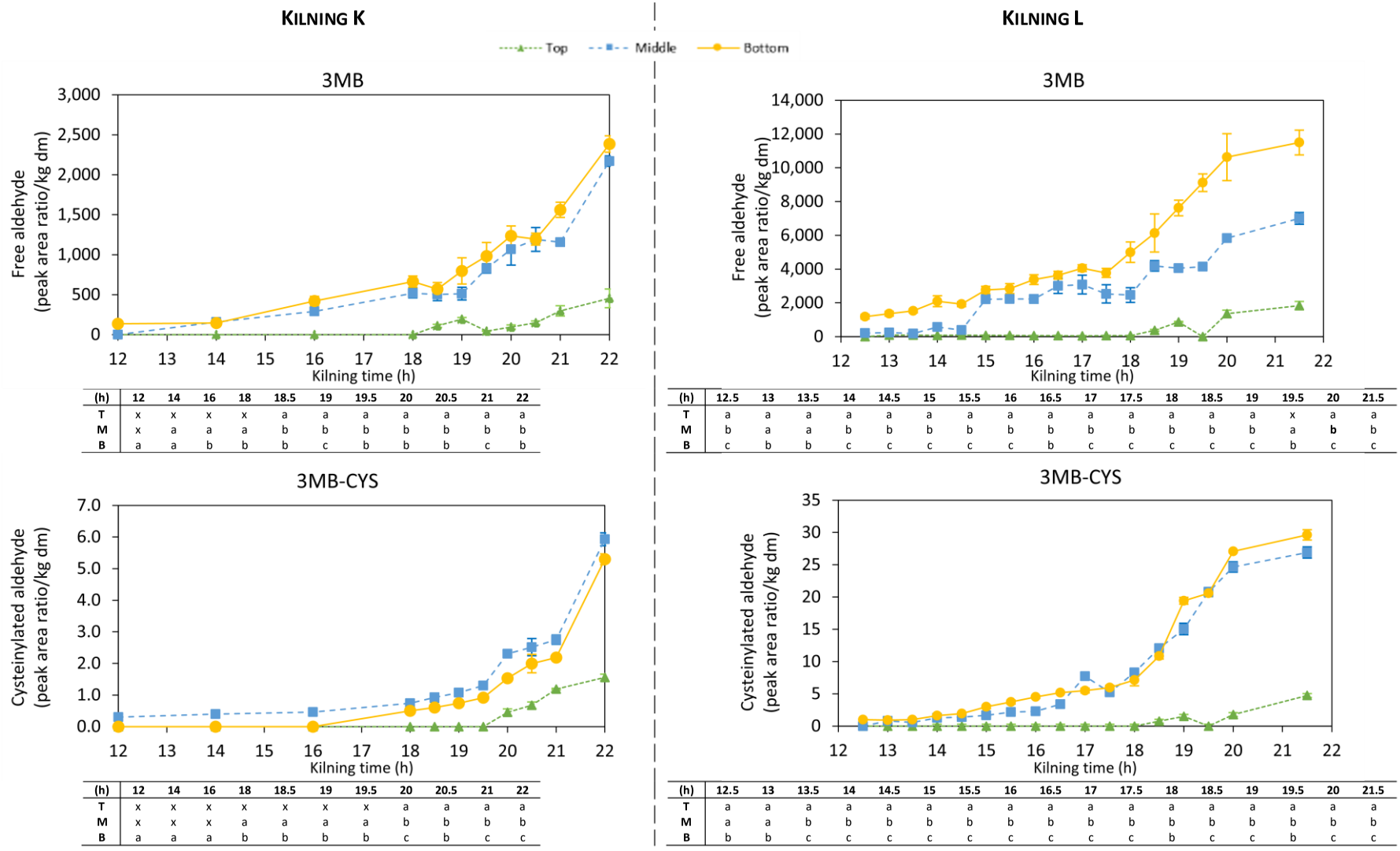
The evolution of furfural (FUR) as a function of kilning time in samples derived from the three layers of the kiln bed, is similar to the one described above for Strecker aldehydes (see Figure 4-5). Thus, always the highest levels of FUR are found in the bottom layer and differences among layers become more pronounced as kilning proceeds. At the end of kilning, approx. 4 times (batch K) and 6 times (batch L) higher levels of FUR were determined in the bottom layer compared to the top layer.

Figure 4-6 represents the evolution of hexanal (HEX) in the three grain bed layers as a function of kilning time. Based on statistical analysis, an unequivocal judgement on the potential effect of process-related gradients in relation to HEX cannot be made (due to fluctuations in levels of this compound during kilning, probably because of the high volatility of HEX). At the end of kilning, levels of HEX were comparable in the three layers and also comparable to the initial levels, determined at the start of monitoring the kilning process. Regarding the non-volatile, cysteinylated hexanal (HEX-CYS), statistically significant differences between the three investigated layers of the grain bed can be noticed for both batches (see Figure 4-6).

At the end of kilning, the highest HEX-CYS content was found in the middle layer, and lowest values were measured in the top layer.

The evolution of *trans*-2-nonenal (T2N) is presented in Figure 4-7. In batch K, levels of T2N were always the lowest in the top layer and were statistically different from the middle and bottom layer, which showed similar behaviour. For batch L, visual inspection of Figure 4-7 allows to observe differences in levels of T2N between bottom and top layers towards the end of kilning. However statistical analysis does not provide conclusive evidence for this observation.

Data presented in this section suggest that process-related gradients (caused by variable exposure to heat load within the thick grain bed), significantly affect generation of both free and cysteinylated aldehydes during the course of kilning. Generally, the highest levels of aldehydes were found in samples derived from the bottom layer, thus the ones exposed to the highest heat load. As far as we know, this is the first scientific reporting and unambiguous analytical evidence of the occurrence of 'staling aldehydes gradients' in nowadays applied pneumatic malting. It is further hypothesised that the existence of such pronounced gradients in final (pilsner) malt quality, may ultimately impair beer quality, and, in particular, beer flavour stability.



(h)	12	14	16	18	18.5	19	19.5	20	20.5	21	22
T	x	x	x	x	a	a	a	a	a	a	a
M	x	a	a	a	b	b	b	b	b	b	b
B	a	a	b	b	b	c	b	b	b	c	b

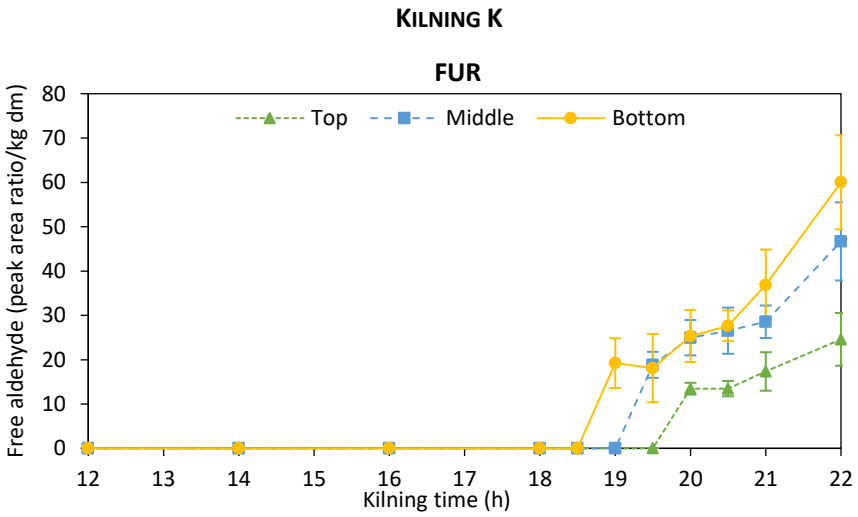
(h)	12.5	13	13.5	14	14.5	15	15.5	16	16.5	17	17.5	18	18.5	19	19.5	20	21.5
T	a	a	a	a	a	a	a	a	a	a	a	a	a	a	x	a	a
M	b	a	a	b	b	b	b	b	b	b	b	b	b	b	a	**b**	b
B	c	b	b	c	c	c	c	c	b	c	c	c	c	c	b	c	c

(h)	12	14	16	18	18.5	19	19.5	20	20.5	21	22
T	x	x	x	x	x	x	x	a	a	a	a
M	x	x	x	a	a	a	a	b	b	b	b
B	a	a	a	b	b	b	b	c	b	c	c

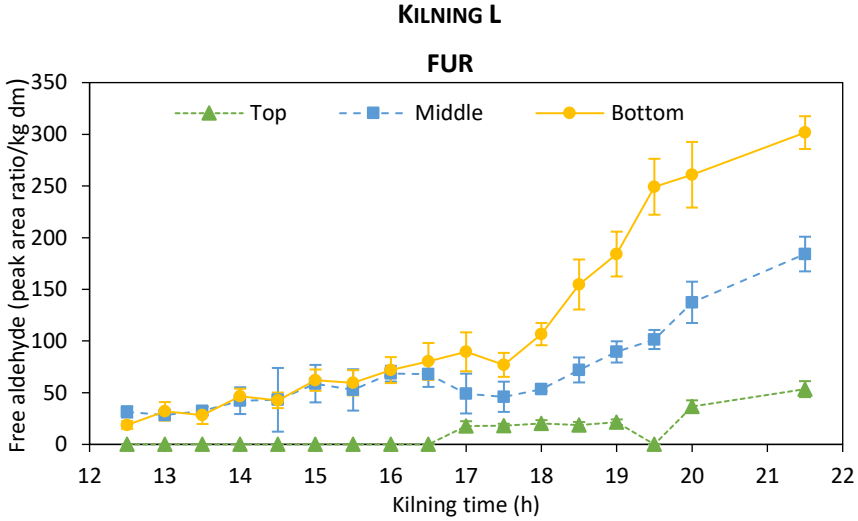
(h)	12.5	13	13.5	14	14.5	15	15.5	16	16.5	17	17.5	18	18.5	19	19.5	20	21.5
T	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
M	a	a	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b
B	b	b	c	c	c	c	c	c	c	c	c	b	c	c	b	c	c

Figure 4-4. Evolution of free and cysteinylated 3-methylbutanal as a function of kilning time in samples collected from the bottom (B), middle (M), and top (T) layer of the kiln - malting batch K (left side), malting batch L (right side).

Results are expressed as mean values (n=3), error bars = standard deviation. Statistical comparisons between bottom, middle and top layer by post-hoc HSD Tukey's test to distinguish among significant different groups ($p \leq 0.05$) (a, b, c). x – statistical comparison not shown because quantification values are below LOD.



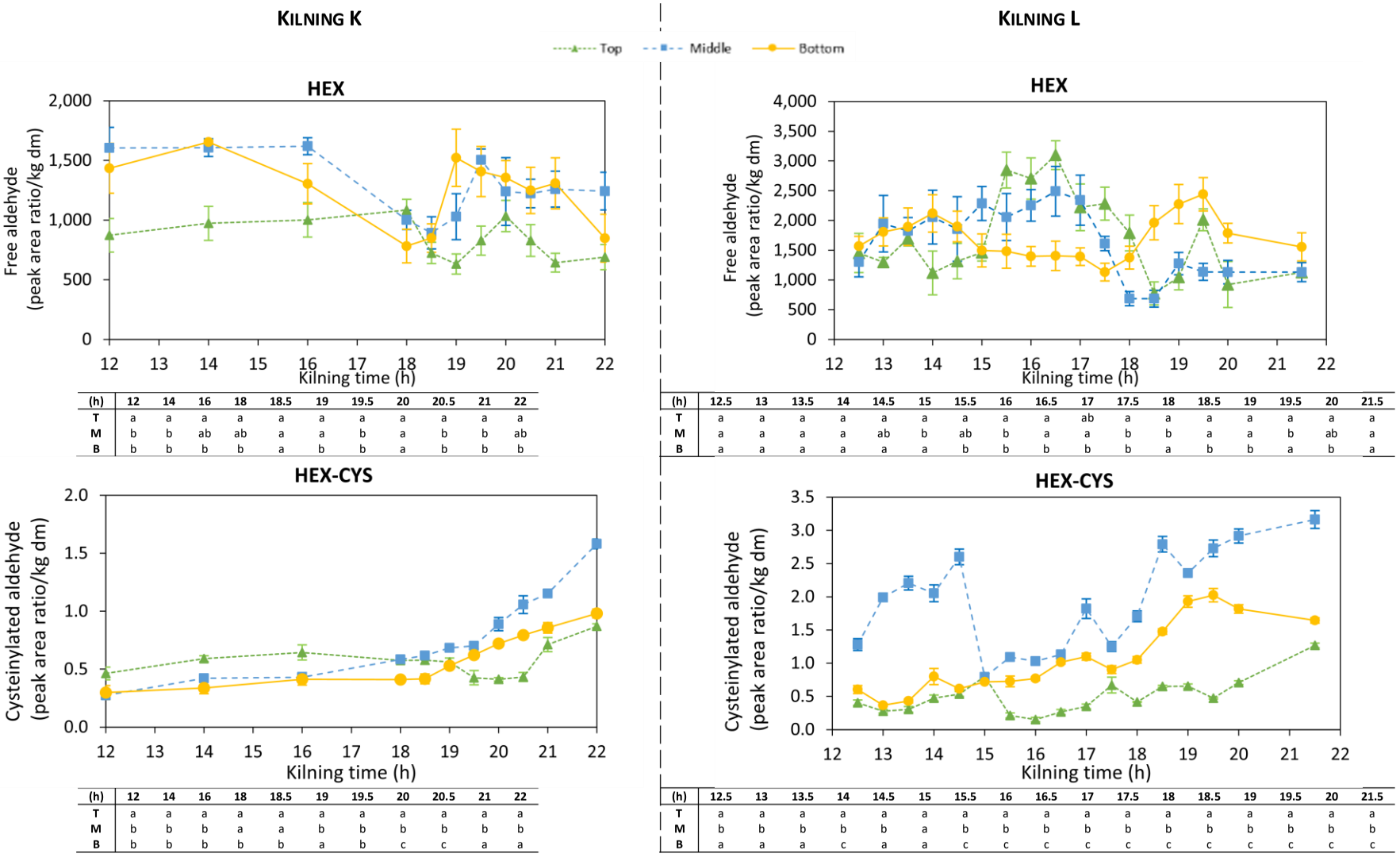
(h)	12	14	16	18	18.5	19	19.5	20	20.5	21	22
T	x	x	x	x	x	x	x	a	a	a	a
M	x	x	x	x	x	x	a	b	b	ab	b
B	x	x	x	x	x	a	a	b	b	b	b



(h)	12.5	13	13.5	14	14.5	15	15.5	16	16.5	17	17.5	18	18.5	19	19.5	20	21.5
T	a	a	a	a	a	a	a	a	a	a	a	a	a	a	x	a	a
M	b	b	b	b	a	b	b	b	b	b	b	b	b	b	a	b	b
B	c	b	b	b	a	b	b	b	b	c	c	c	c	c	b	c	c

Figure 4-5. Evolution of furfural as a function of kilning time in samples collected from the bottom (B), middle (M) and top (T) layer of the kiln - malting batch K (left side), malting batch L (right side).

Results are expressed as mean values (n=3), error bars = standard deviation. Statistical comparisons between bottom, middle and top layer by post-hoc HSD Tukey's test to distinguish among significant different groups ($p \leq 0.05$) (a, b, c). x – statistical comparison not shown because quantification values are below LOD. FUR-CYS<LOD.



KILNING K – HEX (h)	12	14	16	18	18.5	19	19.5	20	20.5	21	22
T	a	a	a	a	a	a	a	a	a	a	a
M	b	b	ab	ab	a	a	b	a	b	b	ab
B	b	b	b	b	a	b	b	a	b	b	b

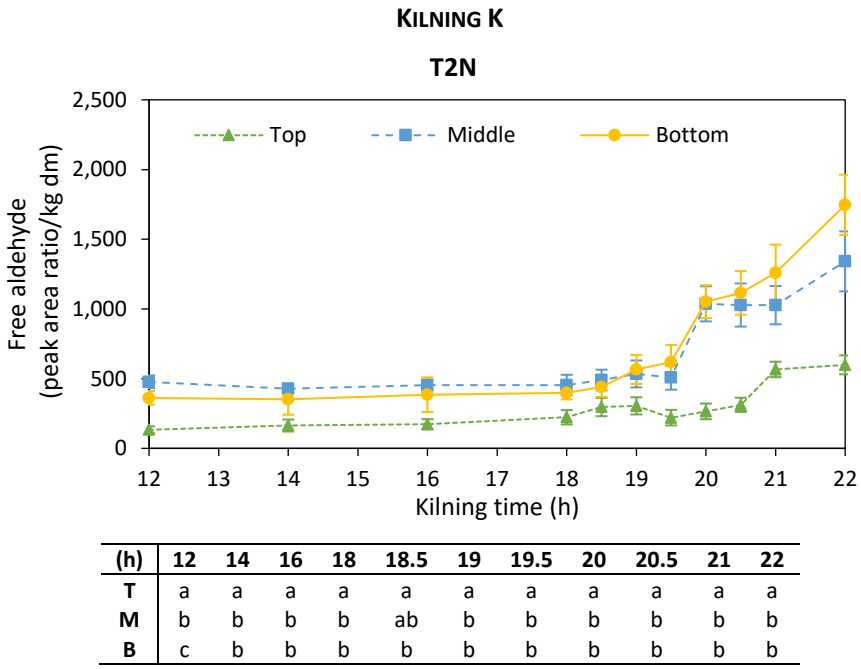
KILNING L – HEX (h)	12.5	13	13.5	14	14.5	15	15.5	16	16.5	17	17.5	18	18.5	19	19.5	20	21.5
T	a	a	a	a	a	a	a	a	a	ab	a	a	a	a	a	a	a
M	a	a	a	a	ab	b	ab	b	a	a	b	b	a	a	b	ab	a
B	a	a	a	a	a	a	b	b	b	b	b	a	b	b	a	b	a

KILNING K – HEX-CYS (h)	12	14	16	18	18.5	19	19.5	20	20.5	21	22
T	a	a	a	a	a	a	a	a	a	a	a
M	b	b	b	a	a	b	b	b	b	b	b
B	b	b	b	b	b	a	b	c	c	a	a

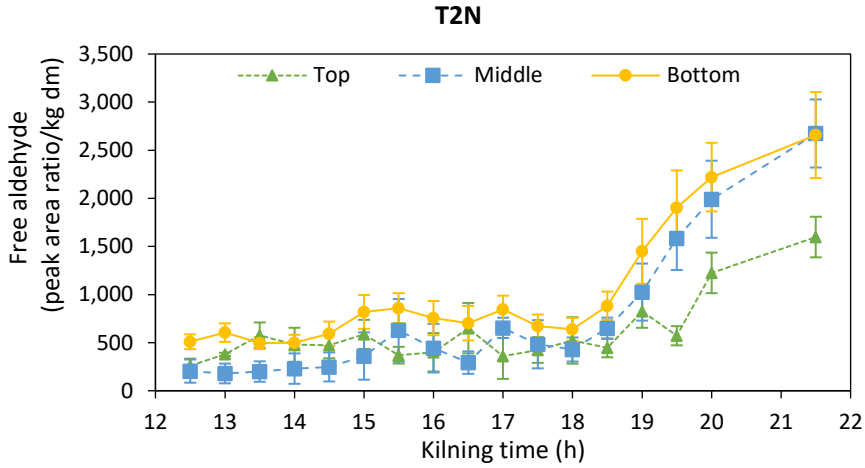
KILNING L – HEX-CYS (h)	12.5	13	13.5	14	14.5	15	15.5	16	16.5	17	17.5	18	18.5	19	19.5	20	21.5
T	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
M	b	b	b	b	b	a	b	b	b	b	b	b	b	b	b	b	b
B	a	a	a	c	a	a	c	c	c	c	c	c	c	c	c	c	c

Figure 4-6. Evolution of free and cysteinylated hexanal as a function of kilning time in samples collected from the bottom (B), middle (M) and top (T) layer of the kiln – malting batch K (left side), malting batch L (right side).

Results are expressed as mean values (n=3), error bars = standard deviation. Statistical comparisons between bottom, middle and top layer by post-hoc HSD Tukey's test to distinguish among significant different groups ($p \leq 0.05$) (a, b, c). x – statistical comparison not shown because quantification values are below LOD.



(h)	12	14	16	18	18.5	19	19.5	20	20.5	21	22
T	a	a	a	a	a	a	a	a	a	a	a
M	b	b	b	b	ab	b	b	b	b	b	b
B	c	b	b	b	b	b	b	b	b	b	b



(h)	12.5	13	13.5	14	14.5	15	15.5	16	16.5	17	17.5	18	18.5	19	19.5	20	21.5
T	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
M	a	a	b	a	a	a	a	a	a	ab	a	a	ab	a	b	ab	b
B	b	b	a	a	a	a	a	a	a	b	a	a	b	a	b	a	b

Figure 4-7. Evolution of *trans*-2-nonenal as a function of kilning time in samples collected from the bottom (B), middle (M) and top (T) layer of the kiln - malting batch K (left side), malting batch L (right side).

Results are expressed as mean values (n=3), error bars = standard deviation. Statistical comparisons between bottom, middle and top layer by post-hoc HSD Tukey's test to distinguish among significant different groups ($p \leq 0.05$) (a, b, c). x – statistical comparison not shown because quantification values are below LOD.

4.3.4. Investigation of the potential relationship between levels of aldehydes during industrial-scale green malt kilning and the evolution of the grain drying process

The results presented in the previous part 4.3.3. demonstrated that process-related physicochemical gradients significantly affect generation of aldehydes during green malt kilning. Therefore, in this section, to better understand the observed differences between the grain bed layers, levels of individual aldehydes are scrupulously compared with the evolution of the grain drying process (measured as grain moisture content), as well as with the evolution of thiobarbituric acid index (TBI).

The evolution of the grain drying process and the TBI varied among grain bed layers. For both batches K and L, grain moisture content decreased during the monitored stage of kilning in the bottom, middle, and top layers from approx. 19% - 4%, 24% - 4%, and 39% - 5%, respectively. For batch K, TBI in bottom, middle, and top layers changed - as a function of increasing kilning time - from 3 up to 7, 1 up to 6, and <LOD up to 5, respectively, whereas for batch L, TBI changed from 6 up to 17, <LOD up to 16, and <LOD up to 9, for bottom, middle and top layers, respectively. Thus, in general, the TBI value is decreasing from bottom layer over middle layer to top layer, indicating a decrease in the rate of formation of carbonyls.

For all (cysteinylated) Strecker aldehydes, similar results were obtained (see Appendix E). Therefore, as an example, the results on 3MB and 3MB-CYS are presented here. The overall pattern of evolution of 3MB during kilning is comparable among the three grain bed layers, and between both batches K and L, even though levels of 3MB strongly differ among layers and batches (see Figure 4-8). A relatively slow, gradual increase in levels of 3MB takes place up to approx. 17.5h - 19h of kilning. From that moment on, however, a sudden increase in the rate of formation of 3MB is observed, and, moreover, this increase in 3MB appears to coincide with an increase in TBI and a simultaneous decline in grain moisture content.
More specifically, in the case of batch K, levels of 3MB start to increase rapidly when grain moisture content reaches approx. 7.0%, 7.0%, and 6.2% in the bottom, middle, and top layer, respectively. Also, at this particular point of kilning, the TBI value starts to increase and amounts to 4.1, 3.8, and 3.4 units in the bottom, middle, and top layer, respectively. Likewise, in batch L, at this critical moment during kilning, a similar behaviour of 3MB is found when grain moisture content declines to approx. 7.2% (bottom), 7.5% (middle), and 8.8% (top), respectively, and TBI values start to increase and amount up to 11.9, 11.0, and 4.8 units in the bottom, middle, and top layer, respectively. The inlet air temperature in both batches was equal to 78°C, while the outlet air was 66°C in batch K and 68°C in batch L, which may explain the above made observations, at least in part.
Compared to 3MB, the evolution of the bound-state 3MB-CYS is quite similar to its free counterpart (see Figure 4-9), which seems logical from of the point of view of chemical equilibrium.
Comparable results to 3MB were found when evaluating the behaviour of the Maillard product, furfural (see Figure 4-10). Also this finding is not surprising, since both Strecker aldehydes and furfural are clearly related to the scheme of the complex, overall Maillard reaction[66] (see also Chapter 1, section 1.1.5.1).
In summary, due to detailed monitoring as a function of kilning time and sampling of different grain bed layers, we were able to pinpoint that the rate of formation of both Strecker aldehydes and furfural strongly increases when grain moisture content declines to approx. 7%

(bottom layer of kiln), and 6% - 9% (top layer of kiln), mainly as a consequence of the application of a considerable amount of heat load in each layer at this point of kilning (air temperature ranging from 66°C (outlet air) to 78°C (inlet air)).
Water activity (a_w) and processing temperature are already known for decades as important determinants of the rate of the Maillard reaction[237–239]. Regarding a_w, it is recognised that Maillard reactions and, in general, maximum browning in food products, mainly occur at a_w values between 0.65-0.70[92,220], which in malt corresponds to approx. 14% of moisture[221] (at lower a_w, Maillard reaction rates decrease, presumably due to somewhat reduced mobility of reactants[240]). Regarding the effect of temperature, it has been reported that within temperature ranges from 40°C up to 70°C, the rate of browning approx. doubles per 10°C[241], while within 60°C - 100°C, the reaction rate increases somewhat faster by approx. 10% per 1°C[92].

Regarding Strecker degradation of amino acids and concomitant formation of α-dicarbonyls (as principal reactants in Strecker degradation and formation of furfural), Liedke[242] reported that formation of α-dicarbonyls is enhanced at a water activity, which is clearly lower (in particular, $a_w<0.3$), compared to the a_w values mentioned above for the overall Maillard reaction (a_w = 0.65-0.70). For malt, a water activity of 0.3 corresponds to a moisture content of approx. 5% - 6%[221], which is quite close to the critical moisture contents as determined by us for formation of Strecker aldehydes, as well as furfural (for the grain in the bottom layer, a moisture content of approx. 7%; for the grain in the top layer, a moisture content of approx. 6% - 9%).

In relation to the impact of temperature, Nobis *et al.*[78], who studied formation of 3-deoxyglucosone during malt kilning, reported that the quantities of this particular α-dicarbonyl compound surpassed the quantities of its Amadori precursor when increasing kilning temperature from 70°C to 80°C. Also this finding from literature is largely in line with our study, as a sudden increase in the formation of Strecker degradation products and furfural, was found by us to occur at temperatures ranging from 66°C - 78°C, depending on the position of the grain bed layer.

In summary, by applying the above described experimental approach - as far as we know - this is the first time that the turning point in formation of aldehydes during green malt kilning has been determined in such a detailed way.

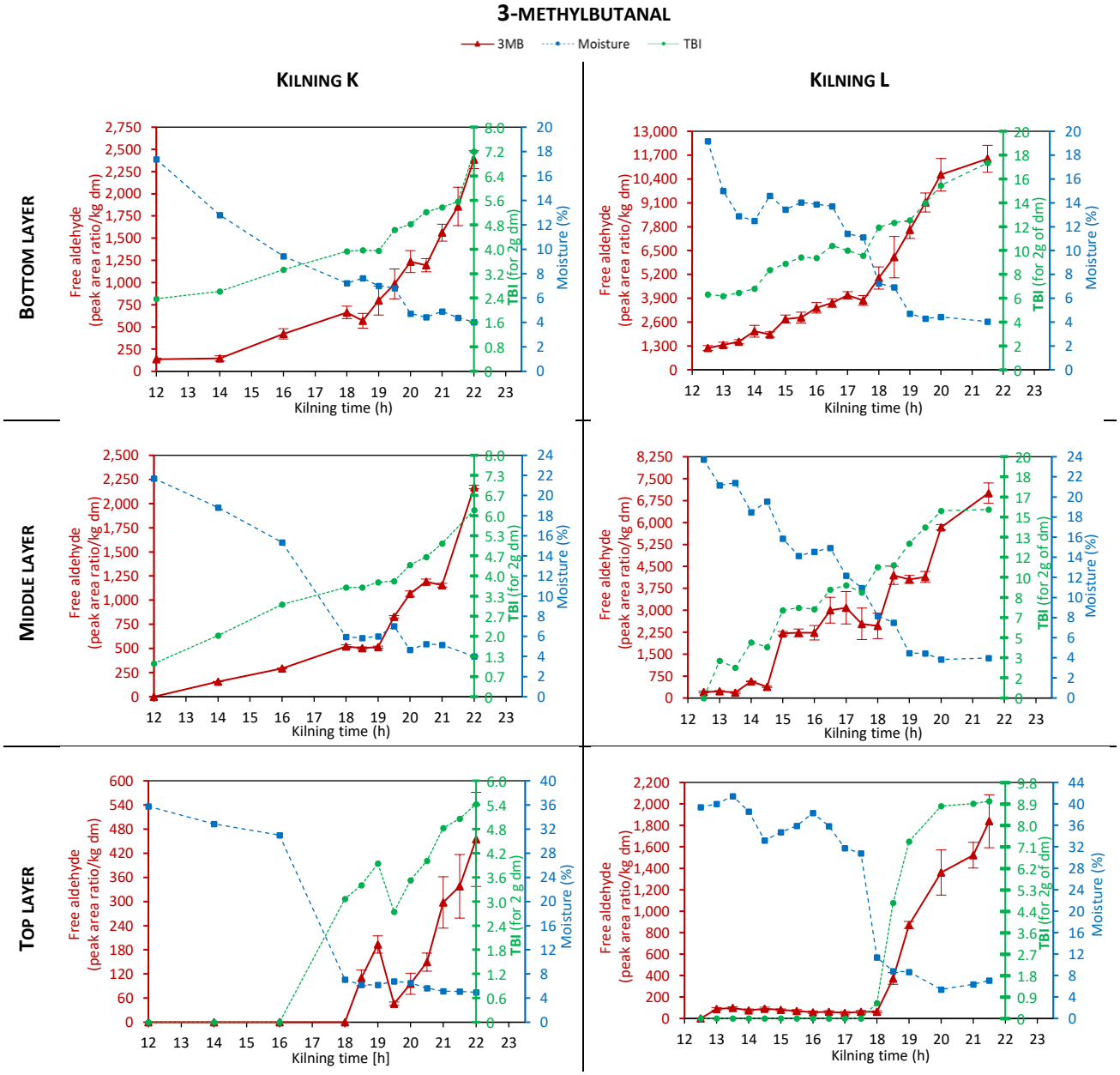


Figure 4-8. Evolution of levels of 3-methylbutanal, grain moisture content, and TBI, determined in samples collected from bottom, middle and top layers of the kiln, as a function of kilning time (batch K left side, batch L right side).

Results are expressed as mean values (n=3 for 3MB; n=2 for moisture content and TBI), error bars = standard deviation.

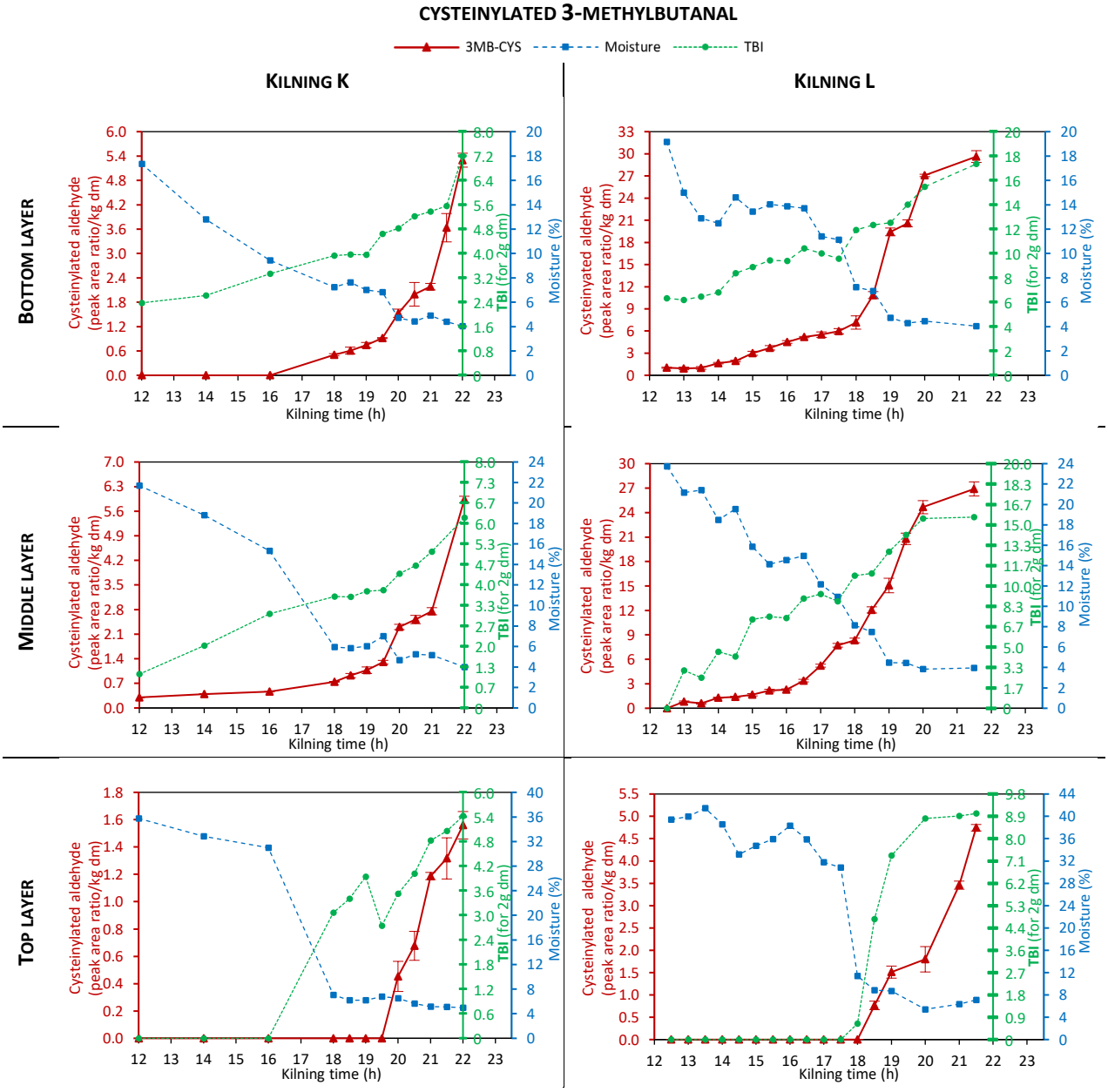


Figure 4-9. Evolution of levels of cysteinylated 3-methylbutanal, grain moisture content, and TBI, determined in samples collected from bottom, middle and top layers of the kiln, as a function of kilning time (batch K left side, batch L right side).

Results are expressed as mean values (n=3 for 3MB-CYS; n=2 for moisture content and TBI), error bars = standard deviation.

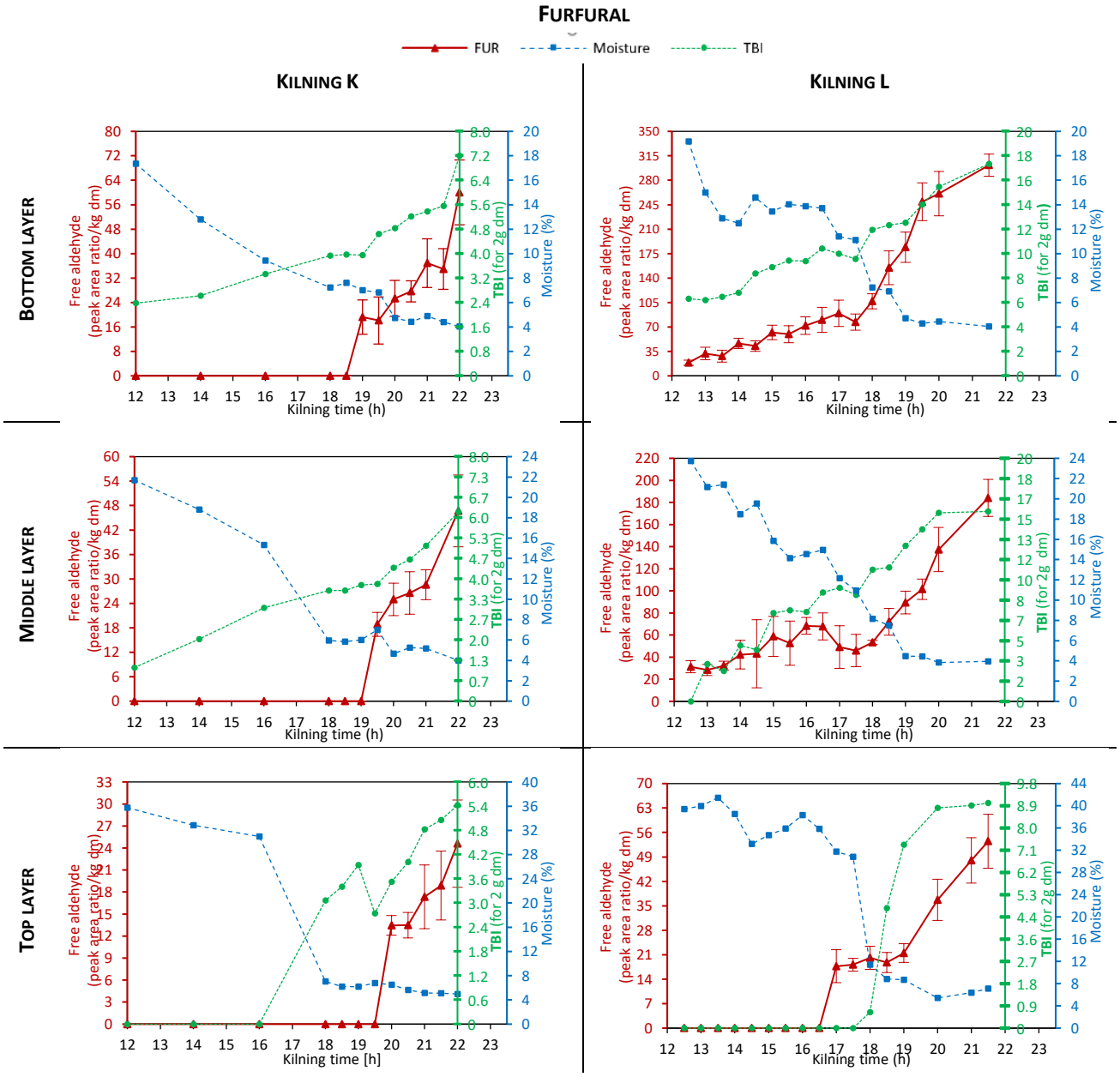


Figure 4-10. Evolution of levels of furfural, grain moisture content, and TBI, determined in samples collected from bottom, middle and top layers of the kiln, as a function of kilning time (batch K left side, batch L right side).

Results are expressed as mean values (n=3 for FUR; n=2 for moisture content and TBI), error bars = standard deviation.

Regarding staling aldehydes and fatty acid oxidation, in particular the evolution of *trans*-2-nonenal (T2N) (see Figure 4-11), observed trends in batch K and L, as well as in the different grain bed layers are comparable, and, furthermore, the patterns resemble those previously shown for 3MB and FUR. Thus, as observed before, the rate of formation of T2N strongly increases when grain moisture content declines to approx. 7% (bottom layer of kiln), and approx. 6% - 9% (top layer of kiln), caused by applying increased heat load (*i.e*. air temperature ranging from 66°C (temp. outlet air at top) to 78°C (temp. inlet air at bottom)). As a result of exposure to higher temperatures, limited enzymatic oxidation of fatty acids[64], as well as a higher rate of autoxidation, may explain the observed increases in T2N at this particular point during kilning.

The evolution of hexanal (HEX) during kilning in relation to the grain drying process and TBI, shows some difference in comparison to the other aldehydes (see Figure 4-12). When considering only the major changes in levels of HEX, the lowest concentrations are found at the end of a first stage of kilning, *i.e.* after approx. 18h - 19h. However, immediately after this moment, a small to moderated increase in HEX can be noticed. These observations are most obvious in the bottom layers of both batches and the observed increase in HEX may be explained in a similar way as was done for T2N.
Regarding cysteinylated hexanal (HEX-CYS), the overall trend is an increase in levels throughout the process, which is even more pronounced during the second stage of kilning (more heat load), compared to the first stage of kilning (see Figure 4-13).

Referring to the second stage of the kilning process, when higher temperatures were applied, obviously, also the most pronounced increases in levels of (bound-state) aldehydes were found. This is very clear for (cysteinylated) Strecker aldehydes, free furfural, free *trans*-2-nonenal, and cysteinylated hexanal. However, regarding free hexanal, a less pronounced (but still noticeable) increase can be seen.

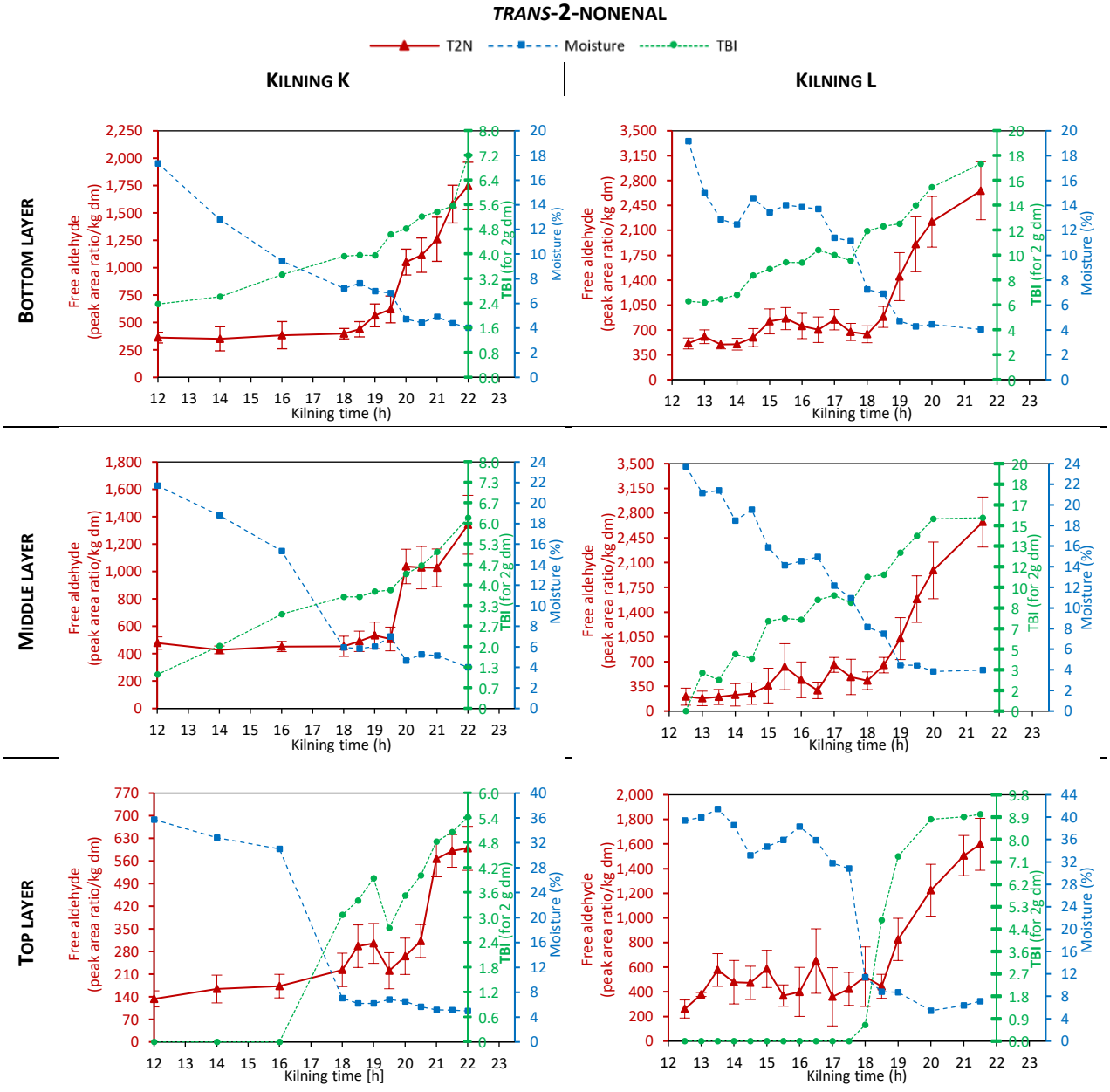


Figure 4-11. Evolution of levels of *trans*-2-nonenal, grain moisture content, and TBI, determined in samples collected from bottom, middle and top layers of the kiln, as a function of kilning time (batch K left side, batch L right side).

Results are expressed as mean values (n=3 for T2N; n=2 for moisture content and TBI), error bars = standard deviation.

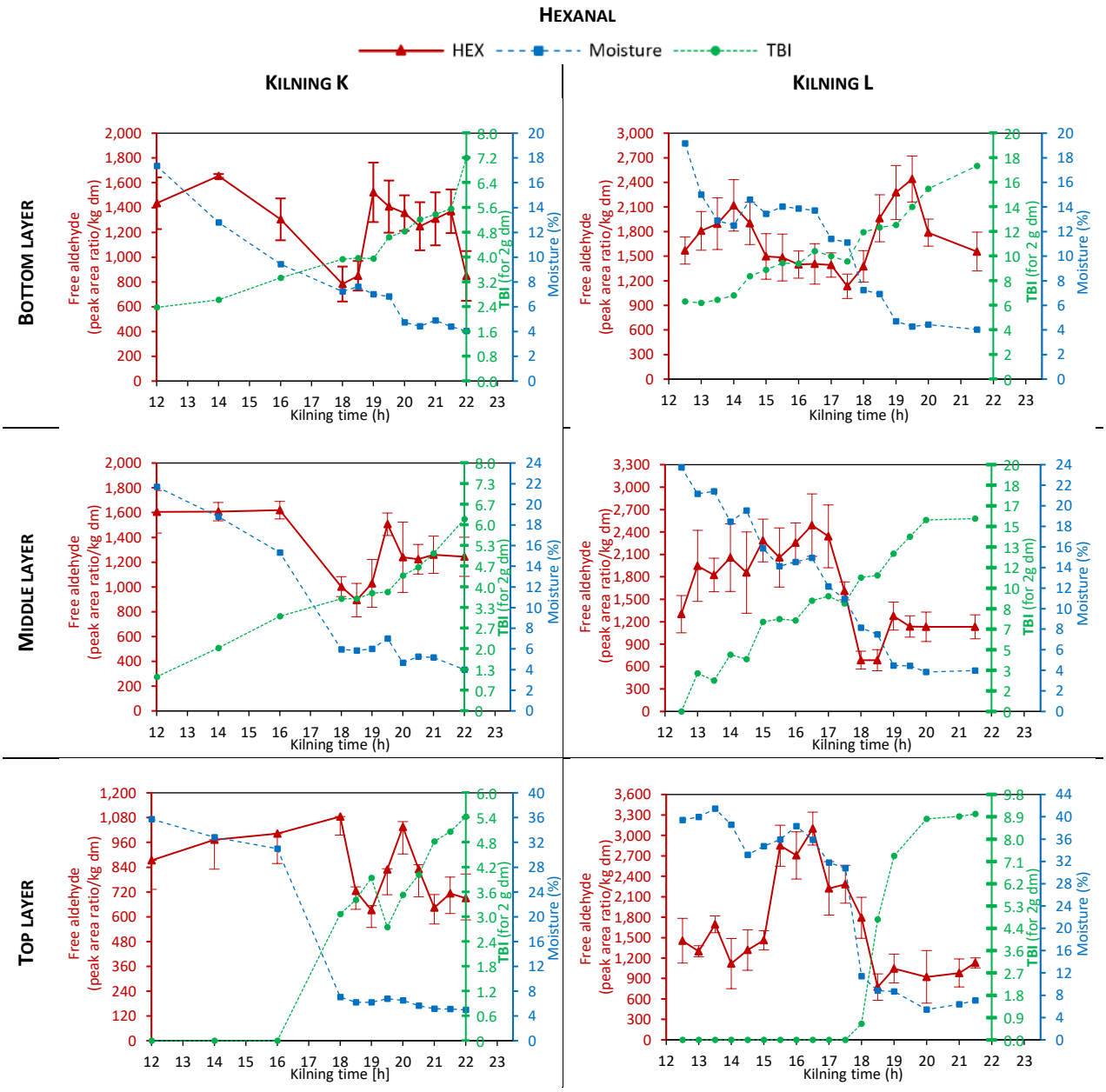


Figure 4-12. Evolution of levels of hexanal, grain moisture content, and TBI, determined in samples collected from bottom, middle and top layers of the kiln, as a function of kilning time (batch K left side, batch L right side).

