On the Origins and Formation Kinetics of Worty Flavours in Alcohol-Free Beers

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On the Origins and Formation Kinetics of Worty Flavours in Alcohol-Free Beers

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of the requirement for the degree of
Doctor of Philosophy in Food and Nutritional Sciences

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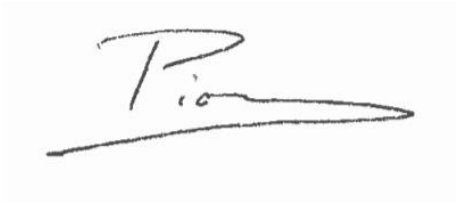
Declaration

I hereby confirm that this is my own work and the use of all materials from other sources has been properly and fully acknowledged.



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Abstract

The interest of consumers towards alcohol-free beer (AFB) has increased over the last few years due to several motivations. However, some of them, such as those brewed by cold contact fermentation (CCF) show a characteristic aroma reminiscent of wort or malt, lacking the appreciated fruity flavour of their alcoholic counterparts. From the current literature, the Strecker aldehydes methional, 2- and 3-methylbutanal are responsible for this aroma. The aim of this PhD project was to identify the role of odour-active compounds in AFB and their contribution to the worty character, as well as to study the formation kinetics of them during malt kilning. By means of the sensomic approach, five key aroma compounds were identified, among 27 odour-active compounds. These were methional (boiled potato-like aroma), 3-methylbutanal (cocoa-like), (*E*)-β-damascenone (apple, jam-like), 5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone (curry, spicy-like), and phenylacetaldehyde (floral, honey-like).

As a part of the sensomic method, perception thresholds of the odour-active compounds were determined in an AFB-like matrix. Differences were observed between our threshold values, both ortho- and retronasal, and the values from the literature in water and ethanol/water mixtures. Moreover, the calculation method employed (best estimate threshold BET or logistic regression) and the presence of false positives had a significant impact on the final results.

In the next part of the project, the formation of (*E*)-β-damascenone and Strecker aldehydes during malt kilning (isothermal curing stage at 65, 78 or 90 °C) was investigated. The former increased over time in malts kilned at 78 and 90 °C, whilst at 65 °C the trend was not very clear. During mashing and wort boiling, the amount of (*E*)-

β-damascenone was affected by a compromise between formation and degradation/evaporation.

The formation of five Strecker aldehydes (the four mentioned above and 2-methylpropanal) was monitored during the curing stage of malt kilning too. These compounds are formed during thermal processes by the Maillard reaction between reducing sugars and amino acids. Multi-response kinetic modelling was used to fit the analytical results to a mechanistic mathematical model. The model developed showed the importance of the formation of two intermediate compounds in the reaction of glucose and amino acids. The first group of intermediates was Amadori rearrangement products (ARP), whereas the second was a pool of short chain dicarbonyls, such as glyoxal and methylglyoxal, that were not determined analytically. The results from the kinetic parameters demonstrated the high sensitivity of the degradation of ARP to changes in temperature because of its high activation energy.

The outcomes from this PhD may have a great importance for brewers and brewing scientists. The understanding of the role of flavour compounds in AFB is the starting point to design mitigation strategies for the formation of worty off-flavours and thus improve the beer's organoleptic characteristics and consumer acceptability.

A Helena por su sonrisa

"Ser un ignorante te da la ventaja
de intentar cosas que no son convencionales,
porque no estás contaminado por una manera de
hacer las cosas (…). Intentas cosas que a lo mejor
a alguien que tiene más práctica ni se le pasa por
la cabeza porque es ridículo."

Translation:
"Being ignorant gives you the advantage
of trying unconventional things, because you are
not contaminated by a certain way of doing things (…).
You try things a person with more experience may not
try because of how ridiculous the idea is."

Juan Ignacio Delgado Alemany, La Vida Moderna (4x105)

Table of Contents

List of Figures

List of Tables

List of Appendices

0

0

List of Abbreviations

2MB	*2-methylbutanal*
2MP	*2-methylpropanal*
3-AFC	*three-alternative forced choice*
3MB	*3-methylbutanal*
AAi	*amino acids with their ARP quantified*
AAj	*amino acids with their ARP non quantified*
ABV	*alcohol by volume*
AEDA	*aroma extract dilution analysis*
AFB	*alcohol-free beer*
AIC	*Akaike information criterion*
Ala	*alanine*
ANOVA	*analysis of variance*
Arg	*arginine*
ARP	*Amadori rearrangement product*
Asn	*asparagine*
Auth.	*authentic*
bDam	*(E)-β-damascenone*
BET	*best estimate threshold*
CCF	*cold contact fermentation*
CoA	*coenzyme A*
df	*film thickness (referred to GC capillary column)*
DF	*degrees of freedom*
dm	*dry matter*
DMS	*dimethyl sulphide*
DT	*detection threshold*
DVB	*divinylbenzene*
Ea	*activation energy*
EBC	*European Brewery Convention*
ECD	*electron capture detector*
e-CFR	*Electronic Code of Federal Regulation of the USA*
EI	*electron impact*
ESI	*elestrospray ionisation*
FAA	*conversion factor for an amino acid AA*
FAN	*free amino nitrogen*
FD	*flavour dilution*
FID	*flame ionisation detector*
FruAAi	*fructosyl derivatives of AAi*
FruAAj	*fructosyl derivatives of AAj*
FruAla	*fructosyl alanine*
Fruc	*fructose*
FruGly	*fructosyl glycine*
FruIle	*fructosyl isoleucine*
FruLeu	*fructosyl leucine*
FruMet	*fructosyl methionine*
FruPhe	*fructosyl phenylalanine*
FruPro	*fructosyl proline*
FruVal	*fructosyl valine*
GC-MS	*gas chromatography-mass spectrometry*
GC-O	*gas chromatography-olfactometry*
GC-ToF-MS	*GC- Accurate-Mass Time of Flight-Mass Spectrometry*
Gln	*glutamine*
Glu	*glutamic acid*
Gluc	*glucose*
Gly	*glycine*
His	*histidine*
HPD	*highest posterior density*
HS-SPME	*headspace-solid phase microextraction*
i.d.	*internal diameter*
Ile	*isoleucine*
Int1	*intermediate compound*
LC	*liquid chromatograph*
Leu	*leucine*
LR	*logistic regression*
LRI	*linear retention index*
LSD	*least significant differences*
Lys	*lysine*
MBT	*3-methyl-2-butene-1-thiol*
Met	*methionine*
Meth	*methional*
MF	*modified frequency*
MRM	*multiple reaction monitoring*
MRP	*Maillard reaction products*
MS/MS	*tandem mass spectrometry*
NCI	*negative chemical ionisation*
OAV	*odour activity value*
°P	*degree Plato*
PDMS	*polydimethylsiloxane*
PEI	*polyetherimide*
PFBHA	*O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride*
PhAc	*phenylacetaldehyde*

Phe	*phenylalanine*	SDP	*sugar degradation products*
POMS	*polyoctylmethylsiloxane*	Ser	*serine*
ppb	*parts per billion*	SIM	*single ion monitoring*
ppm	*parts per million*	SPME	*solid phase microextraction*
Pro	*proline*	Thr	*threonine*
PUG	*partly unmodified grains*	Trp	*tryptophan*
QDA®	*quantitative descriptive analysis*	Tyr	*tyrosine*
RSM	*response surface methodology*	Unk.	*unknown*
SAFE	*solvent-assisted flavour evaporation*	Val	*valine*
SCC	*spinning cone column*	WHO	*World Health Organisation*
SCDC	*short chain dicarbonyl*	WUG	*whole unmodified grains*

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José Piornos

Chapter 0.

Chapter 0. Introductory chapter

The popularity of alcohol-free beers (AFB) has increased over the last few decades, motivated by different reasons, such as healthier lifestyle habits, religious concerns, or restrictive driving legislations. However, the organoleptic characteristics of AFB still lacks the appreciated fruity flavour of most of regular alcoholic beers. AFB brewed by biological methods usually has an aroma reminiscent of wort because of the unfavourable fermentation conditions. This aroma is considered as an off-flavour in beers, and thus a negative attribute that has an impact on consumers' acceptability. One of the most widely used biological methods at industrial scale is cold contact fermentation (CCF). This method consists of fermenting the wort at very low temperature, around -0.5 °C, which reduces yeast activity and thus, the production of ethanol. However, the flavour of this AFB is very similar to that of wort.

The literature showed that the compounds identified as responsible for this "worty" or malty aroma so far were Strecker aldehydes. During fermentation of alcoholic beers, these compounds are transformed into fruity alcohols and esters, but this transformation was very limited under the conditions for CCF. However, further information about the role of aroma compounds other than Strecker aldehydes in the worty flavour of AFB is not available in the scientific literature. According to previous studies, detection thresholds of these compounds determined in water are extremely low. The absence of alcohol, as well as the presence of non-fermented sugars, suggests that the release of compounds from AFB is different to that from water or water/ethanol mixtures.

Strecker aldehydes are formed during thermal processes, such as malt kilning, through the Maillard reaction of amino acids and reducing sugars. Indeed, the Strecker

aldehydes 2-methylpropanal, 2- and 3-methylbutanal, methional and phenylacetaldehyde were already identified in barley malt. The formation kinetics of these compounds during malt kilning is unknown, neither the effect of temperature, precursors' concentration or presence of intermediate species. By understanding how the process conditions during brewing affect the concentration of the key aroma compounds is essential to control it and minimise their impact on the organoleptic characteristics of AFB.

0.1. Hypotheses

The main hypotheses of this PhD thesis are that, along with the Strecker aldehydes methional, 2- and 3-methylbutanal, other odour-active compounds are key contributors to the worty character of AFB, and these are formed during brewing by thermal process. These hypotheses are described in more detail in the following sub-hypotheses:

- *There are important aroma compounds which contribute to worty notes in AFB other than the Strecker aldehydes already identified.*
- *The formation of key aroma compounds during malt kilning is affected by the malt kilning conditions (temperature and time).*
- *The formation of Strecker aldehydes takes place via the degradation of relatively reactive sugars (glucose and fructose) and amino acids, and the formation of several intermediates of different stability and reactivity (such as Amadori rearrangement products and short chain dicarbonyls).*

0.2. Objectives

The main objective of this project was to identify the aroma compounds responsible for the worty off-flavour of AFB brewed by CCF, as well as to study their formation during key steps of the brewing process. This main objective was intended to be achieved by fulfilling the following specific objectives:

- To review the literature available on the topic of AFB in order to identify the relevant flavour compounds in it, the effect of the lack of alcohol on the sensory perception, as well as the effect of the brewing methods on the organoleptic characteristics of the final product.
- To identify and quantify odour-active volatile compounds in an AFB of reference.
- To identify key aroma compounds in AFB by means of the sensomic methodology, and to elucidate their contribution to the worty character.
- To study the formation of (*E*)-β-damascenone during the curing stage of malt kilning, as well as to determine its levels at different stages of mashing and wort boiling prepared with malts cured at different temperatures.
- To develop a kinetic model for the formation of five Strecker aldehydes (2-methylpropanal, 2- and 3-methylbutanal, phenylacetaldehyde and methional) during the curing stage of kilning for the range of temperatures used for Lager malts.