Results are expressed as mean values (n=3 for HEX; n=2 for moisture content and TBI), error bars = standard deviation.

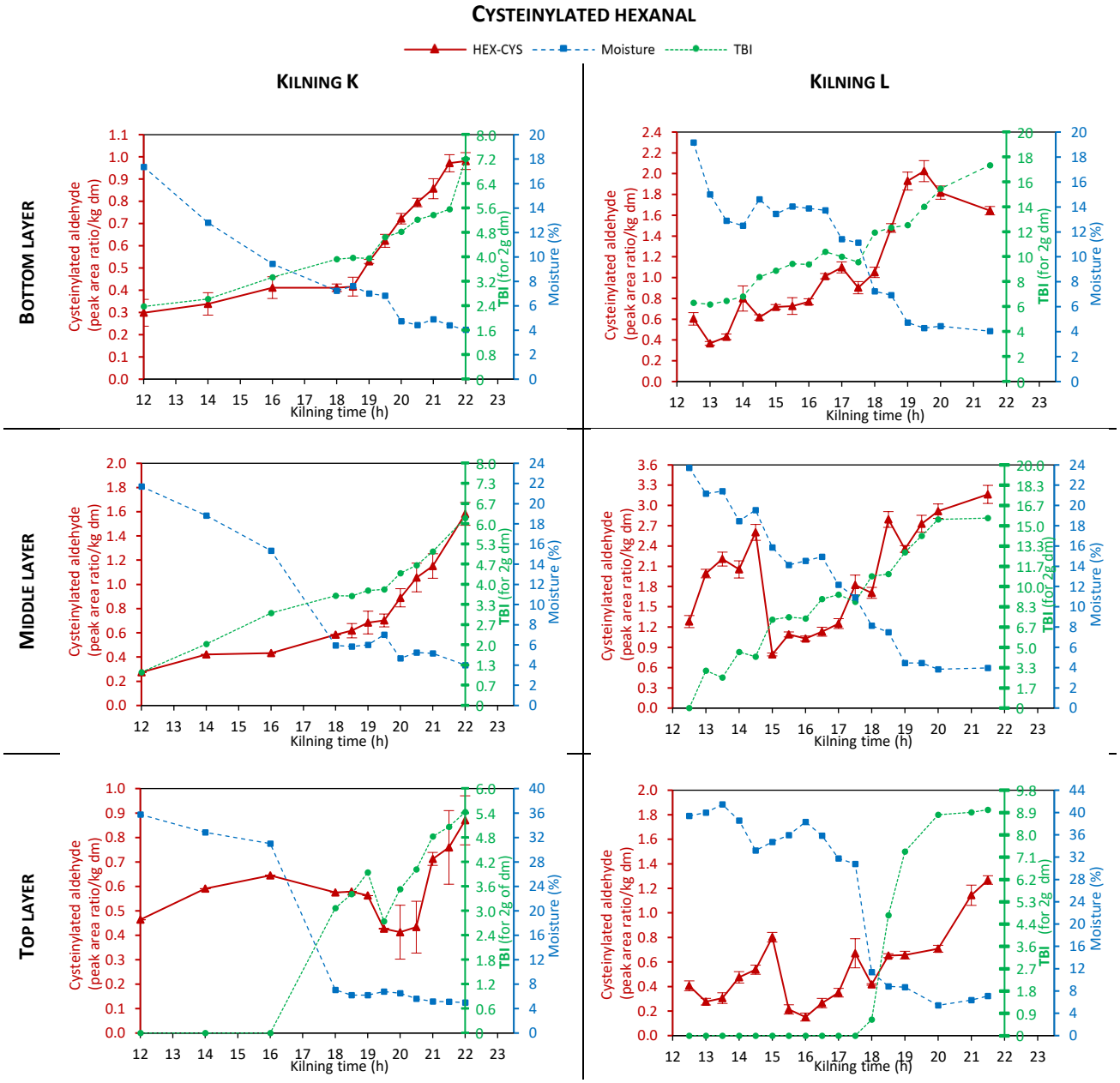


Figure 4-13. Evolution of levels of cysteinylated hexanal, grain moisture content, and TBI, determined in samples collected from bottom, middle and top layers of the kiln, as a function of kilning time (batch K left side, batch L right side).

Results are expressed as mean values (n=3 for HEX-CYS; n=2 for moisture content and TBI), error bars = standard deviation.

Based on the data already presented in this section, it can be envisaged that the grain drying process, expressed in terms of the decrease in grain moisture content (caused by gradually increasing applied heat load), affects the formation of staling aldehydes. To verify this, correlation coefficients between levels of aldehydes, grain moisture content, and TBI, were calculated for the three studied grain bed layers in batches K and L. Aiming at reliable results, only quantifiable data (≥LOQ; see Appendix A and Appendix B) were taken into consideration for these calculations. Consequently, since determinations of cysteinylated aldehydes were often <LOQ, correlation coefficients are not given for these compounds. For the same reason, correlation coefficients among variables are not reported for the top layers in batches K and L. Correlation coefficients are considered significant at a p-value ≤0.05 (Student's t-test) (for calculated correlation coefficients, see Table 4-3).

A first comparison between the physical parameters monitored during kilning, showed that the observed increase in TBI coincides with the decrease in grain moisture content, as strongly negative correlations (r between -0.86 and -0.98) were always found between these two parameters, regardless of the tested grain bed layer or the batch.

Regarding correlation coefficients among individual aldehydes, in general, (very) strong positive correlations were found, in particular for aldehydes measured in the bottom and middle layers of both batches. For example, among Strecker aldehydes very strong relations were found between 2MP and 2MB (r>0.98), 2MP and PHE (r>0.97), as well as PHE and 2MB (r>0.97). Interestingly, furfural (generally recognised as heat load indicator), shows very strong correlations with various Strecker aldehydes, such as 2MP, 2MB, 3MB, and PHE. However, for the fatty acid oxidation indicator, hexanal, no statistically significant relationships (p>0.05) could be determined with any other aldehyde, including *trans*-2-nonenal. *Trans*-2-nonenal by itself, however, does show to correlate very well with several other aldehydes, determined in bottom and middle layers, such as for instance with 2MP (r>0.97), 2MB (r>0.98), PHE (r>0.94), and FUR (r>0.93). These data suggest that even though the chemical origin of *trans*-2-nonenal is clearly different from Strecker aldehydes and furfural, its behaviour during kilning is quite similar (see also Figure 4-8, Figure 4-10 to Figure 4-11).

Finally, when comparing physical parameters (moisture content, TBI) with aldehydes, in the bottom and middle layers of both batches K and L, various strong correlations were found. In particular, strong and statistically significant positive correlations are found between TBI and the aldehydes 2MP (r>0.95), 2MB (r>0.94), PHE (r>0.89), and FUR (r>0.87), respectively. Moreover, strong negative correlations between grain moisture content and the same aldehydes (2MP, 2MB, PHE, FUR), are noticed (r< -0.70). As could be expected, also for *trans*-2-nonenal, strong negative Pearson correlations with moisture content (r≤ -0.7) and strong positive correlations with TBI (r≥0.88) were obtained. However, according to the p-value ≤ 0.05, used in the evaluation of statistical significance, calculated correlation coefficients between *trans*-2-nonenal and moisture content or TBI, were not statistically significant.

Table 4-3. Correlation coefficients among free aldehydes, grain moisture content, and TBI, determined during kilning of batches K and L (bottom and middle layers samples).

Bottom layer	Batch K [1]									
Batch L [2]	**Aldehyde**	**2MP**	**2MB**	**3MB**	**MET**	**PHE**	**FUR**	**HEX**	**T2N**	**Moist** (%)	**TBI** (for 2g dm)
	2MP		0.99	1.00		0.99	0.96	-0.43	0.97	-0.88	0.97
	2MB	0.98		1.00		0.99	0.97	-0.45	0.98	-0.84	0.98
	3MB	0.98	1.00			0.98	0.91	-0.89	0.95	-0.97	0.96
	MET										
	PHE	0.97	0.99	1.00			0.98	-0.15	0.94	-0.83	0.97
	FUR	0.97	0.99	1.00		1.00		-0.92	0.87	-0.72	0.97
	HEX	0.00	0.55	0.51		0.20	0.11		-0.65	0.36	-0.41
	T2N	1.00	0.99	1.00		1.00	0.93	-0.74		-0.73	0.88
	Moist (%)	-0.92	-0.90	-0.93		-0.91	-0.90	-0.20	-0.89		-0.86
	TBI (for 2g dm)	0.98	0.97	0.97		0.96	0.97	-0.06	0.96	-0.89	

Middle layer	Batch K [3]									
Batch L [4]	**Aldehyde**	**2MP**	**2MB**	**3MB**	**MET**	**PHE**	**FUR**	**HEX**	**T2N**	**Moist** (%)	**TBI** (for 2g dm)
	2MP		1.00			0.99	1.00	0.07	0.99	-0.59	0.97
	2MB	0.99				0.99	1.00	-0.51	1.00	-0.81	0.96
	3MB	1.00	0.99								
	MET										
	PHE	0.97	0.97	0.98			1.00	0.38	0.97	-0.80	0.99
	FUR	0.95	0.96	0.95		0.97		-0.52	0.99	-0.80	0.98
	HEX	-0.54	-0.52	-0.48		-0.39	-0.33		0.62	-0.76	-0.484
	T2N	1.00	1.00	1.00		1.00	1.00	-0.91		-0.91	0.92
	Moist (%)	-0.88	-0.82	-0.80		-0.74	-0.70	0.54	-0.76		-0.88
	TBI (for 2g dm)	0.95	0.94	0.93		0.89	0.87	-0.42	0.93	-0.98	

Correlation coefficients indicated in grey background are not statistically significant ($p>0.05$), moreover, data is not presented when values on quantification of aldehydes were <LOQ. Degrees of freedom: '1' = 2-10, '2' = 2-13, '3' = 2-9, '4'= 2-13. Compounds: Moist. = moisture, 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, and T2N = *trans*-2-nonenal.

4.4. Conclusions

The study carried out in Chapter 4 presents a critical evaluation of industrial-scale malting in connection to process-associated physicochemical gradients and formation of staling aldehydes. Moreover, this work intended to indicate the most critical point(s) regarding generation of free and cysteinylated aldehydes during malting, and to better understand the relationship of aldehyde formation with basic malting process variables (applied temperature regime, moisture content of grain).

Monitoring of free and cysteinylated aldehydes throughout two independent, industrial-scale malting processes demonstrated that the content of both free and cysteinylated aldehydes is clearly higher in finished malt compared to its starting raw material, barley. More specifically, during germination, a first, marked increase in levels of (cysteinylated) hexanal and *trans*-2-nonenal was observed. Next, during kilning (in particular, after 18h of kilning), a pronounced increase in levels of (cysteinylated) Strecker aldehydes, furfural, and a further rise in *trans*-2-nonenal, was noticed. These outcomes, as a result of two independent, industrial-scale pale malt productions (indicated as batches K and L) largely confirm our findings obtained in chapter 3 on malting batch G.

In addition to the observed, overall evolution of aldehydes throughout malting, a clear effect of process-associated physiochemical gradients on aldehyde formation was found for the first time. The latter was demonstrated by statistical analysis of levels of aldehydes, measured in samples collected from the bottom, middle, and top layers of the kiln, as a function of duration of industrial-scale kilning. The obtained results imply that the gradients, caused by pneumatic processing in relatively thick grain beds, and thereby provoking physicochemical differences between the various layers, also significantly affect the generation of aldehydes. Clearly, except for hexanal, an unequivocal impact of the location of the grain in the bed was found for all aldehydes. The highest levels of aldehydes were determined in the bottom layer of the grain bed (exposed to the highest heat load), and *vice versa* for the upper layer (exposed to the lowest heat load).

To better understand the observed differences between the layers, levels of aldehydes during kilning were compared to the evolution of the grain drying process (measured as grain moisture), as well as the evolution of TBI (generally accepted as a measure of applied heat load). Irrespective of the grain bed layer, it was found that - except for hexanal - an increase in aldehydes during kilning coincides with an increase in TBI and a decrease in moisture content. In particular for the bottom and middle layers of the kiln, strong correlations were found among free aldehydes, moisture content, and TBI, as a function of kilning time. Based on available data points and measurements, an increase in rates of formation of carbonyl compounds in general (TBI) and aldehydes in particular (except for hexanal), appears to occur when moisture of the grain has reached approx. 6% - 9% whilst considerable heat load is being applied. Consequently, this specific point in malt production seems to be critical from the perspective of the formation of carbonyl compounds, including staling aldehydes.

4.5. Related documents

Appendix A contains quantification of free and cysteinylated aldehydes in samples derived from different stages of the industrial-scale malting process K.

Appendix B contains quantification of free and cysteinylated aldehydes in samples derived from different stages of the industrial-scale malting process L.

Appendix C presents statistical evaluation of free Strecker aldehydes as a function of kilning time in samples collected from the bottom, middle, and top layer of the kiln of batch K and batch L.

Appendix D presents statistical evaluation of cysteinylated Strecker aldehydes as a function of kilning time in samples collected from the bottom, middle, and top layer of the kiln of batch K and batch L.

Appendix E shows evolution of free and cysteinylated Strecker aldehydes in relation the grain drying process and applied heat load during kilning.

Chapter 5

Evaluation of selected process variables of industrial-scale malting as a function of malt quality parameters and potential beer flavour (in)stability

5.1. Introduction

As described in detail in chapter 1, the choice of barley variety as well as the applied malting protocol will determine the overall quality of finished malt, including its potential impact on final beer flavour (in)stability. More specifically, in chapter 1, an overview of the influence of barley selection and malting on the formation of staling aldehydes and their precursors is provided in Table 1-4. Nevertheless, the significance of malting process variables in relation to beer flavour (in)stability remains poorly understood.

In this experimental part of the PhD, the aim is to investigate several industrial-scale malting process variables that may play a major role in final beer flavour (in)stability. Based on the availability of suitable samples from industrial-scale production on the one hand, and our previous findings (see chapters 3, 4) on the other hand, the degree of grain modification and the kilning-off temperature of pale lager malt were selected as potentially highly relevant, industrial variables.

Results obtained in chapters 3 and 4, point to the importance of heat load in relation to aldehyde formation. It was clearly demonstrated that aldehydes increase at the stage of green malt drying at elevated temperature, as well as during kilning-off. Since these experimental findings are also supported by data from literature[26,110,133], kilning-off temperature was selected as a first variable. In addition, in chapter 4, detailed process monitoring of two malting batches pointed to the degree of grain modification as a potential key factor in relation to the formation of staling aldehydes (*i.e.* increased grain modification gave rise to increased levels of aldehydes). It is therefore hypothesised that a higher degree of grain modification indirectly enhances formation of aldehydes, through the development of a reservoir of suitable precursors required for generation of aldehydes during subsequent kilning. Consequently, next to kilning-off temperature, the grain modification was selected as a second, major variable in this study.

Finally, because of the availability of suitable samples from the malting company, it was also decided to include sulphuring of green malt at the drying stage of kilning, which is a commonly applied treatment aimed at reduced formation of cancerogenic nitrosamines[243]. It is hypothesised that sulphuring may affect levels of aldehydes found in malt, due to the antioxidative properties of bisulphite as such[189] and/or to possible binding of bisulphite to free aldehydes[81].

5.2. Materials and Methods

5.2.1. Chemicals

Detailed information on the chemicals used for determination of free and cysteinylated aldehydes may be found in chapter 2, section 2.2.1. The chemicals required for determination of TBI, amino acids and standard malt quality parameters are specified in chapter 3, section 3.2.1.

Determination of trihydroxy fatty acids in malt. Lactic acid (90%, CAS 50-21-5), diethyl ether (99%, CAS 60-29-7), and pyridine (CAS 110-86-1) were purchased from Merck, Germany. Silyl-991 (CAS 25561-30-2) was acquired from Machery-Nagel GmbH, Germany. Heneicosane (98%, CAS 629-94-7) was obtained from Sigma-Aldrich, USA. Brewtan (CAS1401-55-4) was purchased from OmniChem, Belgium

5.2.2. Malts

All malt samples were provided by Boortmalt Antwerp, Belgium. Following malting variables were evaluated:

- degree of grain modification - six-row winter barley variety Etincel was subjected to four independent pale malt productions (malting M, N, O, and P). The difference in the degree of grain modification was achieved by spraying germinating barley from batch O and P with gibberellic acid during the first day of germination. Gibberellic acid was not applied in the preparation of batch M and N. The four batches were kilned-off at 85°C.
- Kilning-off temperature - six-row winter barley variety Etincel was subjected to two independent pale malt productions (malting A and B). The applied malting program was similar, except for total kilning time and kilning-off temperature. Total kilning times for batch A and B were 23h 55 min and 22h 32 min, respectively (green malt A was dried longer at 70°C for about 1h in order to attain the desired water activity). Kilning-off temperatures for batch A and B were 85°C and 95°C, respectively.
- Sulphuring during the drying stage of kilning - six-row winter barley variety Passarel was subjected to two independent pale malt productions (malting C and D). Sulphuring (burning elemental sulphur at the beginning of green malt drying) was applied on batch C, whereas sulphuring was not applied when drying green malt D. Both batches were kilned-off at 85°C.

5.2.3. Determination of free aldehydes in malt

Quantitative determination of free aldehydes in malt samples was performed according to the optimised procedure as described in chapter 3, section 3.2.3.

5.2.4. Determination of cysteinylated aldehydes in malt

Quantitative determination of cysteinylated aldehydes in malt samples was performed according to the optimised procedure as described in chapter 2, section 2.2.9.

5.2.5. Determination of standard quality parameters of malt

Standard quality parameters of malt were assessed according to the European Brewery Convention methods (EBC Analytica, 2018), as described in chapter 3, section 3.2.7.

5.2.6. Determination of TBI of malt

Thiobarbituric acid index (TBI) was measured following the adapted method of Coghe *et al*.[218], which itself is based on the method described by Thalacker and Brikenstock[219] (for more details, see chapter 3, section 3.2.5.).

5.2.7. Determination of amino acids in malt

Amino acids were determined with the use of AccQ•Tag Derivatisation Kit (Waters, USA) followed by AccQ•Tag Ultra UPLC-PDA method (Waters, USA), as described in chapter 3, section 3.2.6.

5.2.8. Determination of trihydroxy fatty acids in malt

Trihydroxy fatty acids (THFAs) were determined in malt samples according to EFBT internal method based on the combination of methods of Möller-Hergt et al.[244] and Wackerbauer and Meyna[245]. The procedure consisted of three main steps: mashing, liquid-liquid extraction of trihydroxy fatty acids and chromatographic separation with detection. In the first stage, 50.0 g ± 0.05 g of milled malt was mixed with the solution (preheated to 70°C), containing: 390 mL of Milli-Q water, 10 mL of Brewtan solution (6 g/L) and 1 mL of 9% lactic acid. The mix was mashed for 10 min at 70°C. Then, the weight was corrected by adding Milli-Q water up to a total weight of 450 g ± 0.2 g. The mash was then filtered using filter paper (Whatman, grade 2555 ½ pre-pleated 320 mm), whilst cooling the filtrate on ice. The first 20 mL of filtrate were transferred to a brown-glass bottle and stored at -20°C. In the next stage, 5 mL of the unfrozen wort sample were vigorously shaken for 3 min with 16 mL of pure diethyl ether. Then, the mixture was centrifuged for 5 min at 9,000 rpm. The upper layer containing extracted fatty acids in diethyl ether was collected in a test tube, where the solvent was evaporated by flushing it with N_2. The extraction of wort was repeated three times. Next, 0.5 mL of internal standard (36.4 mg/L of heneicosane in pure hexane) was added to the test tube and hexane was evaporated using N_2. For derivatisation, 300 µL of Silyl reagent and 100 µL pyridine were added. The sample was heated at 90°C for 1 h. After cooling, the liquid was transferred to an HPLC vial and frozen at -20°C until further analysis. Separation and detection were done by GC-FID Thermo Quest CE Trace 2000 (Interscience, Benelux), equipped with cyano-phenyl-methyl deactivated retention gap (2.5 m × 0.53 mm i.d., Varian, The Netherlands), and a fused silica analytical capillary column (CP-Sil 5 CB LOW BLEED/MS; 50 m × 0.25 mm i.d., 0.25 µm film thickness, Varian, The Netherlands). Samples (2 µL) were manually injected using a Hamilton syringe (10 µL, Model 701 N Syringe). The oven temperature was kept at 40°C for 5 min, then raised to 290°C at 6°C/min and held at 290°C for 20 min. Helium was used as a carrier gas at a flow rate of 1.0 mL/min. Data processing was performed by Chromcard software 1.07. The final value (based on two replicates) was expressed as milligram of THFAs per kilogram of malt dry basis.

5.2.9. Statistical analysis

Statistically significant differences were analysed by Student's t-test or one-way ANOVA (p-value ≤0.05 was considered as statistically significant) (applied software: Excel 365, Microsoft, USA; SPSS Statistics 26, IBM, USA). For principal component analysis (PCA), following software was used: Unscrambler 10.5.1, Camo Analytics, Japan.

5.3. Results and Discussion

5.3.1. Potential impact of grain modification on the formation of aldehydes during malting

Six-row winter barley (variety Etincel) was subjected to four independent, industrial-scale pale malt productions, further indicated as malting batches M, N, O, and P. Whereas gibberellic acid (GA) was not applied in the preparation of batch M and N, batch O and P were sprayed with GA during the first day of germination. Addition of GA is performed to stimulate formation of hydrolytic enzymes (in particular, α-amylase and proteases) during germination[140,246], which allows shortening germination time and may result in malt of a higher degree of modification[247]. However, more intense grain modification (increased cytolysis and proteolysis) will provide an increase in levels of low-molecular-weight compounds (in particular, amino acids and reducing sugars), and thus result in more precursors, suitable for later generation of aldehydes during subsequent kilning. Based on previous observations (chapter 4), this study on four independent industrial-scale pale malting batches, represents more profound investigation of the potential indirect effect of grain modification on levels of aldehydes found in finished malt.

As shown in Table 5-1 (comparison of standard quality parameters of malt), generally, the replicates (batch M and N, as well as batch O and P) appear to be largely comparable. Addition of GA to batch O and P during the first day of germination, resulted in somewhat higher α-amylase activity and higher proteolytic modification (*i.e.* higher content of TSP, KI, and FAN). Cytolytic modification (assessed by β-glucan content and friability) was however comparable between the four batches. Thus, in summary, batch O and P, show a higher degree of grain modification, largely in terms of proteolysis.

Table 5-1. Comparison of standard quality parameters determined in malts obtained without addition of gibberellic acid (lower grain modification; batch M and N) and with addition of gibberellic acid (higher grain modification; batch O and P).

Malting variable/ Malt quality parameter	Lower grain modification		Higher grain modification			
	Batch M	Batch N	Batch O	Batch P		
	Mean	Mean	Mean	Mean		SD
Moisture (%)	4.2[b]	4.1[b]	3.7[a]	4.1[b]	±	0.1
Extract yield (% dm)	80.6[a]	80.4[a]	80.6[a]	80.6[a]	±	0.4
Colour (EBC)	3.1[a]	2.5[a]	3.4[a]	3.3[a]	±	0.4
TBI (for 10 g dm)	8.7[ac]	7.5[c]	10.0[a]	11.4[b]	±	0.5
Friability (%)	93.6[b]	90.4[a]	87.2[a]	94.2[b]	±	1.7
Homogeneity (%)	98.8[b]	98.6[b]	96.4[a]	99.2[b]	±	0.5
β-Glucan (mg/L)	54[b]	118[a]	122[a]	42[b]	±	20
Viscosity (mPas)	1.54[a]	1.57[a]	1.57[a]	1.52[a]	±	0.02
Total protein (% dm)	10.3[a]	10.2[a]	10.3[a]	10.1[a]	±	0.3
Total soluble protein (% dm)	4.3[ab]	4.1[b]	4.6[a]	4.7[a]	±	0.16
Kolbach Index (%)	42.2[bc]	40.3[c]	44.7[ab]	46.9[b]	±	1.4
Free amino nitrogen (mg/L)	154[bc]	145[c]	175[ab]	178[b]	±	9
Diastatic power (°WK)	463[a]	453[a]	443[a]	505[a]	±	25
α-Amylase activity (DU)	58[a]	52[b]	67[a]	74[a]	±	5.5
pH	5.96[a]	5.94[a]	5.95[a]	5.95[a]	±	0.05

Results are presented as mean values (n=2 or 3, depending on the measured malt quality parameter); standard deviations (SD) in the column on the right, represent standard deviations of the applied by the maltings analytical method for each malt quality parameter. Statistical comparisons between the batches M, N, O, and P performed by post-hoc HSD Tukey's test to distinguish among statistically significant different ($p \leq 0.05$) groups (a, b, c).

Quantitative determination of free aldehydes in malts derived from batch M, N, O, and P, clearly shows that grain modification plays a role in their formation (see Figure 5-1A). GA addition appears to have a pronounced effect on the formation of Strecker aldehydes and furfural (levels of these aldehydes almost doubled), whereas an increase in hexanal and *trans*-2-nonenal is somewhat less clear, since the batches O and P are statistically different from batch N, but not from batch M. It is assumed that the results obtained for Strecker aldehydes and furfural are related to the fact that gibberellic acid triggers formation of α-amylase[140] (providing substrates for the Maillard reaction), as well as proteases[246], thereby increasing levels of *i.a.* amino acids, which can further undergo Strecker degradation. Regarding the fatty acid oxidation products hexanal and *trans*-2-nonenal, exogenous GA would not have an effect on LOX-activity[248]. Based on the results presented in Figure 5-1A, although in general, higher levels of hexanal and *trans*-2-nonenal were noticed in the GA treated batches, statistically significant different levels were not registered between GA treated batches and GA untreated batches. Furthermore, in accordance with free Strecker aldehydes, higher quantities of cysteinylated Strecker aldehydes were determined in the malts showing a higher degree of modification (malts O and P, see Figure 5-1B). Also, similarly to free hexanal, levels of cysteinylated hexanal were not statistically different upon GA treatment.

In summary, the results presented above largely confirm our previous observations made in chapter 4. Therefore, it can be concluded that grain modification significantly impacts formation of aldehydes during malting, *e.g.* via formation of suitable precursors for later generation of aldehydes during subsequent kilning.

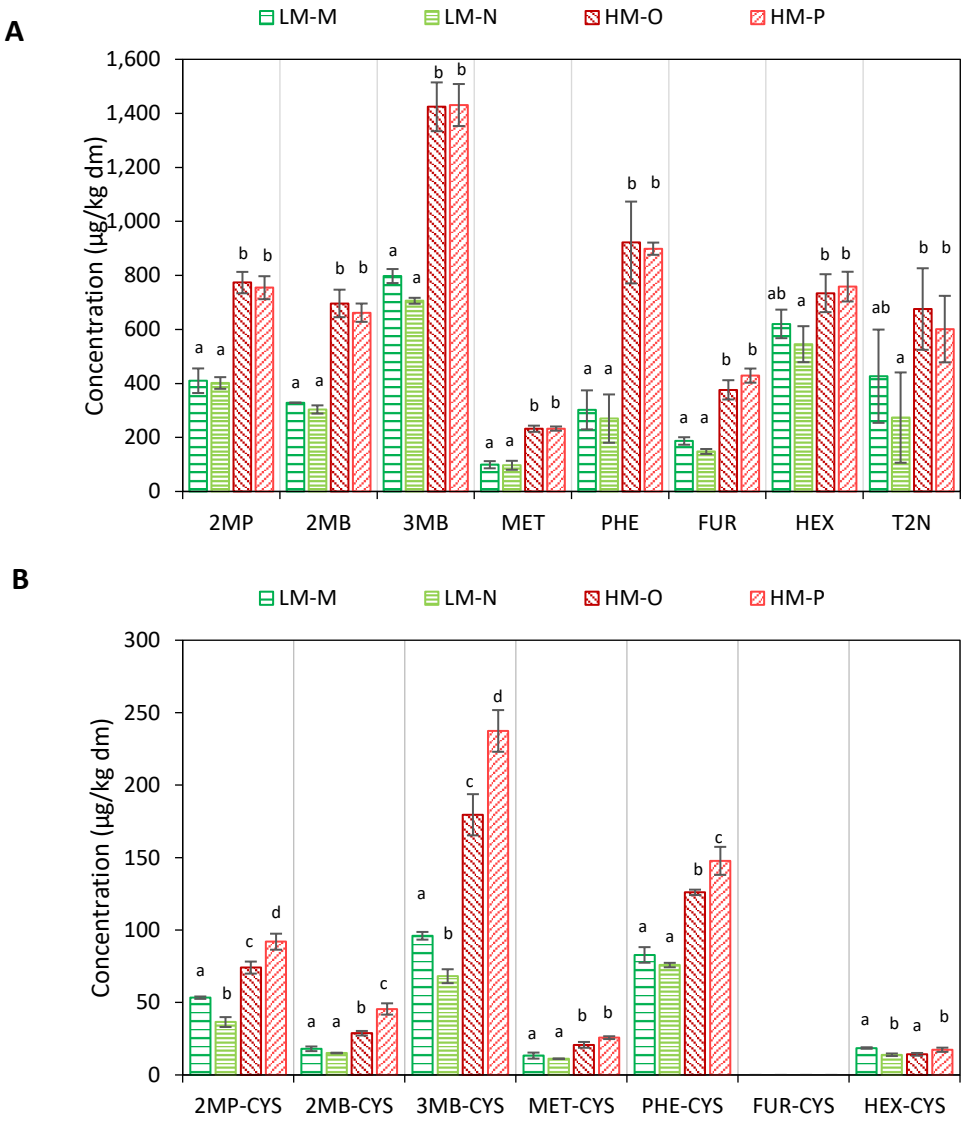


Figure 5-1. Levels of free aldehydes (A) and cysteinylated aldehydes (B) determined in malts of low degree and high degree of grain modification, respectively.

Samples: LM-M - low degree of grain modification batch M; LM-N - low degree of grain modification batch N; HM-O - high degree of grain modification batch O; HM-P - high degree of grain modification batch P. Compounds quantified: 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, and T2N = *trans*-2-nonenal, 2MP-CYS = cysteinylated 2-methylpropanal, 2MB-CYS = cysteinylated 2-methylbutanal, 3MB-CYS = cysteinylated 3-methylbutanal, MET CYS = cysteinylated methional, PHE-CYS = cysteinylated phenylacetaldehyde, FUR-CYS = cysteinylated furfural (<LOD), and HEX-CYS = cysteinylated hexanal (cysteinylated *trans*- 2-nonenal is not presented as the reference compound was not available).Results are expressed as mean values (n=3), error bars = standard deviation. Statistical comparisons between samples LM-M, LM-N, HM-O, and HM-P by post-hoc HSD Tukey's test to distinguish among statistically significant different ($p \leq 0.05$) groups (a, b, c).

5.3.2. Potential impact of kilning-off temperature on the formation of aldehydes during malting

Six-row winter barley (variety Etincel) was subjected to two independent, industrial-scale pale malt productions, further indicated as malting batches A and B. Kilning-off temperatures for batch A and B were set to 85°C and 95°C, respectively. A commonly applied kilning-off temperature for pale malt production is 85°C (as in the case of batch A). However, for batch B, presumably to arrive at a pale type of malt with a somewhat increased colour, a higher kilning-off temperature (95°C) was exceptionally applied by the malting company. Moreover, in case of batch B, to anticipate more Maillard reactions (*i.e.* to obtain more colour), increased proteolysis was aimed at during the industrial processing (see Table 5-2).

From the perspective of beer staling potential of malt, a higher kilning-off temperature will probably have a direct impact on the formation of aldehydes as more Maillard reactions may lead to higher levels of furfural and Strecker aldehydes[77,133]. Also, it is known that the rate of autoxidation leading to fatty acid oxidation products, increases at elevated temperatures[66,249]. However, free aldehydes will also evaporate more when exposed to higher temperatures.

Based on the discussion above and our previous observations on the relevance of heat load in relation to the formation of aldehydes (see chapters 3, 4), the specific aim of this work is to evaluate the effect of the kilning-off temperature (85°C and 95°C, respectively) on levels of aldehydes in finished malts.

Results on standard quality parameters, individual amino acids, and trihydroxy fatty acids (THFAs), obtained from malts derived from batch A (kilned-off at 85°C) and batch B (kilned off at 95°C) are summarised in Table 5-2.

Table 5-2. Comparison of standard malt quality parameters, individual amino acids, and trihydroxy fatty acids, determined in malts kilned-off at 85°C and 95°C, respectively.

Malting variable/ Malt quality parameter	Kilning-off at 85°C Batch A			Kilning-off at 95°C Batch B			Statistical significance
	Mean		SD	Mean		SD	
Moisture (%)	4.2	±	0.1	3.8	±	0.1	*
Extract yield (% dm)	79.3	±	0.4	80.4	±	0.4	*
Colour (EBC)	3.2	±	0.4	5.5	±	0.4	*
TBI (for 10 g dm)	12.8	±	0.2	30.4	±	0.1	*
Friability (%)	84.4	±	1.7	88.4	±	1.7	*
Homogeneity (%)	96.2	±	0.5	97.4	±	0.5	*
β-Glucan (mg/L)	94	±	20	62	±	20	
Viscosity (mPas)	1.52	±	0.02	1.51	±	0.02	
Total protein (% dm)	10.9	±	0.3	11.0	±	0.3	
Total soluble protein (% dm)	4.19	±	0.16	4.88	±	0.16	*
Kolbach Index (%)	38.4	±	1.4	44.3	±	1.4	*
Free amino nitrogen (mg/L)	134	±	9	178	±	9	*
Individual amino acids (mg/kg dm)							
Methionine	175	±	14	140	±	19	*
Valine	514	±	58	588	±	31	
Isoleucine	265	±	30	330	±	17	*
Leucine	620	±	71	772	±	30	*
Phenylalanine	494	±	43	714	±	84	*
Cysteine + Cystine	36	±	2.5	25	±	1.0	*
Diastatic power (°WK)	460	±	25	420	±	25	
α-Amylase activity (DU)	51.0	±	5.5	54.0	±	5.5	
pH	5.90	±	0.05	5.80	±	0.05	*
Trihydroxy fatty acids (mg/kg dm)	29.5	±	3.2	21.5	±	1.1	*

Results are presented as mean values (n=2 or 3, depending on the measured malt quality parameter) ± standard deviation (SD). Statistical comparison between batches by Student's t-test; * = statistically significant (p-value ≤ 0.05).

It can be seen that kilning-off temperature significantly impacts particular standard quality parameters of malt. As expected, an increased temperature clearly resulted in a lower moisture content, higher heat load index (TBI), and higher malt colour. Both malts show a satisfying degree of cytolytic modification, as can be derived from the relevant parameters (friability, β-glucan content, viscosity) (recommended values for high-quality pale malt are provided in Table 3-3, chapter 3). Malts A and B also show comparable diastatic power and α-amylase activity. However, in malt B (kilned-off at 95°C), a relatively higher proteolytic modification was observed (higher TSP, FAN, and KI). Also, in this malt, higher levels of individual amino acids (except for methionine and cysteine/cystine) were determined. The measured lower levels of methionine and cysteine/cystine in batch B may be due to the presence of sulphur in the side chains of these amino acids. In particular, cysteine (containing a thiol group) is highly sensitive to oxidation[66,250] and it is also prone to binding to *e.g.* aldehydes[81]. Although somewhat less than cysteine, methionine (containing a S-methyl thioether group) is also sensitive to oxidation[66,251], and, compared to cysteine, it also shows higher binding affinity towards *e.g.* metals[252]. In addition, lower concentrations of trihydroxy

fatty acids (THFAs) were measured in malt kilned-off at 95°C. It is hypothesised that this may be due to heat-induced, non-enzymatic conversion of THFAs into hexanal and *trans*-2-nonenal. In summary, data on the quality parameters of the malts, demonstrate that for malt B a higher colour was obtained, in combination with a high diastatic power/α-amylase activity (similar to the enzymatic potential of malt A).

Quantitative determination of aldehydes in malts kilned-off at 85°C and 95°C, clearly shows the effect of a higher kilning-off temperature on the formation of free and cysteinylated aldehydes (Figure 5-2). In particular, higher levels of free Strecker aldehydes, cysteinylated Strecker aldehydes, and furfural were found in malt B (concentrations almost tripled). Also, significantly higher levels of hexanal and *trans*-2-nonenal were determined in malt B.

In summary, it has been demonstrated that a relatively high kilning-off temperature (95°C), in combination with higher proteolytic modification, clearly resulted in elevated levels of free and cysteinylated aldehydes in finished malt, compared to the standard protocol for pale malt production with kilning-off at 85°C. Moreover, the results presented above, confirm our previous findings obtained in chapters 3 and 4, where it was observed that more heat load imparts generation of aldehydes.

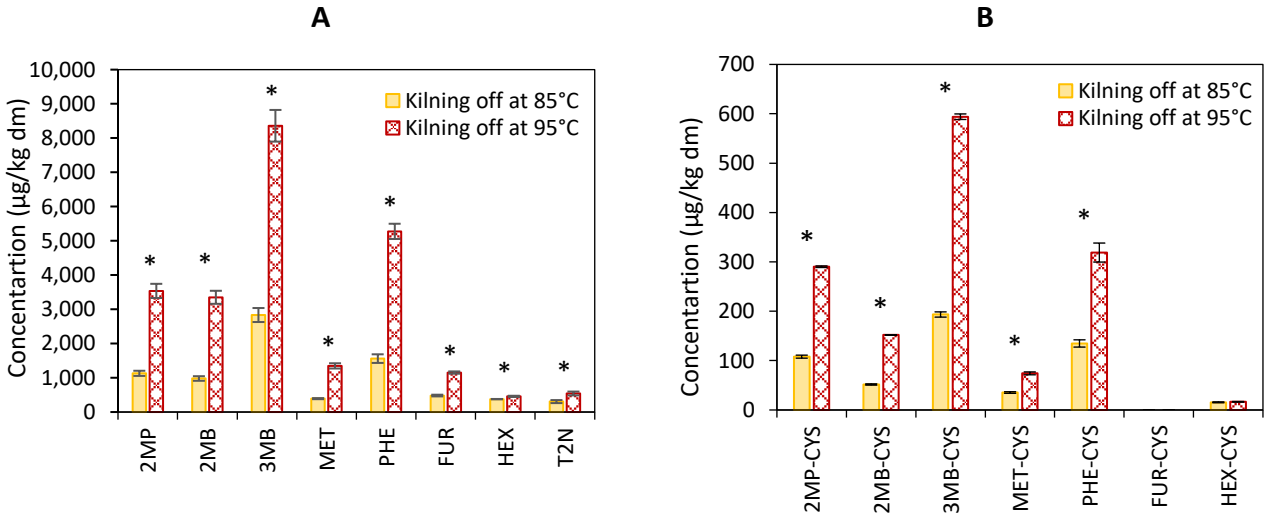


Figure 5-2. Levels of free aldehydes (A) and cysteinylated aldehydes (B) determined in malts kilned-off at 85°C and 95°C.

Compounds quantified: 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, and T2N = *trans*-2-nonenal, 2MP-CYS = cysteinylated 2-methylpropanal, 2MB-CYS = cysteinylated 2-methylbutanal, 3MB-CYS = cysteinylated 3-methylbutanal, MET CYS = cysteinylated methional, PHE-CYS = cysteinylated phenylacetaldehyde, FUR-CYS = cysteinylated furfural (<LOD), and HEX-CYS = cysteinylated hexanal (cysteinylated *trans* 2-nonenal is not presented as the reference compound was not available). Results are expressed as mean values (n=3), error bars = standard deviation. Statistical comparison between batches by Student's t-test; * = statistically significant (p-value ≤ 0.001).

5.3.3. Potential impact of sulphuring of green malt on the formation of aldehydes during malting

Six-row winter barley (variety Passarel) was subjected to two independent, industrial-scale pale malt productions, further indicated as malting batches C and D. In the production process of batch C, sulphuring (burning elemental sulphur to obtain sulphur dioxide) was applied at the beginning of green malt drying, whereas batch D was produced without sulphuring. Sulphuring is often applied in industrial maltings during the first stage of green malt drying to reduce levels of carcinogenic nitrosamines in finished malt[87,243]. However, sulphuring may have side effects on malt quality, *e.g*. increased levels of soluble nitrogen and a decreased malt colour[140,189], presumably due to less Maillard reactions[253]. From the perspective of beer flavour (in)stability, sulphuring applied during drying of green malt could enhance binding of free aldehydes, leading to the formation of aldehyde-bisulphite adducts[81]. In this experiment, the potential effect of sulphuring on levels of aldehydes in finished malt is evaluated.

Results on standard quality parameters, individual amino acids, and trihydroxy fatty acids (THFAs), obtained from malts derived from batch C (with sulphuring of green malt) and batch D (without sulphuring) are summarised in Table 5-3.

Table 5-3. Comparison of standard malt quality parameters, individual amino acids, and trihydroxy fatty acids, determined in malts with and without sulphuring during drying of green malt.

Malting variable/ Malt quality parameter	Without sulphuring			With sulphuring			Statistical significance
	Mean		SD	Mean		SD	
Moisture (%)	3.4	±	0.1	3.6	±	0.1	
Extract yield (% dm)	80.9	±	0.4	81.4	±	0.4	
Colour (EBC)	3.2	±	0.4	3.7	±	0.4	
TBI (for 10 g dm)	21.3	±	0.3	22.4	±	0.2	*
Friability (%)	90.4	±	1.7	86.8	±	1.7	
Homogeneity (%)	98.4	±	0.5	98.3	±	0.5	
β-Glucan (mg/L)	238	±	20	222	±	20	
Viscosity (mPas)	1.57	±	0.02	1.54	±	0.02	
Total protein (% dm)	10.3	±	0.3	10.5	±	0.3	
Total soluble protein (% dm)	4.75	±	0.16	4.75	±	0.16	
Kolbach Index (%)	46.1	±	1.4	45.2	±	1.4	
Free amino nitrogen (mg/L)	165	±	9	163	±	9	
Individual amino acids (mg/kg dm)							
Methionine	182	±	5	181	±	15	
Valine	577	±	25	608	±	65	
Isoleucine	334	±	15	353	±	38	
Leucine	752	±	36	808	±	87	
Phenylalanine	678	±	25	619	±	32	
Cysteine + Cystine	35	±	0.7	32	±	3.2	
Diastatic power (°WK)	410	±	25	400	±	25	
α-Amylase activity (DU)	54.0	±	5.5	51.0	±	5.5	
pH	6.01	±	0.05	5.97	±	0.05	
Trihydroxy fatty acids (mg/kg dm)	25.3	±	2.3	24.3	±	5.6	

Results are presented as mean values (n=2 or 3, depending on the measured malt quality parameter) ± standard deviation (SD). Statistical comparison between batches by Student's t-test; * = statistically significant (p-value ≤ 0.05).

As shown in Table 5-3, there appears to be no difference between sulphured and non-sulphured malt, regarding standard quality parameters. According to literature[140,189], a reduced malt colour and an increased level of soluble nitrogen were expected in sulphured malt, however, this was not found in our study. Regarding quantification of free aldehydes, no significant differences between malts produced with and without sulphuring were observed (see Figure 5-3). Regarding cysteinylated aldehydes, large differences between the malts were not observed, although somewhat lower levels of 2MP-CYS and 3MB-CYS were noticed in the sample with sulphuring.

In summary, sulphuring of green malt during drying had no major impact on levels of aldehydes, neither on malt standard quality parameters.

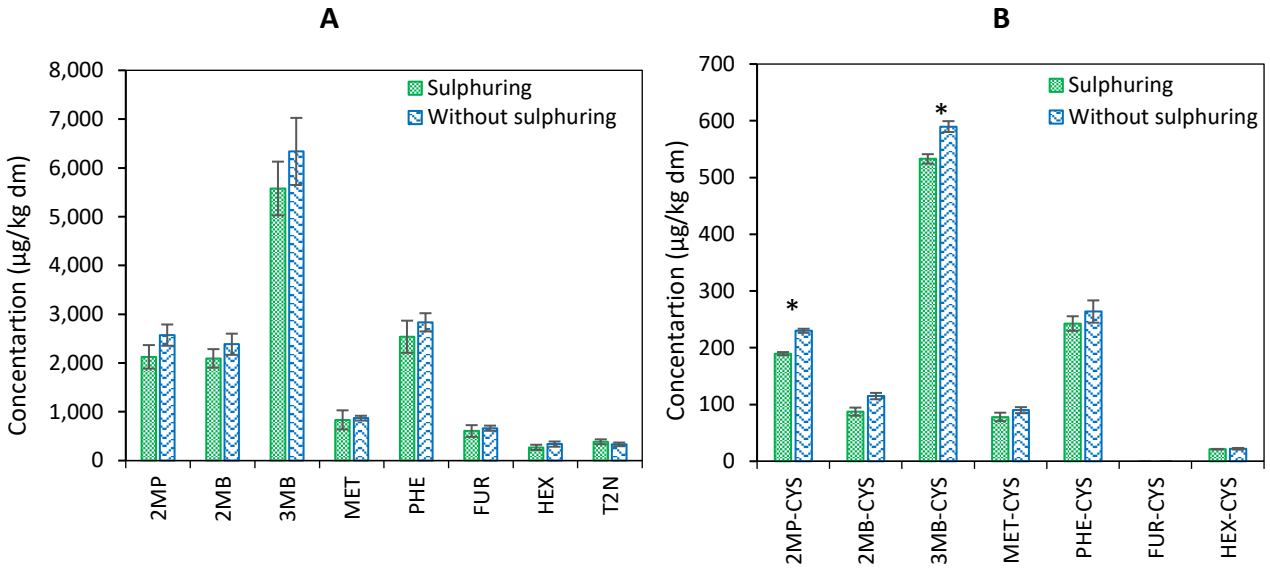


Figure 5-3. Levels of free aldehydes (A) and cysteinylated aldehydes (B) determined in malts with and without sulphuring of green malt during drying.

Compounds quantified: 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, and T2N = *trans*-2-nonenal, 2MP-CYS = cysteinylated 2-methylpropanal, 2MB-CYS = cysteinylated 2-methylbutanal, 3MB-CYS = cysteinylated 3-methylbutanal, MET CYS = cysteinylated methional, PHE-CYS = cysteinylated phenylacetaldehyde, FUR-CYS = cysteinylated furfural (<LOD), and HEX-CYS = cysteinylated hexanal (cysteinylated *trans* 2-nonenal is not presented as the reference compound was not available). Results are expressed as mean values (n=3), error bars = standard deviation. Statistical comparison between batches by Student's t-test; * = statistically significant (p-value ≤ 0.05).

5.3.4. Multivariate data analysis

In order to identify the main sources of variation in the data set, the collected measurements including content of (cysteinylated) aldehydes and quality parameters of malts A-D and M-P were subjected to Principal Component Analysis (PCA). The bi-plot of PC-1 and PC-2 accounts for 70% of the data variation (see Figure 5-4). When analysing the position of the scores on the bi-plot, it can be seen that malts of lower grain modification (LM-M and LM-N) group together and are clearly separated from malts of higher grain modification (HM-O and HM-P). This observation is in conformity with the previous conclusion made in section 5.3.1. that the replicates are largely comparable. Further, the wide difference in the positions of the malts kilned-off at 85°C and 95°C, respectively (located in opposite quadrants of the bi-plot) points

to a high impact of heat load on the analysed characteristics of the malts (see also outcomes of section 5.3.2). In respect of sulphuring, as mentioned previously in section 5.3.3., little differences regarding aldehydes and malt quality parameters were found, which is reflected in the bi-plot by the very close positioning of the samples S and NS.

In conclusion, regarding the eight, analysed industrial-scale maltings, grain modification and, in particular, kilning-off temperature appear to have a large impact on the standard malt quality parameters and levels of aldehydes.

Regarding the position of loadings on the bi-plot, free Strecker aldehydes and furfural can be mainly associated with thiobarbituric acid index (TBI), as well as colour (Col), total soluble protein (TSP), Kolbach Index (KI), and free amino nitrogen (FAN). Similar relationships were also found in chapter 3.

Interestingly, the loadings of the fatty acid oxidation aldehydes, hexanal and *trans*-2-nonenal, are clearly different from the other marker aldehydes. This differential behaviour in comparison to the other aldehydes and to each other, fully corresponds to our previous observations (see chapters 3 and 4).

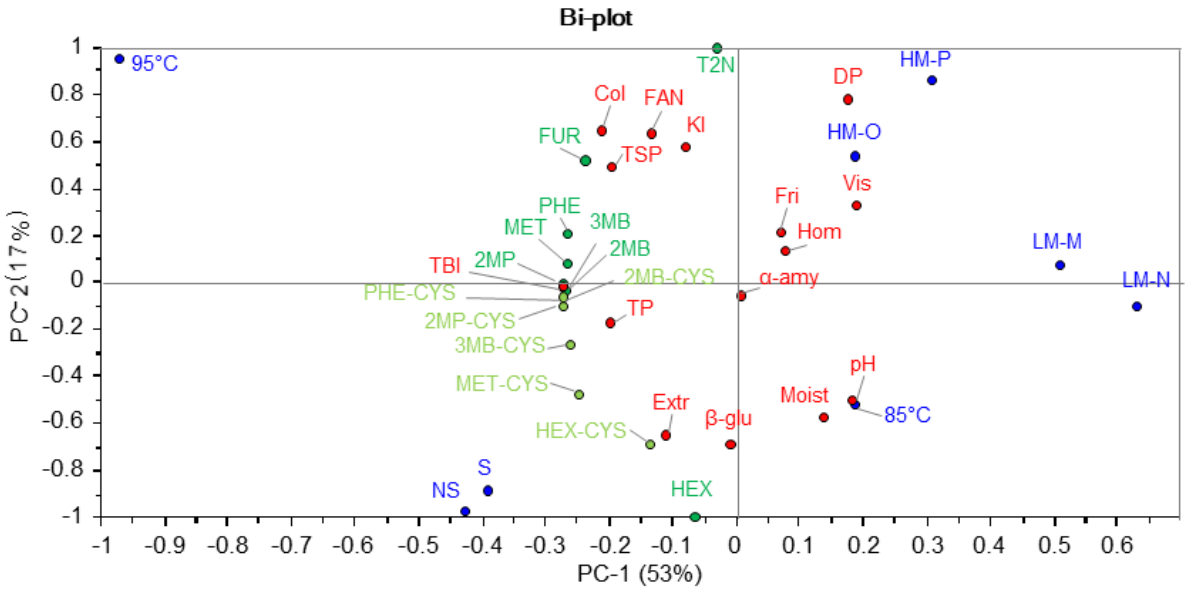


Figure 5-4. Bi-plot of PCA on levels of free aldehydes, cysteinylated aldehydes, and malt quality parameters, determined in eight different types of industrial-scale pale lager malt.

Samples: LM-M - low grain modification batch M (variety Etincel); LM-N - low grain modification batch N (variety Etincel); HM-O - high grain modification batch O (variety Etincel); HM-P - high grain modification batch P (variety Etincel); 95°C - malt kilned-off at 95°C (variety Etincel); 85°C - malt kilned-off at 85°C (variety Etincel); S - malt with sulphuring (variety Passarel); NS-malt without sulphuring (variety Passarel). Compounds quantified: 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, and T2N = *trans*-2-nonenal, 2MP-CYS = cysteinylated 2-methylpropanal, 2MB-CYS = cysteinylated 2-methylbutanal, 3MB-CYS = cysteinylated 3-methylbutanal, MET CYS = cysteinylated methional, PHE-CYS = cysteinylated phenylacetaldehyde, FUR-CYS = cysteinylated furfural (<LOD), and HEX-CYS = cysteinylated hexanal (cysteinylated *trans* 2-nonenal is not presented as the reference compound was not available). Analytical parameters: Ext: extract, Col: colour, TP: total protein, TSP: total soluble protein, KI: Kolbach Index, Vis: viscosity, Fri: friability, Hom: homogeneity, DP: diastatic power, FAN: free amino nitrogen, β-glu: β-glucan, α-amy: α-amylase activity.

5.4. Conclusions

This research study presents further evaluation of industrial-scale malting as a function of a selection of malting variables, which, based on literature and own findings obtained in this PhD, may play a pivotal role regarding levels of (cysteinylated) aldehydes and related standard quality parameters in finished malt. In particular, grain modification, kilning-off temperature, and sulphuring during drying of green malt were selected as malting process variables.

In general, regarding formation of aldehydes, it is concluded from this work that grain modification and kilning-off temperature are to be considered key parameters. Kilning-off at a relatively high temperature (95°C) in pale malt production, clearly resulted in elevated levels of free and cysteinylated aldehydes in finished malt, when compared to the standard protocol of kilning-off at 85°C. Moreover, increased grain modification enhanced formation of aldehydes, but presumably in a more indirect way, *i.e*. via enzymatic formation of suitable precursors for later generation of aldehydes during subsequent kilning. Results derived from sulphuring of green malt during the first drying stage of kilning (mainly aimed to reduce formation of nitrosamines), did not show a truly significant impact on levels of (cysteinylated) aldehydes in the finished malt.

Regarding possible relationships between free aldehydes, cysteinylated aldehydes, and available malt quality parameters, multivariate data analysis demonstrated that higher levels of free and cysteinylated aldehydes are found in malts showing a higher thiobarbituric acid index (TBI), colour, total soluble protein (TSP), Kolbach Index (KI), and free amino nitrogen (FAN). More specifically, these malts were derived from processes involving increased grain modification or increased kilning-off temperature.

In conclusion, the data obtained in chapter 5, demonstrate that particular malting process variables (*i.e.* grain modification and kilning-off temperature) strongly impact – either indirectly (degree of modification) or directly (kilning-off temperature) – generation of (cysteinylated) aldehydes. Consequently, grain modification and curing temperature, were selected as key factors to design and further elaborate on an experimental setup, involving micro-malting towards improved malt quality (see further chapter 6).

Chapter 6

Micro-malting in search of ‘reduced beer staling potential - optimal brewing quality’

6.1 Introduction

Results obtained in the previous chapters of this PhD (see chapters 3 - 5) on malting samples derived from an industrial-scale, demonstrated that particular malting process variables strongly influence generation of (cysteinylated) aldehydes. In this part of the PhD, in order to better understand the interaction between selected key factors of malt production on the formation of staling aldehydes and standard quality parameters of malt, micro-malting combined with dedicated, experimental design is applied.

Regarding selection of key factors, results presented in chapters 3 and 4 on monitoring of the evolution of staling aldehydes throughout industrial-scale malting processes, point to the importance of heat load as a pertinent parameter influencing formation of aldehydes. In both chapters, a pronounced increase in levels of aldehydes was observed during the stage of drying at increased temperature (when the moisture content of the grain becomes relatively low and higher operating temperatures are being applied). Moreover, based on the outcomes of chapter 5 on finished malts ('*Evaluation of selected process variables of industrial-scale malting as a function of malt quality parameters and potential beer flavour (in)stability'*), higher levels of aldehydes were determined when malts were exposed to increased heat load during kilning. Similar findings regarding heat load have been reported in literature[26,110,133]. Results obtained in chapters 4 and 5 also demonstrated that a higher degree of grain modification may (indirectly) enhance formation of aldehydes, presumably via development of an extended reservoir of suitable precursors (*e.g.* amino acids[217]) and/or intermediates (*e.g.* α-dicarbonyls[78]) for generation of aldehydes during subsequent kilning. Consequently, grain modification and kilning-off temperature, have been selected as key factors to study their interactional effect, in particular, on the levels of staling aldehydes in a well-defined set of micro-malting experiments. Finally, the obtained, experimental data is used for theoretical modelling of malting conditions towards producing pale lager malt of reduced beer staling potential (*i.e.* finished malt showing significantly lower levels in free aldehydes and their precursors), whilst at least preserving - if not improving - overall malt brewing quality.

6.2. Materials and methods

6.2.1. Chemicals

Detailed information on the chemicals used for determination of free and cysteinylated aldehydes may be found in chapter 2, section 2.2.1. Chemicals required for determination of TBI, amino acids and standard malt quality parameters are specified in chapter 3, section 3.2.1. Chemicals used for determination of trihydroxy fatty acids are described in chapter 5, section 5.2.1.

Determination of lipoxygenase activity in malt. Linoleic acid (99%, CAS 60-33-3), Tween 20 (CAS 9005-64-5), acetic acid (≥99%, CAS 64-19-7) and boric acid (≥99%, CAS 10043-35-3) were purchased at Sigma-Aldrich, USA. Brij™ 98 (CAS 9004-98-2) was acquired from ACROS Organics, USA, whereas sodium acetate (CAS 12709-3) was purchased at Merck, Germany.

Determination of S-methylmethionine. Dimethyl sulphide (DMS; ≥99%, CAS 75-18-3) and ethyl methanesulfonate (EMS; ≥99%, CAS 62-50-0) was purchased at Sigma-Aldrich, USA, whereas fibre 85 µm Carboxen/Polydimethylsiloxane (CAR/PDMS) at Supelco, USA.

Determination of total polyphenols. Carboxymethylcellulose (CMC; CAS 9004-32-4) and ethylenediaminetetraacetic acid (EDTA; CAS 60-00-4), ammonia (≥99%, CAS 7664-41-7) and ammonium iron (III) citrate (CAS 1185-57-5) were all purchased from Sigma-Aldrich, USA.

6.2.2. Experimental design of micro-malting, statistical analysis, and numerical optimisation

The experimental design of micro-malting, statistical analysis, and numerical optimisation were carried out with the use of Design-Expert software (Stat-Ease Inc., USA). The influence of two malting process factors – degree of grain modification (variable A) and kilning-off temperature (variable B) – was investigated on 43 responses regarding malt quality. Different degrees of grain modification were achieved by varying the degree of steeping (grain moisture content after 24h of germination). Selected levels of variable A were 40%, 44%, 46%, and 48% of moisture content, aiming at under-, well- and overmodified malts[128,162,254]. Selected levels of variable B were 60°C, 73°C, and 85°C. The choice of temperatures is partly based on commonly applied industrial-scale kilning for the production of pale lager malt. The first stage of drying is typically carried out at 60°C (thus, in this study, this temperature was selected as the minimal value of variable B), and usually, kilning-off is set at 85°C (thus, in this study, it was chosen as a maximal value of variable B)[129]. In our experimental design, the temperature of 73°C was selected as midpoint between minimum and maximum values.

A central composite design using two factors, including three replicates at the central point of the design (degree of steeping: 44%, kilning-off temperature: 73°C), was chosen for fitting the response surface by the statistical software Design-Expert. Consequently, the experimental design (see Table 6-1) consisted of 22 malt samples, produced according to 12 different malting protocols.

Table 6-1. Overview of the design matrix of the experimental setup resulting in 22 malts produced according to 12 different malting protocols.

Experiment	Variable A Degree of steeping (%)	Variable B Kilning-off temperature (°C)
1	44	73
2	48	73
3	46	60
4	40	60
5	48	60
6	44	73
7	40	60
8	44	60
9	48	85
10	46	85
11	44	73
12	46	85
13	44	85
14	40	85
15	46	60
16	44	60
17	40	85
18	44	85
19	40	73
20	48	85
21	48	60
22	46	73

On the micro-malted samples, the following parameters (responses) were determined and measured values were entered to the software:

(1) standard quality parameters of malt: moisture content, extract yield, colour, thiobarbituric acid index (TBI), friability, homogeneity, partly unmodified grains (PUG), β-glucan content, viscosity, total protein (TP), total soluble protein (TSP), Kolbach Index (KI), free amino nitrogen (FAN), diastatic power (DP), α-amylase activity, pH, S-methylmethionine content (SMM), and malting yield;

(2) free aldehydes: 2-methylpropanal (2MP), 2-methylbutanal (2MB), 3-methylbutanal (3MB), methional (MET), phenylacetaldehyde (PHE), furfural (FUR), hexanal (HEX), *trans*-2-nonenal (T2N), and the sum of free aldehydes;

(3) cysteinylated aldehydes: cysteinylated forms of 2-methylpropanal (2MP-CYS), 2-methylbutanal (2MB-CYS), 3-methylbutanal (3MB-CYS), methional (MET-CYS), phenylacetaldehyde (PHE-CYS), furfural (FUR-CYS), hexanal (HEX-CYS), and the sum of cysteinylated aldehydes;

(4) compounds related to *de novo* formation of aldehydes: amino acids (valine, isoleucine, leucine, methionine, and phenylalanine), trihydroxy fatty acids (THFAs), lipoxygenase activity (LOX), and total polyphenol content.

For each analytical parameter, a response surface model (*i.e.* predicted model equation) was fitted, based on measurements on 22 malt samples. The model was statistically evaluated with analysis of variance (ANOVA, F-test), p-value of ≤0.05 (significant) and p-value of ≤0.01 (highly significant). The goodness of fit (variability among data points of one response) was assessed by the coefficient of determination (r^2). Predicted models are visualised in the form of 3D models.

Strength of potential relationships between parameters was assessed based on Pearson's correlation coefficient (r): very strong (r=0.90 - 1.00), strong (r=0.70 - 0.89), moderate (r=0.50 - 0.69), weak (r=0.30 - 0.49), and negligible (r <0.30)[255].

Theoretical, numerical optimisation of malting conditions was performed by introducing to the software preselected values of variable A and variable B. Based on this information and previously obtained equations of response models, predicted levels of responses were generated as output.

6.2.3. Micro-malting

Six-row, winter barley variety Etincel was provided by Boortmalt Antwerp, Belgium. Barley was screened over a 2.2 mm slotted sieve (sample cleaner MLN, Pfeuffer, Germany). Next, 500 g of sample was placed in a malting cage and then micro-malted in a steep-germinator and a kiln (Custom Laboratory Products, UK). At the end of the process, malt rootlets were removed with a deculmer (sample cleaner MLN, Pfeuffer, Germany).
Malting process protocols depended on two selected variables: variable A – degree of steeping and variable B – kilning-off temperature. The resulting 12 micro-malting protocols are presented in Table 6-2. Regarding steeping, the number of wet and dry phases, as well as duration of immersion in water varied among malting protocols (variants I, II, III, IV). In all cases, the temperature during steeping was set at 16°C. During the whole steeping period, periodic turning was performed for 2 min out of 10 min. During wet steeps, periodic aeration was performed for 1 min out of 10 min. Germination was also carried out at 16°C, with periodic turning as indicated for steeping. The degree of steeping was assessed 24h after onset of germination by measuring green malt moisture content (EBC 4.2 method). Drying conditions were similar for all malting protocols, whereas kilning-off differed among the applied protocols (variants i, ii, iii). The same cooling time (1h) was set for all malts.

Table 6-2. Twelve micro-malting protocols – four variants of steeping programs and three variants of kilning-off programs.

Steeping variants (16°C)	**Germination** [a]	**Drying** [b]	**Kilning-off variants** [c]	**Cooling**
I (40%) 5h WP – 15h DP – 0.25 h WP **II (**44%) 8h WP – 15h DP – 9 h WP **III (**46%) 8h WP – 15h DP – 7 h WP – 15h DP – 0.5h WP **IV (**48%) 8h WP– 15h DP – 8 h WP – 15h DP – 3h WP	96h 16°C	6h 60°C 6h 65°C 3h 70°C	**i** - 6h 60°C **ii** - 6h 73°C **iii** - 2h 80°C 2h 83°C 2h 85°C	1h

40%, 44%, 46%, 48% = desired degree of steeping; WP = wet period, DP = dry period;
[a] = application of 100% fresh air,
[b] = application of 100% fresh air, rate of airflow was set to 100% for the first 12h, reduced to 90% during the next 3h.
[c] = rate of airflow was set to 50%, increase in air recirculation every 2h, starting from 50% of recirculated air, followed by 75%, up to 100%, respectively.

6.2.4. Determination of free and cysteinylated aldehydes in malt

Quantitative determination of free aldehydes in malt samples was performed according to the optimised procedure as described in chapter 3, section 3.2.3. Quantitative determination of cysteinylated aldehydes in malt samples was performed according to the optimised procedure as described in chapter 2, section 2.2.9.

6.2.5. Determination of standard quality parameters of malt

Standard quality parameters of malt were assessed according to the European Brewery Convention methods (EBC Analytica, 2018), as described in chapter 3, section 3.2.7.

6.2.6. Determination of TBI of malt

Thiobarbituric acid index (TBI) was measured following the adapted method of Coghe *et al*.[218], which itself is based on the method described by Thalacker and Brikenstock[219] (for more details, see chapter 3, section 3.2.5.).

6.2.7. Determination of SMM in wort

S-methylmethionine (SMM) determination in wort was carried out following the protocol described by De Rouck *et al*.[256]. This method affords indirect quantification of SMM by its heat-induced decomposition into dimethyl sulphide (DMS), which can be quantified via headspace solid-phase microextraction (HS-SPME), followed by gas chromatography with flame ionization detector (GC-FID). Direct quantification of DMS in wort samples was performed by external calibration. DMS was dissolved in 4 mL of Milli-Q water in a range from 0.1 µg/L to 8.0 µg/L, and 50 µL of internal standard (1 µg/L; ethyl methyl sulphide, 96% (EMS)) was added to each calibration sample. The peak area ratio of DMS over EMS was used to calculate DMS concentrations. For determination of DMS in wort, 4 mL of wort was added to a GC-vial, spiked with 50 µL of internal standard (EMS, 1 µg/L), and subjected to GC-FID analysis. For SMM quantification, 4 mL of wort was transferred to a GC-vial and then incubated at 100°C for 160 min (to release DMS). After cooling down to room temperature, samples were spiked with 50 µL of internal standard (EMS, 1 µg/L) and were subjected to GC-FID analysis. All samples were pre-equilibrated for 2 min at 30°C and extracted by HS-SPME for 15 min at 30°C with agitation at 250 rpm, using an 85 µm fibre with Carboxen/Polydimethylsiloxane (CAR/PDMS) coating (Supelco, USA). The fibre was thermally desorbed in the injection port of the GC for 3 min. Separations were performed on an RTX-1 fused silica capillary column (30 m × 0.32 mm i.d., 3 µm film thickness Restek) coupled with a pulsed flame photometric detector (PFPD 5380, OI Analytical, Texas, USA) operated in the sulphur mode. GC conditions were as follows: carrier gas (helium) flow of 1.2 mL/min; inlet temperature 250°C, injection in split mode (split ratio 10:1); oven temperature 35°C for 3 min, further raised to 250°C at 5°C/min and held at 250°C for 5 min. The PFPD was set at 250°C and 560 V with air 1 and air 2 at 10 mL/min and hydrogen at 12.5 mL/min. Data were processed with Chromcard 2.3.2 (Thermo Electron Corporation, Italy) and WinPulse 32 2.0 (OI Analytical, USA). The SMM content was calculated by subtracting the DMS level of the sample without heat exposure, from the DMS value of the sample exposed to heat.

6.2.8. Determination of amino acids in malt

Amino acids valine, isoleucine, leucine, methionine, and phenylalanine were determined with the use of AccQ•Tag Derivatisation Kit (Waters, USA) followed by AccQ•Tag Ultra UPLC-PDA method (Waters, USA), as described in chapter 3, section 3.2.6.

6.2.9. Determination of total polyphenols in wort

Total polyphenol content in wort was determined based on the spectrophotometric EBC method 8.12. The method is based on the reaction between polyphenols and iron ions in an alkaline medium, forming a red complex, which absorbance is measured at λ=600 nm. Sample preparation consisted of mixing 10 mL of Congress wort with 8 mL of CMC/EDTA reagent (10 g/L CMC, 2 g/L EDTA), followed by addition of 0.5 mL of ammonium hydroxide (25% (v/v) in Milli-Q water) and 0.5 mL of colour reagent (35 g/L ammonium iron citrate in Milli-Q water). The mixture was diluted with Milli-Q water up to a final volume of 25 mL. Blank sample was prepared in a similar way, however, without addition of colour reagent. Samples were incubated for 10 min at room temperature, and, subsequently transferred to UV cuvettes. Absorbance was measured at λ=600 nm against the blank sample. Results of measurements were multiplied by a factor of 820 yielding the concentration in mg/L, which was further recalculated to mg/kg dm of malt.

6.2.10. Determination of trihydroxy fatty acids in malt

Trihydroxy fatty acids (THFAs) in malt samples were determined by the EFBT internal method, based on a combination of the methods of Möller-Hergt *et al.*[244] and Wackerbauer *et al.*[245], as described in chapter 5, section 5.2.8.

6.2.11. Determination of total lipoxygenase activity in malt

Extraction of lipoxygenase enzymes (LOX) from malt samples and spectrophotometric measurement of their activity was carried out according to the EFBT internal method, based on the methods of De Buck *et al*.[257] and Boeykens *et al*. internal method. Sample preparation consisted of mixing 5 g of finely milled malt with 50 mL of acetate buffer (0.1 *M*, pH 5) containing detergent Brij 98 (0.1%), and stirring the mixture for 40 min at 250 rpm. Next, the homogenate was centrifuged (11,000 rpm, 2 min), and the supernatant was used as crude enzyme extract. Stock solution of substrate was prepared on ice by dispersing 620 µL of linoleic acid in 10 mL of deaerated borate buffer (0.2 *M*, pH 9.0) and 2 mL NaOH (0.1 *M*). The solution was mixed and diluted with borate buffer up to a total volume of 100 mL. A sample of 10 mL of stock solution was further diluted with 3 drops of Tween20 and 150 mL of deaerated Milli-Q water. Next, the pH was adjusted to pH 7.7 with HCl (0.1 *M*) and Milli-Q water was added up to a total volume of 200 mL. To measure enzyme activity, 200 µL of crude enzyme extract was added to a UV cuvette containing a mixture of 2.7 mL of diluted substrate solution (previously flushed with air) and 400 µL of mixed buffer (25 mL of borate buffer mixed with 75 mL of acetate buffer). The blank sample was prepared in a similar way, 0.2 mL of acetate buffer was added instead on enzyme. Absorption (λ=234 nm) was measured precisely 2 min after addition of enzyme extract against the blank sample. The final result of LOX-activity expressed as U/g of dm of malt, was obtained by multiplication of the measured absorbance value by a factor of 165 and correcting for malt dry mass.

6.2.12. Determination of malting yield

The real malting yield (% dm) was calculated as described by Briggs[128]. First, the final malt weight obtained was divided by the initial barley weight. Next, for calculating the real malting yield and the real losses, the moisture content of both barely and finished malt were taken into account.

6.3. Results and discussion

6.3.1. Influence of malting conditions on responses related to standard quality parameters of malt and levels of aldehydes/aldehyde precursors

6.3.1.1. Overview of data processing related to modelling of responses and correlations among analytical parameters

The effect of grain modification and kilning-off temperature in micro-malting was evaluated through 43 responses (see materials and methods, section 6.2.3.) measured on finished malts, among them: levels of free and cysteinylated aldehydes, levels of several compounds related to *de novo* formation of aldehydes, and standard quality parameters of malt. Next, raw data obtained on the 43 different responses were modelled (as described in section 6.2.3.; using Design-Expert software), as a function of variable A (degree of steeping) and variable B (kilning-off temperature). The outcomes of modelling are presented in Table 6-3, showing the regression analysis for each response (*i.e.* the equation of the model), analysis of variance (F-test to evaluate the significance of model), and calculation of the coefficient of determination (r^2) for each model. Two types of equations – linear or quadratic – were obtained for the calculated models. The F-tests of responses showed that the corresponding models are highly significant (p-value ≤0.01). Only the response on 'extract yield' did not provide a significant model because of little variation among raw data (see Appendix F), and no model was obtained for cysteinylated furfural since values were always <LOD.
All of the responses (except for the final moisture content of malt, and, evidently, as mentioned before, extract yield) were significantly influenced by variable A (degree of steeping). Variable B (kilning-off temperature), however, only significantly impacted levels of (cysteinylated) aldehydes (except for free and cysteinylated hexanal), LOX-activity, TBI, colour, diastatic power, SMM content, moisture content, TP, and malting yield. This implies that - at least within the applied experimental setup - finished malt quality parameters related to cytolysis and proteolysis were not significantly impacted by the kilning-off temperature programme. In summary, the above findings confirm the importance of the degree of steeping and kilning-off temperature as highly relevant, key parameters in malting. The obtained, significant models allow to further study the interactional effects of the independent variables A and B on each measured, individual response (see further, sections 6.3.1.2 – 6.3.1.3).

In addition to modelling, correlation coefficients among process variables A (steeping degree) and B (kilning-off temperature), standard malt quality parameters, levels of (cysteinylated) aldehydes, and compounds related to *de novo* formation of aldehydes were calculated. Data are shown in the correlation matrix in Figure 6-1, as a heatmap (for detailed discussion on relevant correlation coefficients, see sections 6.3.1.2 – 6.3.1.3).

Table 6-3A. Influence of micro-malting conditions on responses related to standard quality parameters of malt, levels of aldehydes, and levels of compounds involved in de *novo* formation of aldehydes.

Response	p-value			r^2	Model type	Predicted model equation
	Model	Variable A	Variable B			
Standard quality parameters of malt						
Moisture content	< 0.0001**	0.8662	< 0.0001**	0.8646	L	Moisture = 7.59 - 0.003 × A - 0.043 × B
Extract yield	0.3782	0.6697	0.3348	0.2632	-	-
Colour	< 0.0001**	< 0.0001**	0.001**	0.9270	Q	Colour = 108 - 4.06 × A - 0.56 × B + 0.01 × AB + 0.04 × A^2 + 0.002 × B^2
TBI	< 0.0001**	< 0.0001**	< 0.0001**	0.9637	Q	TBI = 395 -16.32 × A + 1.76 × B - 0.03 × AB + 0.17 × A2 - 0.003 × B2
Friability	< 0.0001**	< 0.0001**	0.6595	0.9578	L	Friability = -190 + 5.77 × A + 0.03 × B
Homogeneity	< 0.0001**	< 0.0001**	0.1400	0.9844	Q	Homogeneity = -1150 - 50 × A + 0.51 × B - 0.03 × AB - 0.49 × A^2 - 0.004 × B^2
Partly unmodified grain	< 0.0001**	< 0.0001**	0.1242	0.9846	Q	PUG = 1231 - 50 × A + 0.43 × B + 0.02 × AB + 0.49 × A^2 - 0.004 × B^2
β-Glucan content	< 0.0001**	< 0.0001**	0.4349	0.9295	Q	β-Glucan content = -14310 + 811 × A - 40 × B - 0.08 × AB - 9.99 × A2 + 0.31 ×B2
Viscosity	< 0.0001**	< 0.0001**	0.5496	0.9138	Q	Viscosity = 25 - 0.91 × A - 0.05 × B + 0.0001 × AB + 0.009 × A2 + 0.0003 × B2
Total protein	< 0.0001**	< 0.0001**	0.0413*	0.7439	L	TP = 12.82 - 0.05 × A - 0.004 × B
Total soluble protein	< 0.0001**	< 0.0001**	0.3705	0.9057	L	TSP = -6.46 + 0.23 × A + 0.004 × B
Kolbach index	< 0.0001**	< 0.0001**	0.2924	0.8807	L	KI = -67.93 + 2.36 × A + 0.06 × B
Free amino nitrogen	< 0.0001**	< 0.0001**	0.4766	0.9778	Q	FAN = 1230 - 69 × A + 5.83 × B - 0.009 × AB + 0.94 × A2 - 0.04 × B2
Diastatic power	< 0.0001**	< 0.0001**	< 0.0001**	0.7743	L	Diastatic power = -205 + 22 × A - 4.38 × B
α-Amylase activity	< 0.0001**	< 0.0001**	0.0772	0.7907	L	α-Amylase activity = -88 + 3.59 × A - 0.21 × B
pH	< 0.0001**	< 0.0001**	0.0689	0.8944	L	pH = 8.10 - 0.05 × A - 0.002 × B
S-methylmethionine	< 0.0001**	< 0.0001**	< 0.0001**	0.9064	Q	SMM = -471 + 18 × A + 1.92 × B - 0.04 × AB - 0.16 × A^2 - 0.002 × B^2
Malting yield	< 0.0001**	< 0.0001**	0.0142*	0.7974	L	Malting yield = 151.96 - 1.21 × A - 0.10 × B
Free aldehydes						
2MP	< 0.0001**	< 0.0001**	< 0.0001**	0.9684	Q	2MP = 32204 - 1346 × A - 148 × B + 1.31 × AB + 15.31 × A^2 + 0.88 × B^2
2MB	< 0.0001**	< 0.0001**	< 0.0001**	0.9816	Q	2MB = 27224 - 998 × A - 229 × B + 2.58 × AB + 10.35 × A^2 + 1.07 × B^2
3MB	< 0.0001**	< 0.0001**	< 0.0001**	0.9773	Q	3MB = 78102 - 3405 × A - 270 × B + 3.05 × AB + 38.63 × A^2 + 1.57 × B^2
MET	< 0.0001**	< 0.0001**	< 0.0001**	0.9723	Q	MET = 17332 - 458 × A - 239 × B + 2.87 × AB + 3.30 × A^2 + 0.89 × B^2
PHE	< 0.0001**	< 0.0001**	< 0.0001**	0.9739	Q	PHE = 87763 - 2901 × A - 810 × B + 9.98 × AB + 26.55 × A^2 + 3.02 × B^2
FUR	< 0.0001**	< 0.0001**	< 0.0001**	0.9665	Q	FUR = 36014 - 1367 × A - 214 × B + 3.25 × AB + 13.52 × A2 + 0.61 × B2
HEX	< 0.0001**	< 0.0001**	0.1369	0.9070	Q	HEX = 14813 - 853 × A + 87 × B - 1.16 × AB + 11.13 × A^2 - 0.23 × B^2

Table 6-3B. Influence of micro-malting conditions on responses related to standard quality parameters of malt, levels of aldehydes, and levels of compounds involved in de novo formation of aldehydes.

Response	p-value			r^2	Model type	Predicted model equation
	Model	Variable A	Variable B			
Free aldehydes continuation						
T2N	< 0.0001**	0.0003**	< 0.0001**	0.7183	L	T2N = -1954 + 35.45 × A + 10.88 × B
Sum of free aldehydes	< 0.0001**	< 0.0001**	< 0.0001**	0.9847	Q	Sum of ALD = 3.17×10^5 - 12230 × A - 1963×B + 23.97 × AB + 127×A^2 + 8.23×B^2
Cysteinylated aldehydes						
2MP-CYS	< 0.0001**	< 0.0001**	< 0.0001**	0.9866	Q	2MP-CYS = 4403 - 103 × A - 71 × B + 0.70 × AB + 0.72 × A^2 + 0.31 × B^2
2MB-CYS	< 0.0001**	< 0.0001**	< 0.0001**	0.9711	Q	2MB-CYS = 2580 - 69 × A - 35 × B + 0.35 × AB + 0.57 × A^2 + 0.16 × B^2
3MB-CYS	< 0.0001**	< 0.0001**	< 0.0001**	0.9483	Q	3MB-CYS = 12318 - 322 × A - 173 × B + 1.68 × AB + 2.59 × A^2 + 0.79 × B^2
MET-CYS	< 0.0001**	0.0002**	< 0.0001**	0.9511	Q	MET-CYS = 1048 - 9.11 × A - 26.80 × B + 0.21 × AB - 0.04 × A^2 + 0.13 × B^2
PHE-CYS	< 0.0001**	< 0.0001**	< 0.0001**	0.9602	Q	PHE-CYS = 5587 - 202 × A - 42 × B + 0.48 × AB + 2.03 × A^2 + 0.19 × B^2
FUR-CYS	-	-	-	-	-	-
HEX-CYS	0.005*	0.0118*	0.3825	0.6181	Q	HEX-CYS = 339 - 23 × A + 4.21 × B - 0.07 × AB + 0.33 × A^2 - 0.01 × B^2
Sum of cysteinylated aldehydes	< 0.0001**	< 0.0001**	< 0.0001**	0.9704	Q	Sum of CYS-ALD = 26281 - 729 × A - 344 × B + 3.36 × AB + 6.21 × A^2 + 1.57 × B^2
Potential precursors and compounds related to *de novo* formation of aldehydes						
Valine	< 0.0001**	< 0.0001**	0.3031	0.8580	Q	Val = -5948 + 178 × A + 52 × B - 0.37 × AB - 1.42 × A^2 - 0.24 × B^2
Isoleucine	< 0.0001**	< 0.0001**	0.2090	0.8797	Q	Ile = -2655 + 63 × A + 31 × B - 0.20 × AB - 0.34 × A^2 - 0.16 × B^2
Leucine	< 0.0001**	< 0.0001**	0.1616	0.8548	Q	Leu = -4699 + 113 × A + 57 × B - 0.32 × AB - 0.70 × A^2 - 0.30 × B^2
Methionine	< 0.0001**	< 0.0001**	0.2801	0.8074	Q	Met = -1004 + 31.28 × A + 8.75 × B - 0.06 × AB - 0.26 × A^2 - 0.04 × B^2
Phenylalanine	< 0.0001**	< 0.0001**	0.1689	0.8760	Q	Phe = -270 - 76 × A + 49 × B - 0.29 × AB + 1.43 × A^2 - 0.26 × B^2
Trihydroxy fatty acids	0.0009**	0.0003**	0.5293	0.5216	L	THFA = 128 - 1.91 × A - 0.07 × B
LOX-activity	< 0.0001**	< 0.0001**	< 0.0001**	0.9708	Q	LOX-activity = 8.56 - 3.28 × A + 1.71 × B - 0.02 × AB + 0.07 × A^2 - 0.03 × B^2
Total polyphenol content	< 0.0001**	< 0.0001**	0.2270	0.8227	Q	TPC = 7044 – 324.76 × A + 4.79 × B + 0.39 × AB + 3.63 × A^2 - 0.14 × B^2

Variable A = degree of steeping (% moisture), variable B = kilning-off temperature (°C); * = p-value ≤0.05, significant; ** = p-value ≤0.01, highly significant; '-' = p-value >0.05, not significant, r^2 = coefficient of determination; assigned model type for each response: Q = quadratic, L = linear. In the predicted model equation: A = degree of steeping, B = kilning-off temperature. Compounds: 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, T2N = *trans*-2-nonenal, 2MP-CYS = cysteinylated 2-methylpropanal, 2MB-CYS = cysteinylated 2-methylbutanal, 3MB-CYS = cysteinylated 3-methylbutanal, MET-CYS = cysteinylated methional, PHE-CYS = cysteinylated phenylacetaldehyde, FUR-CYS = cysteinylated furfural (<LOD), and HEX-CYS = cysteinylated hexanal (cysteinylated *trans*-2-nonenal is not presented as the reference compound was not available), TBI = thiobarbituric acid index, LOX-activity = lipoxygenase activity

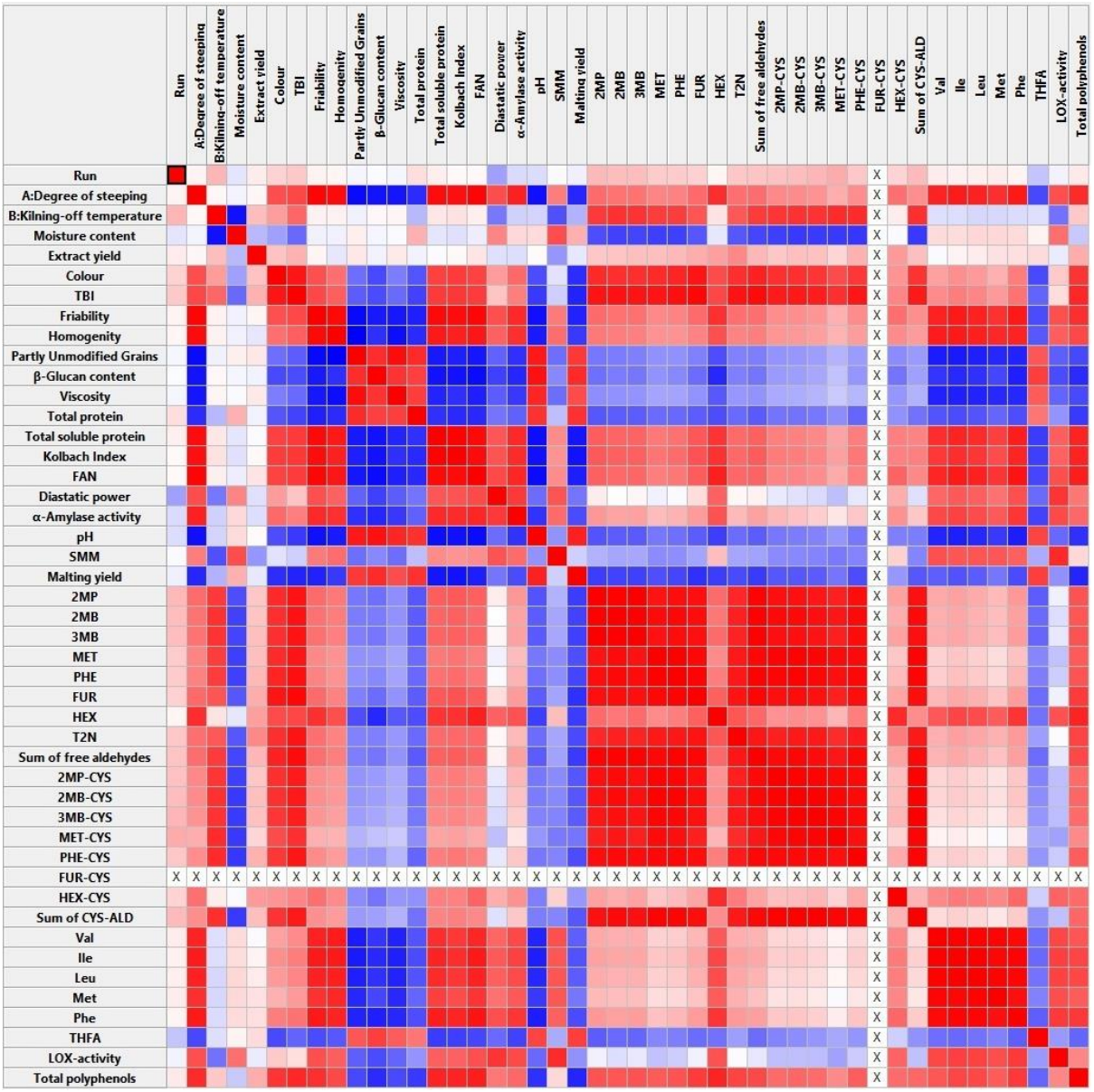


Figure 6-1. Correlation matrix presented as heatmap, illustrating calculated correlation coefficients among process variables A (steeping degree) and B (kilning-off temperature), standard malt quality parameters, levels of (cysteinylated) aldehydes, and compounds related to *de novo* formation of aldehydes.

Colour intensity refers to the strength of possible relationships (higher colour intensity points to increased strength of correlation). Red = positive correlation; blue = negative correlation; white = no correlation; x = correlation not available as FUR-CYS <LOD. Run = default indication of performed experiment (imposed by software Design-Expert). Parameters and compounds: TBI = thiobarbituric acid index, FAN = free amino nitrogen, SMM = S-methylmethionine, 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, T2N = *trans*-2-nonenal, 2MP-CYS = cysteinylated 2-methylpropanal, 2MB-CYS = cysteinylated 2-methylbutanal, 3MB-CYS = cysteinylated 3-methylbutanal, MET-CYS = cysteinylated methional, PHE-CYS = cysteinylated phenylacetaldehyde, FUR-CYS = cysteinylated furfural, HEX-CYS = cysteinylated hexanal, (levels in cysteinylated *trans*-2-nonenal cannot be presented as the reference compound was not available), Val = valine, Ile = isoleucine, Leu = leucine, Phe = phenylalanine, Met = methionine, THFA = trihydroxy fatty acids, LOX-activity = lipoxygenase activity.

6.3.1.2. Impact of degree of steeping and kilning-off temperature on standard quality parameters of malt

In this section, more detailed evaluation of the influence of the degree of steeping (variable A) and kilning-off temperature (variable B) on standard quality parameters of malt is performed (for raw data obtained on the finished malts, see Appendix F). To facilitate evaluation, the overall quality of the different variants of malt is judged according to a set of recommended standard quality parameters (for more information on these parameters, see Table 3-3, chapter 3).

As mentioned in the previous section, the degree of steeping significantly affected all analysed malt quality parameters (except for final moisture content of malt and extract yield). In particular, parameters related to cytolysis (friability, β-glucan content, viscosity) and proteolytic modification (TSP, FAN, KI) were impacted by variable A, as well as α-amylase activity. Clearly, a higher degree of steeping results in higher grain modification and α-amylase activity. According to modelling performed in this case study on micro-malting (for more details on modelling, see Table 6-3 and Appendix G) and recommended values for pale lager malt quality (see Table 3-3**,** chapter 3), satisfactory cytolytic modification was found when the degree of steeping was >46%, whilst acceptable proteolytic modification was obtained when the degree of steeping was 45% - 47%.

Moisture content of finished malt was influenced only by the applied kilning-off temperature (variable B) in that lower values for moisture were found in malts kilned-off at higher temperatures. All malt variants were within the recommended range for moisture (3% - 5%). As expected, and already mentioned before, kilning-off temperature mainly impacts malt quality parameters that are connected to the applied heat load (*e.g.* malt colour, diastatic power).

Regarding standard malt quality parameters, both variables A and B impacted diastatic power, colour, TBI, SMM content, and malting yield. Diastatic power increased with an increase in the degree of steeping, and decreased with higher kilning-off temperatures. Higher levels of diastatic power were determined in more modified malts because of higher moisture content as such, and, presumably also because germination of these samples was somewhat longer due to a second dry rest during steeping. Somewhat lower levels of diastatic power, as observed when applying higher kilning-off temperatures, can be ascribed to inactivation of the relatively heat-sensitive β-amylase[258–260], a predominant enzyme of the diastatic power test. Nevertheless, diastatic power in all malt samples exceeded the minimum recommended value (>220 °WK). Regarding colour, higher grain modification resulted in higher levels of potential precursors as can be concluded from the results obtained on levels of amino acids (see Appendix F). Moreover, a higher kilning-off temperature gave rise to more colour, due to an increased rate of colour formation. All malts with a degree of steeping >44%, met the recommended range for pale lager malt (2 EBC - 4.5 EBC), regardless of the kilning-off temperature. Further, TBI values increased with an increase in variable A, as well as variable B. Thus, as mentioned in chapter 4, it appears that - at least for pale lager malts - measured TBI values do not necessarily directly reflect the amount of heat load applied during kilning. Apparently, independent from heat load, also grain modification as such impacts the TBI value of finished malt. Regarding S-methylmethionine (SMM; the well-known precursor of dimethyl sulphide (DMS), a beer off-flavour perceived at levels above 30-100 µg/L as 'cooked vegetables'[261]), its content increased with the degree of steeping and decreased with higher

kilning-off temperatures. This is in accordance with literature, as SMM is synthesised during germination, and its content depends mostly on barley variety and grain modification[228,262]. Regarding required specifications, SMM content of finished pale lager malt may vary between 2 mg/kg - 8 mg/kg[228]. Among analysed malt samples, relatively low modified malts (<46% of moisture content), kilned-off at higher temperatures do meet the traditional brewery requirements regarding SMM content. This may be explained by the fact that an increased kilning-off temperature reduces SMM content, as SMM starts to decompose fastly at >70°C into the volatile DMS, which is then further removed on the kiln[263,264]. Finally, malting yield reflecting biological losses and, therefore, growth of the embryo, was affected by both variables A and B. In fact, any variable relevant to malting will - to some extent - impact biological growth. From the model obtained on 'malting yield' (see Table 6-3), it is clear that in our study the degree of steeping is the predominant variable, compared to kilning-off temperature. Evidently, grain modification which parallels biological growth, should have a major impact on the malting yield.

In general, when applying a degree of steeping of >46%, malts of satisfying quality (according to generally accepted recommendations) have been obtained through micro-malting. Levels in SMM might still represent some issue, but adapted brewing schemes should easily provide an answer to this, for instance, by mashing-off at increased temperatures[256]. Regarding kilning-off temperature, at least within the temperature range applied in our case study, this variable does not seem to impose limitations in respect of overall pale lager malt quality. Consequently, opportunities to arrive at pale lager malt of improved quality seem feasible, in particular by applying less heat load at kilning.

6.3.1.3. Impact of degree of steeping and kilning-off temperature on levels of (cysteinylated) aldehydes and compounds related to *de novo* formation of aldehydes

This part evaluates the influence of the degree of steeping (variable A) and kilning-off temperature (variable B) on levels of (cysteinylated) aldehydes and compounds related to *de novo* formation of aldehydes.

The 3D plots presented in Figure 6-2 model response surfaces of the sum of (cysteinylated) aldehydes and of individual (cysteinylated) aldehydes as a function of the degree of steeping and kilning-off temperature. For all aldehydes, a quadratic model provided the best fit, except for *trans*-2-nonenal (linear model). Regarding free aldehydes, their sum ranges between 1,285 µg/kg dm - 16,811 µg/kg dm, and it increases with an increase in the degree of steeping as well as kilning-off temperature. Likewise, levels of all individual free aldehydes increase with higher grain modification (achieved by a higher degree of steeping), which may be ascribed to an increase in levels of potential precursors (*e.g.* amino acids, see Appendix F) for the later generation of aldehydes during kilning. In fact, careful inspection of Appendix F shows that increased levels of aldehydes in the finished malts are clearly associated with parameters representing proteolysis, such as Kolbach Index and FAN. Moreover, as expected, levels of individual free aldehydes (except for hexanal) increase when kilning-off at a higher temperature. These data are in conformity with our previous findings in chapters 3 - 5 and with results available in literature, where higher levels of free aldehydes were found in malt samples exposed to higher heat load[26,110,133]. An exception is, however, hexanal, levels of

which seem to be mainly influenced by the degree of steeping and, to a much lesser extent, by the kilning-off temperature. Thus, the 3D model representing hexanal is totally different from the models of all other aldehydes (see Figure 6-2). This might be (partly) related to the relatively high volatility of hexanal, which may (to some extent) obscure oxidative formation of hexanal during kilning-off. Moreover, the observed behaviour of hexanal during micro-malting is in line with our previous results (obtained on industrial-scale samples) shown in chapters 3 - 5, where the effect of heat load on hexanal formation was also found to be minor. Data obtained by modelling are confirmed by calculated correlation coefficients (see Figure 6-1), as it appears that levels of all staling aldehydes (except for hexanal) are mainly influenced by kilning-off temperature ($r = 0.65 - 0.80$), and, to a lesser extent, by the degree of steeping ($r = 0.48 - 0.58$). Conversely, levels of hexanal appear to be mainly determined by the degree of steeping ($r = 0.82$), whereas kilning-off temperature does not seem to show impact ($r = 0.10$). Finally, regardless of the analysed malt, 3MB appears to be the major free aldehyde followed by PHE, 2MP, and 2MB (also observed in chapters 3 - 5 for all industrial-scale malt samples).