0.3. Structure of the thesis

In this PhD thesis,

Chapter 1 presents a comprehensive review on the literature regarding several aspects related to the flavour compounds in AFB.

Chapter 2 presents a study on the identification and quantification of key odourants in AFB and their relative contribution to wortiness.

Chapter 3 shows a study on the determination of orthonasal and retronasal detection thresholds of the identified compounds in an AFB-like matrix, required for the sensomics process.

Chapter 4 presents a preliminary study on the formation of (*E*)-β-damascenone during malt kilning, mashing and wort boiling.

Chapter 5 shows a study on the formation of five Strecker aldehydes during malt kilning at different temperatures and provides a kinetic model accounting the rate limiting reactions and intermediate species.

Chapter 6 summarises the main conclusions of this PhD thesis, along with possible future work for continuing this line of research.

Figure 0.1 shows graphically the structure of the thesis and its chapters. The main outcomes from each chapter are written in bold.

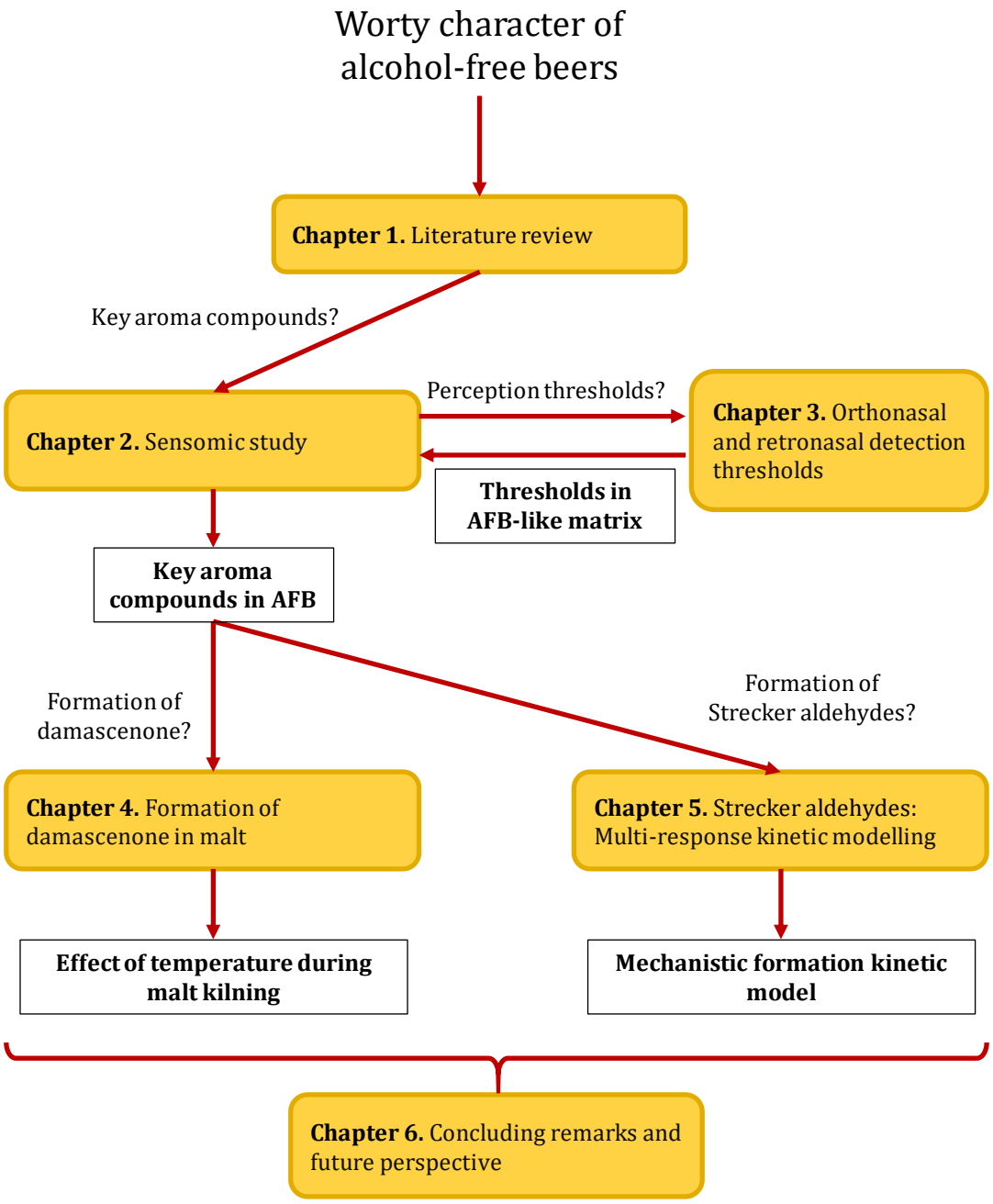

Figure 0.1 General structure of the thesis. Main outcomes in bold.

Chapter 1.

Chapter 1. Alcohol-Free and Low-Alcohol Beers: Flavour Chemistry and Sensory Characteristics

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This chapter has been drafted following the format of the Comprehensive Reviews in Food Science and Food Safety journal, where it is intended to be submitted.

Abstract

Alcohol-free beers have gained popularity in the last decades because they are a healthier alternative to alcoholic beers. Consumers are becoming more aware of the benefits of reducing the alcohol consumption, and this has increased the sales of non-alcoholic alternatives. However, there are still many challenges for the brewing industry to produce an alcohol-free beer that resembles the pleasant fruity flavour of regular beers. The aim of this literature review is to give a comprehensive overview of alcohol-free beer focusing, in particular, on flavour chemistry. The formation of the most important flavour compounds has also been reviewed, focusing on Strecker aldehydes, higher alcohols and esters. The role of ethanol as a direct and indirect flavour-active compound has been examined, as well as the influence of the most common brewing methods on the flavour profile. The choice of a physical (dealcoholisation) or a biological method has a great impact on the organoleptic characteristics of the final product, and thus the physical and chemical principles of these different methods have been discussed in this study. Other aspects, such as the legal definition of alcohol-free beers in different countries and the consumer perception, have been addressed.

1.1. Introduction

Beer is one the most popular alcoholic beverages across the world. Besides its pleasant fruity flavour and role as a thirst-quencher, consumption of beer presents benefits for health, such as reduced risk of cardiovascular disease, antioxidant effects and a source of minerals, amongst many other (Pilarski & Gerogiorgis, 2019). However, the population is becoming more conscious of the risks of abusive consumption of alcohol and in consequence, there is a clear trend to reduce alcohol intake (Shield, Parry, & Rehm, 2013). Due to this and other grounds, such as religious reasons or prohibition of alcoholic beverages in certain countries, and clear quality improvement of the alcohol-free products, alcohol-free and low-alcohol beers have experienced a remarkable growth in the market. Citizens are becoming more aware of the dangers of driving under the influence of alcohol. Indeed, this was a reason for most of Dutch consumers to choose a non-alcoholic beer (57% of the respondents), followed by 40% who selected alcohol-free beer because they liked the taste, 39% were abstaining from alcohol and 32% chose it as a thirst quencher (Nederlandse Brouwers, 2017). The best example of the success of alcohol-free beer is Spain, leader in production and consumption of alcohol-free beers in Europe. Alcohol-free beer is considered a beverage of choice for 46% of beer drinkers in Spain, where it comprised 17.1% of total beer consumed in the country in 2018 (Ministerio de Agricultura, 2018). In other countries such as the Netherlands the consumption of alcohol-free beers is much more modest, where it only represented 3.3% of the total sales in 2016, but growing every year (Nederlandse Brouwers, 2016).

Nonetheless, there are still challenges that the brewers have to overcome in order to improve the low-alcohol alternatives and reach the levels of sales of the regular beers. Although many choose alcohol-free beer because they appreciate the flavour, others often

comment that it does not taste as good as a standard beer. In a survey carried out within South Korean beer drinkers, the main reason (56% of the respondents) not to drink an alcohol-free beer was because "it does not taste good" (Statista Research Department, 2019). Indeed, one of the main challenges when brewing an alcohol-free beer is its flavour. The fruity flavour of regular beers is caused by the presence of esters that are formed during regular fermentation, which are much scarcer in their alcohol-free counterparts. Furthermore, highly odour-active aldehydes are usually found in concentrations well above their perception thresholds in alcohol-free beers brewed by certain biological methods. Hence, the brewing method chosen plays a key role in the flavour profile of the final product.

The literature available on the topic is often focused on the optimisation of the control of ethanol formation or removal. However, the impact of the brewing method on the flavour compounds and sensory properties is secondary in many studies, not to mention the forgotten role of ethanol as a contributor to flavour and sensory perception. The aim of this paper is to give an overview of alcohol-free and low-alcohol beers addressing the following questions: what are the legal definitions of alcohol-free and low-alcohol beer, what are the current consumer trends in the matter, how the relevant flavour compounds are formed, what effects does the lack of alcohol have, what are the different production strategies or methodologies for these beers, and how do they affect the sensory properties of the final product. In order to present a more comprehensive approach, this review covers beers with an alcohol content below 3.5% alcohol by volume (ABV).

1.2. What is an alcohol-free beer?

The definition of an alcohol-free beer is not as straightforward as it may sound and varies quite significantly between countries. Most commonly, alcohol-free beers are not defined separately from regular beers, but as a subcategory within (Figure 1.1). In the USA, according to the title 27 of the Electronic Code of Federal Regulations (e-CFR) of the United States (Title 27 Alcohol, Tobacco Products and Firearms, e-CFR, USA), beer is defined as a *"fermented beverage (...) containing one-half of one percent or more of alcohol by volume, brewed or produced from malt"* (27 e-CFR.25.11). Beers containing less than 0.5% ABV fall into the category of *malt beverages* (27 e-CFR.7.10), so they are called "non-alcoholic malt beverages". The term *alcohol-free* is used for the beverages containing 0% ABV.

On the other side of the Atlantic Ocean, alcohol-free or low-alcohol beers are not defined in EU regulations. According to the Article 9(1k) of the Regulation No 1169/2011,

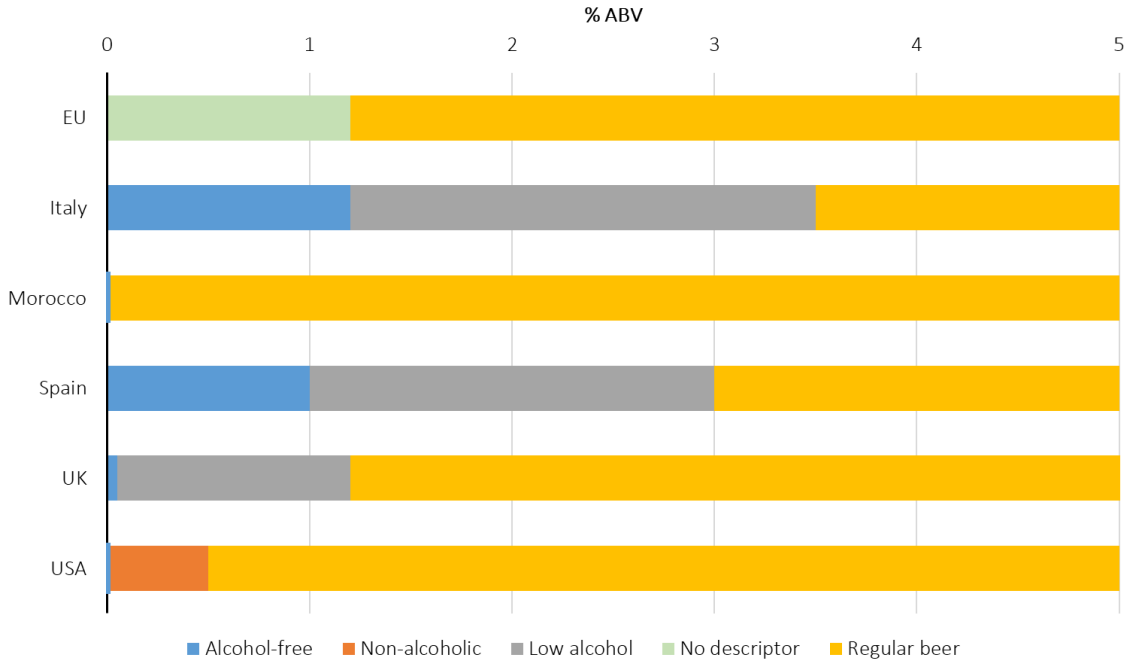


Figure 1.1 Alcohol content ranges for definitions of beers with low content of alcohol in different countries.

beverages containing less than 1.2% alcohol do not need to state the alcohol content on the label. In addition to the European legislation, the countries within the EU may apply their own definition of alcohol-free beers. For instance, in Spain alcohol-free beers or "beer without alcohol" (*cerveza sin alcohol*, in Spanish) are those containing less than 1% ABV, whereas beers with an alcohol content between 1 and 3% are called low-alcohol beers (Boletín Oficial del Estado, 2016). In Italian legislation, the term "*birra analcolica*" is used for those with less than 1.2% ABV and "*birra light*" or "*birra leggera*" for those from 1.2 to 3.5%ABV (Decreto del Presidente della Repubblica n. 272, 1998). The Department of Health & Social Care of the UK suggested different descriptors for beers of low alcohol content, but these are not mandatory (Department of Health & Social Care, 2018). "*Alcohol-free beer*" is a term used for beers with up to 0.05% ABV, and "*low alcohol*" is referred to the range between 0.05 and 1.2% ABV. If the beer has been produced by dealcoholisation of a regular beer, the alcohol content must not exceed 0.5% ABV for it to be labelled as "*de-alcoholised*".

Legislation is stricter in other countries like Morocco, where the term *alcohol-free beer* (*bière sans alcool*, in French) only applies to those with an alcohol content equal to 0% ABV (Administration des Douanes et Impôts Indirects du Royaume du Maroc, 1977). Further information regarding the denomination of beers and other alcoholic beverages can be found in the International Alliance for Responsible Drinking's website, where they published a table detailing the alcohol beverage labelling requirements for over 100 countries.

1.3. Consumer behaviour and market insights

Abstention from alcohol has risen over the last few decades. According to a study of Global Market Insights (2019), the non-alcoholic beer market size was estimated to be

13.5 billion US dollars in 2016, with a value projection of 25 billion US dollars in 2024. This reflects the growth of the variety of products worldwide. The world leader in releasing new alcohol-free and low-alcohol beers (defined as below 3.5% ABV) is China where 29% of the new beers launched in 2016 belong to this category. In Spain this percentage was 12%, 11% in Germany, 9% in Poland (Mintel, 2017) and an average of 8% globally.

This tendency towards abstention from alcohol has increased in the UK from 10% non-drinkers in 1998 to 21% in 2013 (Office for National Statistics, 2013). Moreover, this growing trend has been observed to be more intense within the youngest groups of the population. Alcohol abstention in people between 16 and 24 years old increased from 18% in 2005 to 29% in 2015 according to a study carried out in the UK (Ng Fat, Shelton, & Cable, 2018). According to the World Health Organization (2019), similar behaviour has been observed in young people from 15 to 24 years old in most European countries. In seventeen European countries, the consumption of alcohol by people of this age group decreased, with Croatia, the Netherlands and Romania reporting the greatest reduction. On the contrary, in 12 other countries the consumption increased, and in only four of them (Belgium, Latvia, Malta and Slovenia) the increase was greater than 10% (Figure 1.2).

The increase in sales of alcohol-free beers seems to be a consequence of this trend change, but it has also been reflected in the sales of their alcoholic counterparts. As early as the 1990s, a decrease in the consumption of alcoholic beverages was observed in both the Canadian and USA markets. In 1990 in Canada, the sales dropped by 0.5% while the

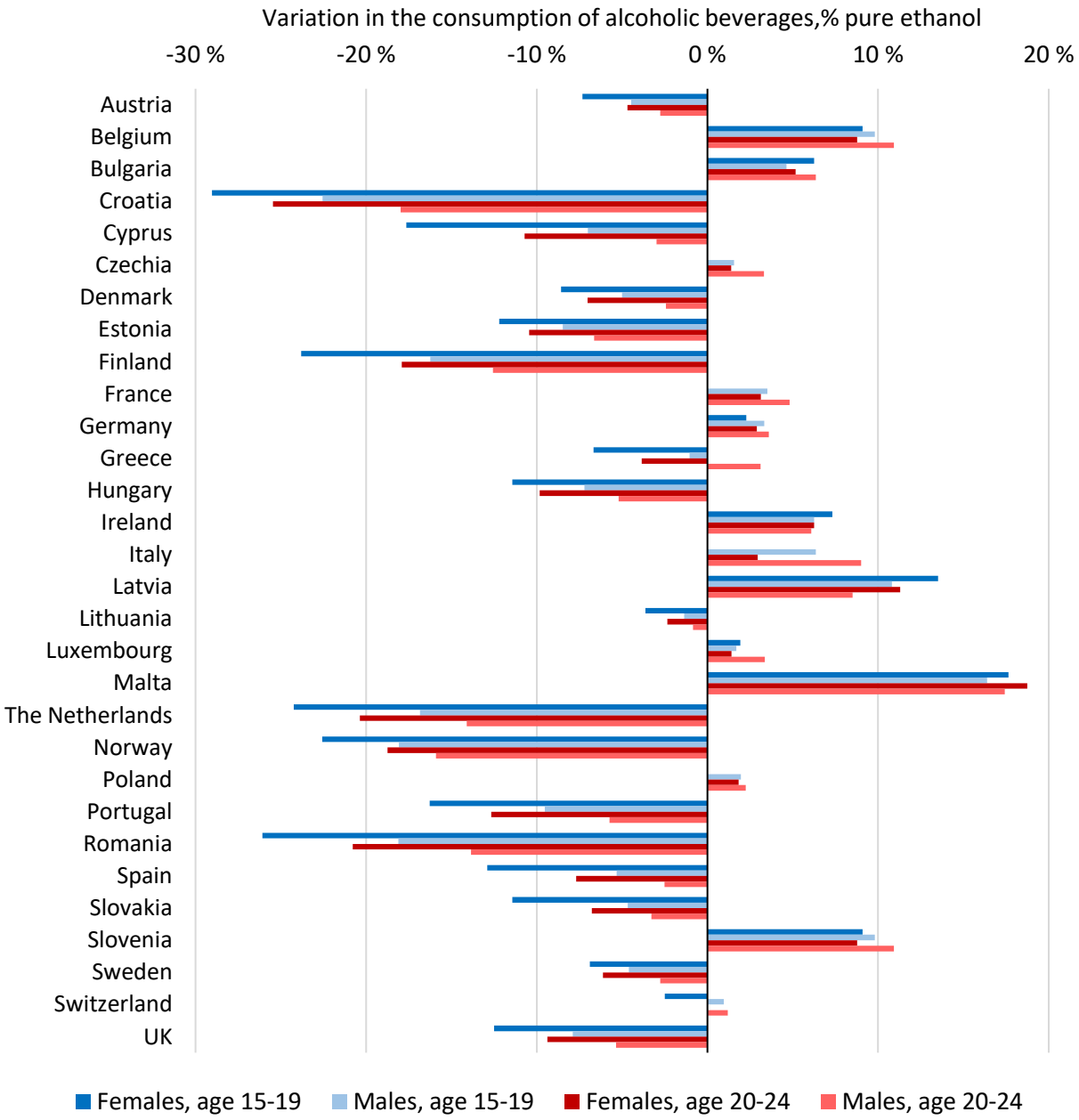


Figure 1.2 Variation of alcohol consumption (in terms of pure ethanol) in young people in Europe between 2010 and 2016. Source: Compilation based on data from WHO-Regional Office for Europe (2019).

alcohol-free beer sales increased by 48%. In 1991 in the USA, the former decreased by 2% and the latter increased by 33% (Stein, 1993). The trend has not changed since then, despite the increasing trend in other countries like the Netherlands, where in the last ten years the consumption of alcohol-free beer has increased more than 400% (Nederlandse Brouwers, 2018). According to a recent study, 47% of the beer consumers in the

Netherlands declared that they drink alcohol-free beer (below 0.5% ABV) at least once a month (Nederlandse Brouwers, 2019). Also, 75% of the respondents stated that they consumed more alcohol-free beer than two years before the survey.

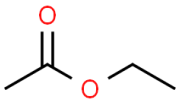
In the UK the sales of low-/no-alcohol lager beers have increased from £30 million in the period between June 2016-May 2017 to £50 million in the same period of 2018/2019 (Mintel, 2019b). Besides, in 2019 British consumers stated that 33% of them reduced their consumption of alcohol in the last 12 months, and 20% did not drink alcohol at all (Mintel, 2019b). The reasons for this were mainly to improve their health (47% of the respondents), to manage their body weight (38%) or to save money (34%). 16% of UK beer drinkers would like to have a broader offer of low-/no-alcohol beers in the market, this percentage jumping to a 25% for people aged 18-24 (Mintel, 2019a). This segment of consumers in Germany showed a similar opinion towards alcohol-free and low-alcohol beers. Almost a third of the respondents (31%) agreed that these beers taste "as good" as regular beers with an alcohol content of 4-6% ABV (Mintel, 2017). Furthermore, in a country of a historical tradition of beer production and consumption, this study showed that only 9% of Germans said that they would feel embarrassed to order one of these beers.