Concerning levels of cysteinylated aldehydes[l], their sum ranges between 27 µg/kg dm - 1,556 µg/kg dm. The lowest values were found for low modified malts kilned-off at 60°C, whereas the highest values were obtained for highly modified malts kilned-off at 85°C. Similar to free aldehydes, a quadratic model provided the best fit for each individual cysteinylated aldehyde. Also regarding the impact of variables A and B, individual cysteinylated aldehydes follow patterns that are similar to those of their free counterparts. Regardless of the analysed malt, 3MB-CYS appears to be the major cysteinylated aldehyde followed by PHE-CYS, 2MP-CYS, and 2MB-CYS.

The 3D models shown in Figure 6-2 allow to presume that a slight decrease in the degree of steeping and/or kilning-off temperature may lead to a considerable decrease in levels of both free and cysteinylated aldehydes, and, as a consequence, may result in a significantly reduced beer staling potential of malt.

[l] Regarding cysteinylated furfural, MS signals were always <LOD. As already mentioned in chapter 3, (slightly) acidic conditions such as those in malt, are not favourable for the formation of the furfural-cysteine adduct, because of the relatively low electrophilic character of the aldehyde functional group of furfural (due to the aromatic nature of the R group of furfural). Furthermore, data on cysteinylated *trans*-2-nonenal cannot be given since the reference compound is not available.

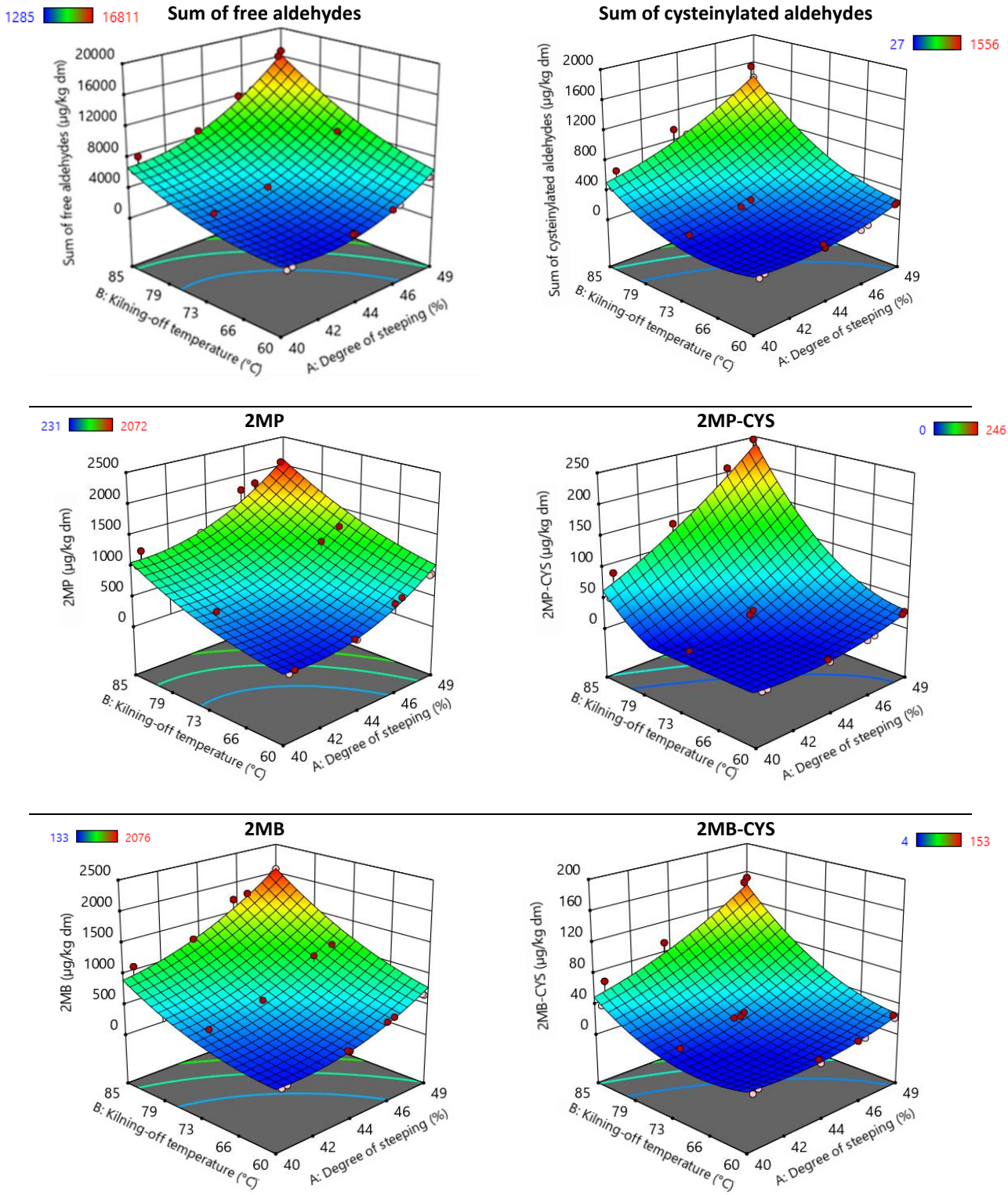


Figure 6-2A. 3D plots modelling the influence of the degree of steeping (ranging from 40% to 48%) and kilning-off temperature (ranging from 60°C to 85°C) on levels of free aldehydes (left side) and cysteinylated aldehydes (right side).

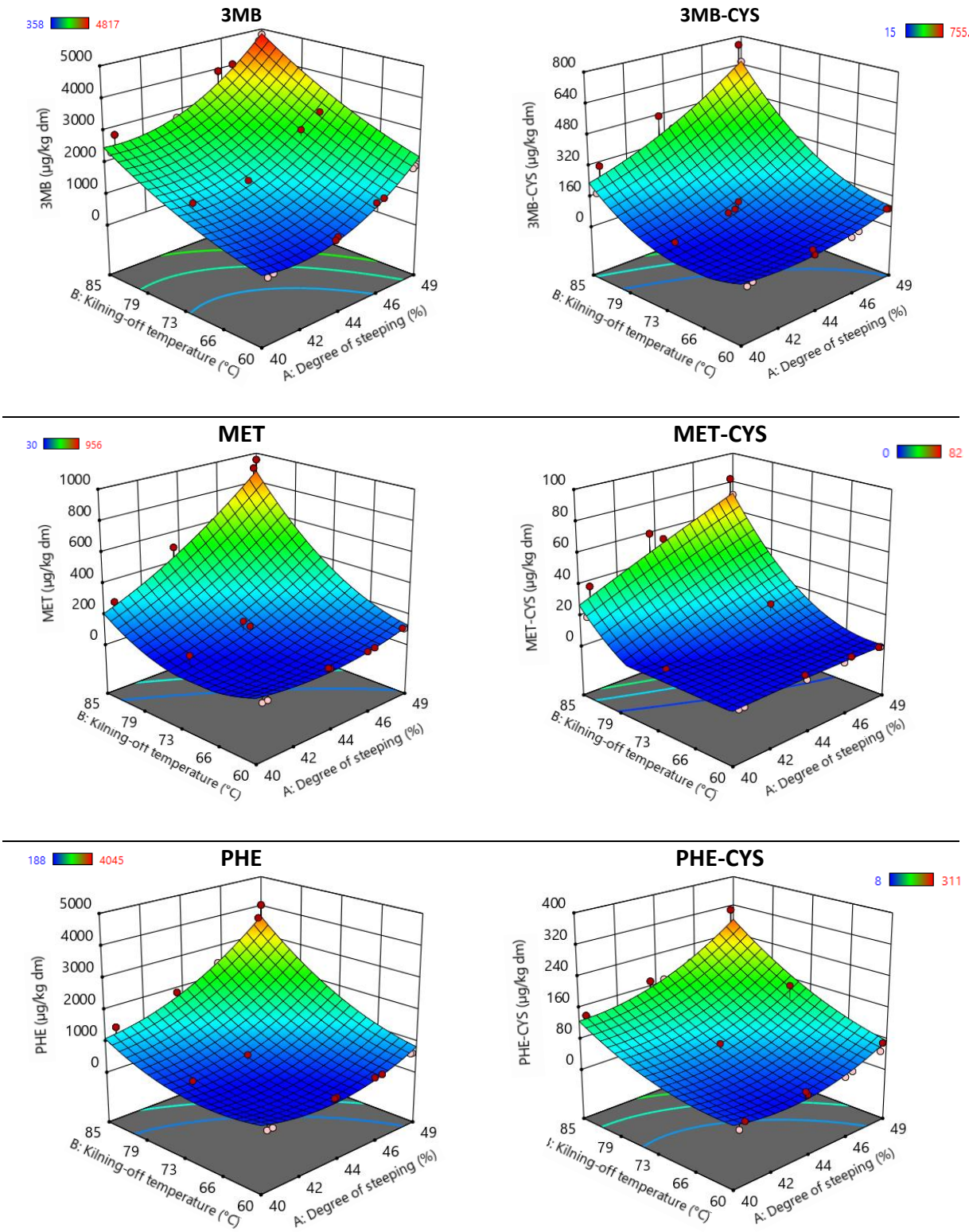


Figure 6-2B. 3D plots modelling the influence of the degree of steeping (ranging from 40% to 48%) and kilning-off temperature (ranging from 60°C to 85°C) on levels of free aldehydes (left side) and cysteinylated aldehydes (right side).

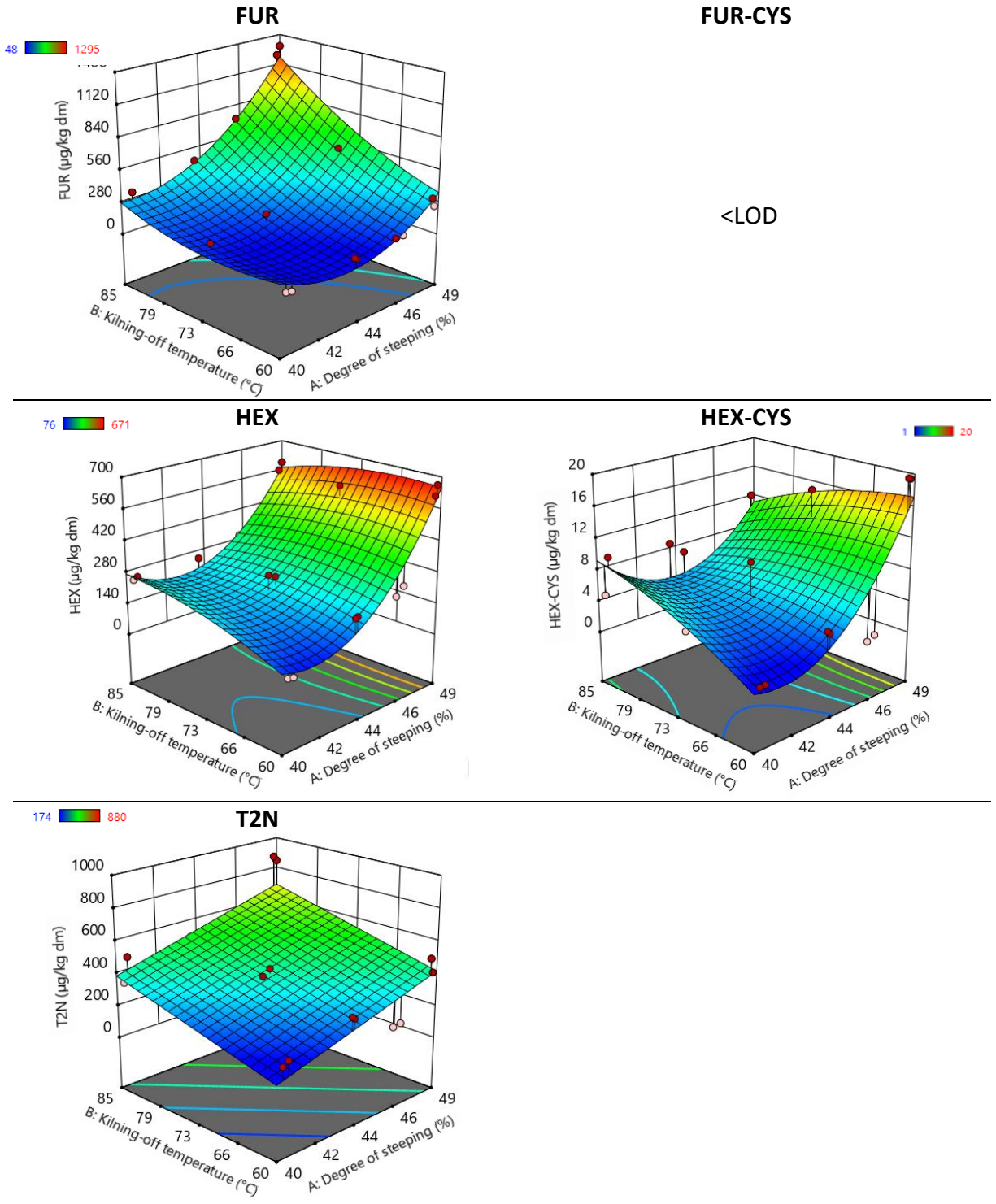


Figure 6-2C. 3D plots modelling the influence of the degree of steeping (ranging from 40% to 48%) and kilning-off temperature (ranging from 60°C to 85°C) on levels of free aldehydes (left side) and cysteinylated aldehydes (right side).

Red points = design points above predicted value, pink points = design points below predicted value. Compounds: 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, T2N = *trans*-2-nonenal, 2MP-CYS = cysteinylated 2-methylpropanal, 2MB-CYS = cysteinylated 2-methylbutanal, 3MB-CYS = cysteinylated 3-methylbutanal, MET-CYS = cysteinylated methional, PHE-CYS = cysteinylated phenylacetaldehyde, FUR-CYS = cysteinylated furfural (<LOD), and HEX-CYS = cysteinylated hexanal (cysteinylated *trans*-2-nonenal is not presented as the reference compound was not available). LOD = limit of detection.

The 3D plots modelling the influence of variables A and B on the levels of compounds related to *de novo* formation of aldehydes are shown in Figure 6-3**.** Regarding potential precursors of Strecker aldehydes (*i.e.* amino acids valine, isoleucine, leucine, methionine, and phenylalanine), levels of individual amino acids increased with an increase in the degree of steeping, which can be ascribed to intensified proteolysis in more modified malts. However, in contrast to Strecker aldehydes, kilning-off temperature did not seem to impact significantly levels of amino acids. Thus, levels of amino acids are mainly impacted by the degree of steeping, whereas levels of Strecker aldehydes are impacted by both the degree of steeping and kilning-off temperature. Weak relationships (r=0.31 - 0.34) among levels of amino acids and levels of their corresponding aldehydes were found. However, strong to very strong correlations were obtained when analysing relationships for samples that were kilned-off at one and the same temperature, thereby excluding kilning-off temperature as a variable. The following correlation coefficients among levels of amino acids and corresponding aldehydes were obtained at kilning-off at 85°C, 73°C, and 60°C: r>0.79, r>0.77, and r>0.91, respectively. These strong correlations point to the relevance of the degree of steeping and, therefore, the degree of grain modification as a key impact factor in relation to the formation of Strecker degradation aldehydes, independent from the applied kilning-off temperature.

Levels of trihydroxy fatty acids (THFAs, potential precursors of fatty acid oxidation aldehydes) decreased with an increase in the degree of steeping. This may suggest incorporation of THFAs into various (bio)chemical pathways (*i.a.* formation of hexanal and *trans*-2-nonenal) to a somewhat higher extent in more modified grains, although only weak to moderate correlations between levels of THFAs and fatty acid oxidation aldehydes were found. More specifically, correlation coefficients between THFAs and hexanal or *trans*-2-nonenal amounted to r= -0.55 (moderate) and r= -0.38 (weak), respectively (see Figure 6-1). In this respect, it should be mentioned that THFAs, besides being intermediates of fatty acid oxidation towards aldehydes, play a much broader role in plant physiology. For instance, it is well-known that THFAs show antifungal and antibacterial activity[265,266] (for an overview of biological activities exposed by oxidised fatty acids, including THFAs, reference is made to[265,267]).

Regarding LOX-activity, catalysing enzymatic oxidation of unsaturated fatty acids, it was significantly affected by both variables. The lowest LOX-activity was determined in malt of 40% degree of steeping and kilned-off at 85°C, whereas the highest values were obtained in malt of 48% degree of steeping and kilned-off at 60°C. LOX-activity appears to be positively correlated with hexanal content (r=0.68), while no correlation was found with *trans*-2-nonenal. Therefore, it is suggested that formation of hexanal during malting occurs predominantly via enzymatic oxidation of unsaturated fatty acids, whereas *trans*-2-nonenal would be mainly formed via autoxidation. This hypothesis can be supported by the observed differences in the behaviour of hexanal and *trans*-2-nonenal during malting as previously found in chapters 3 and 4, as well as results reported by Dong *et al.*[110], suggesting that formation of hexanal in malt occurs mostly during germination, as opposed to *trans*-2-nonenal, mainly formed during kilning.

In connection to oxidative transformations, the total polyphenol content of malt was assessed, as some of these compounds are known to show radical-scavenging or reducing properties[87,152,176,268]. Total polyphenol content increased significantly with an increase in the degree of steeping, which - from the biological point of view - may be ascribed to more pronounced release of phenolic compounds during germination under conditions that

enhance modification[165], and (technically spoken) also to higher friability (see Appendix F), both of which would facilitate extraction of polyphenols from finished malt. Interestingly, total polyphenol content correlated positively with hexanal (r=0.87) and *trans*-2-nonenal (r=0.73), which seems contradictory to findings reported by Guido *et al.*[29] who suggested an inhibitory action of malt polyphenols towards LOX-activity. In contrast, our results rather indicate prooxidative activity of polyphenols under the conditions studied. Moreover, total polyphenol content was also found to be strongly correlated with furfural (r=0.77) and moderately correlated with Strecker aldehydes (r=0.64 - 0.67). Thus, in general, the polyphenol content of our finished, experimental malts correlate quite well with levels of aldehydes. However, whether there is a true 'cause-effect' relationship between increased levels in polyphenols and aldehydes during malting, requires further investigation.

Finally, regarding potential relationships among aldehydes and other analytical parameters of malt, 3-methylbutanal and hexanal were selected as representative staling aldehydes, since 3-methylbutanal is always present in the highest concentration and the behaviour of hexanal is clearly different from the other aldehydes. Figure 6-1 demonstrates that 3-methylbutanal is correlated with malt quality parameters affected mainly by the kilning-off temperature, such as TBI (r=0.92) and colour (r=0.81), whereas moderate relationships (r=0.60 - 0.65) are found with indicators of proteolytic modification (TSP, KI, and FAN). Regarding hexanal, strong correlations (r= +/-0.70 up to +/-0.88) are noticed with indicators of grain modification (*i.e.* friability, β-glucan content, TSP, KI, FAN), as well as with TBI and colour. The observed relationships among staling aldehydes and malt quality parameters in this micro-malting study are in conformity with our previous findings obtained on industrial-scale samples (see chapters 3 - 5).

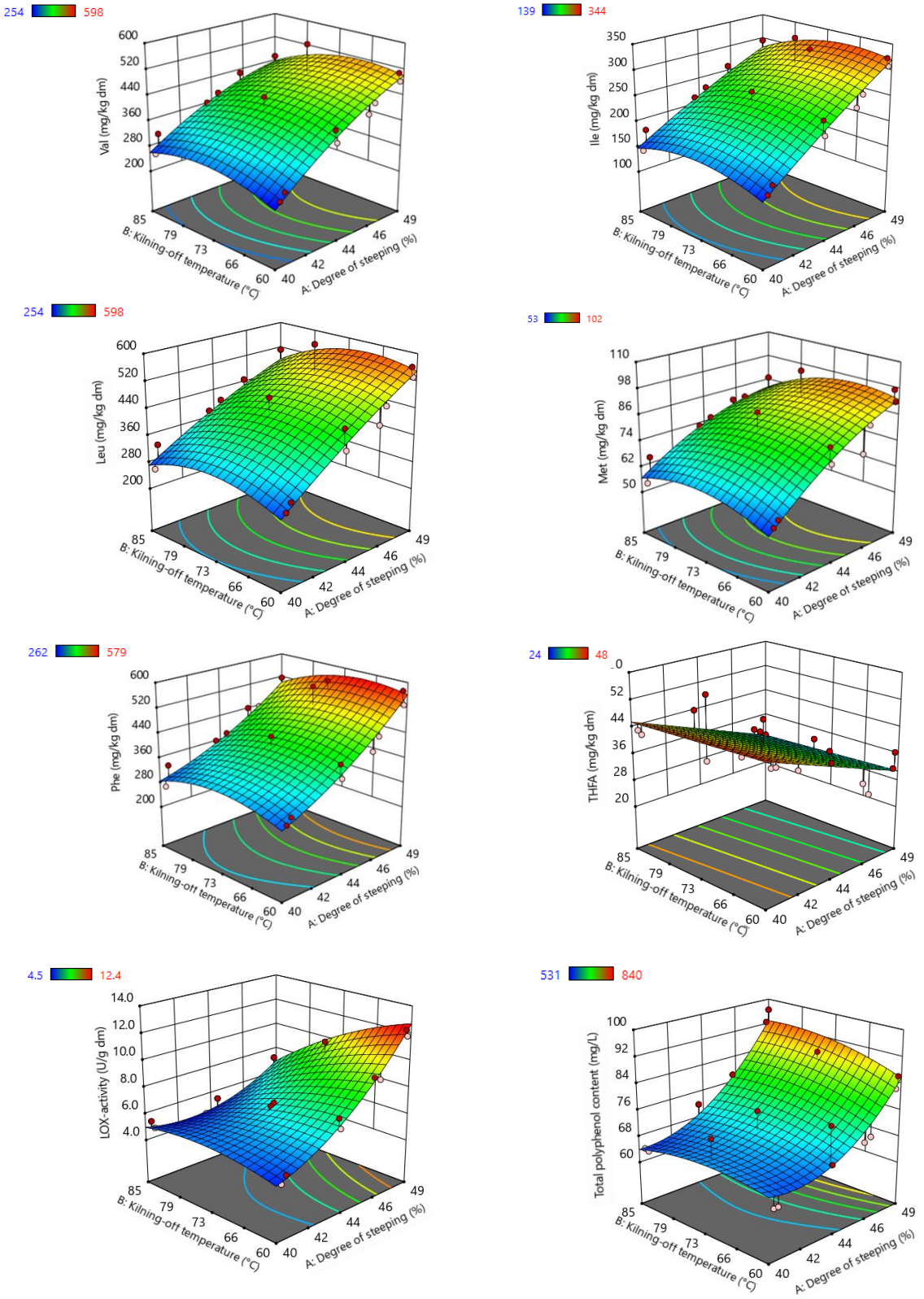


Figure 6-3. 3D plots modelling the influence of the degree of steeping (ranging from 40% to 48%) and kilning-off temperature (ranging from 60°C to 85°C) on levels of compounds related to *de novo* formation of aldehydes.

Red points = design points above predicted value, pink points = design points below predicted value. Compounds: Val = valine (potential precursor of 2-methylpropanal), Iso = isoleucine (potential precursor of 2-methylbutanal), Leu = leucine (potential precursor of 3-methylbutanal), Met = methionine (potential precursor of methional), PHE = phenylalanine (potential precursor of phenylacetaldehyde), THFA = trihydroxy fatty acids, LOX-activity = lipoxygenase activity.

6.3.2. Theoretical optimisation of malting conditions towards malt of reduced beer staling potential and high brewing quality

The obtained predictive models for individual responses as a function of varying degrees of steeping and kilning-off temperatures (see Table 6-3), are used in this section for theoretical, numerical optimisation of malting conditions towards the challenge of production of malt of reduced beer staling potential, whilst at least preserving (if not improving) its high brewing quality. Results of this optimisation study are presented in Table 6-4. Clearly, numerical values obtained are valid solely for this particular case study as optimisation is based on only one specific barley variety and one barley batch, micro-malted under the selected, experimental conditions.

Optimisation trails were performed by modification of the degree of steeping and kilning-off temperature, independently from each other. Two series of theoretical outputs were obtained by varying the degree of steeping and simultaneously maintaining kilning-off temperature constant (outputs 1-3), and vice versa (outputs 4-6).

In the first series of trials, software was instructed to set the degree of steeping to 45%, 46%, or 47%, and kilning-off temperature to 75°C (to facilitate SMM decomposition into volatile DMS). Generally, compared to the industrial-scale malts A-P analysed in this PhD thesis, relatively low (5,719 µg/kg dm) up to moderate (8,358 µg/kg dm) total levels of free aldehydes were predicted in the outputs 1-3. Generated results indicate that a decrease in the degree of steeping would lead to a decline in levels of staling aldehydes. Regarding standard quality parameters of malt, a lower steeping degree resulted in clearly lower grain modification and colour. In particular, output 1 (degree of steeping 45%) as well as output 2 (degree of steeping 46%) would give rise to malts of relatively low beer staling potential, however, it would also lead to insufficient cytolytic modification as apparent from low values for friability and high values for β-glucan content. The observations made on the first series of outputs 1-3 suggest that reduction of levels of aldehydes in malt is possible via application of a lower degree of steeping, however, sufficient attention remains to be paid to grain modification in order to assure high brewing quality of the malt.

In the second series of the optimisation trials, the degree of steeping was set to 47% as it resulted in satisfying modification in this study, while the kilning-off temperature was varied from 60°C up to 80°C. In the generated outputs 4-6, the predicted beer staling potential of malt can be regarded as relatively low (4,235 µg/kg dm) up to relatively high (10,555 µg/kg dm). Results indicate that a decrease in kilning-off temperature would lead to a decline in levels of staling aldehydes. Interestingly, levels of hexanal remain comparable, regardless of the applied kilning-off temperature. In particular outputs 4 and 5 look promising when aiming at malt with reduced beer staling potential. Especially output 4 delivered relatively low levels in staling aldehydes, whereas the KI representing proteolysis and, thus, formation of amino acids is similar (47%), compared to outputs 5 and 6 of this series. Furthermore, predicted standard quality parameters of outputs 4-6 largely meet the recommended values for high-quality pale lager malt. Only levels of β-glucan content can

be regarded as relatively high (approx. 220 - 260 mg/L), as well as SMM content[II]. However, kilning-off at lower temperatures may lead to a significantly higher diastatic power (including higher α-amylase activity), which should be considered as a clear improvement in overall malt quality. In summary, results obtained for outputs 4-6, suggest that levels of staling aldehydes may be reduced by applying lower kilning-off temperatures, whilst preserving acceptable overall malt quality and increasing its amylolytic potential.

Finally, aiming at reduced levels of aldehydes and high brewing quality of malt, output 7 was generated by applying a degree of steeping of 47% and kilning-off at 62°C (see Table 6-4). The predicted beer staling potential of this hypothetical malt can be estimated as relatively low (4,571 µg/kg dm), grain modification as quite sufficient (acceptable friability, β-glucan content, FAN, KI), and amylolytic activity as high (high diastatic power and α-amylase activity). Predictions of output 7 confirm our observations made in the first series (outputs 1-3). Furthermore, the theoretical example of output 7, together with outputs 4 and 5, demonstrates the feasibility of producing pale lager malt of reduced beer staling potential and improved overall quality of malt, by combining sufficient grain modification during germination with less heat load at kilning.

[II] Regarding somewhat higher values of β-glucan, compensations can be carried out in the maltings and/or the brewery. Regarding malting, β-glucan content may be reduced by selection of a barley variety with a lower initial β-glucan level[270], by addition of gibberellic acid during germination[128], or by microbial community management (for instance, via inoculation of the steeping water with *Lactobacillus plantarum* or *Pediococcus pentosaceus*)[271–273]. On the brewhouse level, β-glucan content may be reduced by application of relatively heat-stable microbial β-glucanase during mashing[129]. Regarding high values of SMM in finished malt, reduction in its levels may be considered by application of, for instance, somewhat lower germination temperatures thereby suppressing growth of the embryo and concomitant SMM formation[261,264]. Likewise, in the brewery, novel wort production techniques - promoting DMS formation during mashing-off/mash separation and efficient removal of residual DMS at onset of boiling[256] or incorporation of a stripping column after wort boiling (https://www.krones.com/en/products/machines/wort-stripping.php) - allow efficient DMS removal from wort.

Table 6-4. Examples of outputs of theoretical optimisation of the malting process, showing prediction of malting conditions (degree of steeping, kilning-off temperature), levels of (cysteinylated) aldehydes, and values for standard quality parameters of malt.

Parameter	Output 1	Output 2	Output 3	Output 4	Output 5	Output 6	Output 7	Recommended values for high-quality pale lager malt
Degree of steeping (%)	45	46	47	47	47	47	47	-
Kilning-off temp. (°C)	75	75	75	60	70	80	62	-
Aldehydes (µg/kg dm)								
2MP	886	1,032	1,208	723	1,002	1,458	765	-
2MB	823	960	1,118	560	878	1,411	606	-
3MB	1,998	2,337	2,754	1,472	2,248	3,338	1,602	-
MET	209	267	331	83	204	503	93	-
PHE	893	1,156	1,473	467	986	2,111	522	-
FUR	267	375	510	182	370	680	210	-
HEX	277	350	446	434	454	427	442	-
T2N	457	492	528	364	473	582	386	-
Sum of free aldehydes	**5,719**	**6,910**	**8,358**	**4,235**	**6,572**	**10,555**	**4,571**	-
Sum of CYS -aldehydes	349	436	535	159	331	817	168	-
Quality parameters								
Moisture (%)	4.2	4.2	4.2	4.9	4.4	4.0	4.8	3-5 [1,2,4,6,7]
Colour (EBC)	2.2	2.4	2.8	2.5	2.6	3.1	2.5	2-4.5 [1,2,4,5,6,7]
TBI (for 10 g of dm)	7.7	9.4	11.4	6.8	9.7	13.3	7.3	-
Friability (%)	72	77	83	83	83	83	83	> 80 [1,4,5,6,7] > 85 [3]
Homogeneity (%)	92	96	98	98	98	98	99	> 95 [4,5]
PUG (%)	8	4	2	2	2	2	1	< 5 [4,5]
β-Glucan content (mg/L)	458	354	231	262	226	251	250	< 250 [1,2]
Viscosity (mPas)	1.65	1.58	1.54	1.57	1.53	1.56	1.56	< 1.6 [1,2,4,5,7]
TP (% dm)	10.2	10.1	10.1	10.1	10.1	10.0	10.1	9.5-11.0 [3,4,7] < 10 [2,5]
TSP (mg/L)	4.3	4.5	4.7	4.7	4.7	4.8	4.7	3.8-4.8 [2,3,6,7] 3.7-4.4 [5]
KI (%)	42	45	47	47	47	47	46	35-41 [2] 38-44 [3,5] 37-45 [1,4,6,7]
FAN (mg/L)	160	177	196	189	195	194	191	160-250 [1,2,3,4,5,6]
Diastatic power (WK)	449	470	492	558	514	470	549	>220 [2,3,4,5,6,7]
α-Amylase activity (DU)	58	62	65	68	66	64	68	>30 [1,4] >40 [3,5]
SMM (mg/kg dm)	9.2	9.6	9.6	15.1	11.5	7.6	14.4	2-8 [2]
Malting yield (%)	90	88	87	89	88	87	89	>80 [1]

Results of theoretical optimisation are based on models presented in Table 6-3. Compounds: 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, T2N = *trans*-2-nonenal, CYS-aldehydes = cysteinylated aldehydes, TBI = thiobarbituric acid index, PUG = partly unmodified grains, TP = total protein, TSP = total soluble protein, KI = Kolbach Index, FAN = free amino nitrogen, SMM = S-methylmethionine. Sources from literature and/or maltings: 1 – Briggs *et al.*[247]; 2 Kunze[129]; 3 – Eβlinger[230]; 4 – O'Rourke[228]; 5 – Davies[184]; 6 – corporative webpage Dingemans NV; 7 – corporative webpage Castle malting.

6.4. Conclusions

This study on micro-malting of barley aimed at better understanding of the influence of grain modification and kilning-off temperature on the formation of staling aldehydes. Based on a central composite design using two factors (grain modification, kilning-off temperature), 22 different malts were prepared, following 12 different malting protocols. Finished malts were evaluated by measuring a multitude of responses related to standard quality parameters of malt, levels of (cysteinylated) aldehydes, and levels of potential precursors of aldehydes (in total, 43 different responses were measured). Next, experimental data was used for theoretical modelling of malting conditions towards producing pale lager malt of reduced beer staling potential, whilst at least preserving - if not improving - overall malt brewing quality.

Modelling of the different responses as a function of the two selected malting variables, indicated that more extensive grain modification results in higher cytolytic and proteolytic modification, as well as increased diastatic power, including α-amylase activity. Application of higher kilning-off temperatures, and thus increased heat load gives rise to more colour, increased TBI values, and increased levels of (cysteinylated) aldehydes. Moreover, it was shown that both investigated key factors of malt production significantly affect formation of aldehydes. Thus, a decrease in the degree of steeping and/or kilning-off temperature led to a decrease in levels of free and cysteinylated aldehydes, and vice versa. The above findings demonstrate the impact of the degree of steeping and, even more, the impact of kilning-off temperature on final malt quality.

Regarding possible relationships between aldehydes and available malt quality parameters, it was demonstrated that higher levels of aldehydes coincide with malts showing higher thiobarbituric acid index (TBI), colour, TSP, KI, and FAN. These particular correlations derived from micro-malting are in full conformity with our previous findings obtained on industrial-scale samples (see chapters 3 - 5). Moreover, it was observed that cysteinylated aldehydes represent approx. 10% of the levels of free aldehydes.

Theoretical optimisation of the malting protocol demonstrated that it appears to be feasible to produce pale lager malt of improved quality (*i.e.* malt showing strongly reduced beer staling potential and high brewing quality), by combining sufficient grain modification during germination with less heat load at kilning. Nevertheless, to verify the applicability of our findings obtained in this case study, further micro-malting investigations involving various barley varieties, barley batches, *etc.,* should be carried out, as well as several trials on pilot-scale.

6.5. Related documents

Appendix F contains an overview of raw data on standard quality parameters, levels of (cysteinylated) aldehydes, and compounds related to *de novo* formation of aldehydes.

Appendix G contains a visual presentation of 41 models obtained by plotting individual responses (malt quality parameters) as a function of variable A (degree of steeping) and variable B (kilning-off temperature).

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The literature study presented in chapter 1 and published by Filipowska *et al*.[233] indicates the importance of malt quality in relation to beer flavour (in)stability. Interestingly, it has been reported that the same volatile compounds that are directly involved in beer flavour deterioration, *i.e*. staling aldehydes, also develop during malting. In particular germination and kilning appear to be involved in the generation of staling aldehydes and their precursors, thereby contributing to the beer staling potential of malt. Nevertheless, little is known on the process parameters enhancing development of staling aldehydes during malting, as well as on the formation of bound-state precursors of aldehydes in the complex matrix of malt. Therefore, for this technology-driven PhD, it was anticipated that investigation of the malt properties and the malting process itself, may lead to better understanding of the formation of the staling potential of malt and possibly also of the beer ageing phenomenon.

Aiming at successful assessment of the pale lager malt production process, the study conducted in chapter 2 focused on optimisation of sample preparation for HS-SPME-GC-MS determination of staling aldehydes in various types of malting samples (*i.e.* green malt, partially kilned malt, finished pale lager malt). Further, quantification of both free and cysteinylated aldehydes in industrial-scale pale malt production samples allowed obtaining an integrated view on the evolution of volatile, free and non-volatile, bound-state aldehydes throughout malting (chapters 3 and 4). Clearly, the total content of (cysteinylated) aldehydes was higher in finished malt compared to its starting material, barley. Moreover, evaluation of three independently conducted pale lager malt productions (batches G, K, and L) as a function of process duration pointed to germination and kilning as the most critical stages of malting in relation to aldehyde formation. More specifically, the initial stage of germination implies enzymatic fatty acid oxidation as reflected by the formation of hexanal and *trans*-2-nonenal, whereas the stage of drying at elevated temperature (in particular when arriving at a critical moisture content of approx. 6% - 9%) result in intensified generation of (cysteinylated) Strecker aldehydes and furfural. Moreover, a rapid increase in levels of (cysteinylated) Strecker aldehydes, furfural, and *trans*-2-nonenal continued through kilning-off.

Evaluation of the kilning process as a function of the position of the grains in the bed (bottom, middle, top layer), unravelled a clear effect of process-associated physiochemical gradients (caused by pneumatic processing in relatively thick grain beds) on the formation of aldehydes (chapter 4). Except for hexanal, the highest levels of free and bound-state aldehydes were found in samples derived from the bottom layer of the grain bed (exposed to the highest heat load). Conversely, samples taken at the same time from the upper layer (exposed to significantly less heat load) showed the lowest levels of aldehydes. It can therefore be concluded from chapters 3 and 4 that, in particular, exposure to heat load (depending on the stage of malting and on the position of the grain in the bed) plays a critical role in the development of (cysteinylated) aldehydes during malt production.

Next, the analysis of malting process variables on levels of staling aldehydes in finished malts (chapter 5), showed that the degree of grain modification and kilning-off temperature impact (directly or indirectly) generation of (cysteinylated) aldehydes during malting.

In summary, based on the experiments performed on industrial-scale samples, it can be concluded that the degree of grain modification and applied heat load are key factors regarding generation of aldehydes and, therefore, the staling potential of pale lager malt.

The final experimental part on micro-malting and modelling (chapter 6), resulted in a more detailed evaluation of the effect of varying degrees of grain modification and kilning-off temperatures on levels of (cysteinylated) aldehydes and other malt quality parameters. It was concluded that grain modification impacts in particular levels of hexanal, whereas kilning-off temperature mainly affects levels of (cysteinylated) Strecker aldehydes, furfural, and *trans*-2-nonenal. Theoretical modelling of malting conditions towards malt of reduced levels of aldehydes and improved brewing quality led to the conclusion that targeted adjustment of both grain modification and kilning-off temperature may result in malt of superior quality (*i.e.* reduced levels of staling aldehydes and high brewing quality). It is proposed that, upon further research, these observations may find future applications in the malting and brewing industry.

Finally, from the analytical point of view, it was observed throughout this doctoral study that a particular set of malt quality parameters, *i.e.* thiobarbituric acid index, colour, total soluble protein, Kolbach Index, and free amino nitrogen is associated with the development of aldehydes, especially Strecker degradation aldehydes. Therefore, it is proposed that these analytical parameters, commonly available for maltsters and brewers, may assist in estimating the staling potential of pale lager malt.

Regarding perspectives for future work, the insights obtained in this PhD study give rise to a range of several new research questions, both applied and more fundamental.

From the more fundamental point of view, staling aldehydes present in malt are unlikely to be a direct cause of beer flavour deterioration during storage. Therefore, it is recommended to investigate other compounds that are delivered with malt to the brewing process. For example, reactants involved in Strecker degradation (*e.g.* α-dicarbonyls), intermediates of Maillard reactions (*e.g.* 3-deoxysone derived from pentose), and intermediates of oxidation of unsaturated fatty acids (*e.g.* trihydroxy fatty acids), which may contribute to *de novo* formation of aldehydes in a beer package during storage. In the same line of thoughts, it is recommended to further investigate possible binding of aldehydes during malting and brewing to molecules different from L-cysteine (*e.g.* peptides, polyphenols).

A further research idea is in relation to the differential behaviour (as observed throughout this PhD) between the fatty acid oxidation aldehydes hexanal and *trans*-2-nonenal. It seems that during malt production, *trans*-2-nonenal would be predominantly formed via autoxidation, whereas hexanal would be mainly developed via enzymatic oxidation. To clarify the relative importance of enzymatic fatty acid oxidation vs. autoxidation in malting and brewing, more fundamental research is required.

Another research suggestion is mainly based on observations made in chapter 6, where it appeared that sufficient cytolytic modification to assure untroubled processing in the brewhouse may lead to excess in staling aldehydes. On the other hand, it was shown that levels of aldehydes can be minimised by reducing proteolytic modification, which largely parallels cytolysis. Therefore, it would be interesting to further unravel the barley grain physiology to better understand as to what extent the expression of enzymes contributing

to proteolytic and cytolytic modification is (more or less) connected or controlled independently. Such fundamental knowledge could contribute to the objective of producing pale lager malt of minimised staling potential (by selectively limiting proteolysis) and excellent brewing quality (via controlled, but still sufficient cytolysis).

From the technological perspective, key factors of malt production in relation to aldehyde formation, as observed in this PhD, *i.e.* applied heat load, moisture content during green malt drying, and process-associated gradients, require more research attention.
An increase in levels of aldehydes during malting was found to be strongly related to the exposure of the grain to heat load. Therefore, it is suggested to assess the application of lower temperatures, in particular at kilning-off. Presumably, this may result in improved overall quality of malt (*i.e.* higher enzymatic potential and lower levels of staling aldehydes), as well as environmental and economic benefits, due to reduced energy consumption. One step further – brewing with green (unkilned) malt – would allow minimising applied heat load by omitting kilning. A recent PhD study performed jointly at the University of Nottingham and KU Leuven demonstrated that levels of staling aldehydes in aged green malt beers were lower than in their kilned malt counterparts[269]. Consequently, more applied research into the direction of reduced heat load in malting is highly recommended. Within the same train of thoughts, as demonstrated in chapter 4, it appears feasible to pinpoint a critical point, *i.e.* a particular moment and grain moisture content during kilning, where the formation of staling aldehydes starts to increase strongly. Therefore, it is suggested to conduct further research on shorter kilning schedules, *i.e.* drying until the 'critical point', and to assess the potential value of such type of experimental malts, regarding both malting and brewing quality.
Most challenging is to find a feasible solution allowing to limit physicochemical gradients as these are inherent to pneumatic processing in relatively thick grain beds, in particular when turning during kilning is omitted, and when dry, warm air is supplied from the floor level of the kiln. Alternative approaches and techniques of water removal should be considered and revised with regard to their applicability on an industrial scale and impact on overall malt quality, including levels of staling aldehydes.

Finally, since micro-malting experiments performed in chapter 6 started from a single batch of one particular barley variety, in future research, the use of more barley varieties, different batches, *etc.,* is highly recommended, in order to find out whether predictions in the presented case study would still apply to other starting materials for malting. Furthermore, the applicability of the result should be validated by further testing on pilot-scale, where malting gradients, as well as environmental and economic aspects, can be evaluated. Also brewing trails should be involved to assess the reduced staling potential of innovative, pilot-scale malts. If outcomes on pilot-scale would be promising, implementation on the industrial-scale may be considered.

REFERENCES

1. De Cooman L, Aerts G, Overmeire H, & De Keukeleire D (2000) Alterations of the profiles of iso-α-acids during beer ageing, marked instability of trans-iso-α-acids and implications for beer bitterness consistency in relation to tetrahydroiso-α-acids *J Inst Brew* 106(3) 169–178, doi:10.1002/j.2050-0416.2000.tb00054.x.
2. Malfliet S, Van Opstaele F, De Clippeleer J, Syryn E, Goiris K, De Cooman L, & Aerts G (2008) Flavour instability of pale lager beers: Determination of analytical markers in relation to sensory ageing *J Inst Brew* 114(2) 180–192, doi:10.1002/j.2050-0416.2008.tb00324.x.
3. Belgian Brewers (2019) European beer trends - Annual Report.
4. Paternoster A, Jaskula-Goiris B, De Causmaecker B, Vanlanduit S, Springael J, Braet J, De Rouck G, & De Cooman L (2019) The interaction effect between vibrations and temperature simulating truck transport on the flavor stability of beer *J Sci Food Agric* 99(5) 2165–2174, doi:10.1002/jsfa.9409.
5. Hashimoto N (1966) Report of the Research Laboratories of Kirin Brewery Co.
6. Jaskula-Goiris B, De Causmaecker B, De Rouck G, De Cooman L, & Aerts G (2011) Detailed multivariate modelling of beer staling in commercial pale lagers *Brew Sci* 64(11–12) 119–139.
7. Ditrych M, Filipowska W, De Rouck G, Jaskula-Goiris B, Aerts G, Andersen ML, & De Cooman L (2019) Investigating the evolution of free staling aldehydes throughout the wort production process *Brew Sci* 72 10–17, doi:10.23763/BrSc18-21ditrych.
8. Bustillo Trueba P, Jaskula-Goiris B, Ditrych M, Filipowska W, De Brabanter J, De Rouck G, Aerts G, De Cooman L, & De Clippeleer J (2021) Monitoring the evolution of free and cysteinylated aldehydes from malt to fresh and forced aged beer *Food Res Int* 140 110049, doi:10.1016/j.foodres.2020.110049.
9. Saison D, Vanbeneden N, De Schutter DP, Daenen L, Mertens T, Delvaux F, & Delvaux FR (2010) Characterisation of the flavour and the chemical composition of lager beer after ageing in varying conditions *Brew Sci* 63(3–4) 41–53.
10. Vanderhaegen B, Neven H, Verachtert H, & Derdelinckx G (2006) The chemistry of beer aging - A critical review *Food Chem* 95 357–381, doi:10.1016/j.foodchem.2005.01.006.
11. Paternoster A, Jaskula-Goiris B, Buyse J, Perkisas T, Springael J, Braet J, De Rouck G, & De Cooman L (2020) The relationship between flavour instability, preference and drinkability of fresh and aged beer *J Inst Brew* 126(1) 59–66, doi:10.1002/jib.582.
12. Dalgliesh CE (1977) Flavour stability. *Proceedings 16th European Brewery Convention.* Amsterdam, p.623−659.
13. Zufall C, Racioppi G, Gasparri M, & Franquiz J (2005) Flavour stability and ageing characteristics of light-stable beers. *Proceedings 30th European Brewery Convention*. Prague, p.1–8.
14. Vanderhaegen B, Delvaux F, Daenen L, Verachtert H, & Delvaux FR (2007) Aging characteristics of different beer types *Food Chem* 103 404–412, doi:10.1016/j.foodchem.2006.07.062.
15. Vanderhaegen B, Neven H, Coghe S, Verstrepen KJ, Verachtert H, & Derdelinckx G (2003) Evolution of chemical and sensory properties during aging of top-fermented beer *J Agric Food Chem* 51(23) 6782–6790, doi:10.1021/jf034631z.
16. Saison D, De Schutter DP, Uyttenhove B, Delvaux F, & Delvaux FR (2009) Contribution of staling compounds to the aged flavour of lager beer by studying their flavour thresholds *Food Chem* 114(4) 1206–1215, doi:10.1016/j.foodchem.2008.10.078.
17. Baert JJ, De Clippeleer J, Hughes PS, De Cooman L, & Aerts G (2012) On the origin of free and bound staling aldehydes in beer *J Agric Food Chem* 60(46) 11449–11472,

doi:10.1021/jf303670z.