1.4. Flavour chemistry of beer

Although beer can be produced from just four ingredients, i.e. barley malt, hops, yeast and water, the result in terms of flavour compounds is a very complex mixture of hundreds of different volatiles and non-volatiles. Depending on their concentration and how far this is from their perception threshold, their contribution to the overall aroma of beer vary. The perception threshold of a flavour compound is defined as the minimal concentration at which this can be perceived. The ratio of concentration and threshold is

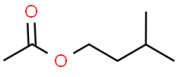
known as odour activity value (OAV) and is a useful indicator of whether a compound is likely to be a contributor to the aroma of the product. For this reason, from the hundreds of flavour compounds identified in different beers, the number of impact character compounds is much more limited (Meilgaard, 1975b). The most important flavour compounds in beer can be classified into aldehydes, higher alcohols, esters, vicinal diketones, sulfur compounds and hop-derived compounds. Figure 1.3**Error! Reference source not found.** shows a selection of the most important flavour compounds in beer, their odour quality and perception threshold in water.

Aldehydes are generally formed by different chemical mechanisms during malt kilning, mashing and wort boiling. Aldehydes have been identified as the main contributors to the characteristic malty and worty aroma of alcohol-free beers, especially those brewed by biological methods (Perpète & Collin, 1999b). This is due to the incomplete transformation of aldehydes into alcohols and esters because of the process conditions non adequate for their formation (See Section 1.7.2). Albeit many flavour-active aldehydes have been identified in alcohol-free beers and regular beers, such as acetaldehyde, hexanal, (*E*)-2-nonenal, benzaldehyde, furfural and 5-hydroxymethyl-furfural, the most relevant ones are 2-methylbutanal, 3-methylbutanal, and methional (Gernat, Brouwer, & Ottens, 2020). These three aldehydes, along with 2-methylpropanal and phenylacetaldehyde, have very low perception thresholds, from 105 µg/L water for phenylacetaldehyde to 0.43 µg/L water for methional (Czerny et al., 2008; Saison, De Schutter, Uyttenhove, Delvaux, & Delvaux, 2009). Moreover, their concentrations in Lager beers (Table 1.1) are much lower than in low-alcohol beers and, consequently, their contribution to the overall aroma of alcohol-free beers is considerably higher.

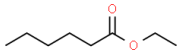


ethyl acetate

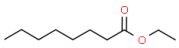
green apple, fruity
1,110 ppb [a]



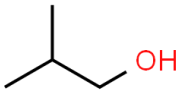
3-methybutyl acetate
(isoamyl acetate)
banana, solvent
510 ppb [a]



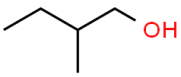
ethyl hexanoate
(ethyl caproate)
fruity
210 ppb [b]



ethyl octanoate
(ethyl caprylate)
fruity
900 ppb [b]

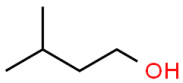


2-methylpropanol
(isobutanol)
malty
550 ppb [c]

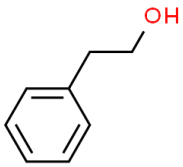


2-methylbutanol

malty, solvent
1,200 ppb [c]

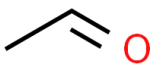


3-methylbutanol
(isoamyl alcohol)
malty
220 ppb [c]

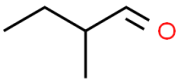


2-phenylethanol

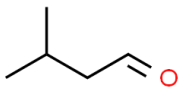
flowery, honey
140 ppb [c]



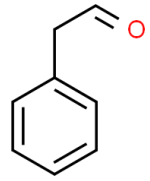
acetaldehyde
fresh, green
25 ppb [c]



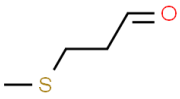
2-methylbutanal
malty
1.5 ppb [c]



3-methylbutanal
malty
0.50 ppb [c]

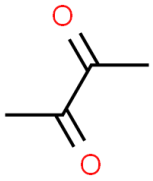


phenylacetaldehyde
honey
105 ppb [a]

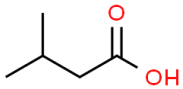


methional

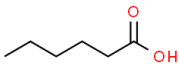
cooked potato
0.43 ppb [c]



2,3-butanedione
(diacetyl)
buttery
1.0 ppb [c]



3-methylbutanoic acid
(isovaleric acid)
sweaty
490 ppb [c]



hexanoic acid
(caproic acid)
goat, vegetable oil
4,800 ppb [d]

Figure 1.3 Key aroma compounds commonly found in beer and their aroma quality and perception threshold in water (ppb = µg/L). Esters in orange, alcohols in green, aldehydes and ketones in blue, and acids in red. Molecule structures retrieved from ChemSpider.com, Royal Society of Chemistry, accessed on 4th February 2020. Ref.: [a] Saison, De Schutter, Uyttenhove, Delvaux, & Delvaux (2009), [b] Kobayashi, Shimizu, & Shioya (2008), [c] Czerny et al. (2008), [d] Wagner, Granvogl, & Schieberle (2016).

Table 1.1 Concentration of Strecker aldehydes in Lager beers from Spain and Czechia. Source: Andrés-Iglesias, Nešpor, et al. (2016).

Compound	Concentration, µg/L	
	Spanish beers	Czech beers
2-methylpropanal	1.71 – 28.3 - 229	4.63 - 24.0 - 58.0
2-Methylbutanal	1.80 - 8.78 - 60.4	3.16 - 11.7 - 34.5
3-Methylbutanal	0.97 - 7.60 - 47.2	3.57 - 13.7 - 38.2

Expressed as minimum value – average – maximum value.

Higher alcohols, common term in brewing for those other than ethanol and methanol, and esters are of key importance in alcohol-free beer flavour, either for their presence or absence. These compounds are responsible to the fruity flavour of regular beers The most important higher alcohols in beer are 3-methylbutanol, 2-methylbutanol, 2-phenylethanol and 2-methylpropanol, all of them derived from amino acids (Fritsch & Schieberle, 2005). In Bavarian wheat beers, several esters were found (Bellut & Arendt, 2019) to be key odourants and showed OAV considerably higher than 1 (i.e. above the threshold). The most important ones were 3-methylbutyl acetate (OAV=231), ethyl 2-methylpropanoate (225) and ethyl butanoate (115). A common defect of some alcohol-free beers brewed by types of yeasts other than *Saccharomyces cerevisiae* is the presence of one or more of these compounds at a high concentration, this being translated into an unbalanced aroma or overpowered by these compounds (Bellut & Arendt, 2019).

Other flavour compounds groups of flavour compounds relevant to beer aroma are vicinal diketones, sulfur compounds and hop-derived compounds. 2,3-Butanedione (diacetyl, butter-like aroma), and in a lower extent 2,3-pentanedione (butter, creamy aroma) are the most relevant vicinal diketones in beer. These are associated with an "unmatured" aroma in fresh beers (Nakatani, Takahashi, Nagami, & Kumada, 1984) or as a defect after long storage (Inoue, 1998). Sulfur compounds are well known flavour compounds because of their very low perception threshold and characteristic aroma. In

beer, the most relevant ones are dimethyl sulphide (DMS; sweetcorn aroma) and 3-methyl-2-butene-1-thiol (MBT; skunky aroma), considered as off-flavours above a certain concentration. Sulphur compounds contribute positively to beer flavour at low levels. They are also well known in flavour science because of the very low perception thresholds, this being 0.84 µg/L for DMS and only 0.01 ng/L for MBT (Czerny et al., 2008; San Juan, Cacho, Ferreira, & Escudero, 2012). DMS concentration is mainly reduced during wort boiling through evaporation (Scheuren, Baldus, Methner, & Dillenburger, 2015), whereas MBT is formed from the degradation of hops iso-α-acids brought on by light (De Keukeleire, Keyerick, Huvaere, Skibsted, & Andersen, 2008; Parker, 2012). On the other hand, hops contribute to beer flavour with a huge variety of aroma compounds, mostly terpenes and sesquiterpenes, such as linalool (floral, piney), myrcene (woody, piney aroma), caryophyllene (earthy, spicy) and humulene (hoppy aroma) (Peacock, 1998).

1.5. Origins and formation of aroma compounds during the brewing process

Due to their role as key aroma compounds both in regular and alcohol-free beers, the present section aims to cover the principal formation pathways that take place during the brewing process. These aroma compounds are mainly derived from amino acids and formed by a variety of mechanisms involving chemical reactions from thermal processes and fermentation. Figure 1.4 provides a simplified overview of the brewing process and the impact on the flavour compounds.

1.5.1. Aldehydes

2-Methylpropanal, 2- and 3-methylbutanal, methional, and phenylacetaldehyde belong to the Strecker aldehydes. These have been found in stages of the brewing process

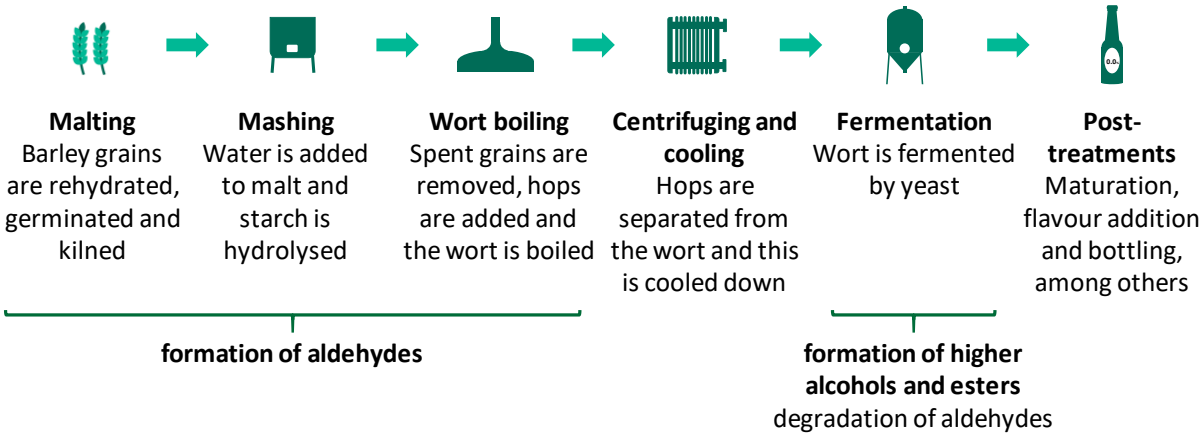


Figure 1.4 Overview of the brewing process and relative impact on flavour compounds. Cereal icon authored by Good Ware (www.flaticon.com).

as early as the malted grains (Beal & Mottram, 1994; De Clippeleer et al., 2010). Malting is a process in which germinated grains, usually barley, are roasted or kilned under a current of hot air. The temperature of the drying gas is controlled, and it influences greatly the characteristics of the finished malt in terms of colour and aroma. Temperatures below 100 °C are applied to achieve pale malts, whereas roasted or "specialty" malts require temperature in the range of 130-230 °C (Yahya, Linforth, & Cook, 2014). The authors also reported that higher kilning temperature increases the concentration of phenylacetaldehyde in finished malts.