18. Herrmann M, Klotzbucher B, Wurzbacher M, Hanke S, Kattein U, Back W, Becker T, & Krottenthaler M (2010) A new validation of relevant substances for the evaluation of beer aging depending on the employed boiling system *J Inst Brew* 116(1) 41–48, doi:10.1002/j.2050-0416.2010.tb00396.x.
19. Evans DJ, Schmedding DJM, Bruijnje A, Heideman T, King BM, & Groesbeek NM (1999) Flavour impact of aged beers *J Inst Brew* 105(5) 301–307, doi:10.1002/j.2050-0416.1999.tb00524.x.
20. Caballero I, Blanco CA, & Porras M (2012) Iso-α-acids, bitterness and loss of beer quality during storage *Trends Food Sci Technol* 26(1) 21–30, doi:10.1016/j.tifs.2012.01.001.
21. Bamforth CW & Letini A (2011) The flavour instability of beer, p. 94-109. In *Beer: A quality perspective. Handbook of alcoholic beverages.* Academic Press. doi:10.1016/B978-0-12-669201-3.00003-8.
22. Jaskula-Goiris B, De Causmaecker B, De Rouck G, Aerts G, Paternoster A, Braet J, & De Cooman L (2019) Influence of transport and storage conditions on beer quality and flavour stability *J Inst Brew* 125(1) 60–68, doi:10.1002/jib.535.
23. Gagula G, Mastanjević K, Mastanjević K, Krstanović V, Horvat D, & Magdić D (2020) The influence of packaging material on volatile compounds of pale lager beer *Food Packag Shelf Life* 24 100496, doi:10.1016/j.fpsl.2020.100496.
24. Paternoster A, Jaskula-Goiris B, Perkisas T, Springael J, De Rouck G, De Cooman L, & Braet J (2019) A model to simulate the overall ageing score impact of temperature and time on the sensorial quality of lager *J Inst Brew* 125(3) 364–373, doi:10.1002/jib.566.
25. Bamforth CW (2000) Making Sense of flavor change in beer *Tech Q Master Brew Assoc Am* 37(2) 165–171.
26. Gastl M, Spieleder E, Hermann M, Thiele F, Burberg F, Kogin A, Ikeda H, Back W, & Narziss L (2006) The influence of malt quality and malting technology on the flavour stability of beer. *Brew Sci* 163–175.
27. Furukawa Suárez A, Kunz T, Cortés Rodríguez N, MacKinlay J, Hughes P, & Methner FJ (2011) Impact of colour adjustment on flavour stability of pale lager beers with a range of distinct colouring agents *Food Chem* 125(3) 850–859, doi:10.1016/j.foodchem.2010.08.070.
28. Mikyška A, Hrabák M, Hašková D, & Šrogl J (2002) The role of malt and hop polyphenols in beer quality, flavour and haze stability *J Inst Brew* 108(1) 78–85, doi:10.1002/j.2050-0416.2002.tb00128.x.
29. Guido LF, Curto AF, Boivin P, Benismail N, Gonçalves CR, & Barros AA (2007) Correlation of malt quality parameters and beer flavor stability: Multivariate analysis *J Agric Food Chem* 55(3) 728–733, doi:10.1021/jf0623079.
30. Bettenhausen HM, Barr L, Broeckling CD, Chaparro JM, Holbrook C, Sedin D, & Heuberger AL (2018) Influence of malt source on beer chemistry, flavor, and flavor stability *Food Res Int* 113 487–504, doi:10.1016/j.foodres.2018.07.024.
31. Olaniran AO, Hiralal L, Mokoena MP, & Pillay B (2017) Flavour-active volatile compounds in beer: production, regulation and control *J Inst Brew* 123(1) 13–23, doi:10.1002/jib.389.
32. De Clippeleer J, De Rouck G, De Cooman L, & Aerts G (2010) Influence of the hopping technology on the storage-induced appearance of staling aldehydes in beer. *J Inst Brew* 116(4) 381–398.
33. Bustillo Trueba P, Jaskula-Goiris B, Sanders M, Vincken J-P, De Clippeleer J, Van der Eycken E, De Brabanter J, De Rouck G, Aerts G, & De Cooman L (2019) First time quantification of cysteinylated aldehydes in malt and brewery samples *Proceedings 37th European Brewery Convention*. Antwerp.

34. Palamand SR, & Hardwick WA (1968) Studies on the relative flavour importance on some beer constituents *Tech Q Master Brew Assoc Am* 6(2) 117–128.

35. Vesely P, Lusk L, Basarova G, Seabrooks J, & Ryder D (2003) Analysis of aldehydes in beer using solid-phase microextraction with on-fiber derivatization and gas chromatography/mass spectrometry *J Agric Food Chem* 51(24) 6941–6944, doi:10.1021/jf034410t.

36. Hashimoto N, & Kuroiwa Y (1975) Proposed pathways for the formation of volatile aldehydes during storage of bottled beer. A*nnual meeting of American Society of Brewing Chemists* p. 104–111, doi:10.1080/00960845.1975.12007147.

37. Lehnhardt F, Gastl M, & Becker T (2018) Forced into aging: Analytical prediction of the flavor-stability of lager beer. A review *Crit Rev Food Sci Nutr* 2642–2653, doi:10.1080/10408398.2018.1462761.

38. Lehnhardt F, Steiner J, Gastl M, & Becker T (2018) Prediction power and accuracy of forced ageing – Matching sensory and analytical results for lager beer *Brew Sci* 71(5–6) 39–48, doi:10.23763/BrSc18-05lenhardt.

39. Lehnhardt F, Becker T, & Gastl M (2020) Flavor stability assessment of lager beer: what we can learn by comparing established methods *Eur Food Res Technol* 246(5) 1105–1118, doi:10.1007/s00217-020-03477-0.

40. Barker RL, Gracey DEF, Irwin AJ, Pipasts P, & Leiska E (1983) Liberation of staling aldehydes during beer storage *J Inst Brew* 89 411–415.

41. Kaneda H, Osawa T, Kawakishi S, Munekata M, & Koshino S (1994) Contribution of carbonyl-bisulfite adducts to beer stability *J Agric Food Chem* 42(11) 2428–2432, doi:10.1021/jf00047a012.

42. Lermusieau G, Noël S, Liégeois C, & Collin S (1999) Nonoxidative mechanism for development of trans-2-nonenal in beer *J Am Soc Brew Chem* 57(1) 29–33, doi:10.1094/ASBCJ-57-0029.

43. Bamforth CW (2004) A critical control point analysis for flavor stability of beer *Tech Q Master Brew Assoc Am* 41(2) 97–103.

44. Suda T, Yasuda Y, Imai T, & Ogawa. Y (2007) Mechanisms for the development of Strecker aldehydes during beer aging *Proceedings 31th European Brewery Convention.* Venice p.1–7.

45. Aron P, & Shellhammer T (2010) A discussion of polyphenols in beer physical and flavour stability *J Inst Brew* 116(4) 369–380, doi:10.1002/j.2050-0416.2010.tb00788.x.

46. Rakete S, Klaus A, & Glomb MA (2014) Investigations on the Maillard reaction of dextrins during aging of pilsner type beer *J Agric Food Chem* 62(40) 9876–9884, doi:10.1021/jf503038c.

47. Wietstock PC, Kunz T, & Methner FJ (2016) Relevance of oxygen for the formation of Strecker aldehydes during beer production and storage *J Agric Food Chem* 64(42) 8035–8044, doi:10.1021/acs.jafc.6b03502.

48. Bruice P (2014) *Organic Chemistry* 7th edition Pearson Education Limited, New York City. ISBN 97812-9216-0344

49. Smit BA, Engels WJM, & Smit G (2009) Branched chain aldehydes: production and breakdown pathways and relevance for flavour in foods *Appl Microbiol Biotechnol* 81(6) 987–999, doi:10.1007/s00253-008-1758-x.

50. Baert JJ, De Clippeleer J, De Cooman L, & Aerts G (2015) Exploring the binding behaviour of beer staling aldehydes in model systems *J Am Soc Brew Chem* 73(1) 100–108, doi:10.1094/asbcj-2015-0109-01.

51. Baert JJ, De Clippeleer J, Jaskula-Goiris B, Van Opstaele F, De Rouck G, & Aerts G (2015) Further elucidation on beer flavor instability: the potential role of cysteine-bound aldehydes *J Am Soc Brew Chem* 73(3) 243–252, doi:10.1094/asbcj-2015-0531-01.

52. Liégeois C, Meurens N, Badot C, & Collin S (2002) Release of deuterated (E)-2-nonenal during beer aging from labelled precursors synthesized before boiling *J Agric Food Chem* 50(26) 7634–7638, doi:10.1021/jf020617v.

53. Solomons TW, & Fryhle CB (2006) *Organic chemistry* 8th edition John Wiley and Sons Inc., Hoboken, NJ, USA. ISBN 9780470524596.

54. Bustillo Trueba P, De Clippeleer J, Van der Eycken E, Guevara Romero JS, De Rouck G, Aerts G, & De Cooman L (2018) Influence of pH on the stability of 2-substituted 1,3-thiazolidine-4-carboxylic acids in model solutions *J Am Soc Brew Chem* 76(4) 272–280, doi:10.1080/03610470.2018.1546094.

55. Drost BW, Van den Berg R, Freijee FJM, Van der Velde EG, & Hollemans M (1990) Flavor stability *J Am Soc Brew Chem* 48(4) 124–131, doi:10.1094/ASBCJ-48-0124.

56. Baert JJ (2015) Unravelling the role of free and bound-state aldehydes in beer flavour instability. *Doctoral dissertation.*

57. Gibson B, Aumala V, Heiniö RL, Mikkelson A, & Honkapää K (2018) Differential evolution of Strecker and non-Strecker aldehydes during aging of pale and dark beers *J Cereal Sci* 83 130–138, doi:10.1016/j.jcs.2018.08.009.

58. Rangel-Aldao R, Bravo A, Galindo-Castro I, Sanchez B, Reverol L, Scherer E, Madrid J, Ramirez JL, Herrera J, Penttila M, Vehkomaki M-L, Vidgren V, Virtanen H, & Home S (2001) Beer flavor stabilization through the control of Maillard reaction intermediates *EBC Symosium'Flavour Flavour Stability'.* Nancy. p.1-9.

59. Guido LF, Boivin P, Benismail N, Gonçalves CR, & Barros AA (2005) An early development of the nonenal potential in the malting process *Eur Food Res Technol* 220(2) 200–206, doi:10.1007/s00217-004-1079-y.

60. Liégeois C, & Collin S (2003) Contribution of malt kilning to the cardboard flavour of aged beers. *Proceedings 29th European Brewery Convention.* Dublin, p.1–9.

61. Dufour JP, Leus M, Baxter AJ, & Hayman AR (1999) Characterization of the reaction of bisulfite with unsaturated aldehydes in a beer model system using nuclear magnetic resonance spectroscopy *J Am Soc Brew Chem* 57(4) 138–144.

62. Garbe LA, Hübke H, & Tressl R (2003) Oxygenated fatty acids and flavour stability - new insights *Proceedings 29th European Brewery Convention.* Dublin, p.1–9.

63. Baxter ED (1984) Recognition of two lipases from barley and green malt *J Inst Brew* 90 277–281, doi:10.1002/j.2050-0416.1984.tb04273.x.

64. Baxter ED (1982) Lipoxidases in malting and mashing *J Inst Brew* 88(6) 390–396, doi:10.1002/j.2050-0416.1982.tb04130.x.

65. Yang G, & Schwarz PB (1995) Activity of lipoxygenase isoenzymes during malting and mashing *J Am Soc Brew Chem* 53(4) 45–49.

66. Belitz H., Grosch W, & Schieberle P (2009) *Food chemistry* , 4th edition Springer-Verlag, Berlin doi:10.1007/978-3-540-69934-7.ISBN 978-3-540-69934-7.

67. Wietstock PC, Kunz T, & Methner FJ (2015) Uptake and release of Ca, Cu, Fe, Mg, and Zn during beer production *J Am Soc Brew Chem* 73(2) 179–184, doi:10.1094/asbcj-2015-0402-01.

68. Hashimoto N, & Eshima T (1977) Composition and pathway of formation of stale aldehydes in bottled beer. *J Am Soc Brew Chem* 35(3) 145–150.

69. Bamforth CW, & Cook DJ (2019) *Food, Fermentation and microorganisms*, 2nd Edition Hoboken, NJ, USA. ISBN: 978-1-405-19872-1.

70. Ferreira IM, & Guido LF (2018) Impact of wort amino acids on beer flavour: A review *Fermentation* 4(2) 23, doi:10.3390/fermentation4020023.

71. Van Boekel MA (2006) Formation of flavour compounds in the Maillard reaction *Biotechnol Adv* 24(2) 230–233, doi:10.1016/j.biotechadv.2005.11.004.

72. Ames JM (1998) Applications of the Maillard reaction in the food industry *Food Chem* 62(4) 431–439, doi:10.1016/S0308-8146(98)00078-8.

73. Laroque D, Inisan C, Berger C, Vouland É, Dufossé L, & Guérard F (2008) Kinetic study on the Maillard reaction. Consideration of sugar reactivity *Food Chem* 111(4) 1032–1042, doi:10.1016/j.foodchem.2008.05.033.

74. Van Boekel MA (2009) Kinetic aspects of the Maillard reaction: a critical review 45(3) 1–29, doi:10.1002/1521-3803(20010601)45:3<150::AID-FOOD150>3.0.CO;2-9.

75. Coghe S, Adriaenssens B, Leonard S, & Delvaux FR (2014) Fractionation of coloured Maillard reaction products from dark specialty malts *J Am Soc Brew Chem* 62(2) 79–86, doi:10.1094/ASBCJ-62-0079.

76. Yahya H, Linforth RST, & Cook DJ (2014) Flavour generation during commercial barley and malt roasting operations: A time course study *Food Chem* 145 378–387, doi:10.1016/j.foodchem.2013.08.046.

77. Channell G., Yahya H, & Cook DJ (2010) Thermal volatile generation in barley malt: On-line MS studies *J Am Soc Brew Chem* 68(4) 175–182, doi:10.1094/asbcj-2010-0715-01.

78. Nobis A, Röhrig A, Hellwig M, Henle T, Becker T, & Gastl M (2019) Formation of 3-deoxyglucosone in the malting process *Food Chem* 290 187–195, doi:10.1016/j.foodchem.2019.03.144.

79. Yaylayan VA (2007) Recent advances in the chemistry of Strecker degradation and Amadori rearrangement: implications to aroma and color formation *Food Sci Technol Res* 9(1) 1–6, doi:10.3136/fstr.9.1.

80. Rizzi GP (2008) The Strecker degradation of amino acids: Newer avenues for flavor formation *Food Rev Int* 24(4) 416–435, doi:10.1080/87559120802306058.

81. Bustillo Trueba P (2020) Beer flavour instability: unravelling formation and/or release of staling aldehydes. *Doctoral dissertation*.

82. Hofmann T, Münch P, & Schieberle P (2000) Quantitative model studies on the formation of aroma-active aldehydes and acids by Strecker-type reactions *J Agric Food Chem* 48(2) 434–440, doi:10.1021/jf990954c.

83. Wietstock PC, & Methner FJ (2013) Formation of aldehydes by direct oxidative degradation of amino acids via hydroxyl and ethoxy radical attack in buffered model solutions *Brew Sci* 66(7–8) 104–113.

84. Stadtman E (1993) Oxidation of free amino acids and amino acid residues in proteins by metal-catalyzed reactions *Annu Rev Biochem* 62 797–821.

85. Cremer DR, Vollenbroeker M, & Eichner K (2000) Investigation of the formation of Strecker aldehydes from the reaction of Amadori rearrangement products with amino acids in low moisture model systems *Eur Food Res Technol* 211(6) 400–403, doi:10.1007/s002170000196.

86. Zufall C, & Tyrell T (2008) The influence of heavy metal ions on beer flavour stability *J Inst Brew* 114(2) 134–142, doi:10.1002/j.2050-0416.2008.tb00318.x.

87. Carvalho DO, Gonçalves LM, & Guido LF (2016) Overall antioxidant properties of malt and how they are influenced by the individual constituents of barley and the malting process *Compr Rev Food Sci Food Saf* 15(5) 927–943, doi:10.1111/1541-4337.12218.

88. Ting PL, Lusk L, Refling J, Kay S, & Ryder D (2008) Identification of antiradical hop compounds *J Am Soc Brew Chem* 66(2) 116–126, doi:10.1094/ASBCJ-2008-0310-01.

89. Wietstock PC, & Shellhammer TH (2011) Chelating properties and hydroxyl-scavenging

activities of hop α- and iso-α-acids *J Am Soc Brew Chem* 69(3) 133–138, doi:10.1094/ASBCJ-2011-0718-01.

90. Bonoli M, Verardo V, Marconi E, & Caboni MF (2004) Antioxidant phenols in barley (Hordeum vulgare L.) flour: comparative spectrophotometric study among extraction methods of free and bound phenolic compounds *J Agric Food Chem Agric Food Chem* 52 5195–5200, doi:10.1021/jf040075c.
91. Özcan MM, Aljuhaimi F, & Uslu N (2018) Effect of malt process steps on bioactive properties and fatty acid composition of barley, green malt and malt grains *J Food Sci Technol* 55(1) 226–232, doi.:10.1007/s13197-017-2920-1.
92. Coghe S, Derdelinckx G, & Delvaux FR (2004) Effect of non-enzymatic browning on flavor, colour and antioxidative activity of dark specialty malts – a review. *Brew Sci* (14) 25–38.
93. De Keukeleire D (2000) Fundamentals of beer and hop chemistry *Quim Nova* 23(1) 108–112.
94. Quifer-Rada P, Vallverdú-Queralt A, Martínez-Huélamo M, Chiva-Blanch G, Jáuregui O, Estruch R, & Lamuela-Raventós R (2015) A comprehensive characterisation of beer polyphenols by high resolution mass spectrometry (LC-ESI-LTQ-Orbitrap-MS) *Food Chem* 169 336–343, doi:10.1016/j.foodchem.2014.07.154.
95. Čechovská L, Konečný M, Velíšek J, & Cejpek K (2012) Effect of Maillard reaction on reducing power of malts and beers *Czech J Food Sci* 30(6) 548–556, doi:10.17221/288/2012-cjfs.
96. Langner E, & Rzeski W (2014) Biological properties of melanoidins: A review *Int J Food Prop* 17(2) 344–353, doi:10.1080/10942912.2011.631253.
97. Hashimoto N (1972) Oxidation of higher alcohols by melanoidins in beer *J Inst Brew* 78(1) 43–51, doi:10.1002/j.2050-0416.1972.tb03428.x.
98. Kunz T, Strähmel A, Cortés N, Kroh LW, & Methner FJ (2013) Influence of intermediate Maillard reaction products with enediol structure on the oxidative stability of beverages *J Am Soc Brew Chem* 71(3) 114–123, doi:10.1094/ASBCJ-2013-0429-01.
99. Hoff S, Lund MN, Petersen MA, Jespersen BM, & Andersen ML (2014) Quality of pilsner malt and roasted malt during storage *J Inst Brew* 120(4) 331–340, doi:10.1002/jib.144.
100. Clarkson SP, Large JP, & Bamforth CW (1992) Oxygen-scavenging enzymes in barley and malt and their effects during mashing *J Inst Brew* 98 111–115.
101. Stephenson WH, Biawa J, Miracle RE, & Bamforth CW (2003) Laboratory-scale studies of the impact of oxygen on mashing *J Inst Brew* 109(3) 273–283.
102. Kanauchi M, & Bamforth CW (2013) Ascorbate peroxidase in malted barley *J Am Soc Brew Chem* 71(2) 97–102, doi:10.1094/ASBCJ-2013-0103-01.
103. Boivin P (2001) A review of pro- and anti-oxidant of malt *EBC Symp. 'Flavour Flavour Stability'.* Nancy. p.1–21.
104. Bamforth CW (2009) Current perspectives on the role of enzymes in brewing *J Cereal Sci* 50(3) 353–357, doi:10.1016/j.jcs.2009.03.001.
105. Kunz T, Müller C, Mato-Gonzales D, & Methner FJ (2012) The influence of unmalted barley on the oxidative stability of wort and beer *J Inst Brew* 118(1) 32–39, doi:10.1002/jib.6.
106. Vaughan A, O'Sullivan T, & Van Sinderen D (2005) Enhancing the microbiological stability of malt and beer - A review *J Inst Brew* 111(4) 355–371, doi:10.1002/j.2050-0416.2005.tb00221.x.
107. Nischwitz R, Lentini A, MacLeod L, Goldsmith M, & Rogers P (2006) Impact of commercial malting and brewing practices on beer flavour stability *Proceedings from 29th Inst. Guild Brewing, Asia Pacific Sect. Brew. Conv.* Hobart, p.1–19.
108. Kunz T, Woest H, Lee EJ, Müller C, & Methner FJ (2011) Improvement of the oxidative wort and beer stability by increased unmalted barley proportion *Brew Sci* 64(7–8) 75–82.

109. Stephan A, & Stettner G (2005) Strategies for minimising the influence of the barley crop year on beer flavour stability *Expr. Multidiscip. Flavour Sci. p.*219–222.

110. Dong L, Piao Y, Zhang X, Zhao C, Hou Y, & Shi Z (2013) Analysis of volatile compounds from a malting process using headspace solid-phase micro-extraction and GC-MS *Food Res Int* 51(2) 783–789, doi:10.1016/j.foodres.2013.01.052.

111. De Clippeleer J, Van Opstaele F, Vercammen J, Francis JG, De Cooman L, & Aerts G (2010) Real-time profiling of volatile malt aldehydes using selected ion flow tube mass spectrometry *LCGC North Am* 28 386–395.

112. Herrmann M, Gastl M, Thiele F, & Back W (2007) Malt volatile compounds (Part I) *Brew Sci* 110–117.

113. Svoboda Z, Mikulíková R, Běláková S, Benešová K, Márová I, & Nesvadba Z (2011) Optimization of modern analytical SPME and SPDE methods for determination of *trans*-2-nonenal in barley, malt and beer *Chromatographia* 73 157–161, doi:10.1007/s10337-011-1958-x.

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

115. Beal AD, & Mottram DS (1994) Compounds contributing to the characteristic aroma of malted barley *J Agric Food Chem* 42(12) 2880–2884, doi:10.1021/jf00048a043.

116. Fickert B, & Schieberle P (1998) Identification of the key odorants in barley malt (caramalt) using GC/MS techniques and odour dilution analyses *Food / Nahrung* 42(06) 371–375.

117. Vandecan S (2010) Formation of flavour components during the production of dark speciality malts. *Doctoral dissertation*.

118. Cortés Rodríguez N, Kunz T, Furukawa Suárez A, Hughes P, & Methner F (2010) Development and correlation between the organic radical concentration in different malt types and oxidative beer stability *J Am Soc Brew Chem* 68(2) 107–113, doi:10.1094/asbcj-2010-0412-01.

119. Hoff S, Lund MN, Petersen MA, Jespersen BM, & Andersen ML (2012) Influence of malt roasting on the oxidative stability of sweet wort *J Agric Food Chem* 60(22) 5652–5659, doi:10.1021/jf300749r.

120. Saison D, De Schutter DP, Delvaux F, & Delvaux FR (2011) Improved flavor stability by aging beer in the presence of yeast *J Am Soc Brew Chem* 69(1) 50–56, doi:10.1094/asbcj-2011-0127-01.

121. Fukuda K, Saito W, Arai S, & Aida Y (1999) Production of a novel proanthocyanidin-free barley line with high quality *J Inst Brew* 105(3) 179–184, doi:10.1002/j.2050-0416.1999.tb00017.x.

122. Yu J, Huang S, Dong J, Fan W, Huang S, Liu J, Chang Z, Tian Y, Hao J, & Hu S (2014) The influence of LOX-less barley malt on the flavour stability of wort and beer *J Inst Brew* 120(2) 93–98, doi:10.1002/jib.122.

123. Hirota N, Kuroda H, Takoi K, Kaneko T, Kaneda H, Yoshida I, Takashio M, Ito K, & Takeda K (2006) Development of novel barley with improved beer foam and flavor stability - the impact of lipoxygenase-1-less barley in the brewing industry *Tech Q Master Brew Assoc Am* 43(2) 131–135.

124. Hoki T, Kanatani R, Saito W, Iimure T, Zhou TS, Takoi K, Tanigawa A, Kihara M, & Ogushi K (2018) Breeding of lipoxygenase-1-less malting barley variety 'Satuiku 2 go' *J Inst Brew* 124(2) 112–120, doi:10.1002/jib.483.

125. 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 Chem* 83(3) 250–254, doi:10.1094/CC-83-0250.

126. Bogdan P, & Kordialik-Bogacka E (2017) Alternatives to malt in brewing *Trends Food Sci Technol* 65 1–9, doi:10.1016/j.tifs.2017.05.001.
127. Trummer J (2018) Innovations in raw materials and technologies *Proceedings from Adv. Brew. Malt. Technol.* Kocierz p. 67–87.
128. Briggs DE (1998) *Malts and Malting* 1st edition Blackie Academic & Professional. ISBN 0412 298007.
129. Kunze W (2014) *Technology brewing and malting* 5th edition VLB Berlin, Berlin. ISBN 9783921690772.
130. Cole N, McLeod L, & Mitchell K (1998) Some effects of steeping and germination parameters on grain hydration and respiration and consequent malt quality *Tech Q Master Brew Assoc Am* 35(2) 8–11.
131. Vandecan SMG, Daems N, Schouppe N, Saison D, & Delvaux FR (2011) Formation of flavor, color, and reducing power during the production process of dark specialty malts *J Am Soc Brew Chem* 69(3) 150–157, doi:10.1094/ASBCJ-2011-0626-01.
132. Mackie AE, & Slaughter JC (2000) Key steps during barley malting that influence the concentration of flavor compounds *J Am Soc Brew Chem* 58(2) 69–72, doi:10.1094/asbcj-58-0069.
133. Huang S, Yu J, Yin H, Lu J, Dong J, Li X, Hu S, & Liu J (2016) Optimization of kilning progress for equilibrating multiple parameters that strictly affect malt flavour and sensory evaluation *J Inst Brew* 122(4) 706–713, doi:10.1002/jib.369.
134. Coghe S, Benoot K, Basteyns A, & Delvaux FR (2004) Influence of specialty malts on wort and beer characteristics *Brew Sci* 1–18.
135. Preuß T, Forster C, Thum B, & Back W (2001) Dark malt – the key to distinct flavour attributes and high flavour stability in dark beer *EBC Symp. 'Flavour Flavour Stability'.* Nancy. p.1–13.
136. Czuchajowska Z, Klamczynski A, Paszczynska B, & Baik BK (1998) Structure and functionality of barley starches *Cereal Chem* 75(5) 747–754, doi:10.1094/CCHEM.1998.75.5.747.
137. Izydorczyk MS, & Biliaderis CG (2000) Structural and functional aspects of cereal arabinoxylans and β-glucans *Dev Food Sci* 41 361–384, doi:10.1016/S0167-4501(00)80016-3.
138. Quinde Z, Ullrich SE, & Baik BK (2004) Genotypic variation in color and discoloration potential of barley-based food products *Cereal Chem* 81(6) 752–758, doi:10.1094/CCHEM.2004.81.6.752.
139. Fox GP (2010) Chemical composition in barley grains and malt quality in *Genetics and improvement of barley malt quality* 1st edition Springer, doi:10.1007/978-3-642-01279-2. ISBN 978-3-642-01279-2.
140. Briggs DE (2002) Malt modification - A century of evolving views *J Inst Brew* 108(2) 395–405.
141. Evers AD, Blakeney L, & O'Brien A.B. (1999) Cereal structure and composition *Aust J Agric Res* 50(5) 629–650, doi:10.1071/AR98158.
142. Lewis BMJ (2001) Who needs malt? Endosperm mashing *Tech Q Master Brew Assoc Am* 38(2) 111–114.
143. Lewis MJ (2003) Malt , the beauty and the beast — an appraisal for craft brewers *Appraisal* 40(3) 186–188.
144. Van Waesberghe IJWM (1991) Practical investigations on the possible impact of mash separation time on beer flavor and its flavor stability influence of the husk fraction *Tech Q Master Brew Assoc Am* 28(1) 33–37.
145. Brinch-Pedersen H, Borg S, Tauris B, & Holm PB (2007) Molecular genetic approaches to

increasing mineral availability and vitamin content of cereals *J Cereal Sci* 46(3) 308–326, doi:10.1016/j.jcs.2007.02.004.

146. Gupta M, Abu-Ghannam N, & Gallaghar E (2010) Barley for brewing: Characteristic changes during malting, brewing and applications of its by-products *Compr Rev Food Sci Food Saf* 9(3) 318–328, doi:10.1111/j.1541-4337.2010.00112.x.

147. De Schepper CF, Michiels P, Langenaeken NA, & Courtin CM (2020) Accurate quantification of small and large starch granules in barley and malt *Carbohydr Polym* 227 1–8, doi:10.1016/j.carbpol.2019.115329.

148. Zurcher A, Krottenthaler M, Rauber M, Schneeberger M, & Back W (2001) Influence of the acrospire of malted barley on flavour stability and other quality parameters of beer *EBC Symp. 'Flavour Flavour Stability'.* Nancy. p.1–9.

149. Herb D, Filichkin T, Fisk S, Helgerson L, Hayes P, Meints B, Jennings R, Monsour R, Tynan S, Vinkemeier K, Romagosa I, Moscou M, Carey D, Thiel R, Cistue L, Martens C, & Thomas W (2017) Effects of barley (Hordeum vulgare L.) variety and growing environment on beer flavor *J Am Soc Brew Chem* 75(4) 345–353, doi:10.1094/asbcj-2017-4860-01.

150. Takashio M, & Shinotsuka K (1998) Preventive production of beer against oxidation-recent advances in brewing technology *Food Sci Technol Int Tokyo* 4(3) 169–177, doi:10.3136/fsti9596t9798.4.169.

151. Bravi E, Marconi O, Perretti G, & Fantozzi P (2012) Influence of barley variety and malting process on lipid content of malt *Food Chem* 135(3) 1112–1117, doi:10.1016/j.foodchem.2012.06.041.

152. Mareček V, Mikyška A, Hampel D, Čejka P, Neuwirthová J, Malachová A, & Cerkal R (2017) ABTS and DPPH methods as a tool for studying antioxidant capacity of spring barley and malt *J Cereal Sci* 73 40–45, doi:10.1016/j.jcs.2016.11.004.

153. Zhao H, Li H, Sun G, Yang B, & Zhao M (2013) Assessment of endogenous antioxidative compounds and antioxidant activities of lager beers *J Sci Food Agric* 93(4) 910–917, doi:10.1002/jsfa.5824.

154. Koren D, Kun S, Hegyesné Vecseri B, & Kun-Farkas G (2019) Study of antioxidant activity during the malting and brewing process *J Food Sci Technol* 56(8) 3801–3809, doi:10.1007/s13197-019-03851-1.

155. Bertholdsson NO (1999) Characterization of malting barley cultivars with more or less stable grain protein content under varying environmental conditions *Eur J Agron* 10(1) 1–8, doi:10.1016/S1161-0301(98)00043-4.

156. Emebiri LC, & Moody DB (2004) Potential of low-protein genotypes for nitrogen management in malting barley production *J Agric Sci* 142(3) 319–325, doi:10.1017/S0021859604004332.

157. Therrien MC, Carmichael CA, Noll JS, & Grant CA (1994) Effect of fertilizer management, genotype, and environmental factors on some malting quality characteristics in barley *Can J Plant Sci* 74(3) 545–547, doi:10.4141/cjps94-098.

158. Yousif AM, & Evans DE (2018) The impact of barley nitrogen fertilization rate on barley brewing using a commercial enzyme (Ondea Pro) *J Inst Brew* 124(2) 132–142, doi:10.1002/jib.478.

159. Gous PW, Warren F, Mo OW, Gilbert RG, & Fox GP (2015) The effects of variable nitrogen application on barley starch structure under drought stress *J Inst Brew* 121(4) 502–509, doi:10.1002/jib.260.

160. Barati V, & Ghadiri H (2017) Assimilate and nitrogen remobilization of six-rowed and two-rowed winter barley under drought stress at different nitrogen fertilization *Arch Agron Soil Sci* 63(6) 841–855, doi:10.1080/03650340.2016.1238075.

161. Cramer ACJ, Mattinson DS, Fellman JK, & Baik BK (2005) Analysis of volatile compounds from

various types of barley cultivars *J Agric Food Chem* 53(19) 7526–7531, doi:10.1021/jf0506939.

162. Brookes PA, Lovett DA, & MacWilliam IC (1976) The steeping of barley. A review of the metabolic consequences of water uptake, and their practical implications *J Inst Brew* 82(1) 14–26, doi:10.1002/j.2050-0416.1976.tb03716.x.

163. Allosio-Ouarnier N, Quemener B, Bertrand D, & Boivin P (2000) Application of high performance anion exchange chromatography to the study of carbohydrate changes in barley during malting *J Inst Brew* 106(1) 45–52, doi:10.1002/j.2050-0416.2000.tb00039.x.

164. Frank T, Scholz B, Peter S, & Engel KH (2011) Metabolite profiling of barley: Influence of the malting process *Food Chem* 124(3) 948–957, doi:10.1016/j.foodchem.2010.07.034.

165. 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 *J Agric Food Chem* 55(26) 10994–11001, doi:10.1021/jf0722710.

166. Müller C, Kunz T, & Methner FJ (2015) The cleaning effect on brewing barley using vibrations during wet steeping *Brew Sci* 68(1–2) 29–37.

167. Ma Z, Zhang L, Liu J, Dong J, Yin H, Yu J, Huang S, Hu S, & Lin H (2020) Effect of hydrogen peroxide and ozone treatment on improving the malting quality *J Cereal Sci* 91 102882, doi:10.1016/j.jcs.2019.102882.

168. Mauch A, Jacob FF, Coffey A, & Arendt EK (2011) Part I. The use of Lactobacillus plantarum starter cultures to inhibit rootlet growth during germination of barley, reducing malting loss and its influence on malt quality *J Am Soc Brew Chem* (56) 227–238, doi:10.1094/ASBCJ-2011-1027-01.

169. Mauch A, Wunderlich S, Zarnkow M, Becker T, Jacob FF, & Arendt EK (2011) Part II. The use of malt produced with 70% less malting loss for beer production: impact on processability and final quality *J Am Soc Brew Chem* (64) 239–254, doi:10.1094/ASBCJ-2011-1107-01.

170. Cole N, MacLeod L, & Mitchell K (1998) Some effects of steeping and germination parameters on grain hydration and respiration and consequent malt quality *Tech Q master Brew Assoc Am* 35(2) 104–107.

171. Kaukovirta-Norja A, & Laasko S (1993) Lipolytic and oxidative changes of barley lipids during malting and mashing *J Inst Brew* 99(5) 395–403, doi:10.1002/j.2050-0416.1993.tb01179.x.

172. Anness BBJ (1984) Lipids of barley, malt and adjuncts *J Inst Brew* 90(5) 315–318, doi:10.1002/j.2050-0416.1984.tb04282.x.

173. Kaukovirta-Norja A, & Laakso S (1998) The effect of kilning on the capability of malt to oxidise lipids *J Inst Brew* 104(6) 327–332, doi:10.1002/j.2050-0416.1998.tb01004.x.

174. Hobke H, Garbe LA, & Tressl R (2005) Characterization and quantification of free and esterified 9- and 13-hydroxyoctadecadienoic acids (HODE) in barley, germinating barley, and finished malt *J Agric Food Chem* 53(5) 1556–1562, doi:10.1021/jf048490s.

175. Silva F, Nogueira LC, Gonçalves C, Ferreira AA, Ferreira IM, & Teixeira N (2008) Electrophoretic and HPLC methods for comparative study of the protein fractions of malts, worts and beers produced from Scarlett and Prestige barley (Hordeum vulgare L.) varieties *Food Chem* 106(2) 820–829, doi:10.1016/j.foodchem.2007.06.047.

176. Dvořáková M, Douanier M, Jurková M, Kellner V, & Dostálek P (2008) Comparison of antioxidant activity of barley (Hordeum vulgare L.) and malt extracts with the content of free phenolic compounds measured by high performance liquid chromatography coupled with CoulArray detector *J Inst Brew* 114(2) 150–159, doi:10.1002/j.2050-0416.2008.tb00320.x.

177. Dong L, Liu R, Dong H, Piao Y, Hu X, Li C, Cong L, & Zhao C (2015) Volatile metabolite profiling of malt contaminated by Fusarium poae during malting *J Cereal Sci* 66 37–45, doi:10.1016/j.jcs.2015.09.006.

178. Chen Y, Zhou Z, Xu K, Zhang H, Thornton M, Sun L, Wang Z, Xu X, & Dong L (2017) Comprehensive evaluation of malt volatile compounds contaminated by Fusarium graminearum during malting *J Inst Brew* 123(4) 480–487, doi:10.1002/jib.453.

179. Lowe DP, & Arendt EK (2004) The use and effects of lactic acid bacteria in malting and brewing with their relationships to antifungal activity , mycotoxins and gushing: A review *J Inst Brew* 110(3) 163–180, doi:/10.1002/j.2050-0416.2004.tb00199.x.

180. Malfliet S (2013) Characterisation of microbial communities and xylanolytic bacteria during malting of barley: impact on malt quality. *Doctoral dissertation.*

181. Fox GP, Panozzo JF, Li CD, Lance RCM, Inkerman PA, & Henry RJ (2003) Molecular basis of barley quality *Aust J Agric Res* 54 1081–1101, doi:10.1071/ar02237.

182. Hattingh M, Alexander A, Meijering I, van Reenen CA, & Dicks LMT (2014) Malting of barley with combinations of Lactobacillus plantarum, Aspergillus niger, Trichoderma reesei, Rhizopus oligosporus and Geotrichum candidum to enhance malt quality *Int J Food Microbiol* 173 36–40, doi:10.1016/j.ijfoodmicro.2013.12.017.

183. Woffenden HM, Ames JM, Chandra S, Anese M, & Nicoli MC (2002) Effect of kilning on the antioxidant and prooxidant activities of pale malts *J Agric Food Chem* 50(17) 4925–4933, doi:10.1021/jf020312g.

184. Davies N (2006) Malt and malt products in *Brewing - New Technologies.* 1st edition Woodhead Publishing p.68-101. ISBN 9780849391590.

185. Kaukovirta-Norja A, Kotiranta P, Aurola A, Reinikainen P, Olkku J, & Laakso S (1998) Influence of water processing on the composition, behavior, and oxidizability of barley and malt lipids *J Agric food Chem* 8561(97) 1556–1562.

186. Kaukovirta-Norja A, Reinikainen P, Olkku J, & Laakso S (1998) Influence of barley and malt storage on lipoxygenase reaction *Cereal Chem* 75(5) 742–746, doi:10.1094/CCHEM.1998.75.5.742.

187. Dumoulinm, M., & Boivin P (2001) Industrial kilning technologies and their influence on organoleptic quality of malt *Proceedings European Brewery Convention*. Budapest, p.200–209.

188. Inns EL, Buggey LA, Booer C, Nursten HE, & Ames JM (2007) Effect of heat treatment on the antioxidant activity, color, and free phenolic acid profile of malt *J Agric Food Chem* 55(16) 6539–6546, doi:10.1021/jf0710231.

189. Guido LF (2016) Sulfites in beer: Reviewing regulation, analysis and role *Sci Agric* 73(2) 189–197, doi:10.1590/0103-9016-2015-0290.

190. Ratti C (2001) Hot air and freeze-drying of high-value foods: A review *J Food Eng* 49(4) 311–319, doi:10.1016/S0260-8774(00)00228-4.

191. Brudzynski A, & Roginski H (1969) Comparative studies of kilned and freeze-dried malts *J Inst Brew* 75(5) 472–476, doi:10.1002/j.2050-0416.1969.tb06385.x.

192. Ferrari-John RS, Katrib J, Zerva E, Davies N, Cook DJ, Dodds C, & Kingman S (2017) Electromagnetic heating for industrial kilning of malt: a feasibility study *Food Bioprocess Technol* 10(4) 687–698, doi:10.1007/s11947-016-1849-0.

193. Fulayter B, Science B, Hawley G, Condensed T, Dictionary C, Perry R, & Kepplinger PE (1997) United States Patent 19 .

194. Pinho O, Ferreira IM, & Santos LH (2006) Method optimization by solid-phase microextraction in combination with gas chromatography with mass spectrometry for analysis of beer volatile fraction *J Chromatogr A* 1121(2) 145–153, doi:10.1016/j.chroma.2006.04.013.

195. De Schutter DP, Saison D, Delvaux F, Derdelinckx G, Rock JM, Neven H, & Delvaux FR (2008) Optimisation of wort volatile analysis by headspace solid-phase microextraction in

combination with gas chromatography and mass spectrometry *J Chromatogr A* 1179(2) 75–80, doi:10.1016/j.chroma.2007.11.103.

196. Kovačevič M, & Kač M (2001) Solid-phase microextraction of hop volatiles - Potential use for determination and verification of hop varieties *J Chromatogr A* 918(1) 159–167, doi:10.1016/S0021-9673(01)00719-1.

197. Vandecan SM, Saison D, Schouppe N, Delvaux F, & Delvaux FR (2010) Optimisation of specialty malt volatile analysis by headspace solid-phase microextraction in combination with gas chromatography and mass spectrometry *Anal Chim Acta* 671(1–2) 55–60, doi:10.1016/j.aca.2010.05.009.

198. Buśko M, Jeleń H, Góral T, Chmielewski J, Stuper K, Szwajkowska-Michałek L, Tyrakowska B, & Perkowski J (2010) Volatile metabolites in various cereal grains *Food Addit Contam - Part A Chem Anal Control Expo Risk Assess* 27(11) 1574–1581, doi:10.1080/19440049.2010.506600.

199. Klensporf D, & Jelen H (2005) Analysis of volatile aldehydes in oat flakes by SPME-GC/MS *Polish J food Nutr Sci* 14(4) 389–395.

200. De Clippeleer J (2013) Flavour stability of pale lager beer. Chemical-analytical characterisation of critical factors related to wort production and hopping. *Doctoral dissertation.*

201. Ridgway K, Lalljie SP, & Smith RM (2007) Sample preparation techniques for the determination of trace residues and contaminants in foods *J Chromatogr A* 1153(1–2) 36–53, doi:10.1016/j.chroma.2007.01.134.

202. Cancilla DA, & Que Hee SS (1992) O-(2,3,4,5,6-Pentafluorophenyl)methylhydroxylamine hydrochloride: a versatile reagent for the determination of carbonyl-containing compounds *J Chromatogr A* 627(1–2) 1–16, doi:10.1016/0021-9673(92)87181-7.

203. Martos PA, & Pawliszyn J (1998) Sampling and determination of formaldehyde using solid-phase microextraction with on-fiber derivatization *Anal Chem Acta* 70(11) 2311–2320, doi:10.1021/ac9711394.

204. Du L, He T, Li W, Wang R, & Xiao D (2015) Analysis of volatile compounds in Chinese Laobaigan liquor using headspace solid-phase microextraction coupled with GC-MS *Anal Methods* 7(5) 1906–1913, doi:10.1039/c4ay02580f.

205. Bueno M, Culleré L, Cacho J, & Ferreira V (2010) Chemical and sensory characterization of oxidative behavior in different wines *Food Res Int* 43(5) 1423–1428, doi:10.1016/j.foodres.2010.04.003.

206. Ershov AY, Nasledov DG, Lagoda I V., & Shamanin V V. (2014) Synthesis of 2-substituted (2R,4R)-3-(3-mercapto-propionyl)thiazolidine-4-carboxylic acids *Chem Heterocycl Compd* 50(7) 1032–1038, doi:10.1007/s10593-014-1560-x.

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

208. Jaskula-Goiris B, De Causmaecker B, De Rouck G, Aerts G, & De Cooman L (2015) Influence of malt specification on beer quality and flavour stability *Proceedings from Chair J. Clerck*. Leuven. Poster.

209. The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (1994) ICH Topic Q2 (R1) Validation of analytical procedures Retrieved online from: http://www.ich.org/fileadmin/Public _ Web _ Site/ICH _ Products/ Guidlines/Quality/Q2 _ R1/Step4/Q2 _ R1 _ Guidline.pdf.

210. Rozet E, Marini RD, Ziemons E, Boulanger B, & Hubert P (2011) Advances in validation, risk and

uncertainty assessment of bioanalytical methods *J Pharm Biomed Anal* 55(4) 848–858, doi:10.1016/j.jpba.2010.12.018.

211. Oona M (2009) *Validation of analytical methods for pharmaceutical analysis* Mourne Training Services, Warrenpoint. ISBN 9780956152817.
212. Schwarz P, Stanley P, & Solberg S (2002) Activity of lipase during mashing *J Am Soc Brew Chem* 60(3) 107–109, doi:10.1094/ASBCJ-60-0107.
213. Kobayashi N, Kaneda H, Kano Y, & Koshino S (1993) The production of linoleic and linolenic acid hydroperoxides during mashing *J Ferment Bioeng* 76(5) 371–375, doi:10.1016/0922-338X(93)90024-3.
214. Dhanani T, Shah S, Gajbhiye NA, & Kumar S (2017) Effect of extraction methods on yield, phytochemical constituents and antioxidant activity of Withania somnifera *Arab J Chem* 10 S1193–S1199, doi:10.1016/j.arabjc.2013.02.015.
215. De Schutter DP (2008) The influence of thermal load during wort boiling on the flavour stability of beer KU Leuven. *Doctoral dissertation.*
216. Peppard TL, & Halsey SA (1981) Malt flavour — transformation of carbonyl compounds by yeast during fermentation *J Inst Brew* 87(6) 386–390, doi:10.1002/j.2050-0416.1981.tb04055.x.
217. Nie C, Wang C, Zhou G, Dou F, & Huang M (2010) Effects of malting conditions on the amino acid compositions of final malt *African J Biotechnol* 9(53) 9018–9025, doi:10.5897/AJB10.370.
218. Coghe S, Martens E, D'Hollander H, Dirinck PJ, & Delvaux FR (2004) Sensory and instrumental flavour analysis of wort brewed with dark specialty malts *J Inst Brew* 110(2) 94–103, doi:10.1002/j.2050-0416.2004.tb00188.x.
219. Thalacker R, & Brikenstock BA (1982) A new criterion in brewing analysis - the thiobarbituric acid number (TBN) *Monatsschrift fur Brauwiss* 35 133–137.
220. Warmbier HC, Schnickels RA, & Labuza TP (1976) Effect of glycerol on nonenzymatic browning in a solid intermediate moisture model food system *J Food Sci* 41 528–531.
221. Barreiro JA, Fernández S, & Sandoval AJ (2003) Water sorption characteristics of six row barley malt (Hordeum vulgare) *Food Sci Technol* 36(1) 37–42, doi:10.1016/S0023-6438(02)00158-5.
222. Laitila A, Wilhelmson A, Mikkola H, Kauppila R, Kotaviita E, Olkku J, Haikara A, & Home S (2003) Importance of drying and sound storage conditions on the microbiological safety and viability of malting barley *Proceedings 29th European Brewery Convention.* Dublin p.124–135.
223. Hugues M, Bovin P, Gauillard F, Nicolas J, Thiry J-M, & Richard-Forget F (1994) Two lipoxygenases from germinated barley-heat and kilning stability *J Food Sci* 59(4) 885–889, doi:10.1111/j.1365-2621.1994.tb08150.x.
224. Wackerbauer K, Meyna S, & Marre S (2003) Hydroxy fatty acids as indicators for ageing and the influence of oxygen in the brewhouse on the flavour stability of beer *Proceedings 29th European Brewery Convention.* Dublin p.1-9.
225. Lide DR (2005) *Handbook of Chemistry and Physics* 88th edition CRC Press, Boca Raton, FL. ISBN 978-1420066791.
226. Mizuno A, Nomura Y, & Iwata H (2011) Sensitive measurement of thermal stress in beer and beer-like beverages utilizing the 2-thiobarbituric acid (TBA) reaction *J Am Soc Brew Chem* 69(4) 220–226, doi:10.1094/ASBCJ-2011-1017-01.
227. Guillén-Sans R, & Guzmán-Chozas M (1998) The thiobarbituric acid (TBA) reaction in foods: A review *Crit Rev Food Sci Nutr* 38(4) 315–350, doi:10.1080/10408699891274228.
228. O'Rourke T (2002) Malt Specifications & Brewing Performance *Brew Int* 2(10) 27–30.
229. Gastl M, Kupetz M, & Becker T (2020) Determination of cytolytic malt modification–Part I: influence of variety characteristics *J Am Soc Brew Chem* 79(1) 53–65,

doi:10.1080/03610470.2020.1796156.

230. Eßlinger HM (2009) *Handbook of brewing. Processes, technology, Markets* 1st edition WILEY-VCH. ISBN 9783527623488.

231. De Causmaecker B, De Rouck G, Aerts G, & De Cooman L (2015) Evaluation of malt process parameters in relation to malt quality *Proceedings from Chair J. Clerck*. Leuven. Poster.

232. Ye L, Huang Y, Li M, Li C, & Zhang G (2016) The chemical components in malt associated with haze formation in beer *J Inst Brew* 122(3) 524–529, doi:10.1002/jib.353.

233. Filipowska W, Jaskula-Goiris B, Ditrych M, Bustillo Trueba P, De Rouck G, Aerts G, Powell C, Cook D, & De Cooman L (2021) On the contribution of malt quality and the malting process to the formation of beer staling aldehydes: a review *J Inst Brew* 127(2) 107–126, doi:10.1002/jib.644.

234. Lloyd WJW (1988) Environmental effects on the biochemical phases of malt kilning *J Am Soc Brew Chem* 46(1) 8–13, doi:10.1094/ASBCJ-46-0008.

235. Muller C, Kleinwächter M, Selmar D, & Methner FJ (2014) The influence of the withering temperature on the resulting proteolytic and cytolytic modification of pale malt *Brew Sci* 67 88–95.

236. Bathgate BG (1983) The relationships between malt friability and wort viscosity *J Inst Brew* 89 416–419.

237. Ames JM (1990) Control of the Maillard reaction in food systems *Trends Food Sci Technol* 1 150–154, doi:10.1016/0924-2244(90)90113-D.

238. Wong CW, Wijayanti HB, & Bhandari BR (2015) Maillard reaction in limited moisture and low water activity environment In: *Water Stress Biol. Chem. Pharm. Food Syst.* 41–63, doi:10.1007/978-1-4939-2578-0_4. ISBN 978-1-4939-2578-0.

239. Nursten HE (2005) *The Maillard reaction - Chemistry, biochemistry and implications*, The Royal Cambridge. ISBN 9780854049646.

240. Derossi A, Severini C, & Cassi D (2011) Mass transfer mechanisms during dehydration of vegetable food: traditional and innovative approaches In: *Advanced Topics in Mass Transer p.* 305–352, doi:10.5772/14725. ISBN 978-953-307-333-0

241. Amaya-Farfan J, & Rodriguez-Amaya DB (2021) The Maillard reactions In: *Chemical Changes During Processing and Storage of Foods* INC, 215–263, https://doi.org/10.1016/b978-0-12-817380-0.00006-3.ISBN 9780128173800.

242. Liedke R (1999) Bildung von α-dicarbonylverbindungen beim abbau von Amadori-umlagerungsprodukten. *Doctoral dissertation.*

243. Wainright T (1986) The chemistry of nitrosamine formation: relevance to malting and brewing *J Inst Brew* 92 49–64.

244. Möller-Hergt G, Roderfeld HJ, & Waterkamp HJ (2001) Changes of hydroxy acids and other relevant compounds in wort and beer depending on storage of malt and barley *EBC Symp. 'Flavour Flavour Stability'.* Nancy. p.1-9.

245. Wackerbauer K, & Meyna S (2001) The occurrence of free and triglyceride-bonded hydroxy fatty acids in barley and malt *EBC Symp. 'Flavour Flavour Stability'.* Nancy. p.1-9.

246. Jacobsen J V., & Varner JE (1967) Gibberellic acid-induced synthesis of protease by isolated aleurone layers of barley *Plant Physiol* 42(11) 1596–1600, doi:10.1104/pp.42.11.1596.

247. Briggs DE, Boulton CA, Brookes PA, & Stevens R (2004) *Brewing science and practice* Woodhead Publishing Limited, Cambridge. ISBN: 9781855734906.

248. Schwarz PB, & Pyler RE (1984) Lipoxygenase and hydroperoxide isomerase activity of malting barley *J Am Soc Brew Chem* 42(2) 47–53, doi:10.1094/asbcj-42-0047.

249. Lee J, Lee Y, & Choe E (2007) Temperature dependence of the autoxidation and antioxidants of soybean, sunflower, and olive oil *Eur Food Res Technol* 226(1–2) 239–246, doi:10.1007/s00217-006-0532-5.

250. De Almeida NE, Lund MN, Andersen ML, & Cardoso DR (2013) Beer thiol-containing compounds and redox stability: Kinetic study of 1-hydroxyethyl radical scavenging ability *J Agric Food Chem* 61(39) 9444–9452, doi:10.1021/jf402159a.

251. Vogt W (1995) Oxidation of methionyl residues in proteins: Tools, targets, and reversal *Free Radic Biol Med* 18(1) 93–105, doi:10.1016/0891-5849(94)00158-G.

252. Deepak RN, Chandrakar B, & Sankararamakrishnan R (2017) Biophysical Chemistry Comparison of metal-binding strength between methionine and cysteine residues : Implications for the design of metal-binding motifs in proteins *Biophys Chem* 224 32–39, doi:10.1016/j.bpc.2017.02.007.

253. Wedzicha B., Bellion IR, & German G (2005) New insight into the mechanism of the Maillard reaction from studies of the kinetics of its inhibition by sulfite *Mail. React. Chem. Food Heal.* 45(1) 82–87, doi:10.1533/9781845698393.2.82.

254. Psota V, Sachambula L, & Paulu A (2015) Sensitivity of the selected malting barley varieties to the degree of steeping *Kvas Prum* 61(10–11) 288–295, doi:10.18832/kp2015026.

255. Mukaka MM (2012) Statistics corner: A guide to appropriate use of correlation coefficient in medical research *Malawi Med J* 24(3) 69–71.

256. De Rouck G, Flores-González AG, De Clippeleer J, De Cock J, De Cooman L, & Aerts G (2010) Sufficient formation and removal of dimethyl sulfide (DMS) without classic wort boiling *Brew Sci* 63(1–2) 31–40.

257. De Buck A, Aerts G, Bonte S, & Dupire S (1999) Rapid evaluation of the two lipoxygenase isoenzyme activities in malt *Proceedings European Brewery Convention.* Brussels p.209–216.

258. Dugulin CA, Clegg SC, De Rouck G, & Cook DJ (2020) Overcoming technical barriers to brewing with green (non-kilned) malt: a feasibility study *J Inst Brew* 126(1) 24–34, doi:10.1002/jib.602.

259. Hämäläinen JJ, & Reinikainen P (2007) A simulation model for malt enzyme activities in kilning *J Inst Brew* 113(2) 159–167, doi:10.1002/j.2050-0416.2007.tb00273.x.

260. Yousif AM, & Evans DE (2020) Changes in malt quality during production in two commercial malt houses *J Inst Brew* 126(3) 233–252, doi:10.1002/jib.609.

261. Bamforth CW (2014) Dimethyl sulfide - Significance, origins, and control *J Am Soc Brew Chem* 72(3) 165–168, doi:10.1094/ASBCJ-2014-0610-01.

262. Dethier M, De Jaeger B, Barszczak E, & Dufour J-P (1991) In Vivo and in Vitro investigations of the synthesis of S-methylmethionine during barley germination *J Am Soc Brew Chem* 49(1) 31–37, doi:10.1094/asbcj-49-0031.

263. Anness BJ, & Bamforth CW (1982) Dimethyl sulphide—a review *J Inst Brew* 88 244–252.

264. Yang B, Schwarz P, & Horsley R (1998) Factors involved in the formation of two precursors of dimethylsulfide during malting *J Am Soc Brew Chem* 56(3) 85–92, doi:10.1094/asbcj-56-0085.

265. Mosblech A, Feussner I, & Heilmann I (2009) Oxylipins: Structurally diverse metabolites from fatty acid oxidation *Plant Physiol Biochem* 47(6) 511–517, doi:10.1016/j.plaphy.2008.12.011.

266. Hou CT, & Forman RJ (2000) Growth inhibition of plant pathogenic fungi by hydroxy fatty acids *J Ind Microbiol Biotechnol* 24(4) 275–276, doi:10.1038/sj.jim.2900816.

267. Prost I, Dhondt S, Rothe G, Vicente J, Rodriguez MJ, Kift N, Carbonne F, Griffiths G, Esquerré-Tugayé MT, Rosahl S, Castresana C, Hamberg M, & Fournier J (2005) Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defence against pathogens *Plant Physiol* 139(4) 1902–1913, doi:10.1104/pp.105.066274.

268. 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 Chem* 107(1) 296–304, doi:10.1016/j.foodchem.2007.08.018.

269. Dugulin CA (2021) Brewing with green malt for an energy and water efficient process : challenges and opportunities. *Doctoral dissertation*.

270. Habschied K, Lalic A, Horvat D, Mastanjevic K, Lukinac J, Jukic M, & Krstanovic V (2020) β - Glucan degradation during malting of different purpose barley varieties *Fermentation* 6(21) 1–9, doi:10.3390/fermentation6010021.

271. Justé A, Malfliet S, Lenaerts M, Cooman L de, Aerts G, Lievens B, & Willems KA (2011) Microflora during malting of barley: Overview and impact on malt quality *Brew Sci* 64(3–4) 22–31.

272. Laitila A, Kotaviita E, Peltola P, Home S, Wilhelmson A, & Brew JI (2007) Indigenous microbial community of barley greatly influences grain germination and malt quality *J Inst Brew* 113(1) 9–20.

273. Laitila A, Sweins H, Vilipola A, Kotaviita E, Olkku J, Home S, & Haikara A (2006) Lactobacillus plantarum and Pediococcus pentosaceus starter cultures as a tool for microflora management in malting and for enhancement of malt processability *J Agric Food Chem* 56 3840–3851, doi:10.1021/jf052979j.

274. Buhr K, Pammer C, & Schieberle P (2009) Influence of water on the generation of strecker aldehydes from dry processed foods *Eur Food Res Technol* 230(3) 375–381, doi:10.1007/s00217-009-1169-y.

LIST OF FIGURES

LIST OF TABLES

APPENDIX A TO CHAPTER 4

Quantification of free and cysteinylated aldehydes in samples derived from different stages of the industrial-scale malting process K

Compounds: 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, T2N = *trans*-2 nonenal, 2MP-CYS = cysteinylated 2-methylpropanal, 2MB-CYS = cysteinylated 2-methylbutanal, 3MB-CYS = cysteinylated 3-methylbutanal, MET-CYS = cysteinylated methional, PHE-CYS = cysteinylated phenylacetaldehyde, FUR-CYS = cysteinylated furfural (<LOD), and HEX-CYS = cysteinylated hexanal (cysteinylated *trans*-2-nonenal is not presented as the reference compound was not available).

Analysed samples: barley; GM = germinating barley (GM0 – onset of germination; GM1-GM4 = germinated for 1, 2, 3, 4 days, respectively); K = samples taken at kilning, after 12 up to 22h of kilning; C = samples taken at cooling, after 0 min to 50 min; FIN = finished malt (without rootlets). During kilning samples were collected from top, middle and bottom grain bed layer, while during cooling samples were taken from top layer.

Results are expressed as mean values (n=3) ± standard deviation. LOD = limit of detection; LOQ = limit of quantification.

Table A4-1. Quantification of free aldehydes in samples derived from different stages of the industrial-scale malting process K.

Free aldehydes (µg/kg dm)	2MP			2MB			3MB		
Raw material									
Barley	<LOD (35)			<LOD (30)			<LOD (136)		
Germination									
GM0	<LOD (35)			<LOD (30)			<LOD (136)		
GM1	<LOD (35)			<LOD (30)			<LOD (136)		
GM2	<LOQ (117)			<LOD (30)			<LOD (136)		
GM3	125 ± 22			<LOD (30)			<LOD (136)		
GM4	187 ± 24			<LOD (30)			<LOD (136)		
Kilning									
	Bottom	Middle	Top	Bottom	Middle	Top	Bottom	Middle	Top
K12.0	<LOQ (117)	<LOQ (117)	<LOD (35)	<LOD (30)	<LOD (30)	<LOD (30)	<LOQ (454)	<LOD (136)	<LOD (136)
K14.0	<LOQ (117)	<LOQ (117)	<LOD (35)	<LOD (30)	<LOD (30)	<LOD (30)	<LOQ (454)	<LOQ (454)	<LOD (136)
K16.0	142 ± 13	104 ± 16	<LOD (35)	<LOQ (99)	<LOQ (99)	<LOD (30)	<LOQ (454)	<LOQ (454)	<LOD (136)
K18.0	199 ± 5	163 ± 25	<LOD (35)	109 ± 9	<LOQ (99)	<LOD (30)	<LOQ (454)	<LOQ (454)	<LOD (136)
K18.5	183 ± 28	165 ± 26	<LOQ (117)	107 ± 22	<LOQ (99)	<LOD (30)	<LOQ (454)	<LOQ (454)	<LOD (136)
K19.0	236 ± 24	161 ± 21	<LOQ (117)	148 ± 35	<LOQ (99)	<LOQ (99)	<LOQ (454)	<LOQ (454)	<LOD (136)
K19.5	277 ± 33	252 ± 13	<LOQ (117)	173 ± 43	131 ± 13	<LOQ (99)	<LOQ (454)	<LOQ (454)	<LOD (136)
K20.0	352 ± 5	305 ± 10	<LOQ (117)	220 ± 19	207 ± 30	<LOQ (99)	<LOQ (454)	<LOQ (454)	<LOD (136)
K20.5	347 ± 37	326 ± 25	<LOQ (117)	244 ± 35	209 ± 28	<LOQ (99)	<LOQ (454)	<LOQ (454)	<LOQ (454)
K21.0	438 ± 1	333 ± 61	<LOQ (117)	296 ± 19	226 ± 21	<LOQ (99)	563 ± 34	<LOQ (454)	<LOQ (454)
K21.5	504 ± 58	-	<LOQ (117)	380 ± 49	-	<LOQ (99)	670 ± 78	-	<LOQ (454)
K22.0/C0	633 ± 48	570 ± 60	126 ± 12	520 ± 26	455 ± 19	131 ± 10	860 ± 37	783 ±23	<LOQ (454)
Cooling									
	Top			Top			Top		
C15	401 ± 28			300 ± 10			598 ± 29		
C30	417 ± 26			282 ± 45			549 ± 92		
C40	300 ± 36			273 ± 30			451 ± 72		
C50	354 ± 49			294 ± 13			527 ± 61		
Finished product									
Malt	375 ± 16			370 ± 25			655 ± 45		

Table A4-2. Quantification of free aldehydes in samples derived from different stages of the industrial-scale malting process K.

Free aldehydes (µg/kg dm)	MET			PHE			FUR		
Raw material									
Barley	<LOD (18)			<LOD (32)			<LOD (17)		
Germination									
GM0	<LOD (18)			<LOQ (107)			<LOD (17)		
GM1	<LOD (18)			<LOQ (107)			<LOD (17)		
GM2	<LOD (18)			<LOQ (107)			<LOD (17)		
GM3	<LOD (18)			<LOQ (107)			<LOD (17)		
GM4	<LOD (18)			<LOQ (107)			<LOD (17)		
Kilning									
	Bottom	Middle	Top	Bottom	Middle	Top	Bottom	Middle	Top
K12.0	<LOD (18)	<LOD (18)	<LOD (18)	<LOQ (107)	<LOQ (107)	<LOQ (107)	<LOD (17)	<LOD (17)	<LOD (17)
K14.0	<LOD (18)	<LOD (18)	<LOD (18)	<LOQ (107)	<LOQ (107)	<LOQ (107)	<LOD (17)	<LOD (17)	<LOD (17)
K16.0	<LOD (18)	<LOD (18)	<LOD (18)	112 ± 6	<LOQ (107)	<LOQ (107)	<LOD (17)	<LOD (17)	<LOD (17)
K18.0	<LOD (18)	<LOD (18)	<LOD (18)	161 ± 25	124 ± 15	<LOQ (107)	<LOD (17)	<LOD (17)	<LOD (17)
K18.5	<LOD (18)	<LOD (18)	<LOD (18)	134 ±15	108 ± 18	<LOQ (107)	<LOD (17)	<LOD (17)	<LOD (17)
K19.0	<LOD (18)	<LOD (18)	<LOD (18)	167 ± 28	112 ± 15	<LOQ (107)	58 ± 17	<LOD (17)	<LOD (17)
K19.5	<LOD (18)	<LOD (18)	<LOD (18)	171 ± 38	153 ± 22	<LOQ (107)	55 ± 23	58 ± 9	<LOD (17)
K20.0	<LOD (18)	<LOD (18)	<LOD (18)	256 ± 33	218 ± 36	<LOQ (107)	76 ± 18	75 ± 12	<LOQ (58)
K20.5	<LOD (18)	<LOD (18)	<LOD (18)	244 ± 39	240 ± 55	<LOQ (107)	83 ± 10	80 ± 15	<LOQ (58)
K21.0	<LOD (18)	<LOD (18)	<LOD (18)	338 ± 52	284 ± 38	<LOQ (107)	111 ± 24	86 ± 11	<LOQ (58)
K21.5	90 ± 12	<LOD (18)	<LOD (18)	370 ± 57	-	<LOQ (107)	106 ± 20	-	74 ± 18
K22.0/C0	108 ± 31	<LOD (18)	<LOD (18)	524 ± 81	508 ± 72	175 ± 68	182 ± 32	141 ± 26	87 ± 5
Cooling									
	Top			Top			Top		
C15	<LOQ (59)			303 ± 28			159 ± 22		
C30	<LOQ (59)			284 ± 59			152 ± 23		
C40	<LOQ (59)			200 ± 44			151 ± 14		
C50	<LOQ (59)			243 ± 67			156 ± 12		
Finished product									
Malt	71 ± 11			380 ± 39			176 ± 16		

Table A4-3. Quantification of free aldehydes in samples derived from different stages of the industrial-scale malting process K.

Free aldehydes (µg/kg dm)	HEX			T2N		
Raw material						
Barley	<LOD (47)			<LOD (37)		
Germination						
GM0	<LOD (47)			<LOD (37)		
GM1	<LOQ (157)			<LOD (37)		
GM2	<LOQ (157)			<LOQ (123)		
GM3	172 ± 26			<LOQ (123)		
GM4	337 ± 73			<LOQ (123)		
Kilning						
	Bottom	Middle	Top	Bottom	Middle	Top
K12.0	509 ± 74	570 ± 61	310 ± 50	<LOQ (123)	<LOQ (123)	<LOQ (123)
K14.0	587 ± 5	571 ± 26	345 ± 50	<LOQ (123)	<LOQ (123)	<LOQ (123)
K16.0	463 ± 60	575 ± 25	356 ± 51	<LOQ (123)	<LOQ (123)	<LOQ (123)
K18.0	278 ± 50	356 ± 28	385 ± 32	<LOQ (123)	<LOQ (123)	<LOQ (123)
K18.5	302 ± 42	317 ± 48	257 ± 31	<LOQ (123)	<LOQ (123)	<LOQ (123)
K19.0	541 ± 85	365 ± 68	224 ± 30	<LOQ (123)	<LOQ (123)	<LOQ (123)
K19.5	500 ± 74	535 ± 32	294 ± 43	<LOQ (123)	<LOQ (123)	<LOQ (123)
K20.0	481 ± 51	440 ± 101	367 ± 46	129 ± 15	<LOQ (123)	<LOQ (123)
K20.5	443 ± 69	434 ± 42	294 ± 47	137 ± 19	127 ± 16	<LOQ (123)
K21.0	465 ± 76	447 ± 53	228 ± 28	155 ± 25	126 ± 19	<LOQ (123)
K21.5	486 ± 62	-	253 ± 34	194 ± 21	126 ± 17	<LOQ (123)
K22.0/C0	301 ± 71	441 ± 56	245 ± 37	215 ± 27	165 ± 27	126 ± 34
Cooling						
	Top			Top		
C15	366 ± 43			178 ± 31		
C30	305 ± 43			161 ± 26		
C40	271 ± 37			141 ± 10		
C50	319 ± 80			136 ± 12		
Finished product						
Malt	191 ± 29			124 ± 15		

Table A4-4. Quantification of cysteinylated aldehydes in samples derived from different stages of the industrial-scale malting process K.

Cysteinylated aldehydes (µg/kg dm)	2MP-CYS			2MB-CYS			3MB-CYS		
Raw material									
Barley	<LOD (25)			<LOD (5)			<LOD (24)		
Germination									
GM0	<LOD (25)			<LOD (5)			<LOD (24)		
GM1	<LOD (25)			<LOD (5)			<LOD (24)		
GM2	<LOD (25)			<LOD (5)			<LOD (24)		
GM3	<LOD (25)			<LOD (5)			<LOD (24)		
GM4	<LOD (25)			<LOD (5)			<LOD (24)		
Kilning									
	Bottom	Middle	Top	Bottom	Middle	Top	Bottom	Middle	Top
K12.0	<LOQ (83)	<LOD (25)	<LOD (25)	<LOD (5)	<LOD (5)	<LOD (5)	<LOD (24)	<LOD (24)	<LOD (24)
K14.0	<LOQ (83)	<LOD (25)	<LOD (25)	<LOD (5)	<LOD (5)	<LOD (5)	<LOD (24)	<LOD (24)	<LOD (24)
K16.0	<LOQ (83)	<LOD (25)	<LOD (25)	<LOD (5)	<LOD (5)	<LOD (5)	<LOD (24)	<LOQ (48)	<LOD (24)
K18.0	<LOQ (83)	<LOD (25)	<LOD (25)	<LOQ (17)	<LOD (5)	<LOD (5)	<LOQ (48)	<LOQ (48)	<LOD (24)
K18.5	<LOQ (83)	<LOQ (83)	<LOD (25)	<LOQ (17)	<LOQ (17)	<LOD (5)	<LOQ (48)	<LOQ (48)	<LOD (24)
K19.0	<LOQ (83)	<LOQ (83)	<LOD (25)	<LOQ (17)	<LOQ (17)	<LOD (5)	<LOQ (48)	<LOQ (48)	<LOD (24)
K19.5	<LOQ (83)	<LOQ (83)	<LOD (25)	<LOQ (17)	<LOQ (17)	<LOD (5)	<LOQ (48)	<LOQ (48)	<LOD (24)
K20.0	<LOQ (83)	<LOQ (83)	<LOD (25)	<LOQ (17)	<LOQ (17)	<LOD (5)	52 ± 3	<LOQ (48)	<LOQ (48)
K20.5	<LOQ (83)	<LOQ (83)	<LOD (25)	19 ± 1	20 ± 2	<LOD (5)	68 ± 10	78 ± 2	<LOQ (48)
K21.0	<LOQ (83)	<LOQ (83)	<LOD (25)	20 ± 2	22 ± 4	<LOD (5)	74 ± 2	85 ± 9	<LOQ (48)
K21.5	<LOQ (83)	<LOQ (83)	<LOD (25)	29 ± 1	27 ± 3	<LOQ (17)	123 ± 12	93 ± 5	<LOQ (48)
K22.0/C0	<LOQ (83)	<LOQ (83)	<LOD (25)	38 ± 4	44 ± 3	<LOQ (17)	180 ± 6	202 ± 7	53 ± 3
Cooling									
	Top			Top			Top		
C15	111 ± 26			25 ± 1			109 ± 8		
C30	127 ± 9			23 ± 1			97 ± 5		
C40	153 ± 18			21 ± 2			89 ± 5		
C50	199 ± 30			20 ± 2			87 ± 5		
Finished product									
Malt	92 ± 6			36 ± 3			218 ± 20		

Table A4-5. Quantification of cysteinylated aldehydes in samples derived from different stages of the industrial-scale malting process K.

Cysteinylated aldehydes (µg/kg dm)	MET-CYS			PHE-CYS			HEX-CYS		
Raw material									
Barley	<LOD (7)			<LOD (11)			<LOD (14)		
Germination									
GM0	<LOD (7)			<LOD (11)			<LOD (14)		
GM1	<LOD (7)			<LOD (11)			<LOD (14)		
GM2	<LOD (7)			<LOD (11)			<LOQ (47)		
GM3	<LOD (7)			<LOD (11)			<LOQ (47)		
GM4	<LOD (7)			<LOD (11)			<LOQ (47)		
Kilning									
	Bottom	Middle	Top	Bottom	Middle	Top	Bottom	Middle	Top
K12.0	<LOD (7)	<LOD (7)	<LOD (7)	<LOD (11)	<LOD (11)	<LOD (11)	<LOD (14)	<LOD (14)	<LOQ (47)
K14.0	<LOD (7)	<LOD (7)	<LOD (7)	<LOD (11)	<LOD (11)	<LOD (11)	<LOD (14)	<LOD (14)	<LOQ (47)
K16.0	<LOD (7)	<LOD (7)	<LOD (7)	<LOD (11)	<LOQ (36)	<LOD (11)	<LOD (14)	<LOD (14)	<LOQ (47)
K18.0	<LOD (7)	<LOD (7)	<LOD (7)	<LOQ (36)	<LOQ (36)	<LOD (11)	<LOD (14)	<LOQ (47)	<LOQ (47)
K18.5	<LOD (7)	<LOD (7)	<LOD (7)	<LOQ (36)	<LOQ (36)	<LOD (11)	<LOD (14)	<LOQ (47)	<LOQ (47)
K19.0	<LOD (7)	<LOD (7)	<LOD (7)	<LOQ (36)	<LOQ (36)	<LOD (11)	<LOQ (47)	<LOQ (47)	<LOQ (47)
K19.5	<LOD (7)	<LOD (7)	<LOD (7)	37 ± 3	<LOQ (36)	<LOD (11)	<LOQ (47)	<LOQ (47)	<LOQ (47)
K20.0	<LOD (7)	<LOD (7)	<LOD (7)	45 ± 1	38 ± 3	<LOQ (36)	<LOQ (47)	<LOQ (47)	<LOQ (47)
K20.5	<LOQ (25)	<LOD (7)	<LOD (7)	50 ± 4	49 ± 4	<LOQ (36)	<LOQ (47)	<LOQ (47)	<LOQ (47)
K21.0	<LOQ (25)	<LOD (7)	<LOD (7)	64 ± 5	50 ± 2	<LOQ (36)	<LOQ (47)	<LOQ (47)	<LOQ (47)
K21.5	<LOQ (25)	<LOQ (25)	<LOD (7)	77 ± 6	71 ± 1	37 ± 2	<LOQ (47)	<LOQ (47)	<LOQ (47)
K22.0/C0	32 ± 2	<LOQ (25)	<LOD (7)	102 ± 2	101 ± 2	41 ± 2	<LOQ (47)	48 ± 1	<LOQ (47)
Cooling									
	Top			Top			Top		
C15	25 ± 1			63 ± 5			<LOQ (47)		
C30	21 ± 1			57 ± 3			<LOQ (47)		
C40	20 ± 2			58 ± 3			<LOQ (47)		
C50	22 ± 1			54 ± 2			<LOQ (47)		
Finished product									
Malt	27 ± 2			110 ± 7			65 ± 4		

APPENDIX B TO CHAPTER 4

Quantification of free and cysteinylated aldehydes in samples derived from different stages of the industrial-scale malting process L

Compounds: 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, T2N = *trans*-2 nonenal, 2MP-CYS = cysteinylated 2-methylpropanal, 2MB-CYS = cysteinylated 2-methylbutanal, 3MB-CYS = cysteinylated 3-methylbutanal, MET-CYS = cysteinylated methional, PHE-CYS = cysteinylated phenylacetaldehyde, FUR-CYS = cysteinylated furfural (<LOD), and HEX-CYS = cysteinylated hexanal (cysteinylated *trans*-2-nonenal is not presented as the reference compound was not available).

Analysed samples: barley; GM = germinating barley (GM0 – onset of germination; GM1-GM4 = germinated for 1, 2, 3, 4 days, respectively); K = samples taken at kilning, after 12 up to 22h of kilning; C = samples taken at cooling, after 0 min to 50 min; FIN = finished malt (without rootlets). During kilning samples were collected from top, middle and bottom grain bed layer, while during cooling samples were taken from top layer.

Results are expressed as mean values (n=3) ± standard deviation. LOD = limit of detection; LOQ = limit of quantification.

Table B4-1. Quantification of free aldehydes in samples derived from different stages of the industrial-scale malting process L.

Free aldehydes (µg/kg dm)	2MP			2MB			3MB		
Barley	<LOD (35)			<LOD (30)			<LOD (136)		
GM0	<LOD (35)			<LOD (30)			<LOD (136)		
GM1	<LOD (35)			<LOD (30)			<LOD (136)		
GM2	<LOQ (117)			<LOD (30)			<LOD (136)		
GM3	125 ± 16			<LOD (30)			<LOD (136)		
GM4	149 ± 16			<LOD (30)			<LOD (136)		
	Bottom	Middle	Top	Bottom	Middle	Top	Bottom	Middle	Top
K12.5	231 ± 15	<LOQ (117)	<LOQ (117)	152 ± 11	<LOD (30)	<LOD (30)	<LOQ (454)	<LOQ (454)	<LOD (136)
K13.0	261 ± 15	<LOQ (117)	<LOQ (117)	179 ± 12	<LOD (30)	<LOD (30)	490 ± 48	<LOQ (454)	<LOD (136)
K13.5	301 ± 33	<LOQ (117)	<LOQ (117)	218 ± 13	<LOD (30)	<LOD (30)	551 ± 51	<LOQ (454)	<LOD (136)
K14.0	383 ± 30	134 ± 28	<LOQ (117)	301 ± 39	<LOQ (99)	<LOD (30)	757 ± 40	<LOQ (454)	<LOD (136)
K14.5	389 ± 9	104 ± 24	<LOQ (117)	295 ± 18	<LOQ (99)	<LOD (30)	694 ± 116	<LOQ (454)	<LOD (136)
K15.0	591 ± 10	387 ± 14	<LOQ (117)	478 ± 34	323 ± 10	<LOD (30)	998 ± 51	798 ± 22	<LOD (136)
K15.5	609 ± 2	401 ± 17	<LOQ (117)	496 ± 40	327 ± 16	<LOD (30)	1,026 ± 78	803 ± 47	<LOD (136)
K16.0	689 ± 6	391 ± 46	<LOQ (117)	587 ± 48	328 ± 34	<LOD (30)	1,219 ± 106	805 ± 89	<LOD (136)
K16.5	704 ± 12	525 ± 87	<LOQ (117)	618 ± 40	478 ± 78	<LOD (30)	1,312 ± 104	1,083 ± 159	<LOD (136)
K17.0	834 ± 34	606 ± 97	<LOQ (117)	743 ± 44	388 ± 81	<LOD (30)	1,463 ± 84	1,113 ± 200	<LOD (136)
K17.5	780 ± 57	495 ± 63	<LOQ (117)	713 ± 42	431 ± 89	<LOD (30)	1,362 ± 72	914 ± 194	<LOD (136)
K18.0	877 ± 51	472 ± 41	<LOQ (117)	727 ± 189	401 ± 44	<LOD (30)	1,804 ± 96	889 ± 157	<LOD (136)
K18.5	1,034 ± 150	892 ± 43	<LOQ (117)	988 ± 225	810 ± 51	<LOQ (99)	2,214 ± 218	1,512 ± 112	<LOQ (454)
K19.0	1,248 ± 49	863 ± 32	196 ± 28	1,338 ± 107	840 ± 34	159 ± 12	2,748 ± 406	1,463 ± 48	<LOQ (454)
K19.5	1,367 ± 183	959 ± 56	257 ± 39	1,695 ± 274	875 ± 56	244 ± 41	3,288 ± 166	1,493 ± 67	490 ± 76
K20.0	1,455 ± 195	1,290 ± 56	298 ± 17	1,857 ± 124	1,324 ± 28	269 ± 22	3,835 ± 187	2,105 ± 33	549 ± 43
K21.5	1,572 ± 156	1,586 ± 131	349 ± 50	1,914 ± 307	1,634 ± 113	336 ± 43	4,147 ± 265	2,526 ± 125	663 ± 89
	Top			Top			Top		
C0	927 ± 114			858 ± 116			1,398 ± 155		
C10	690 ± 76			620 ± 110			1,049 ± 201		
C20	840 ± 73			743 ± 14			1,255 ± 36		
C30	1,002 ± 140			884 ± 144			1,437 ± 218		
C40	1,060 ± 160			1,036 ± 144			1,563 ± 266		
Malt	1,270 ± 234			1,252 ± 263			2,033 ± 138		

Table B4-2. Quantification of free aldehydes in samples derived from different stages of the industrial-scale malting process L.

Free aldehydes (µg/kg dm)	MET			PHE			FUR		
Barley	<LOD (18)			<LOD (32)			<LOD (17)		
GM0	<LOD (18)			<LOD (32)			<LOD (17)		
GM1	<LOD (18)			<LOD (32)			<LOD (17)		
GM2	<LOD (18)			<LOD (32)			<LOD (17)		
GM3	<LOD (18)			<LOD (32)			<LOD (17)		
GM4	<LOD (18)			<LOD (32)			<LOD (17)		
	Bottom	Middle	Top	Bottom	Middle	Top	Bottom	Middle	Top
K12.5	<LOD (18)	<LOD (18)	<LOD (18)	<LOQ (107)	<LOQ (107)	<LOD (32)	58 ± 12	<LOQ (58)	<LOD (17)
K13.0	<LOD (18)	<LOD (18)	<LOD (18)	<LOQ (107)	<LOQ (107)	<LOD (32)	97 ± 20	<LOQ (58)	<LOD (17)
K13.5	<LOD (18)	<LOD (18)	<LOD (18)	<LOQ (107)	<LOQ (107)	<LOD (32)	85 ± 8	<LOQ (58)	<LOD (17)
K14.0	<LOD (18)	<LOD (18)	<LOD (18)	347 ± 72	<LOQ (107)	<LOD (32)	141 ± 31	<LOQ (58)	<LOD (17)
K14.5	<LOD (18)	<LOD (18)	<LOD (18)	332 ± 48	<LOQ (107)	<LOD (32)	129 ± 16	<LOQ (58)	<LOD (17)
K15.0	<LOD (18)	<LOD (18)	<LOD (18)	412 ± 60	488 ± 11	<LOD (32)	188 ± 98	<LOQ (58)	<LOD (17)
K15.5	<LOD (18)	<LOD (18)	<LOD (18)	378 ± 71	466 ± 42	<LOD (32)	180 ± 101	<LOQ (58)	<LOD (17)
K16.0	<LOD (18)	<LOD (18)	<LOD (18)	459 ± 72	419 ± 41	<LOD (32)	218 ± 59	207 ± 23	<LOD (17)
K16.5	<LOD (18)	<LOD (18)	<LOD (18)	511 ± 63	522 ± 60	<LOD (32)	243 ± 87	205 ± 37	<LOQ (58)
K17.0	<LOD (18)	<LOD (18)	<LOD (18)	675 ± 35	640 ± 140	<LOD (32)	271 ± 53	149 ± 58	<LOQ (58)
K17.5	<LOD (18)	<LOD (18)	<LOD (18)	577 ± 40	446 ± 140	<LOD (32)	233 ± 53	139 ± 44	<LOQ (58)
K18.0	<LOD (18)	<LOD (18)	<LOD (18)	723 ± 54	448 ± 136	<LOD (32)	323 ± 68	162 ± 5	<LOQ (58)
K18.5	<LOQ (59)	<LOD (18)	<LOD (18)	974 ± 187	717 ± 138	<LOD (32)	468 ± 102	218 ± 36	<LOQ (58)
K19.0	<LOQ (59)	<LOQ (59)	<LOD (18)	1,276 ± 189	800 ± 159	<LOQ (107)	558 ± 117	271 ± 31	<LOQ (58)
K19.5	<LOQ (59)	<LOQ (59)	<LOD (18)	1,703 ± 82	919 ± 135	<LOQ (107)	755 ± 99	307 ± 28	<LOQ (58)
K20.0	<LOQ (59)	<LOQ (59)	<LOD (18)	1,788 ± 137	1,262 ± 74	315 ± 18	790 ± 58	416 ± 60	<LOQ (58)
K21.5	<LOQ (59)	<LOQ (59)	<LOD (18)	1,940 ± 43	1,690 ± 135	275 ± 41	914 ± 84	558 ± 50	<LOQ (58)
	Top			Top			Top		
C0	158 ± 46			847 ± 161			541 ± 25		
C10	75 ± 37			455 ± 144			286 ± 58		
C20	105 ± 32			638 ± 54			413 ± 55		
C30	125 ± 15			758 ± 85			434 ± 23		
C40	196 ± 46			1,086 ± 198			599 ± 82		
Malt	312 ± 46			1,493 ± 362			705 ± 135		

Table B4-3. Quantification of free aldehydes in samples derived from different stages of the industrial-scale malting process L.

Free aldehydes (µg/kg dm)	HEX			T2N		
Barley	<LOD (47)			<LOD (37)		
GM0	<LOD (47)			<LOD (37)		
GM1	<LOQ (157)			<LOD (37)		
GM2	<LOQ (157)			<LOQ (123)		
GM3	161 ± 31			<LOQ (123)		
GM4	215 ± 42			<LOQ (123)		
	Bottom	Middle	Top	Bottom	Middle	Top
K12.5	557 ± 59	462 ± 88	516 ± 116	<LOQ (123)	<LOQ (123)	<LOQ (123)
K13.0	642 ± 84	691 ± 168	461 ± 28	<LOQ (123)	<LOQ (123)	<LOQ (123)
K13.5	673 ± 112	648 ± 79	602 ± 43	<LOQ (123)	<LOQ (123)	<LOQ (123)
K14.0	752 ± 111	730 ± 160	397 ± 131	<LOQ (123)	<LOQ (123)	<LOQ (123)
K14.5	674 ± 91	659 ± 193	468 ± 105	<LOQ (123)	<LOQ (123)	<LOQ (123)
K15.0	531 ± 98	812 ± 101	518 ± 50	<LOQ (123)	<LOQ (123)	<LOQ (123)
K15.5	527 ± 101	731 ± 140	1,011 ± 107	<LOQ (123)	<LOQ (123)	<LOQ (123)
K16.0	496 ± 59	800 ± 94	961 ± 123	<LOQ (123)	<LOQ (123)	<LOQ (123)
K16.5	499 ± 87	884 ± 148	1,101 ± 85	<LOQ (123)	<LOQ (123)	<LOQ (123)
K17.0	494 ± 53	831 ± 149	789 ± 139	<LOQ (123)	<LOQ (123)	<LOQ (123)
K17.5	402 ± 53	572 ± 42	811 ± 99	<LOQ (123)	<LOQ (123)	<LOQ (123)
K18.0	488 ± 68	244 ± 42	636 ± 107	<LOQ (123)	<LOQ (123)	<LOQ (123)
K18.5	697 ± 102	243 ± 50	275 ± 69	109 ± 18	<LOQ (123)	<LOQ (123)
K19.0	808 ± 117	453 ± 66	371 ± 75	178 ± 42	126 ± 36	<LOQ (123)
K19.5	867 ± 99	403 ± 50	328 ± 137	234 ± 48	195 ± 41	151 ± 26
K20.0	634 ± 58	402 ± 70	348 ± 73	273 ± 44	245 ± 49	185 ± 20
K21.5	553 ± 84	400 ± 57	401 ± 26	326 ± 55	328 ± 44	196 ± 26
	Top			Top		
C0	257 ± 128			345 ± 46		
C10	265 ± 72			234 ± 19		
C20	274 ± 52			232 ± 29		
C30	159 ± 69			201 ± 19		
C40	396 ± 49			220 ± 16		
Malt	358 ± 42			244 ± 43		

Table B4-4. Quantification of cysteinylated aldehydes in samples derived from different stages of the industrial-scale malting process L.

Cysteinylated aldehydes (µg/kg dm)	2MP-CYS			2MB-CYS			3MB-CYS		
Barley	<LOD (25)			<LOD (5)			<LOD (24)		
GM0	<LOD (25)			<LOD (5)			<LOD (24)		
GM1	<LOD (25)			<LOD (5)			<LOD (24)		
GM2	<LOD (25)			<LOD (5)			<LOD (24)		
GM3	<LOD (25)			<LOD (5)			<LOD (24)		
GM4	<LOD (25)			<LOD (5)			<LOD (24)		
	Bottom	Middle	Top	Bottom	Middle	Top	Bottom	Middle	Top
K12.5	<LOD (25)	<LOD (25)	<LOD (25)	<LOD (5)	<LOD (5)	<LOD (5)	<LOQ (48)	<LOD (24)	<LOD (24)
K13.0	<LOD (25)	<LOD (25)	<LOD (25)	<LOD (5)	<LOD (5)	<LOD (5)	<LOQ (48)	<LOQ (48)	<LOD (24)
K13.5	<LOD (25)	<LOD (25)	<LOD (25)	68 ± 5	<LOD (5)	<LOD (5)	<LOQ (48)	<LOQ (48)	<LOD (24)
K14.0	<LOQ (83)	<LOD (25)	<LOD (25)	70 ± 6	<LOD (5)	<LOD (5)	56 ± 3	<LOQ (48)	<LOD (24)
K14.5	<LOQ (83)	<LOQ (83)	<LOD (25)	77 ± 5	<LOD (5)	<LOD (5)	66 ± 3	<LOQ (48)	<LOD (24)
K15.0	<LOQ (83)	<LOQ (83)	<LOD (25)	83 ± 8	<LOD (5)	<LOD (5)	101 ± 9	56 ± 5	<LOD (24)
K15.5	<LOQ (83)	<LOQ (83)	<LOD (25)	78 ± 6	<LOD (5)	<LOD (5)	126 ± 10	73 ± 6	<LOD (24)
K16.0	92 ± 7	<LOQ (83)	<LOD (25)	89 ± 4	<LOD (5)	<LOD (5)	153 ± 6	78 ± 3	<LOD (24)
K16.5	95 ± 1	<LOQ (83)	<LOD (25)	96 ± 6	<LOD (5)	<LOD (5)	176 ± 3	115 ± 5	<LOD (24)
K17.0	108 ± 4	103 ± 4	<LOD (25)	101 ± 4	<LOD (5)	<LOD (5)	187 ± 12	178 ± 6	<LOD (24)
K17.5	114 ± 6	150 ± 6	<LOD (25)	87 ± 1	<LOD (5)	<LOD (5)	203 ± 12	263 ± 7	<LOD (24)
K18.0	138 ± 2	140 ± 4	<LOD (25)	99 ± 4	50 ± 6	<LOD (5)	243 ± 30	283 ± 9	<LOD (24)
K18.5	182 ± 2	215 ± 7	<LOD (25)	135 ± 6	77 ± 5	<LOD (5)	370 ± 16	410 ± 13	<LOD (24)
K19.0	265 ± 13	220 ± 9	<LOD (25)	164 ± 4	93 ± 10	<LOD (5)	661 ± 18	512 ± 30	51 ± 4
K19.5	281 ± 9	295 ± 9	<LOQ (83)	181 ± 4	115 ± 7	<LOQ (17)	701 ± 15	707 ± 23	61 ± 9
K20.0	349 ± 6	333 ± 13	<LOQ (83)	211 ± 7	140 ± 12	<LOQ (17)	921 ± 5	839 ± 27	117 ± 3
K21.5	372 ± 4	420 ± 8	<LOQ (83)	219 ± 13	179 ± 19	26 ± 3	1,008 ± 27	916 ± 29	161 ± 2
	Top			Top			Top		
C0	274 ± 18			49 ± 1			208 ± 15		
C10	248 ± 6			29 ± 2			141 ± 6		
C20	282 ± 7			33 ± 1			159 ± 4		
C30	336 ± 20			42 ± 1			191 ± 12		
C40	354 ± 6			46 ± 2			222 ± 11		
Malt	334 ± 4			100 ± 1			477 ± 21		

Table B4-5. Quantification of cysteinylated aldehydes in samples derived from different stages of the industrial-scale malting process L.