The formation of Strecker aldehydes continues in further stages of the brewing process. Mashing, and specially wort boiling, are processes where high temperature is applied. Mashing is a process where the optimal temperatures for the enzymatic degradation of starches and proteins are applied in the form of a temperature gradient. This way, the concentration of fermentable sugars and amino acids increase significantly due to the action of α- and β-amylases and proteases (Yoshioka & Hashimoto, 1979). During wort boiling, Strecker aldehydes are formed due to the presence of their precursors in the liquid. Due to the boiling conditions, the flavour volatile compounds are

formed through the Maillard reaction and the Strecker degradation, as well as lost by evaporation (Buckee, Malcolm, & Peppard, 1982). These compounds are formed at a higher rate at the beginning of the process, and then their concentration stabilizes due to a balance between formation and evaporation (De Schutter et al., 2008b).

1.5.1.1. The Maillard reaction

Strecker aldehydes are mainly formed from amino acids via the Maillard reaction. In 1912, Louis-Camille Maillard presented his findings in the chemistry of the reaction between several sugars and amino acids (Maillard, 1912). He observed the formation of the colour compounds "melanoidins" from the reaction of the aldehyde group of reducing sugars and the amino group in amino acids, leading to the loss of molecules of CO_2, water, and possibly "the creation of new double bounds by dehydration and cyclic compounds". Some decades later, Hodge (1953) provided a comprehensive account of these reactions, distinguishing three stages: (I) condensation of a reducing sugar and an amino compound to form a Schiff base and a subsequent Amadori rearrangement product, (II) dehydration and fragmentation of sugars and amino acids, and (III) aldol condensation and aldehyde-amine polymerisation.

The Maillard reaction is initiated by the condensation of the carbonyl group in a sugar molecule and an amino group, typically those in amino acids (Figure 1.5a). This first reaction is spontaneous but considerably accelerated at high temperature. The carbonyl group in a reducing sugar is attacked by the amino group, thus forming a Schiff base. This chemical structure can undergo an acid-catalysed rearrangement to form a series of 1-amino-1-deoxyketoses, which are called the Amadori rearrangement products (ARP), via an intermediate eneaminol. The ARPs are relatively stable intermediate compounds in the Maillard reaction. The ARPs formed from aldohexoses are usually more stable than

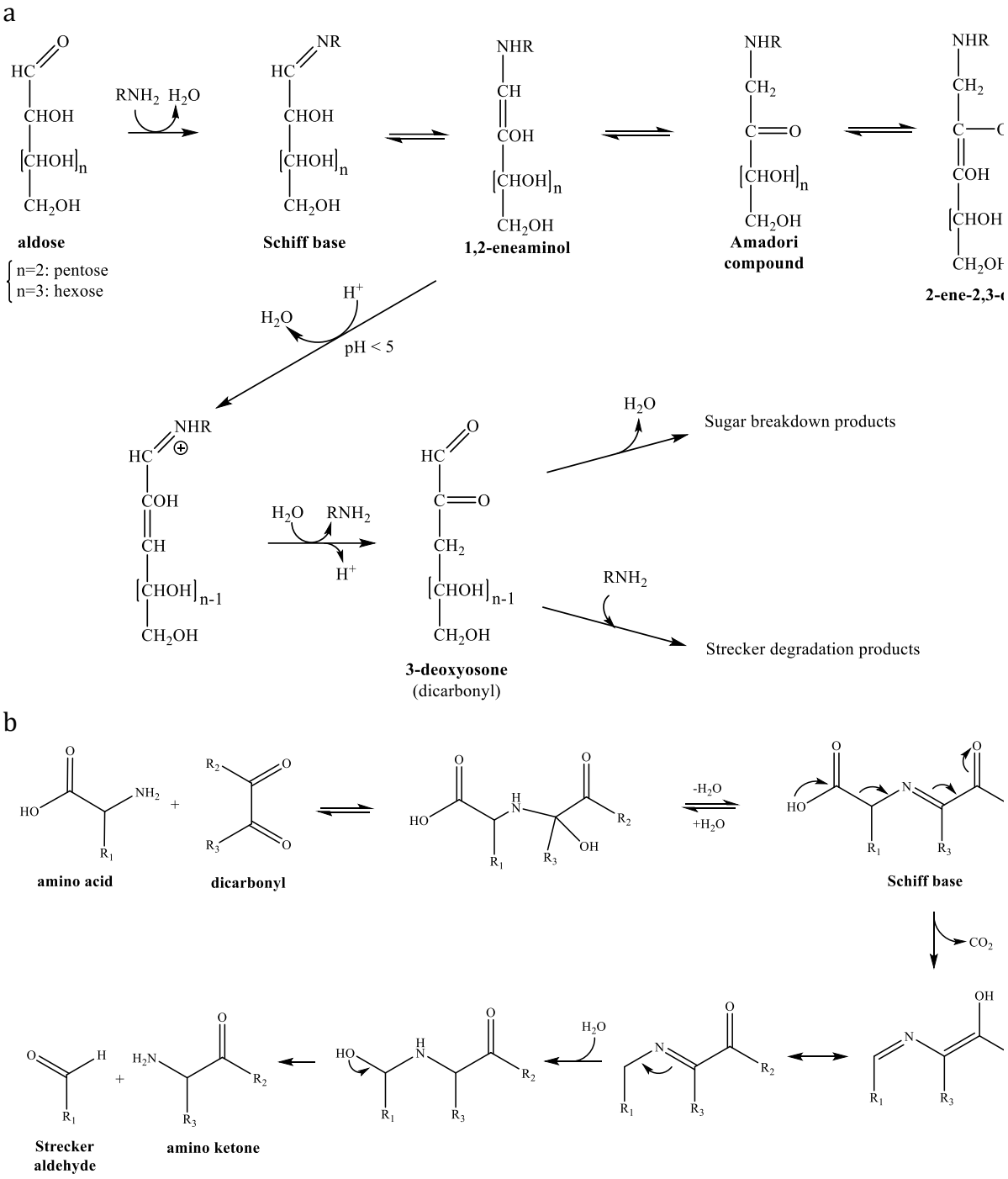


Figure 1.5 Chemical reactions of the Maillard reaction (a) and Strecker degradation (b). Adapted from Gernat et al. (2020).

those from aldopentoses (Parker, 2015). In the case of ketoses, the mechanism is slightly different and a series of 2-amino-2-deoxyaldoses, called Heyns rearrangement products (isomer of ARPs), are formed. ARPs were considered as the starting point of the intermediate stage of the Maillard reaction by Hodge. These intermediates are in

equilibrium with two enol compounds, formed via 1,2-enolisation at low pH and 2,3-enolisation at high pH through keto-enol tautomerisation. The subsequent stages involve the dehydration and regeneration of the amino compound and the formation of the α-dicarbonyl compounds deoxyosones. Since the amino compounds are released from ARPs and Heyns products without any structural change, they are sometimes considered as a catalyser for the initiation of the Maillard reaction.

1.5.1.2. Strecker degradation

Deoxyosones, like 3-deoxyhexosulose and 1-deoxyhexulose from glucose, and other α-dicarbonyl compounds which derive mainly from the fragmentation of the deoxyosones, like glyoxal, methylglyoxal and even 2,3-butanedione, are unstable species which can further react with other compounds or undergo dehydration, cyclisation, and other cleavage reactions (Kocadağlı & Gökmen, 2016). The reaction between α-dicarbonyl compounds and amino acids is called Strecker degradation and it is essential in the formation of aroma volatile compounds in foods submitted to thermal treatments. Figure 1.5b shows the mechanism of the Strecker degradation from an amino acid and a dicarbonyl compound. This reaction was first reported by Adolph Strecker in 1862 when he observed the formation of volatile aldehydes from the reaction between alloxan (5,5-dihydroxypyrimidine-2,4,6(*1H*,*3H*,*5H*)-trione) and amino acids, along with the release of CO_2 (Strecker, 1862). Strecker reported that the CO_2 molecule is released from the carboxyl group of the amino acid, which results on the formation of aldehydes with one less carbon than the original amino acid. Thus, a series of aldehydes are formed from their corresponding amino acids, the most important ones in flavour chemistry being methional (potato-like aroma), 2-methylpropanal (malty), 2-methylbutanal (malty), 3-methylbutanal (malty) and phenylacetaldehyde (honey) due to their low perception

thresholds (Piornos et al., 2019; 0). Their amino acids of origin are methionine, valine, isoleucine, leucine and phenylalanine, respectively.

1.5.1.3. Other lipid-related formation pathways

Other minor pathways for the formation of aldehydes in beer is by the oxidation of unsaturated fatty acids. This reaction usually leads to the formation of linear aldehydes, such as hexanal, (*E*)-2-nonenal, and (*E*,*E*)-2,4-decadienal (De Schutter et al., 2008a; Gernat et al., 2020). However, some lipid-derived reactive carbonyls can further react with amino acids to produce Strecker aldehydes. Hidalgo & Zamora (2016) proposed a formation pathway of Strecker aldehydes from the reaction of an amino acid and a 2-alkenal through an imine intermediate (Figure 1.6). The fat content in malt barley is lower than 3.4%w/w, of which 58% of the fatty acids correspond to linoleic acid (Anness, 1984). This unsaturated fatty acid forms 2-alkenals and 2,4-alkadienals, such as (*E*)-2-octenal and (*E*,*E*)-2,4-decadienal, under high temperature conditions in the presence of O_2 (Schieberle & Grosch, 1981). The relative contribution of this formation pathway to the total amount of Strecker aldehydes is likely to vary depending on the food matrix and

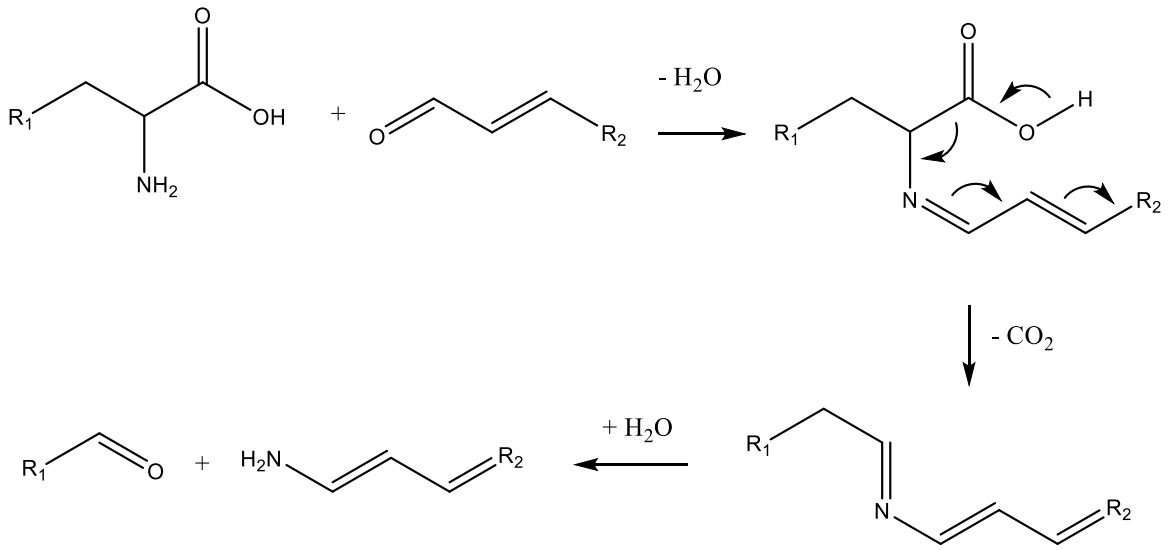


Figure 1.6 Formation pathway of Strecker aldehydes from amino acids and lipid-derived reactive carbonyls. Adapted from Hidalgo & Zamora (2016).

is largely unknown. Nevertheless, the presence of lipids has been proven to contribute positively to the formation of these aldehydes. Gallardo et al. (2008) observed an increase in phenylacetaldehyde in wort when this was supplemented with linoleic acid or its degradation product, (*E*,*E*)-2,4-decadienal. The relative increase in the concentration of phenylacetaldehyde was 1.1-2.5 times and 3.6-4.6 times higher than in the control wort, respectively. These results confirmed the role of alkenals in the formation of Strecker aldehydes by reaction with amino acids.

1.5.1. Higher alcohols

Fermentation is the main step for the formation of higher alcohols and esters in the brewing process. In a similar way to the formation of Strecker aldehydes, higher alcohols are formed from amino acids. In 1907, Felix Ehrlich hypothesised that higher or "fusel" alcohols are formed as fermentation products of amino acids, due to the clear similarity of leucine, isoleucine, valine and phenylalanine, with 3-methylbutanol, 2-methylbutanol, 2-methylpropanol and 2-phenylethanol, respectively (Ehrlich, 1907). He proved his hypothesis by supplementing fermentation media with amino acids, which led to the formation of higher concentrations of these alcohols. Four years later the formation pathway of higher alcohols from amino acids was completed by Neubauer & Fromherz (1911). For this reason, this reaction pathway is called the Ehrlich pathway, or less commonly, the Ehrlich-Neubauer pathway. First, the amino group is substituted by a keto group through a transamination enzymatic reaction, thus forming an α-keto acid (Figure 1.7). In this reversible reaction, glutamate and α-ketoglutarate act as donor and acceptor of the amino group, respectively (Pires, Teixeira, Brányik, & Vicente, 2014). The next step is the non-reversible decarboxylation of the α-keto acid into a Strecker aldehyde (or fusel aldehyde), with the loss of a molecule of CO_2. The aldehyde is lastly reduced to the

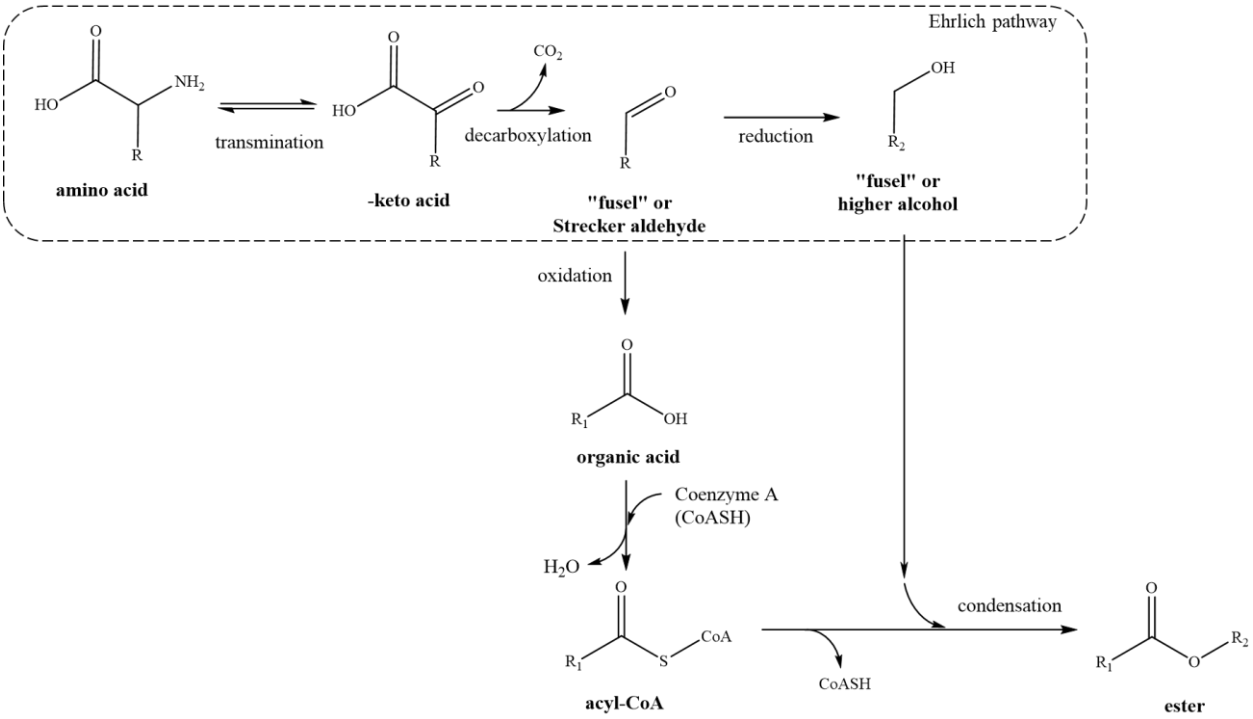


Figure 1.7 Chemical reactions of the Ehrlich pathway and subsequent formation of esters. Adapted from Pires et al. (2014).

corresponding alcohol by action of alcohol dehydrogenases, which also participate in the production of ethanol by reducing acetaldehyde (Bennetzen & Hall, 1982). Fermentation conditions (short time or low temperature) in production processes for alcohol-free and low-alcohol beers such as cold contact fermentation (CCF) and arrested fermentation do not allow enough reduction of the aldehydes and formation of alcohols. Therefore, these aldehydes are key contributors to these beers' aroma. Another mechanism for the formation of higher alcohols in beer is through the anabolic biosynthetic route from carbon source, mainly sugars. In this route, the α-keto acids are formed from carbohydrate metabolism and they act as precursors of aldehydes by decarboxylation as shown in the Ehrlich pathway (Chen, 1978). This formation pathway from carbohydrates has usually been related to fermentation media with low or absent nitrogenous nutrients (Van Gheluwe, Chen, & Valyi, 1975). The contribution of each pathway (catabolic or anabolic) to the final concentration of higher alcohols in the beer is not the same for all

these compounds. 1-Propanol is not significantly affected by the lack of one branched-chain amino acid aminotransferases (essential in the Ehrlich pathway) in mutant yeasts, which means that this compound can be easily formed by the anabolic pathway (Eden, Van Nedervelde, Drukker, Benvenisty, & Debourg, 2001). 2-Methylbutanol and 3-methylbutanol, however, saw their concentration drastically reduced when one of these aminotransferases was absent. The researchers also reported the unexpected formation of higher amounts of these alcohols when two aminotransferases were not coded, which suggested that other formation routes might be involved other than the already known Ehrlich and anabolic pathways.

1.5.2. Esters

The formation of esters in beer by yeast fermentation is closely related to that of alcohols, since they are formed by enzymatic condensation of organic acids and alcohols (Pires et al., 2014). Esters can be divided into two groups: acetate esters (those formed from acetic acid and an alcohol), and ethyl esters (those formed from ethanol and an organic acid). Firstly, the formation of organic acids in beer takes place at the end of the Ehrlich pathway, by oxidation of Strecker aldehydes via the action of aldehyde dehydrogenases (Figure 1.7) (Hazelwood, Daran, Van Maris, Pronk, & Dickinson, 2008). Although organic acids deriving from amino acids, such as 2-methylpropanoic acid, 2- and 3-methylbutanoic acid, or 2-phenylacetic acid, are flavour-active, their contribution to beer flavour is not very important, mainly due to their high perception thresholds, usually well above 1000 μg/L (Czerny et al., 2008; Piornos et al., 2019).

The reaction between alcohols and acids to form esters occurs through the formation of an intermediate acyl-CoA molecule from the organic acid. Acetyl-CoA is a special case of acyl-CoA that can enter the Krebs cycle in aerobic conditions, while in the absence of

oxygen, it can be esterified with a molecule of ethanol to produce ethyl acetate (fruity, solventy aroma), among other esters (Pires et al., 2014). Acetate esters, like ethyl acetate, 3-methylbutyl acetate, 2-phenylethyl acetate and others, are the most abundant esters in beers. The synthases responsible for this condensation reaction have been identified as alcohol acetyl-transferases (Yoshioka & Hashimoto, 1981). The production of ethyl esters by the condensation reaction of acyl-CoA molecules and ethanol is due to the activity of two acyl-CoA:ethanol *O*-acyltransferases (Saerens et al., 2006). For more information regarding the enzymes involved in the production of higher alcohols and esters by yeast, the reader is directed to the review on this topic by Pires et al. (2014).

Several fermentation parameters affect the performance of the yeast for producing fruity alcohols and esters. Amongst them, the most noticeable are the yeast strain and fermentation temperature. The effect of yeast strain is covered in Section 1.7.2.1 of this review paper. Fermentation temperature has a critical effect on the expression of a gene responsible for encoding a permease that allows the transport of amino acids into the yeast cell (Didion, Grauslund, Kielland-Brandt, & Andersen, 1996). As one might expect, this has a great impact in the formation of higher alcohols via the Ehrlich pathway, and thus in the formation of esters. Higher fermentation temperature improves the formation of higher alcohols, esters and ethanol in beer (Takahashi, Yoshioka, Hashimoto, & Kimura, 1997), and this effect has been observed even at slight increases of temperature, such as from 8.5 to 11.5 °C (Kucharczyk & Tuszyński, 2018). For instance, the concentration of 3-methylbutyl acetate increased significantly from 1.46 to 1.82 mg/L when the fermentation temperature increased from 8.5 to 11.5 °C, whereas amyl alcohols (2- and 3-methylbutanol) increased from 67.5 to 74.2 mg/L.

1.6. The role of ethanol in the sensory perception of beer flavour

Ethanol also contributes to the sensory and physico-chemical characteristics of beer. The sensory differences between low-alcohol or alcohol-free beers and alcoholic beers are not limited to the flavour compounds.