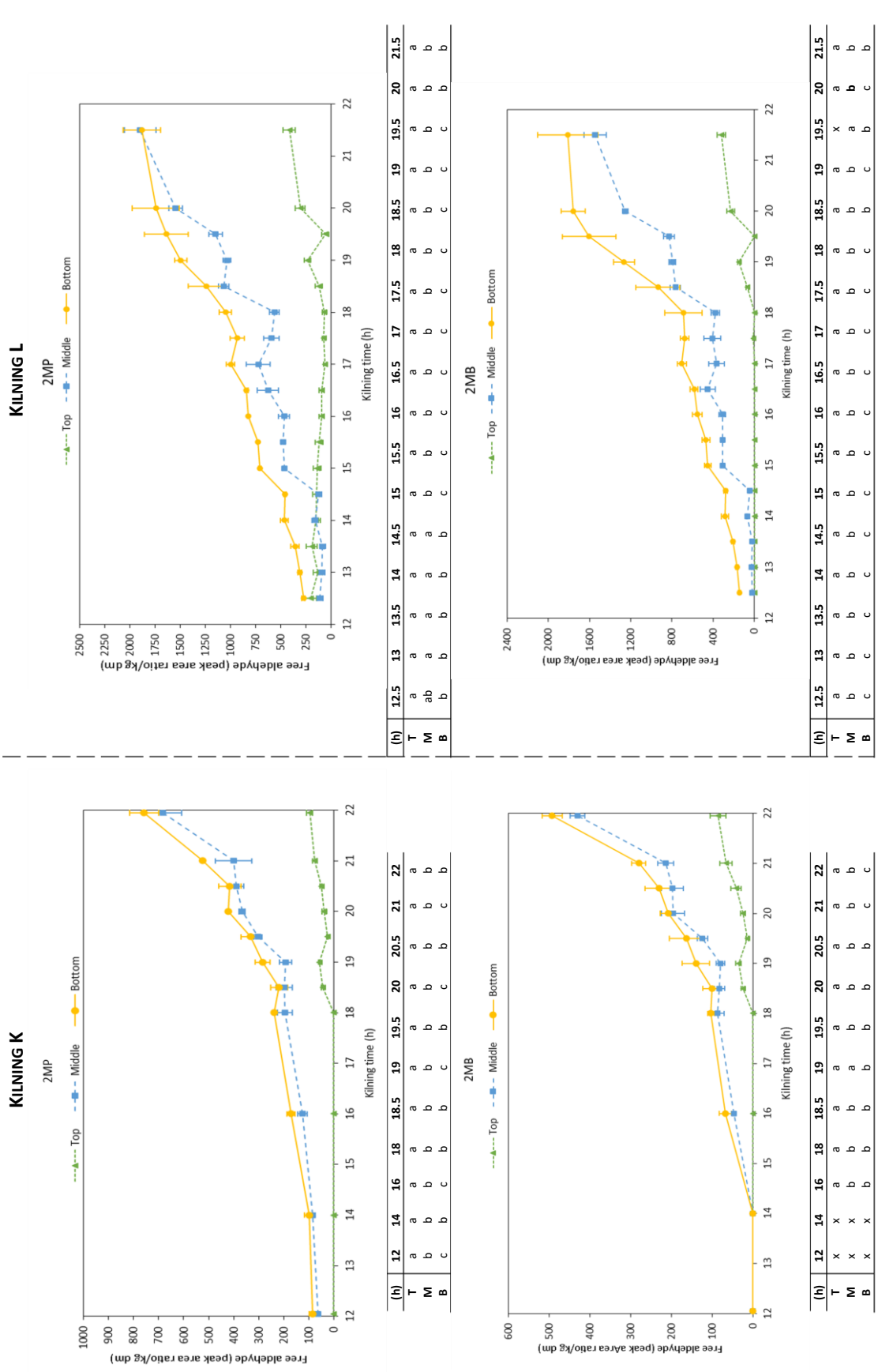
Cysteinylated aldehydes (µg/kg dm)	MET-CYS			PHE-CYS			HEX-CYS		
Barley	<LOD (7)			<LOD (11)			<LOD (14)		
GM0	<LOD (7)			<LOD (11)			<LOD (14)		
GM1	<LOD (7)			<LOD (11)			<LOD (14)		
GM2	<LOD (7)			<LOD (11)			<LOD (14)		
GM3	<LOD (7)			<LOD (11)			<LOD (14)		
GM4	<LOD (7)			<LOD (11)			<LOD (14)		
	Bottom	Middle	Top	Bottom	Middle	Top	Bottom	Middle	Top
K12.5	<LOD (7)	<LOD (7)	<LOD (7)	39 ± 5	<LOD (11)	<LOD (11)	<LOQ (47)	<LOQ (47)	<LOD (14)
K13.0	<LOD (7)	<LOD (7)	<LOD (7)	36 ± 5	<LOQ (36)	<LOD (11)	<LOD (14)	61 ± 2	<LOD (14)
K13.5	<LOQ (25)	<LOQ (25)	<LOD (7)	43 ± 4	<LOQ (36)	<LOD (11)	<LOD (14)	67 ± 3	<LOD (14)
K14.0	<LOQ (25)	<LOQ (25)	<LOD (7)	56 ± 1	44 ± 1	<LOD (11)	<LOQ (47)	63 ± 4	<LOD (14)
K14.5	<LOQ (25)	<LOQ (25)	<LOD (7)	58 ± 3	51 ± 4	<LOD (11)	<LOQ (47)	79 ± 3	<LOD (14)
K15.0	<LOQ (25)	<LOQ (25)	<LOD (7)	77 ± 6	56 ± 2	<LOD (11)	<LOQ (47)	24 ± 1	<LOD (14)
K15.5	26 ± 4	±	<LOD (7)	92 ± 9	73 ± 3	<LOD (11)	<LOQ (47)	33 ± 1	<LOD (14)
K16.0	28 ± 5	±	<LOD (7)	104 ± 1	69 ± 3	<LOD (11)	<LOQ (47)	31 ± 1	<LOD (14)
K16.5	32 ± 2	±	<LOD (7)	102 ± 3	90 ± 7	<LOD (11)	<LOQ (47)	34 ± 2	<LOD (14)
K17.0	32 ± 5	±	<LOD (7)	123 ± 4	106 ± 2	<LOD (11)	<LOQ (47)	38 ± 2	<LOD (14)
K17.5	35 ± 2	±	<LOD (7)	120 ± 1	155 ± 5	<LOD (11)	<LOQ (47)	55 ± 4	<LOQ (47)
K18.0	40 ± 4	±	<LOD (7)	141 ± 5	172 ± 8	<LOD (11)	<LOQ (47)	52 ± 2	<LOQ (47)
K18.5	51 ± 5	±	<LOQ (25)	192 ± 13	207 ± 1	<LOQ (36)	<LOQ (47)	85 ± 3	<LOQ (47)
K19.0	73 ± 1	±	<LOQ (25)	249 ± 2	222 ± 13	50 ± 5	59 ± 2	72 ± 1	<LOQ (47)
K19.5	67 ± 3	±	<LOQ (25)	263 ± 3	308 ± 11	57 ± 9	62 ± 3	83 ± 4	<LOQ (47)
K20.0	88 ±4	±	<LOQ (25)	313 ± 9	337 ± 11	74 ± 8	55 ± 2	89 ± 3	<LOQ (47)
K21.5	89 ± 2	±	<LOQ (25)	353 ± 4	469 ± 20	86 ± 2	50 ± 1	97 ± 4	<LOQ (47)
	Top			Top			Top		
C0	38 ± 2			116 ± 2			<LOQ (47)		
C15	26 ± 3			106 ± 2			<LOQ (47)		
C20	27 ± 1			115 ± 1			<LOQ (47)		
C40	34 ± 2			138 ± 2			<LOQ (47)		
C50	39 ± 2			150 ± 3			<LOQ (47)		
Malt	65 ± 3			209 ± 3			<LOQ (47)		

APPENDIX C TO CHAPTER 4

Statistical evolution of free Strecker aldehydes as a function of kilning time in samples collected from the bottom, middle and top layer of the kiln - malting batch K, malting batch L.

Compounds: 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, and PHE = phenylacetaldehyde. Methional is not presented as not most of values were <LOD.

Results are expressed as mean values (n=3), error bars = standard deviation. Statistical comparisons between bottom, middle and top layer by post-hoc HSD Tukey's test to distinguish among significant different groups ($p \leq 0.05$) (a, b, c). x – statistical comparison not shown because quantification values are below LOD.



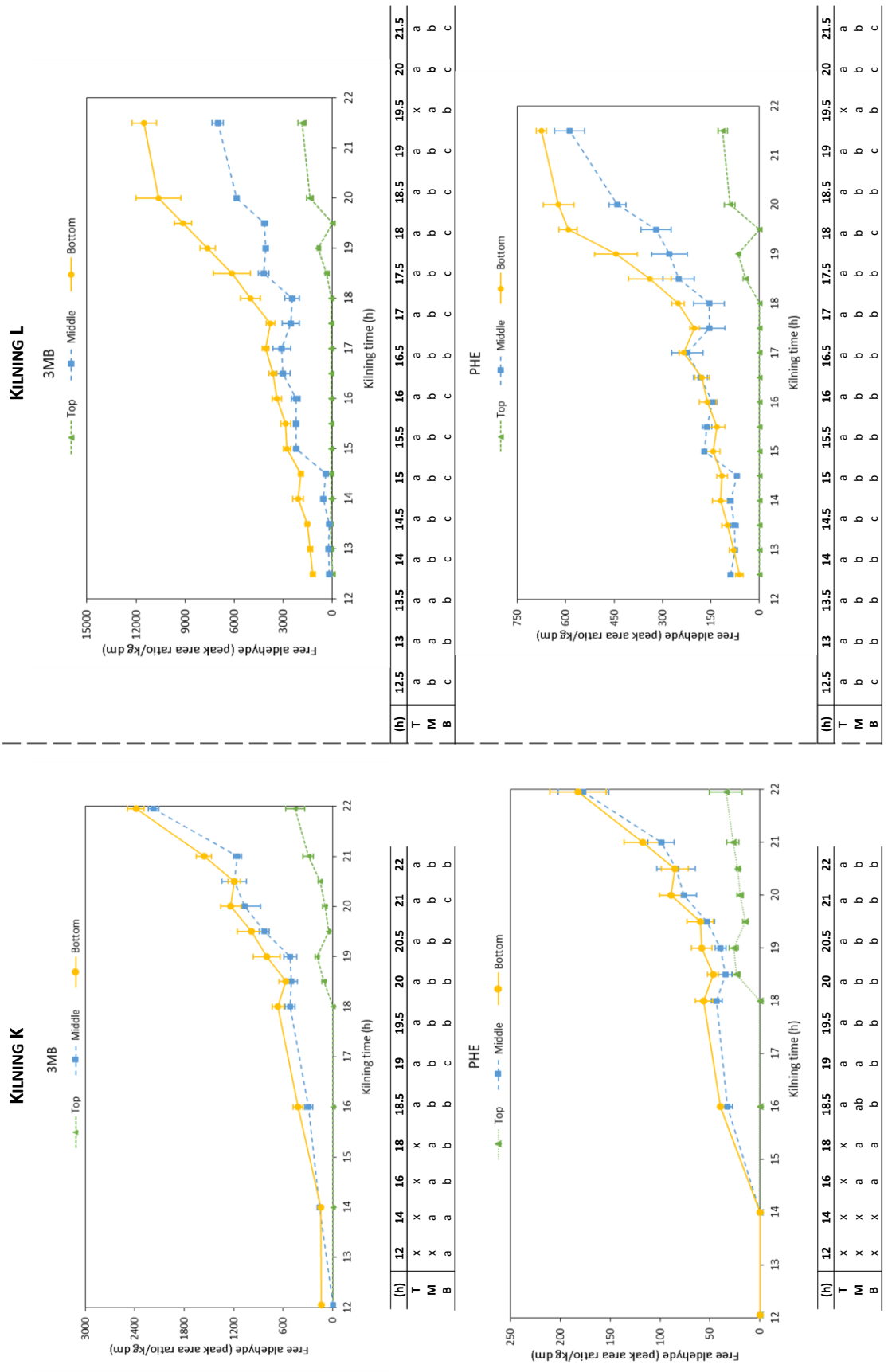
(h)	12.5	13	13.5	14	14.5	15	15.5	16	16.5	17	17.5	18	18.5	19	19.5	20	21.5
T	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
M	ab	a	a	a	a	b	b	b	b	b	b	b	b	b	b	b	b
B	b	b	b	b	b	c	c	c	c	c	c	c	c	c	c	b	b

(h)	12.5	13	13.5	14	14.5	15	15.5	16	16.5	17	17.5	18	18.5	19	19.5	20	21.5
T	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
M	b	b	b	b	b	b	b	b	b	b	b	b	b	b	a	b	b
B	c	c	c	c	c	c	c	c	c	c	c	c	b	c	b	c	b

(h)	12	14	16	18	18.5	19	19.5	20	20.5	21	22
T	x	x	a	a	a	a	a	a	a	a	a
M	x	x	b	b	b	a	b	b	b	b	b
B	x	x	b	b	b	b	b	b	b	c	c

(h)	12	14	16	18	18.5	19	19.5	20	20.5	21	22
T	x	x	a	a	a	a	a	a	a	a	a
M	x	x	b	b	b	a	b	b	b	b	b
B	x	x	b	b	b	b	b	b	b	c	c

Figure C4-1. Statistical evolution of free Strecker aldehydes as a function of kilning time in samples collected from the bottom (B), middle (M) and top (T) layer of the kiln - malting batch K (left side), malting batch L (right side).



(h)	12.5	13	13.5	14	14.5	15	15.5	16	16.5	17	17.5	18	18.5	19	19.5	20	21.5
T	a	a	a	a	a	a	a	a	a	a	a	a	a	a	x	a	a
M	b	a	a	b	b	b	b	b	b	b	b	b	b	b	a	**b**	b
B	c	b	b	c	c	c	c	c	b	c	c	c	c	c	b	c	c

(h)	12.5	13	13.5	14	14.5	15	15.5	16	16.5	17	17.5	18	18.5	19	19.5	20	21.5
T	a	a	a	a	a	a	a	a	a	a	a	a	a	a	x	a	a
M	b	b	b	b	b	b	b	b	b	b	b	b	b	b	a	b	b
B	c	b	b	b	c	b	b	b	b	b	b	c	b	c	b	c	c

(h)	12	14	16	18	18.5	19	19.5	20	20.5	21	22
T	x	x	x	x	a	a	a	a	a	a	a
M	x	x	a	a	ab	a	b	b	b	b	b
B	x	x	a	a	b	b	b	b	b	b	b

(h)	12	14	16	18	18.5	19	19.5	20	20.5	21	22
T	x	x	x	x	a	a	a	a	a	a	a
M	x	x	a	a	a	b	b	b	b	b	b
B	x	x	a	b	b	c	b	b	b	b	b

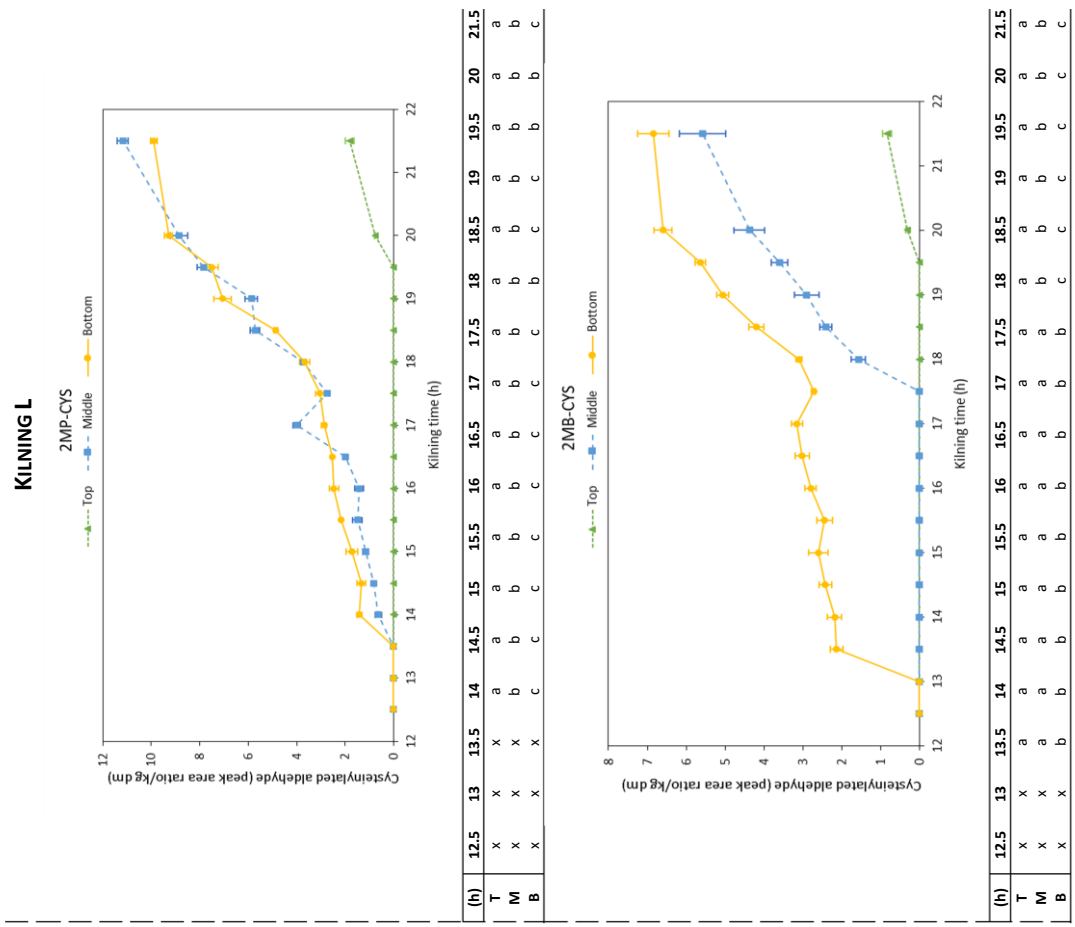
Figure C4-2. Statistical evolution of free Strecker aldehydes as a function of kilning time in samples collected from the bottom (B), middle (M) and top (T) layer of the kiln - malting batch K (left side), malting batch L (right side).

APPENDIX D TO CHAPTER 4

Statistical evolution of cysteinylated Strecker aldehydes as a function of kilning time in samples collected from the bottom, middle and top layer of the kiln - malting batch K, malting batch L.

Compounds: 2MP-CYS = cysteinylated 2-methylpropanal, 2MB-CYS= cysteinylated 2-methylbutanal, 3MB-CYS = cysteinylated 3-methylbutanal, and PHE-CYS = cysteinylated phenylacetaldehyde. Cysteinylated methional is not presented as not most of values were <LOD

Results are expressed as mean values (n=3), error bars = standard deviation. Statistical comparisons between bottom, middle and top layer by post-hoc HSD Tukey's test to distinguish among significant different groups ($p \leq 0.05$) (a, b, c). x – statistical comparison not shown because quantification values are below LOD.

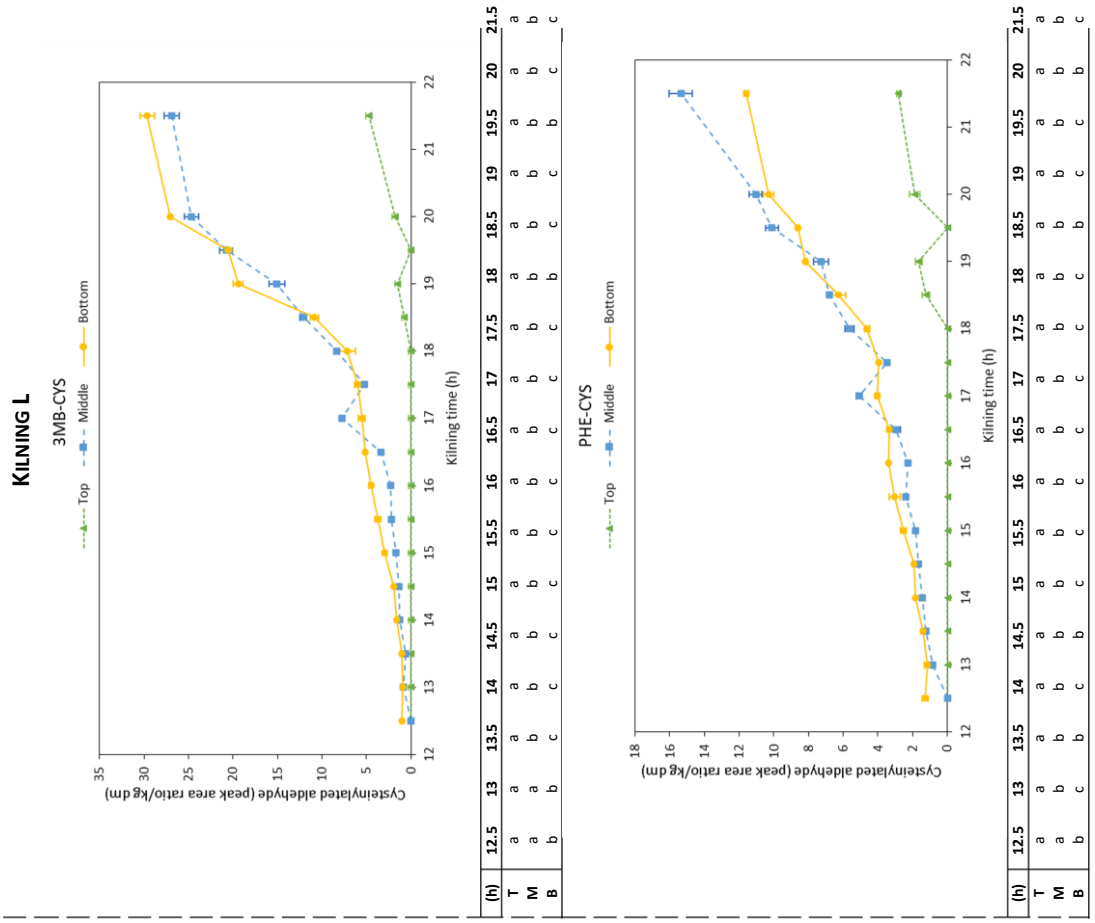


(h)	12.5	13	13.5	14	14.5	15	15.5	16	16.5	17	17.5	18	18.5	19	19.5	20	21.5
T	x	x	x	a	a	a	a	a	a	a	a	a	a	a	a	a	a
M	x	x	x	b	b	b	b	b	b	b	b	b	b	b	b	b	b
B	x	x	x	c	c	c	c	c	c	c	c	b	c	c	b	b	c

(h)	12	14	16	18	18.5	19	19.5	20	20.5	21	22
T	x	x	x	x	x	x	x	x	x	a	a
M	x	a	a	a	a	a	a	a	a	b	b
B	a	b	b	b	b	b	b	b	b	c	b

(h)	12	14	16	18	18.5	19	19.5	20	20.5	21	22
T	x	x	x	x	x	x	x	x	x	a	a
M	x	x	x	x	a	a	a	a	a	b	b
B	x	x	x	a	a	a	a	b	a	c	b

Figure D4-1. Statistical evolution of cysteinylated Strecker aldehydes as a function of kilning time in samples collected from the bottom (B), middle (M) and top (T) layer of the kiln - malting batch K (left side), malting batch L (right side).



(h)	12.5	13	13.5	14	14.5	15	15.5	16	16.5	17	17.5	18	18.5	19	19.5	20	21.5
T	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
M	a	a	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b
B	b	b	c	c	c	c	c	c	c	c	c	b	c	c	b	c	c

(h)	12.5	13	13.5	14	14.5	15	15.5	16	16.5	17	17.5	18	18.5	19	19.5	20	21.5
T	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
M	a	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b
B	b	c	b	c	b	c	c	c	c	c	c	c	b	c	c	b	c

(h)	12	14	16	18	18.5	19	19.5	20	20.5	21	22
T	x	x	x	x	x	x	x	a	a	a	a
M	x	x	x	a	a	a	a	b	b	b	b
B	a	a	a	b	b	b	b	c	b	c	c

(h)	12	14	16	18	18.5	19	19.5	20	20.5	21	22
T	x	x	x	x	x	x	x	a	a	a	a
M	x	x	x	a	a	a	a	b	b	b	b
B	x	x	x	b	b	a	a	b	b	b	b

Figure D4-2. Statistical evolution of cysteinylated Strecker aldehydes as a function of kilning time in samples collected from the bottom (B),

APPENDIX E TO CHAPTER 4

Evolution of free and cysteinylated Strecker aldehydes in relation the grain drying process and applied heat load during kilning. Samples collected from bottom, middle and top grain bed layer of batch K and batch L.

Compounds: 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, 2MP-CYS = cysteinylated 2-methylpropanal, 2MB-CYS= cysteinylated 2-methylbutanal, 3MB-CYS = cysteinylated 3-methylbutanal, MET-CYS = cysteinylated methional and PHE-CYS = cysteinylated phenylacetaldehyde.

Results are expressed as mean values (n=3 for aldehydes, n=2 for moisture content and TBI), error bars = standard deviation. Statistical comparisons between bottom, middle and top layer by post-hoc HSD Tukey's test to distinguish among significant different groups ($p \leq 0.05$) (a, b, c). x – statistical comparison not shown because quantification values are below LOD.

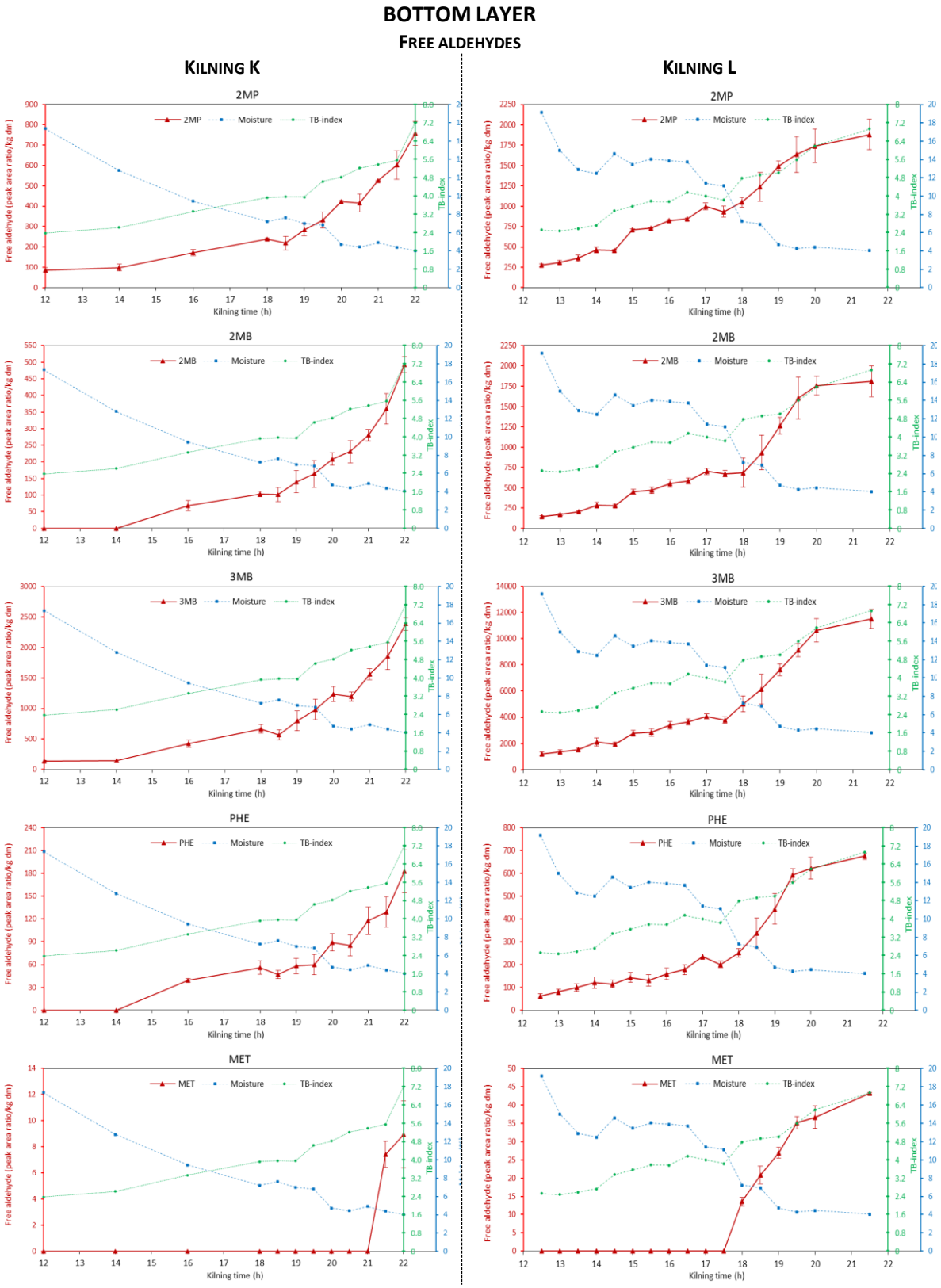


Figure E4-1. Evolution of free Strecker aldehydes in relation the grain drying process and applied heat load during kilning. Samples collected from the bottom layer.

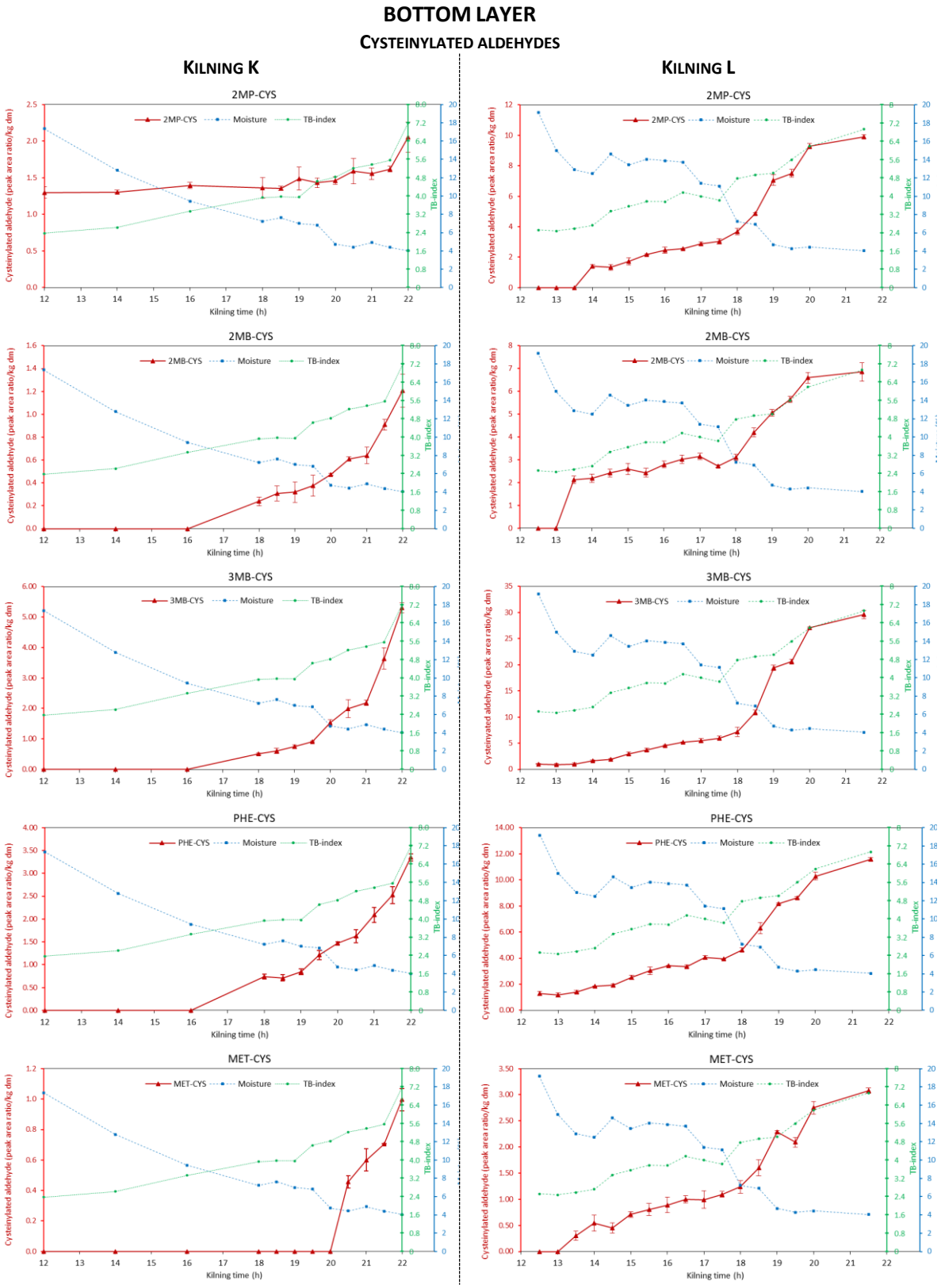


Figure E4-2. Evolution of cysteinylated Strecker aldehydes in relation the grain drying process and applied heat load during kilning. Samples collected from the bottom layer.

MIDDLE LAYER

FREE ALDEHYDES

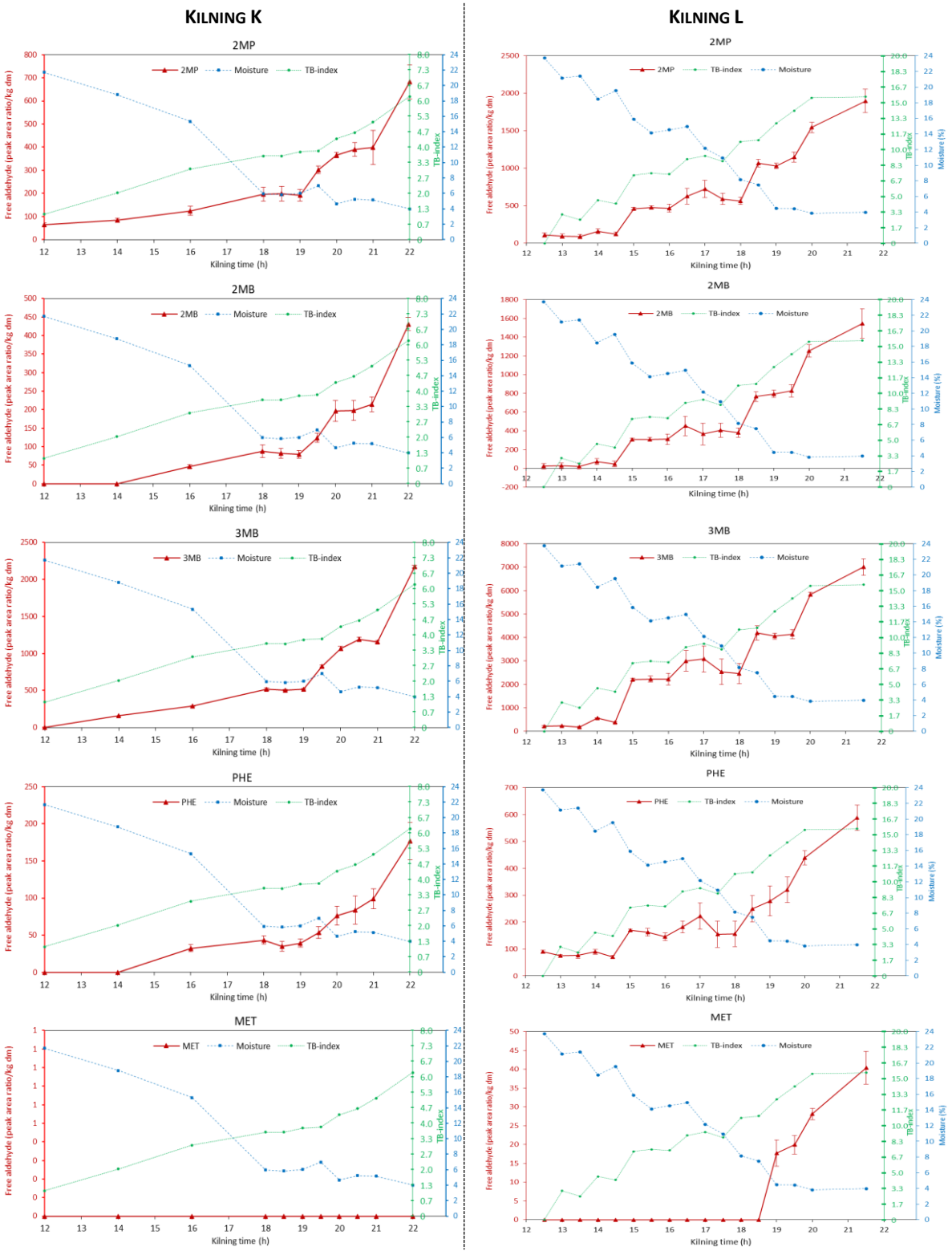


Figure E4-3 Evolution of free Strecker aldehydes in relation the grain drying process and applied heat load during kilning. Samples collected from the middle layer.

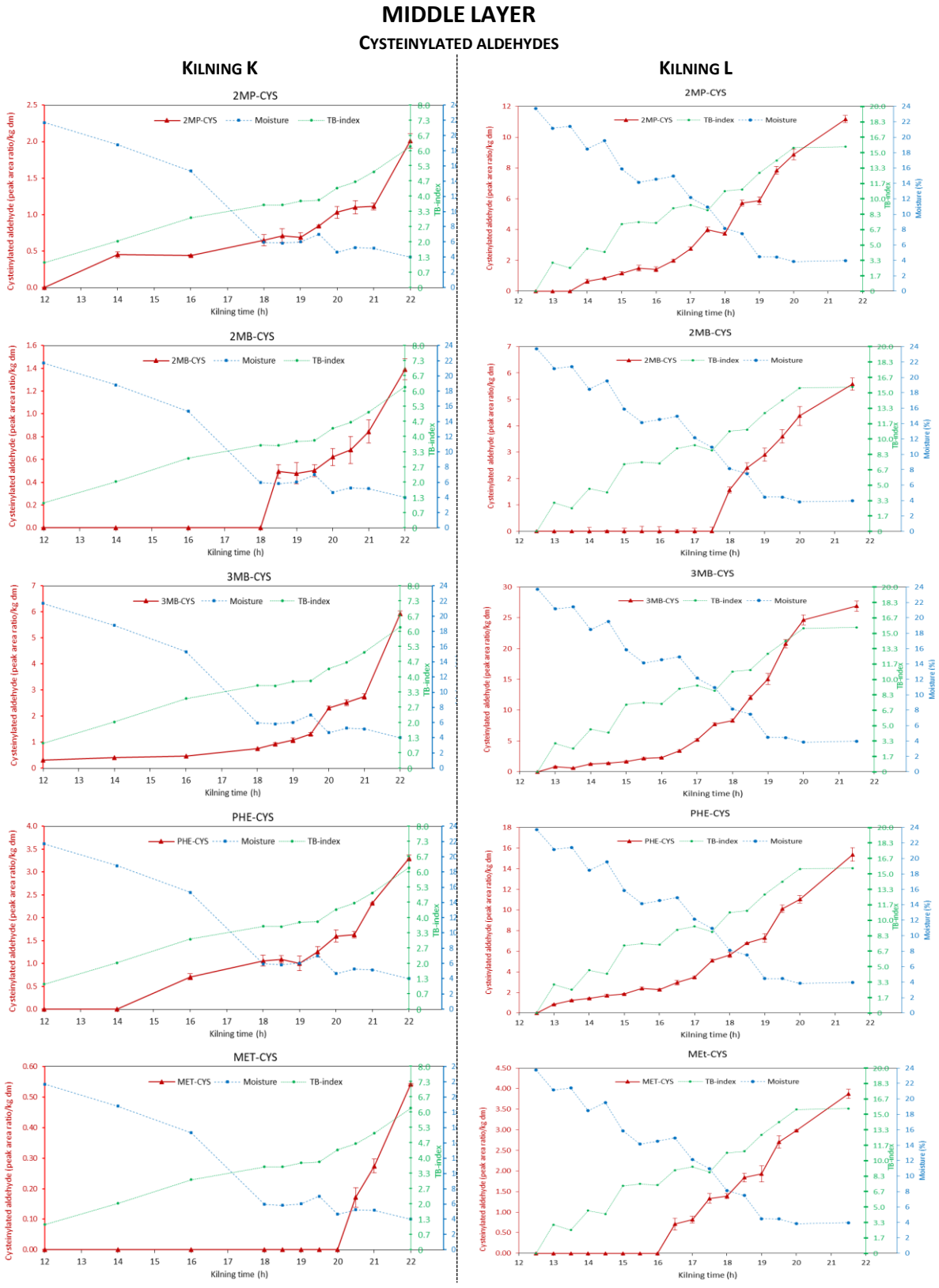


Figure E4-4 Evolution of cysteinylated Strecker aldehydes in relation the grain drying process and applied heat load during kilning. Samples collected from the middle layer.

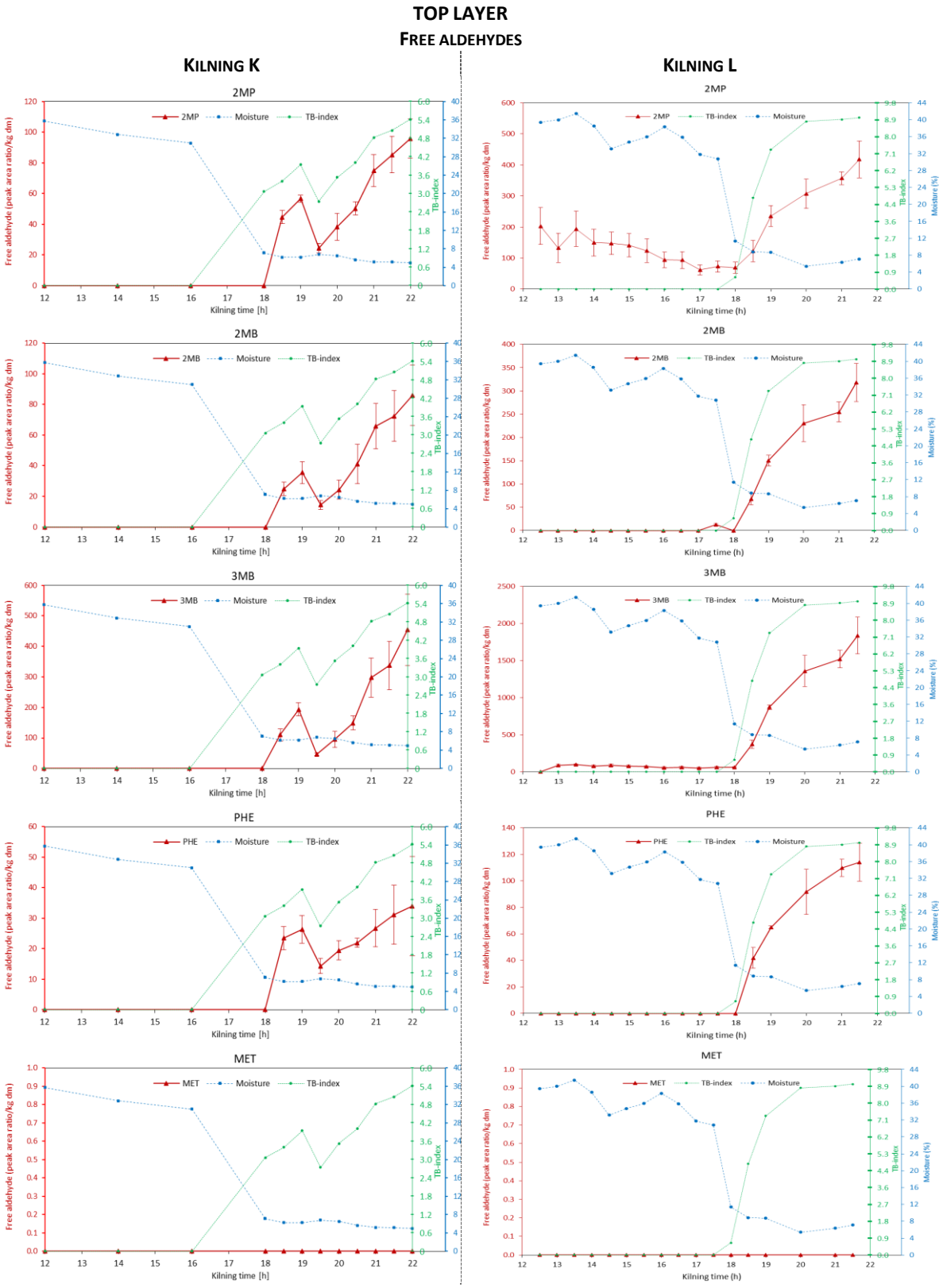


Figure E4-5 Evolution of free Strecker aldehydes in relation the grain drying process and applied heat load during kilning. Samples collected from the top layer.

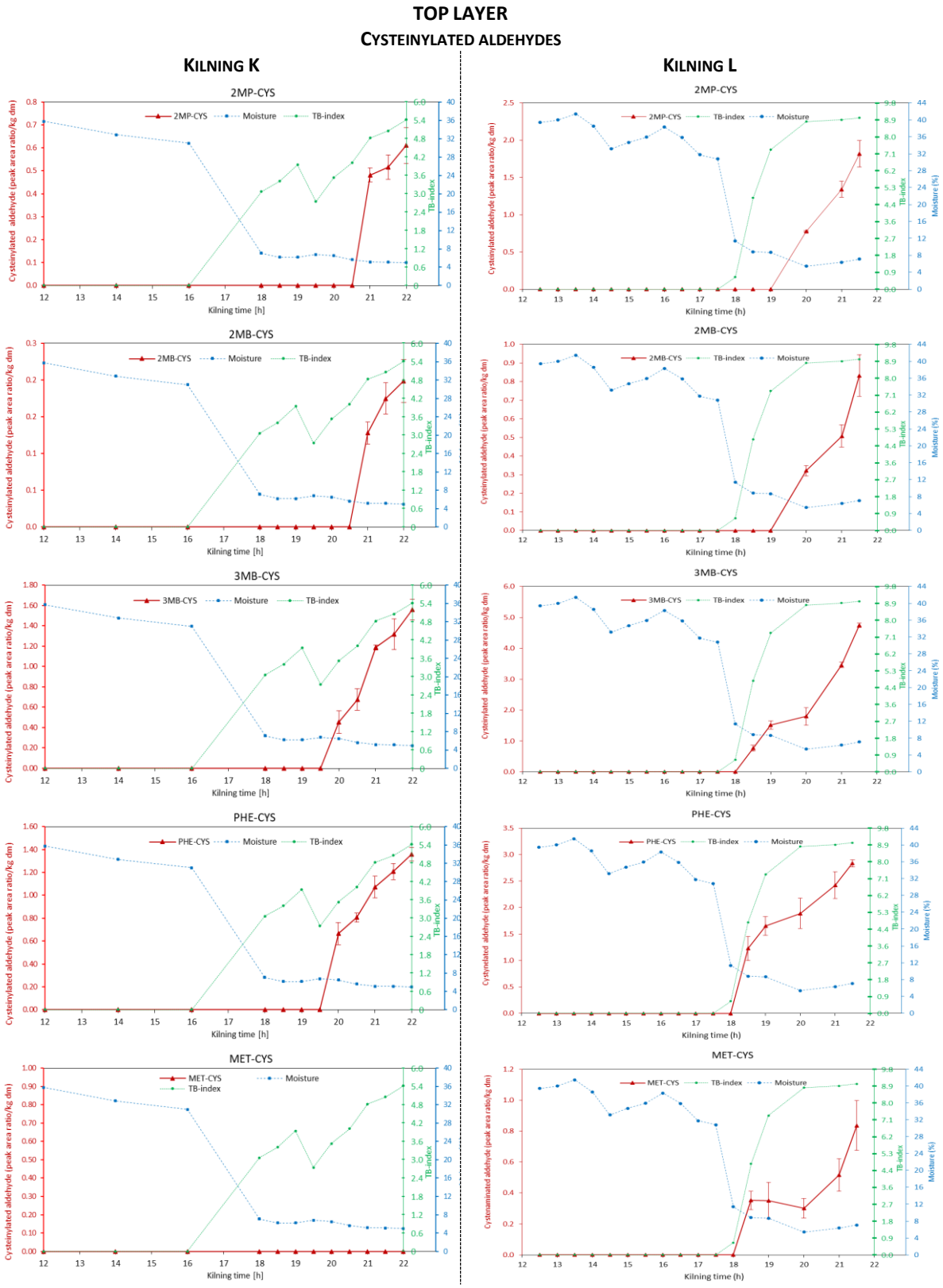


Figure E4-6 Evolution of cysteinylated Strecker aldehydes in relation the grain drying process and applied heat load during kilning. Samples collected from the top layer.

APPENDIX F TO CHAPTER 6

Overview of raw data on standard quality parameters, levels of (cysteinylated) aldehydes, and compounds related to de novo formation of aldehydes.

Data were obtained on 22 malt samples, produced according to 12 different micro-malting protocols.

Abbreviations: Variable A = degree of steeping (% moisture), variable B = kilning-off temperature (°C). TBI = thiobarbituric acid index, PUG = partly unmodified grain, TP = total protein, TSP = total soluble protein, KI = Kolbach Index, FAN = free amino nitrogen, SMM = S-methylmethionine, 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, T2N = trans-2-nonenal, 2MP-CYS = cysteinylated 2-methylpropanal, 2MB-CYS = cysteinylated 2-methylbutanal, 3MB-CYS = cysteinylated 3-methylbutanal, MET-CYS = cysteinylated methional, PHE-CYS = cysteinylated phenylacetaldehyde, FUR-CYS = cysteinylated furfural (<LOD), and HEX-CYS = cysteinylated hexanal (cysteinylated *trans*-2-nonenal is not presented as the reference compound was not available), THFA = trihydroxy fatty acids, LOX-activity = lipoxygenase activity, LOD = limit of detection.

Results are presented as mean values (n=2 or 3, depending on the measured malt quality parameter); standard deviations (SD) of the applied analytical methods are as follows: moisture content ± 0.1, extract yield ± 0.4, colour ± 0.4, friability ± 1.7, homogeneity ± 0.5, partly unmodified grain ± 0.5, β-glucan content ± 20, viscosity ± 0.02, total protein ± 0.3, total soluble protein ± 0.16, Kolbach Index ± 1.4, free amino nitrogen ± 9, diastatic power ± 25, α-amylase activity ± 5.5, pH ± 0.05; for other methods SD are presented in the tables.