1.6.1. Ethanol as a taste compound

Ethanol stimulates the gustatory system being perceived as sweet and bitter (Blizard, 2007; Scinska et al., 2000) and the olfactory system with its characteristic aroma (Czerny et al., 2008), but also the lingual branch of the trigeminal nerve (Clark, Hewson, Bealin-Kelly, & Hort, 2011; Danilova & Hellekant, 2002). Through the trigeminal stimulus, ethanol contributes to the mouthfeel and warming/burning sensation (Clark et al., 2011; Wilson, O'Brien, & MacAirt, 1973), as well as to the perception of body and fullness (Gawel, Van Sluyter, & Waters, 2007). There is more consensus regarding the bitterness of ethanol, perceived as such even at 0.3% v/v in water (Scinska et al., 2000), whereas sweetness does not seem to be affected by ethanol (Gawel et al., 2007). However, these authors found that even small increases in the ethanol content in Riesling wine, from 11.6 to 13.6% v/v, had a significant positive effect on hotness in mouth.

Besides, ethanol provokes a sensation of irritation in the nasal cavity, but this is overcome by the trigeminal burning sensation at concentrations above 5% (units not specified) (Rothe & Schrödter, 1996). It has been proven that ethanol stimulates the trigeminal receptors, some of them being also responsive to mechanical and cooling stimuli (Danilova & Hellekant, 2002). These responses were observed by applying aqueous ethanol solutions at concentrations of 0.7 M, i.e. 32.2 g/L or approximately 4% v/v. When administered together with a sweetener like sucrose or a bitter compound like

quinine, ethanol acts as an enhancer of sweetness or a suppressor of bitterness (Danilova & Hellekant, 2000). This was evidence that ethanol interacts with taste receptors, besides the trigeminal system.

The content of ethanol has also been related to the perception of 'body' in beer. *Body* is a term usually used in several languages (*cuerpo* in Spanish, *corpo* in Italian, *σώμα* in Greek, or *Körper* in German) to describe a texture sensory characteristic, but there is no consensus on its definition. The definitions found in literature are in most cases rather vague, ambiguous, or relating body to other sensory and physical attributes. Several authors have come out with their own definition of body. Clapperton (1974) described body in beer as "*a taste of substantial character, a sense of substance as felt by the palate*". In his glossary of food texture terms, Jowitt (1974) defined body as "*that textural property producing the mouthfeel sensation of substance*". Some authors use the terms 'body' and 'fullness' indistinctively, being described as "*the feeling of thickness/fullness as beer is moved around in the mouth*" (Ramsey et al., 2018). Others consider body within the category of fullness and related to density and viscosity (Langstaff, Guinard, & Lewis, 1991b), even though they found density and viscosity were poorly correlated with the alcohol content (correlation coefficients 0.41 and 0.50, respectively) (Langstaff, Guinard, & Lewis, 1991a). The extremely low concentration of ethanol in alcohol-free beers has been also indicated as the reason behind a watery mouthfeel and lack of body. Ramsey et al. (2018) demonstrated that the addition of ethanol to a commercial alcohol-free beer (0.06% ABV determined analytically) up to 5.25% ABV increased significantly ($p<0.05$) the perception of fullness/body. Moreover, the overall liking of these beers was found to be dependent on the alcohol content. Consumers from this study were classified into three different clusters: some consumers preferred the high alcohol beer (22.8% of

consumers), other the low or no alcohol beer samples (27.7%), and the third cluster was called “enthusiasts” (49.5%) because of their high liking scorings independent of the alcohol content of the beer.

1.6.2. Effect of ethanol on the release of flavour compounds

The presence of ethanol has been demonstrated to influence the perception of aroma compounds present in beverages. In order to perceive an aroma compound, this must escape the food matrix, diffuse into the air and reach the olfactory mucosa (Espinosa Díaz, 2004). The release of the compound from the food matrix (a liquid in the case of beer) into the air is governed by the difference of partial pressures of the aroma compound between the liquid matrix and the air, i.e. it is dependent on its concentration in both phases (Mackay, 1980). The air-liquid partition coefficient K_{al} is a useful parameter to quantify volatility. It can be expressed in terms of the concentration of the compound (compound “i”) in the air and liquid phase, C_i^{air} and C_i^{liq}, respectively (Tsachaki et al., 2008):

$$K_{al} = \frac{C_i^{air}}{C_i^{liq}}$$

When expressed in terms of molar fractions, i.e. the ratio of number of moles of the volatile substance to total number of moles of all substances in a phase, the partition coefficient (K_i) can be related to the partial pressure of the aroma compound (Athès, Peña y Lillo, Bernard, Pérez-Correa, & Souchon, 2004):

$$K_i = \frac{y_i}{x_i} = \gamma_i \frac{P^0_{i(T)}}{P_T}$$

Where y_i and x_i are the molar fractions of i in the air and liquid phases, respectively, γ_i is the activity coefficient, which represents the deviation from an ideal mixture, $P^0_{i(T)}$ is the partial pressure of the pure compound i at a given temperature T, and P_T is the total pressure of the system.

As mentioned above, the composition of the liquid phase affects the partition coefficient and thus the release of volatile compounds into the air. Athès et al. (2004) determined the partition coefficients (at 25 °C) of two aroma compounds commonly found in beers, ethyl hexanoate and 3-methylbutanol, in water and ethanol/water mixtures. They found that the values of K_{al} were significantly lower in 10% v/v and 20% v/v ethanol aqueous solutions, around 35-38% and 58-66%, when compared to 100% water. This has been associated with the role of ethanol as a "cosolvent" along with water (Tsachaki et al., 2008). These authors related the presence of ethanol with a higher solubility of aroma compounds in comparison to an ethanol-free matrix. Nonetheless, solubility seems to be a consequence of the molecular interaction between the aroma compound (the solute) and the matrix (the solvent), as well as volatility is. The affinity of the aroma molecule to the components of the matrix is closely related to factors such as the size (molecular weight, molecular or hydrodynamic volume) and nature (functional groups) of both solute and solvent molecules, and resulting physico-chemical characteristics (water solubility, hydrophobicity) (Mackay, 1980; Philippe et al., 2003; Tsachaki et al., 2008). Obviously, a higher affinity between an aroma compound and a solvent, which applies for many aromas in ethanol with respect to water, is translated into higher retention of the volatile. Hence, the concentration of the volatile compound in the gas phase is lower, which consequently affects the aroma perception. A concentration as low as 0.5%v/v ethanol in water was found to induce the retention (8-12%) of 2- and

3-methylbutanal at the liquid phase when compared to 100% water (Perpète & Collin, 2000b). These aldehydes were retained up to 32-39% when the concentration of ethanol reached 5% v/v. Moreover, the detection threshold of methional in a Lager-like matrix was higher (0.5 μg/L) than in an alcohol-free model solution (0.1 μg/L).

Many have tried to mitigate the effects of the lack of ethanol in alcohol-free beers by the addition of replacers or "ethanol-mimic" components aiming to produce a similar sensory experience in the product. Glycerol (1,2,3-propanetriol) has been proposed as an ethanol replacer due to its capacity to retain undesirable worty aldehydes in alcohol-free beers (Perpète & Collin, 2000b). A concentration of 4.5% glycerol was capable of reducing the release of 2- and 3-methylbutanal into the headspace by up to a 40%. To conclude, the role of ethanol in beer and other beverages is very important in terms of taste, mouthfeel and body, as well as on the release of aroma compounds. Finding a replacement that is able to compensate for these important factors has become a great challenge for brewers, product developers and scientists.

1.7. Effect of production strategy

Alcohol-free beers can be produced following diverse methods. Brányik, Silva, Baszczyňski, Lehnert, & Almeida e Silva (2012) classified the methods for alcohol-free beer production into two main groups: physical and biological processes. Physical methods are based on the removal of ethanol from regular beer by means of thermal processes (vacuum rectification, evaporation) or the application of membrane technology (dialysis, reverse osmosis). On the other hand, biological processes aim to control or avoid ethanol production during fermentation. This can be done by using selected yeast strains ensuring low ethanol production, by reducing the amount of fermentable sugars transferred into wort throughout mashing, or by altering the

fermentation conditions in order to reduce yeast activity and thus the production of ethanol.

The role of the aroma volatiles in alcohol-free beers is quite different depending on the brewing method used as well as the nature of the compounds. In the case of beers produced by physical methods, their character is driven by the loss of esters and higher alcohols during processing. These compounds are mainly formed by yeasts during fermentation and then lost either by distillation or through the semipermeable membrane. The aroma profile of alcohol-free beers brewed by biological methods, however, is determined from the presence of aldehydes well above their perception threshold, while they are low in fruity esters and higher alcohols.

1.7.1. Physical methods

1.7.1.1. Thermal processes

The most widespread physical methods for the removal of alcohol from beer are based on thermal dealcoholisation. According to Brányik et al. (2012), in the early stages of the development of these methods, beer was distilled under atmospheric pressure, involving high temperature applied to beer in order to evaporate ethanol. Consequently, the subsequent organoleptic properties of the alcohol-free beer were unacceptable due to thermal damage of the product. The principle behind thermal separation resides in the difference in volatility between ethanol and the rest of the components of the beer (mostly water) (Müller, Bellut, Tippmann, & Becker, 2017). However, compounds with lower or similar volatilities to ethanol will be co-distilled with the alcohol. Therefore, these techniques lead to the partial or total removal of volatile aroma compounds from the beer (Liguori et al., 2015), thus affecting the organoleptic characteristics of the final

product. Since boiling point for any chemical species depends on pressure, researchers turned to the development of dealcoholisation processes at milder temperature by reducing the pressure. Among the thermal techniques used for the dealcoholisation of beer, vacuum distillation and vacuum evaporation are the most common.

Vacuum distillation

Vacuum distillation consists of the separation of ethanol from the aqueous beer matrix by heat treatment based on the differences in volatility between the two solvents. By reducing the pressure of the system, the boiling temperature of ethanol (and the rest of the volatile compounds present) decreases. This way, ethanol can be stripped off the beer at lower temperature, hence reducing the thermal damage of the product (Mangindaan, Khoiruddin, & Wenten, 2018). The simplest setup for distilling off ethanol by vacuum distillation is a single step process. Andrés-Iglesias, Blanco, García-Serna, Pando, & Montero (2016) employed a rotavapor for the lab-scale dealcoholisation of sixteen commercial lager beers from the Spanish market (from 4.6 to 6.5% ABV). The process was carried out using two different conditions: pressure of, and 200 mbar at 67 °C. Beers distilled at 200 mbar at 67 °C showed higher aroma loss than those processed 102 mbar at 50 °C (Table 1.2). Interestingly, the concentration of 2-phenylethanol increased after distillation, due to the continued formation of this relatively less-volatile compound at those temperatures. Unfortunately, the authors did not report the final ethanol content of the dealcoholised beers, nor the sensory characteristics.