Table F6-1.Standard quality parameters of malt

Experiment	Variable A	Variable B	Moisture	Extract yield	Colour	Friability	Homoge-neity	PUG	β-glucan	Viscosity	TP	TSP	KI	FAN	Diastatic power	α-amylase activity	pH	Malting yield
	%	°C	%	% dm	EBC	%	%	%	mg/L	mPas	% dm	mg/L	% dm	mg/L	WK	DU	-	% dm
1	43.7	73	4.4	80.5	2.0	65.4	87.8	12.2	503	1.75	10.1	4.0	39.7	147	444	45	5.98	92.1
2	47.8	73	4.1	80.3	2.9	94.6	99.8	0.2	104	1.52	9.9	5.4	54.2	225	576	73	5.74	83.4
3	46.1	60	4.9	77.4	2.5	78.2	98.0	2.0	289	1.58	10.1	4.7	46.7	175	575	73	5.87	88.4
4	40.3	60	4.8	79.8	2.1	40.2	60.4	39.6	659	2.26	10.5	3.3	33.9	96	455	47	6.14	96.2
5	48.3	60	5.0	80.3	2.7	86.2	99.6	0.4	88	1.54	10.0	4.4	44.0	216	632	70	5.82	89.8
6	44.1	73	3.8	80.0	2.0	66.6	87.4	12.6	574	1.77	10.2	3.8	36.8	136	441	53	6.03	92.3
7	40.6	60	4.7	79.9	2.3	47.2	66.2	33.8	725	2.27	10.4	3.2	30.5	105	407	49	6.12	95.4
8	43.9	60	4.8	79.6	1.7	63.8	87.0	13.0	539	1.70	10.4	3.9	37.1	138	474	55	6.03	92.0
9	48.6	85	3.4	80.8	4.3	88.0	99.1	0.1	81	1.55	10.0	5.3	53.2	227	492	70	5.75	81.6
10	46.8	85	3.9	79.9	3.3	75.8	97.6	2.4	338	1.58	10.2	4.6	44.7	178	449	75	5.86	88.0
11	44.3	73	4.2	80.1	2.0	68.2	88.8	11.2	653	1.80	10.2	3.9	38.2	140	377	55	6.02	91.9
12	46.0	85	3.8	79.7	3.3	83.4	99.0	1.0	306	1.59	9.9	4.6	46.6	182	466	62	5.85	88.8
13	43.7	85	3.6	79.3	2.3	63.0	86.2	13.8	704	1.88	10.1	3.9	38.2	139	343	45	6.02	92.1
14	40.4	85	4.0	80.1	1.9	44.4	64.2	35.8	678	2.45	10.4	3.3	34.3	97	330	42	6.12	92.2
15	46.5	60	4.9	79.8	2.5	81.8	98.0	2.0	354	1.58	10.1	4.5	45.0	177	576	70	5.88	88.5
16	43.8	60	5.0	79.9	2.0	66.0	86.8	13.2	668	1.80	10.3	4.0	38.6	138	441	49	6.03	92.1
17	40.6	85	4.0	80.3	2.4	51.6	72.2	27.8	815	2.08	10.4	3.3	31.8	111	334	43	6.08	93.9
18	44.5	85	3.8	79.8	2.3	66.2	89.2	10.8	469	1.73	10.2	4.0	39.1	150	305	53	5.85	92.1
19	40.9	73	4.3	79.9	2.0	44.2	65.8	34.2	628	1.92	10.4	3.4	32.5	111	382	42	6.11	95.0
20	48.4	85	3.9	80.1	4.6	93.8	100.0	0.0	114	1.58	9.9	5.2	52.4	213	507	60	5.76	81.1
21	48.2	60	5.0	80.3	3.2	89.4	100.0	0.0	94	1.54	10.2	5.1	50.3	209	515	74	5.82	89.8
22	46.7	73	4.5	79.2	2.3	81.0	98.6	1.4	199	1.59	10.2	4.8	45.9	190	446	59	5.73	87.6

Table F6-2.Standard quality parameters of malt – continuation

Experiment	Variable A	Variable B	TBI		SMM	
	%	°C	for 10 g dm		mg/kg dm	
			Mean	SD	Mean	SD
1	43.7	73	6.14	0.08	7.73	0.53
2	47.8	73	13.28	0.02	10.70	1.16
3	46.1	60	5.03	0.05	16.18	1.82
4	40.3	60	2.34	0.07	4.98	0.36
5	48.3	60	8.39	0.21	12.17	0.43
6	44.1	73	5.56	0.10	8.56	1.65
7	40.6	60	2.44	0.03	4.98	0.56
8	43.9	60	3.94	0.03	10.35	0.91
9	48.6	85	20.13	0.30	3.49	0.92
10	46.8	85	12.68	0.10	5.06	0.36
11	44.3	73	5.31	0.03	7.30	0.48
12	46.0	85	13.03	0.04	6.79	1.22
13	43.7	85	8.84	0.11	5.37	4.07
14	40.4	85	4.49	0.10	2.61	0.78
15	46.5	60	5.46	0.10	16.49	1.12
16	43.8	60	4.42	0.04	11.64	1.32
17	40.6	85	6.79	0.02	2.74	0.10
18	44.5	85	10.72	0.03	5.95	0.91
19	40.9	73	4.94	0.03	4.31	1.74
20	48.4	85	20.37	0.03	4.59	0.77
21	48.2	60	10.03	0.01	16.36	0.91
22	46.7	73	8.75	0.14	13.75	0.19

Table F6-3. Quantitative determination of free aldehydes

Experiment	Variable A	Variable B	2MP		2MB		3MB		MET		PHE		FUR		HEX		T2N		Sum of free ald.
	%	°C	µg/kg dm		µg/kg dm		µg/kg dm		µg/kg dm		µg/kg dm		µg/kg dm		µg/kg dm		µg/kg dm		µg/kg dm
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean
1	43.7	73	637	55	616	22	1,525	96	173	23	654	43	203	20	281	36	400	76	4,513
2	47.8	73	1,337	64	1,164	37	2,992	117	265	37	1,437	117	572	90	587	106	498	266	8,852
3	46.1	60	655	87	489	28	1,265	66	68	16	391	32	117	23	245	79	174	105	3,352
4	40.3	60	231	16	133	7	358	69	30	10	188	18	58	18	81	55	206	47	1,285
5	48.3	60	905	91	688	34	1,963	127	119	22	681	42	265	24	671	191	421	155	5,713
6	44.1	73	586	40	532	13	1,305	56	124	18	508	44	143	19	265	107	434	155	3,897
7	40.6	60	260	27	147	6	406	72	31	11	195	17	48	17	76	14	229	80	1,392
8	43.9	60	322	20	265	8	692	15	62	12	286	32	82	15	223	58	320	126	2,284
9	48.6	85	1,974	129	2,076	116	4,817	260	956	57	4,045	104	1,295	14	597	65	851	143	16,811
10	46.8	85	1,820	151	1,772	130	4,060	336	624	8	2,487	194	734	8	349	11	538	120	12,384
11	44.3	73	602	37	549	12	1,351	65	125	14	549	28	152	20	220	43	368	99	3,916
12	46.0	85	1,764	159	1,730	127	3,954	315	601	49	2,470	63	726	26	301	49	517	158	12,063
13	43.7	85	1,223	85	1,244	74	2,791	216	506	43	1,883	57	453	6	249	47	499	93	8,848
14	40.4	85	978	52	829	18	2,271	66	177	17	946	74	258	13	239	74	332	102	6,030
15	46.5	60	708	62	523	17	1,323	18	74	13	404	22	118	25	280	78	180	111	3,575
16	43.8	60	344	22	288	27	619	280	70	8	275	99	98	13	218	19	334	45	2,276
17	40.6	85	1,206	87	1,077	39	2,795	142	262	22	1,357	51	341	23	248	107	486	153	7,772
18	44.5	85	1,143	103	1,154	69	2,574	200	438	30	1,692	59	404	12	202	67	499	143	8,106
19	40.9	73	612	39	450	10	1,423	40	78	15	462	44	125	24	187	91	214	74	3,551
20	48.4	85	2,072	144	1,994	105	4,628	244	902	24	3,627	214	1,218	21	562	13	880	153	16,142
21	48.2	60	895	79	713	25	1,905	69	127	16	695	22	341	17	627	137	510	244	5,813
22	46.7	73	1,181	101	1,070	69	2,600	159	194	17	950	23	313	22	290	48	380	160	6,813

Table F6-4. Quantitative determination of cysteinylated aldehydes

Experiment	Variable A	Variable B	2MP-CYS		2MB-CYS		3MB-CYS		MET-CYS		PHE-CYS		FUR-CYS		HEX-CYS		Sum of CYS-ald.
	%	°C	µg/kg dm		µg/kg dm		µg/kg dm		µg/kg dm		µg/kg dm		µg/kg dm		µg/kg dm		µg/kg dm
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean
1	43.7	73	16	8	25	1	88	6	<LOD	-	75	8	<LOD	-	<LOD	-	204
2	47.8	73	71	7	45	2	203	6	11	1	166	12	<LOD	-	16	2	512
3	46.1	60	<LOD	-	14	1	34	2	<LOD	-	25	1	<LOD	-	<LOD	-	73
4	40.3	60	<LOD	-	<LOD	-	16	3	<LOD	-	<LOD	-	<LOD	-	<LOD	-	16
5	48.3	60	31	2	24	2	105	4	<LOD	-	74	6	<LOD	-	20	2	254
6	44.1	73	23	2	24	2	95	2	<LOD	-	48	2	<LOD	-	<LOD	-	190
7	40.6	60	<LOD	-	<LOD	-	26	1	<LOD	-	23	8	<LOD	-	<LOD	-	49
8	43.9	60	<LOD	-	<LOD	-	27	1	<LOD	-	21	4	<LOD	-	<LOD	-	48
9	48.6	85	229	10	153	4	659	28	71	4	268	11	<LOD	-	8	1	1,388
10	46.8	85	208	6	108	11	480	18	54	1	197	4	<LOD	-	<LOD	-	1,053
11	44.3	73	28	2	28	1	128	6	<LOD	-	60	5	<LOD	-	9	1	253
12	46.0	85	161	4	95	1	390	12	49	3	196	10	<LOD	-	<LOD	-	891
13	43.7	85	139	4	94	2	479	11	60	3	176	5	<LOD	-	9	1	957
14	40.4	85	46	1	36	3	167	8	18	1	137	12	<LOD	-	<LOD	-	404
15	46.5	60	12	3	14	3	51	2	<LOD	-	33	1	<LOD	-	<LOD	-	110
16	43.8	60	<LOD	-	12	2	59	4	<LOD	-	31	2	<LOD	-	<LOD	-	102
17	40.6	85	87	4	66	2	305	5	37	3	122	8	<LOD	-	9	1	626
18	44.5	85	133	5	82	2	393	21	54	1	171	12	<LOD	-	<LOD	-	833
19	40.9	73	<LOD	-	11	1	38	3	<LOD	-	41	2	<LOD	-	<LOD	-	90
20	48.4	85	247	5	147	3	756	26	83	4	311	10	<LOD	-	12	1	1,556
21	48.2	60	28	1	29	2	109	3	<LOD	-	55	3	<LOD	-	20	2	241
22	46.7	73	51	4	38	3	167	8	17	1	78	7	<LOD	-	<LOD	-	351

Table F6-45 Compounds related to *de novo* formation of staling aldehydes

Experiment	Variable A	Variable B	Valine		Isoleucine		Leucine		Methionine		Phenylalanine		THFA		LOX-activity		Total polyphenols	
	%	°C	mg/kg dm		mg/kg dm		mg/kg dm		mg/kg dm		mg/kg dm		mg/kg dm		U/g dm		mg/kg dm	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	43.7	73	442	13	263	11	479	14	88	3	437	13	44.0	2.2	6.2	0.3	658	16
2	47.8	73	490	54	313	38	538	59	89	10	563	73	35.2	12.7	10.2	0.7	768	18
3	46.1	60	420	17	251	10	430	17	74	3	420	13	31.3	2.9	9.7	1.2	603	1
4	40.3	60	262	1	152	0	284	1	57	1	296	0	45.2	6.4	4.7	0.2	531	1
5	48.3	60	487	1	310	1	536	2	93	3	534	1	37.0	2.8	11.9	1.4	741	12
6	44.1	73	387	8	231	2	418	4	80	2	382	1	42.8	2.5	6.6	0.6	570	3
7	40.6	60	284	3	167	2	307	3	60	1	314	1	45.0	0.9	5.2	0.8	532	17
8	43.9	60	368	4	219	2	391	4	75	1	370	4	40.8	2.2	6.9	0.5	579	5
9	48.6	85	456	9	296	6	514	10	87	3	518	16	29.4	0.3	6.7	0.7	840	3
10	46.8	85	410	4	245	2	427	4	81	2	440	4	24.4	2.0	5.3	1.1	670	8
11	44.3	73	384	15	224	9	401	16	75	0	373	22	46.2	0.1	6.8	0.2	583	14
12	46.0	85	432	2	261	1	449	2	81	0	443	4	31.2	3.9	4.9	0.6	680	1
13	43.7	85	364	1	215	0	382	0	73	1	363	4	44.1	4.1	4.5	0.4	627	28
14	40.4	85	250	3	139	1	254	3	53	0	262	3	42.7	5.9	5.3	0.6	550	11
15	46.5	60	448	13	273	8	478	14	86	1	461	5	27.4	2.7	9.4	0.5	611	6
16	43.8	60	408	12	249	7	455	14	83	2	416	4	44.3	5.6	7.7	0.1	676	7
17	40.6	85	311	12	179	7	325	13	65	5	329	7	41.1	3.4	4.7	1.1	539	31
18	44.5	85	386	8	229	5	405	8	76	1	378	0	48.0	2.4	5.4	0.6	605	14
19	40.9	73	289	46	167	27	315	50	63	7	313	22	39.1	0.5	4.9	1.8	627	3
20	48.4	85	392	8	249	5	429	9	74	3	435	13	25.5	1.1	7.3	0.6	802	1
21	48.2	60	514	57	326	36	566	62	98	9	579	64	32.3	2.3	12.4	1.4	711	19
22	46.7	73	567	17	344	10	598	18	102	3	558	11	26.9	2.7	8.4	1.9	670	12

APPENDIX G TO CHAPTER 6

Visual presentation of 41 models obtained by plotting individual responses (malt quality parameters) as a function of variable A (degree of steeping) and variable B (kilning-off temperature).

Abbreviations: Variable A = degree of steeping (% moisture), variable B = kilning-off temperature (°C).

Compounds: 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, MET = methional, PHE = phenylacetaldehyde, FUR = furfural, HEX = hexanal, T2N = trans-2-nonenal, 2MP-CYS = cysteinylated 2-methylpropanal, 2MB-CYS = cysteinylated 2-methylbutanal, 3MB-CYS = cysteinylated 3-methylbutanal, MET-CYS = cysteinylated methional, PHE-CYS = cysteinylated phenylacetaldehyde, FUR-CYS = cysteinylated furfural (<LOD), and HEX-CYS = cysteinylated hexanal (cysteinylated trans-2-nonenal is not presented as the reference compound was not available), TBI = thiobarbituric acid index, LOX-activity = lipoxygenase activity.

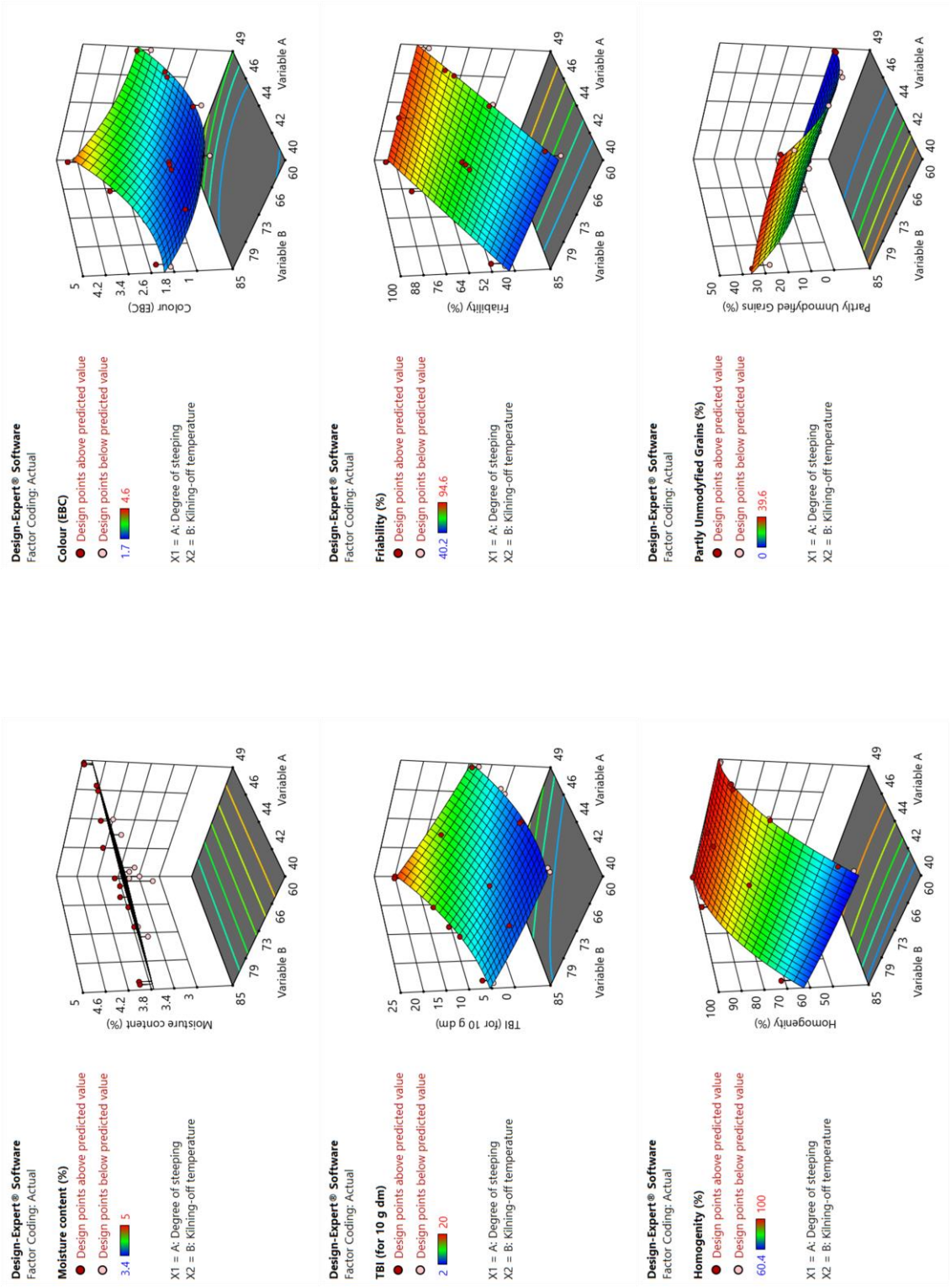


Figure G6-1. Standard quality parameters of malt as a function of the degree of steeping (variable A) and kilning-off temperature (variable B)

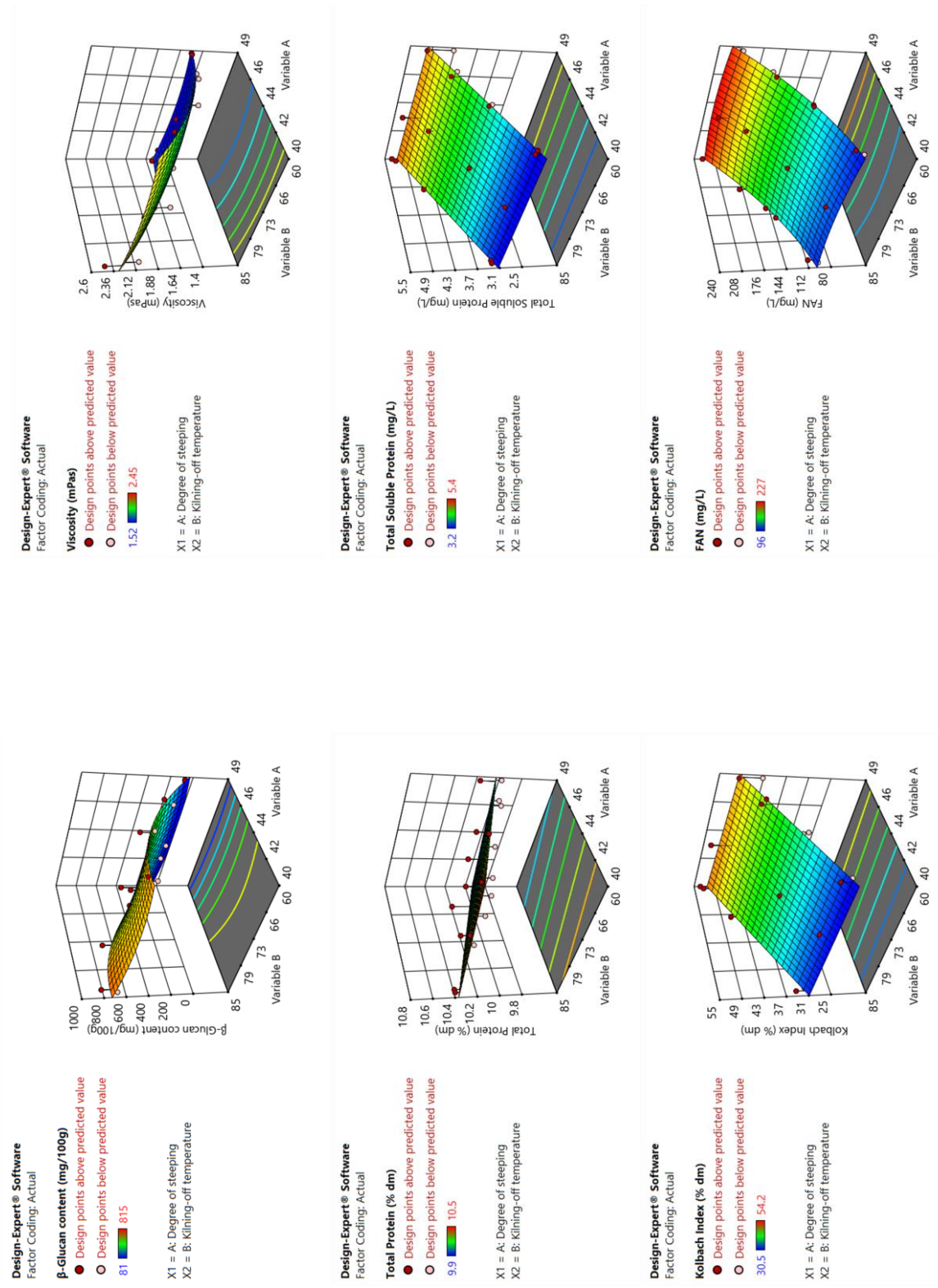


Figure G6-2. Continuation. Standard quality parameters of malt as a function of the degree of steeping (variable A) and kilning-off temperature (variable B)

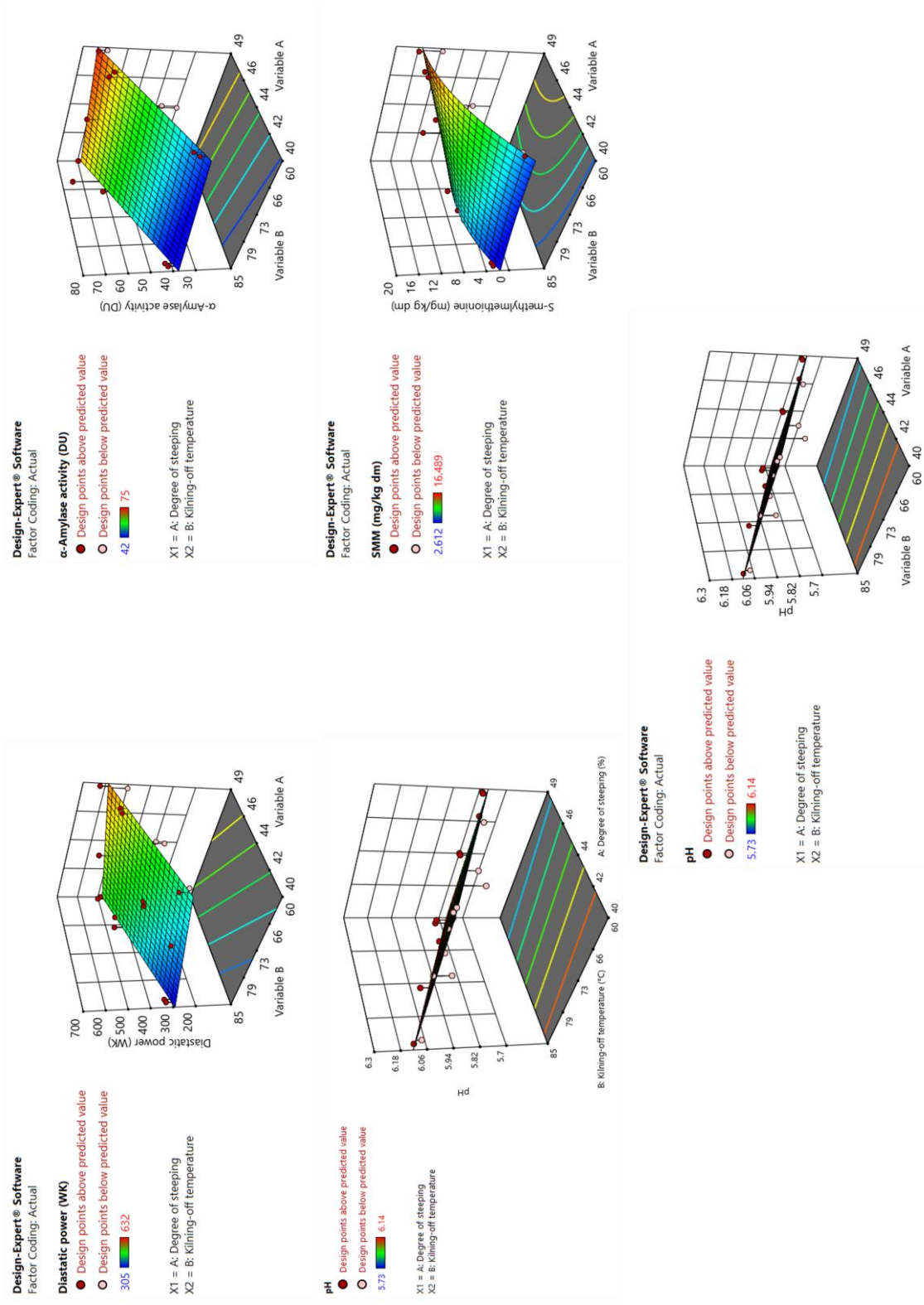


Figure G6-3.continuation. Standard quality parameters of malt as a function of the degree of steeping (variable A) and kilning-off temperature (variable B)

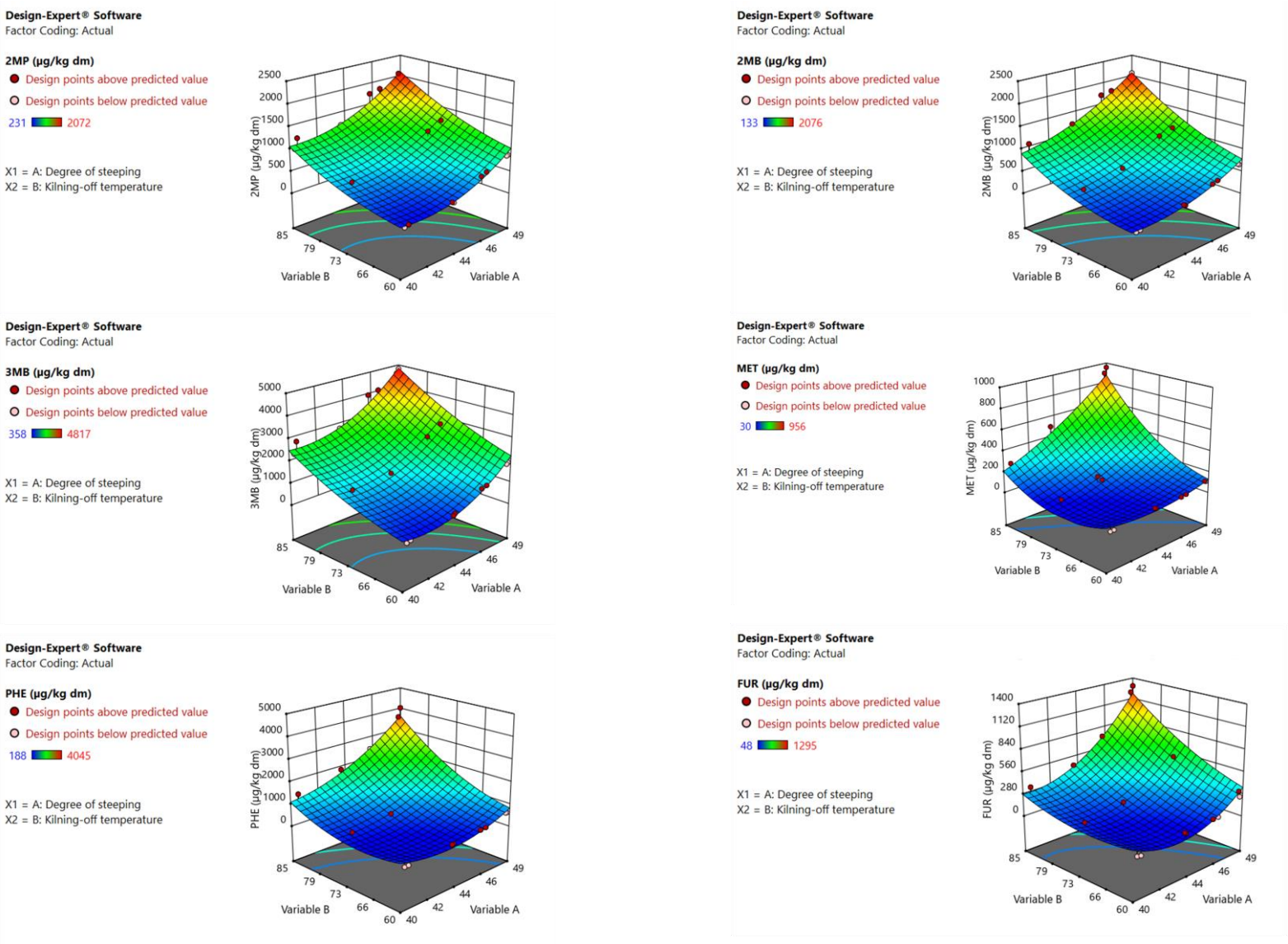


Figure G6-4.Levels of free aldehydes as a function of the degree of steeping (variable A) and kilning-off temperature (variable B)

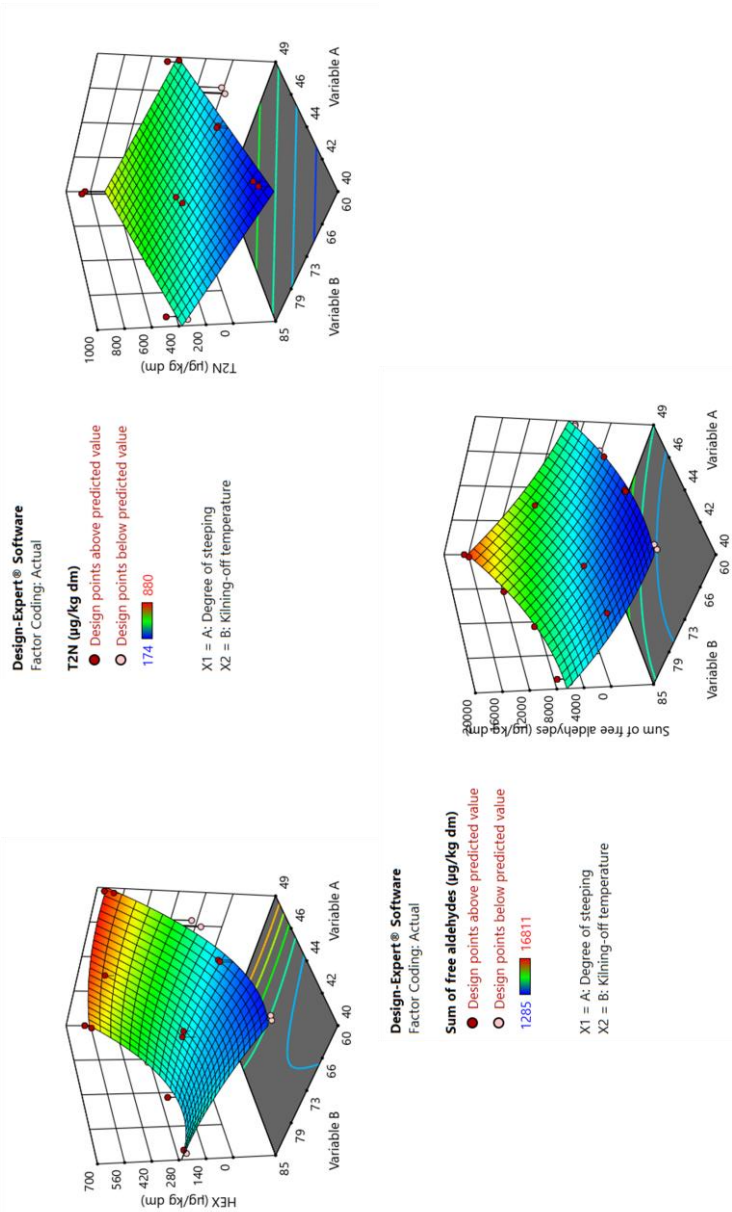


Figure G6-5.continuation. Levels of free aldehydes as a function of the degree of steeping (variable A) and kilning-off temperature (variable B)

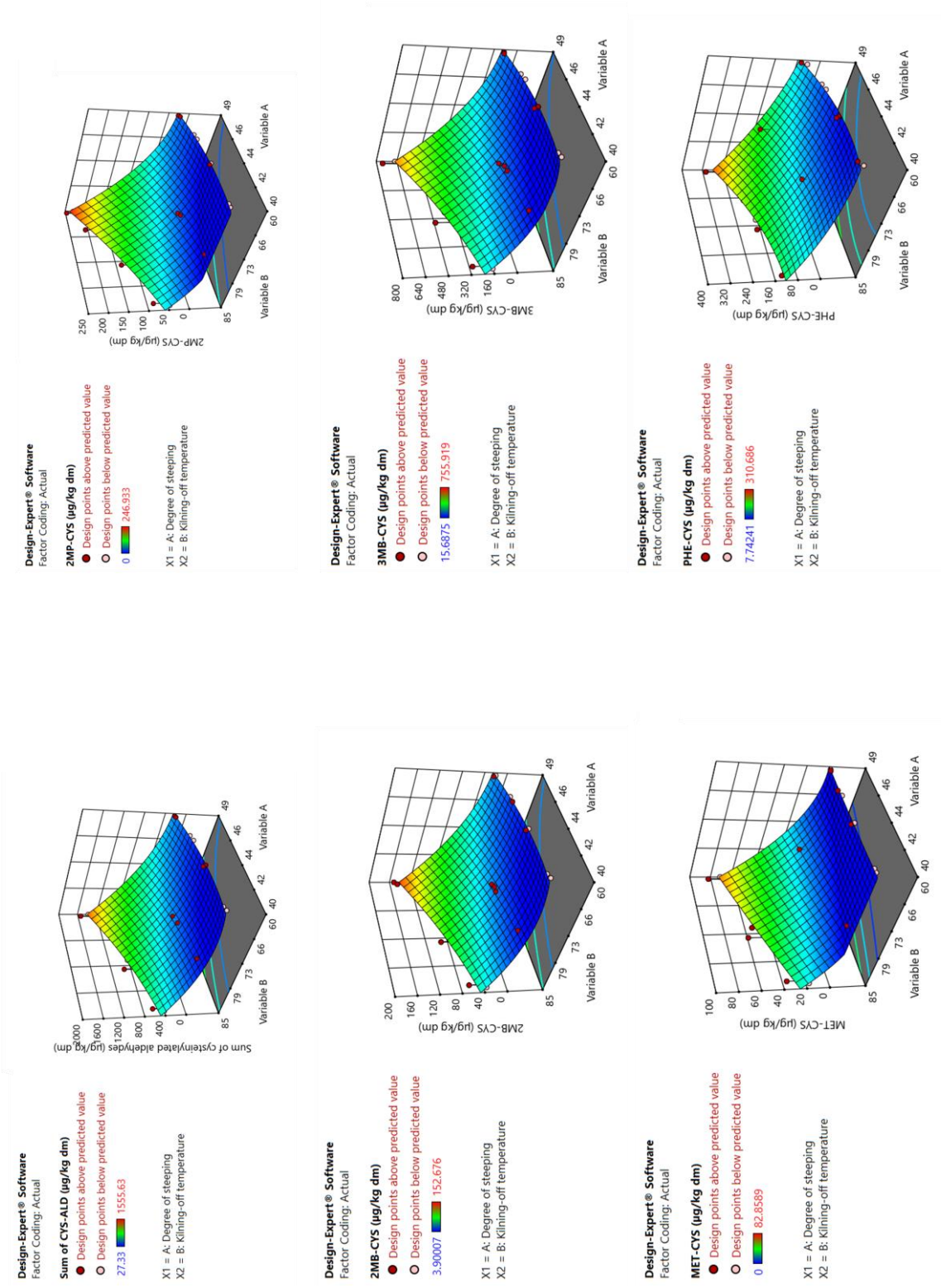
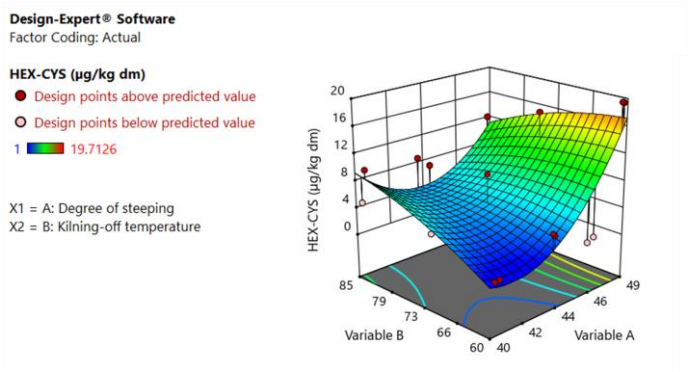


Figure G6-6. Levels of cysteinylated aldehydes as a function of the degree of steeping (variable A) and kilning-off temperature (variable B)



Compounds related to *de novo* formation of aldehydes

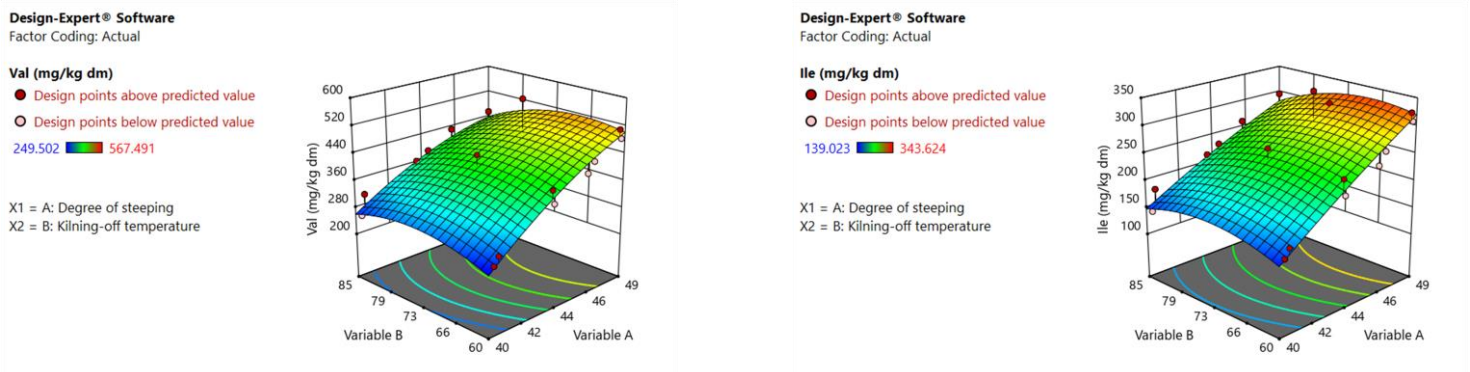


Figure G6-7. continuation. Levels of cysteinylated aldehydes and compounds related to *de novo* formation of aldehydes as a function of the degree of steeping (variable A) and kilning-off temperature (variable B)

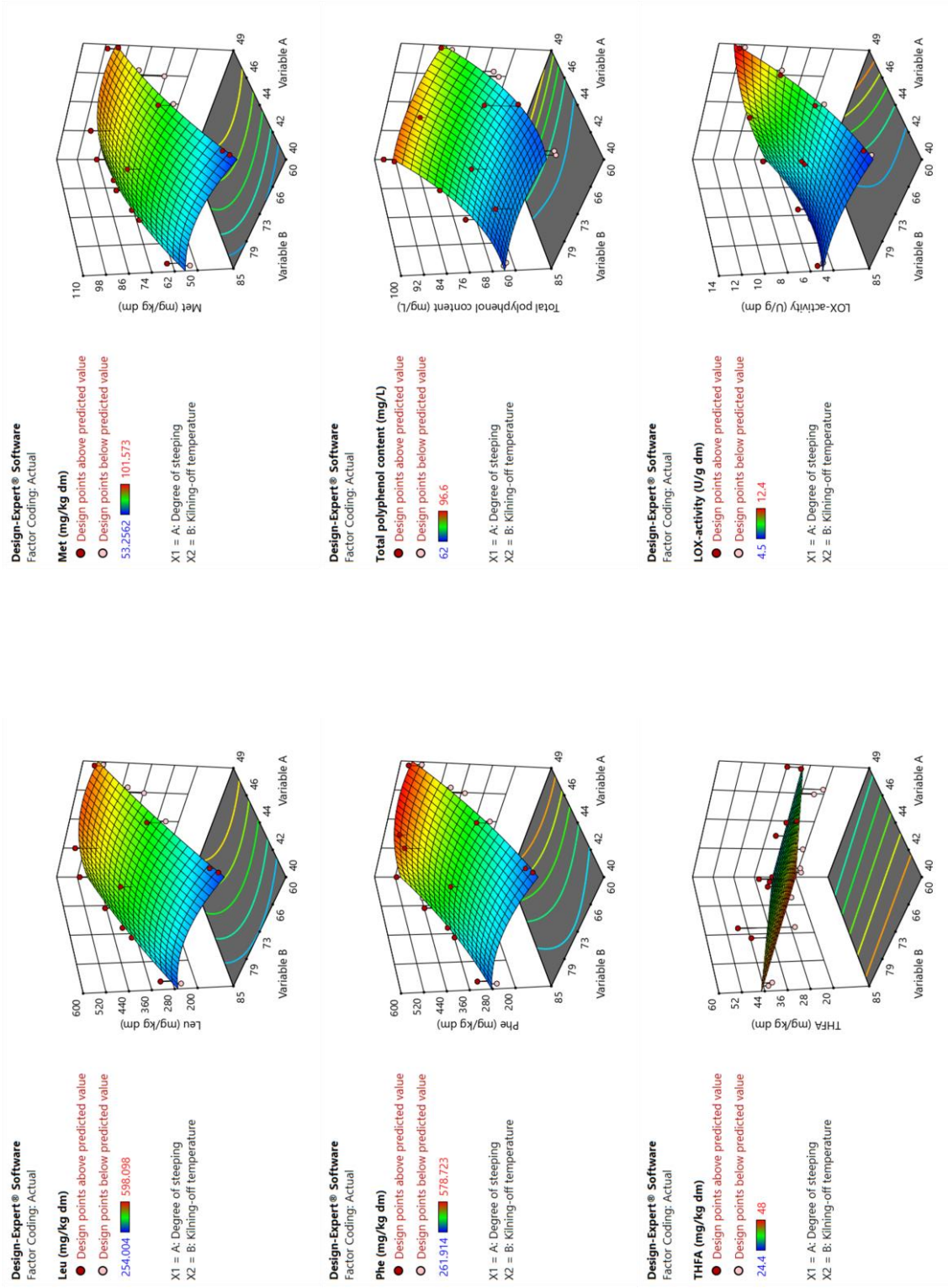


Figure G6-8. Level of compounds related to *de novo* formation of aldehydes as a function of the degree of steeping (variable A) and kilning-off temperature (variable B)

LIST OF PUBLICATIONS

PEER-REVIEWED ARTICLES

Filipowska W., Jaskula-Goiris B., Ditrych M., Bustillo Trueba P., De Rouck G., Aerts G., Powell C., Cook D. & De Cooman L. (2021) On the contribution of malt quality and malting process to the formation of beer staling aldehydes. A review, *Journal of the Institute of Brewing*, 127 (2) 107-126. doi:10.1002/jib.644

Bustillo Trueba P., Jaskula-Goiris B., Ditrych M., **Filipowska W.,** De Brabanter J., De Rouck G., Aerts G., De Cooman L. & De Clippeleer J. (2021) Monitoring the evolution of free and cysteinylated aldehydes from malt to fresh and forced aged beer. *Food Research International,* 140 110049. doi:10.1016/j.foodres.2020.110049

Filipowska W., Jaskula-Goiris B., Ditrych M., Schlich J., De Rouck G., Aerts G. & De Cooman L. (2019) Determination of optimal sample preparation for aldehyde extraction from pale malts and their quantification via headspace solid-phase microextraction followed by gas chromatography and mass spectrometry. *Journal of Chromatography A*, 1612 460647. doi:10.1016/j.chroma.2019.460647

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

ORAL AND POSTER PRESENTATIONS

Filipowska, W., Bolat I., Van D'Huynslager G., Bustillo Trueba P., Ditrych, M., Jaskula-Goiris, B., De Rouck, G. & De Cooman, L. *Identification of critical factors in malt production related to beer flavour (in)stability.* Poster presentation at 36th the Institute of Brewing and Distilling Biennial Convention Asia-Pacific in Perth, Australia; 23-24th February 2021

Filipowska, W., Bustillo Trueba P., Ditrych, M., Jaskula-Goiris, B., Bolat I., Van D'huynslager G., De Rouck, G., Aerts G. & De Cooman, L. *Monitoring of beer staling aldehydes during the malting process.* Oral presentation at 37th European Brewery Convention; Antwerp, Belgium, 2nd-6th June 2019

Filipowska, W., De Zutter, L., Schlich, J., Jaskula-Goiris, B., Bolat, I., Van D'Huynslager, G., Aerts, G., De Rouck, G. & De Cooman, L. *The influence of malting technology on the formation of beer staling aldehydes.* Oral presentation at 6th Young Scientists Symposium on Malting, Brewing and Distilling, Bitburg, Germany; 12-14th September 2018. Honourable mention for 'Best Presentation'

Filipowska W., Ditrych M., De Rouck G., Jaskula-Goiris B. & De Cooman L. *The significance of malt in beer flavour (in)stability.* Oral presentation at 19th School of Fermentation Technology in Kocierz, Poland; 14-16th May 2018

Filipowska W., Ditrych M., De Rouck G., Jaskula-Goiris B., De Clippeleer J., Aerts G. & De Cooman L. *Determination of staling aldehydes in malt.* Poster presentation at 13th Trends in Brewing in Ghent, Belgium; 8-12th April 2018