Vacuum distillation has been also used for the dealcoholisation of wines. Gómez-Plaza, López-Nicolás, López-Roca, & Martínez-Cutillas (1999) produced a dealcoholised white wine with an alcohol content of 0.3% ABV (initially 10.6% ABV) using a continuous one-step distillation setup at industrial scale (1000 L/h inlet stream). Under the conditions applied (67 to 80 mbar, at 25 °C), the aroma compounds behaved similarly as in the dealcoholisation of beers, with substantial or complete losses of the most volatile compounds, such as butyl acetate, ethyl hexanoate, 3-methylbutanol, and α-terpineol. The authors reported that the wine produced was not acceptable to the consumers. However, the volatiles could be easily trapped by condensation at -10 °C and potentially re-added to the wine.

Thin layer evaporators

Another configuration for a distillation apparatus is "thin layer evaporator". In these evaporators, the liquid to be stripped flows as a thin film, which increases the gas-liquid interphase area and thus the mass transfer into the gas phase. This way, the residence time in the device is reduced, preventing the liquid being thermally damaged. This

Table 1.2 Concentration of aroma compounds in alcoholic beers and difference after distillation under different conditions. Source: Andrés-Iglesias et al. (2016).

		% Aroma loss	
	Regular beer	**Distillation at 102 mbar, 50 °C**	**Distillation at 200 mbar, 67 °C**
Distillation time		29.1 ± 3.4 min	18.4 ± 3.4 min
Aroma compounds			
1-Propanol	13.1 ± 3.2 mg/L	-45.8 ± 12.1%	-59.8 ± 5.6%
Ethyl acetate	17.7 ± 5.8 mg/L	-89.6 ± 3.1%	-95.2 ± 2.1%
2-Methylpropanol	12.6 ± 3.7 mg/L	-51.5 ± 9.3%	-73.0 ± 4.7%
3-Methylbutanol	46.1 ± 8.3 mg/L	-53.3 ± 5.6%	-81.8 ± 3.5%
2-Methylbutanol	15.9 ± 6.1 mg/L	-45.2 ± 21.1%	-79.8 ± 9.8%
3-Methylbutyl acetate	1.9 ± 1.0 mg/L	-90.8 ± 2.3%	-94.7 ± 2.2%
2-Phenylethanol	38.8 ± 11.4 mg/L	+27.4 ± 25.1%	-45.0 ± 18.1%

technology has evolved dramatically since the first beer thin film evaporators. In 1916, Becker & Montgomery patented an evaporator for the dealcoholisation of beer in which the beer at 75 °C flowed as a thin film on the outer side of a conical steam jacket, the alcohol and other vapours being released to the open atmosphere (US 1,171,306, 1916). In the 1920s, several configurations of evaporators with internal static cones were developed (US 1,396,232, 1921; US 1,541,296, 1925). An evolution of these evaporators is development the of centrifugal thin film evaporator, which has been used widely for the dealcoholisation of beer, as well as the concentration of juices, coffee and tea extracts, and others (Shinn, 1971). The contact time between the liquid and the heating surfaces has been reported to be around one second, with temperatures as low as 35 °C, and a liquid layer of approximately 0.1 mm deep (Flavourtech, 2018). The use of multiple cones increases the evaporation capacity of the apparatus.

Spinning Cone Column

The most commonly used equipment for dealcoholisation of beer by vacuum evaporation is Spinning Cone Column (SCC). This process was first developed in 1936 for the separation of isotopomers (Bae, Kim, & Lee, 2020) and it is now used on a regular basis for the dealcoholisation of beverages like wine and beer. The beer is transferred into a vertical column containing stationary and rotating inverted cones placed alternately. The forces involved in the movement of the liquid down to the next cone below are a combination of gravitational force, down the stationary inverted cones, and centrifugal force, up to the rotating inverted cones. The beer flows over the upper surface of the rotating cones as a thin liquid film of around 0.1 mm, and comes into contact with the gas (usually steam) flowing upwards allowing ethanol evaporation (Bae et al., 2020). This system requires less temperature than vacuum distillation (40-45 °C) and the

residence time of the beer through the equipment is only approximately 20 s to reach ethanol concentrations below 0.05% ABV in a single pass (Alfa Laval, 2019). For the dealcoholisation of wines (final ethanol content below 0.04% ABV), temperatures as low as 28 °C for one stage process and 24 °C for two stages have been reported (WO 2012/007601 A1, 2012). Thanks to the mild temperatures used, SCC has been also used for the extraction of flavour compounds from foods, like green and black tea (US 2007/0077343 A1, 2007) or citrus fruits (US 6,287,618 B1, 2001).

1.7.1.2. Membrane processes

Membrane technology is currently used for a wide variety of applications in different fields. The dealcoholisation of alcoholic beverages is based on the selectivity of semipermeable membranes which allow certain compounds to pass through. These processes have the advantage of requiring low temperature, mild pressure, and no mobile parts, which lessens maintenance needs. However, membranes are not selective enough to prevent other small compounds from permeating along with ethanol. This usually causes a significant loss of aroma compounds of low molecular weight and the deterioration of sensory characteristics of the dealcoholised beer. Amongst the membrane processes, the following have been utilised for the dealcoholisation of beer: pervaporation, reverse osmosis, osmotic distillation, and dialysis.

Pervaporation

Pervaporation technology has been applied for the dealcoholisation of beers as well as other beverages. In this two-step separation technique, permeation is combined with evaporation: a liquid mixture (feed) is put in contact with a membrane, and the permeate is removed by evaporation at low pressure (Gorri, Norkobilov, & Ortiz, 2017).

Pervaporation is also employed for the recovery of aroma compounds from an alcoholic beer for its use in the aroma reconstitution of a dealcoholised beer. Del Olmo, Blanco, Palacio, Prádanos, & Hernández (2014) extracted the aroma compounds from an alcoholic beer by pervaporation and added them into a low-alcohol beer. The latter was submitted to a sensory evaluation, in which 90% of the panellists preferred the "enriched" low-alcohol beer to the one without the aromas added. Catarino, Ferreira, & Mendes (2009) optimized the dealcoholisation of beer through a polyoctylmethylsiloxane/polyetherimide (POMS/PEI) composite asymmetric membrane by means of surface response methodology. The researchers found that low temperature (optimal temperature 12.4 °C) improved the efficiency of the process, as well as the aroma selectivity for higher alcohols and esters and their retention in the beer. The authors also recommended recovering the permeate containing the aroma compounds by condensation at a temperature below -80°C (WO 2008/099325 A2, 2008).

Pervaporation has also been used in combination with SCC dealcoholisation of beer (Catarino & Mendes, 2011). In the early steps of the process, the aroma compounds in an alcoholic beer (5.67% ABV) were separated in a pervaporation unit. Then, the retentate stream, i.e. the dearomatised semi-dealcoholised beer (0.1-1.1% ABV), was treated in a SCC (50 mbar, 50 °C) unit for further dealcoholisation. After SCC dealcoholisation, the dealcoholised beer (0.02% ABV) was blended with 5-10% v/v fresh alcoholic beer and the aromas separated from the pervaporation process (0.3% v/v approx.). The final product was a beer with 0.45%ABV with a lower concentration of aroma compounds than the original beer, but similar ratios of aroma compound/ethanol. The addition of the pervaporated aroma to the beer was essential to minimize the almost total depletion of them after SCC dealcoholisation; compounds such as propanol, 2-methylpropanol, 3-

methylbutyl acetate, ethyl acetate, and 2- and 3-methylbutanol, were not detectable in the dealcoholised beer.

Reverse osmosis

Reverse osmosis is based on the selective permeability properties of membranes specially designed for the permeability of defined compounds by terms of their molecular weight and polarity. Commonly used in desalination and purification of water from household to industrial scale use, this technique has also been applied for the removal of ethanol from beer. Beer is pumped and filtered under pressure through the membrane, this way allowing ethanol (permeate) to be separated from beer (retentate). Catarino, Mendes, Madeira, & Ferreira (2006) succeeded in dealcoholising beer (initial ethanol content 5.5% ABV approximately) using membrane technology at mild temperatures (assays performed at 4-20 °C) and pressures of 2.03 or 4.06 MPa. Different membrane materials were assessed: cellulose acetate, polyamide, and fiberglass-polyamide. Although the final concentrations were not low enough to be considered as alcohol-free beer (final alcohol concentration <0.5% v/v), the alcohol rejection rate through the membrane reached up to 63%. The authors mentioned higher retention of aroma compounds at lower temperatures (below 5 °C) but further research had to be done in order to optimise the aroma profile and final product quality.

Osmotic distillation

A similar process to reverse osmosis is osmotic distillation or osmotic evaporation, where the solutes to be stripped from the inlet flow are in gaseous state and pass through a microporous membrane and then are dissolved into the permeate stream (liquid). The use of hydrophobic membranes, e.g. made of polypropylene or polytetrafluoroethylene,

prevents the liquid water or ethanol passing through, so the process is only driven by the phase equilibrium between liquid-gas-liquid (Müller, Bellut, Tippmann, & Becker, 2016). This process requires normal pressure and low temperature, which helps reduce the thermal damage of the beer (Valdés, Romero, Saavedra, Plaza, & Bubnovich, 2009). Liguori et al. (2015) used this technology for the dealcoholisation of beer. The process was run at 10 °C isothermally using several filtration cycles, but also recycling the permeate stream (containing alcohol) aiming to reduce water consumption, in a similar way to a multi-step counter-current configuration, where the two streams are put in contact in multiple steps and in opposite directions. The final ethanol content achieved was 0.46% ABV. However, the aroma compounds present in the final product were reduced considerably (Table 1.3) if compared with initial values (77% reduction of higher alcohols, 99% for esters and 93% for aldehydes). No description of the organoleptic characteristics of the dealcoholised beer was reported, but the researchers admitted that the sensorial quality needed to be improved.

Dialysis

Based on a similar principle as reverse osmosis, dialysis has been utilised to remove alcohol from beer as a post-fermentation treatment. Beers dealcoholised by this process can achieve ethanol concentrations below 0.5% ABV (Montanari, Marconi, Mayer, & Fantozzi, 2008). The principle of the mass transfer is the difference of solute concentrations in two solutions (in this application, beer and dialysate) separated by a semipermeable dialysis membrane (Sohrabvandi, Mousavi, Razavi, Mortazavian, & Rezaei, 2010). The mass transfer throughout the membrane is governed by Fick's law for the dialysis process (Müller et al., 2017). Although it is a technique based on the existence of a concentration gradient, a pressure gradient (higher in the beer stream) also helps to

Table 1.3 Effect of the dealcoholisation by osmotic distillation on the aroma compounds of beer. Source: Liguori et al. (2015). Data rounded up to three significant figures. n.d. = not detected.

Aroma compounds, mg/L	Original beer	Alcohol-free beer	Loss,%
Alcohols			
1-Propanol	18.1	n.d.	100
2-Methylpropanol	3.02	0.78	74
2- and 3-Methylbutanol	33.0	1.74	94
Furfuryl alcohol	2.18	0.86	60
2-Phenylethanol	26.6	15.7	40
Total alcohols	83.0	19.1	77
Esters			
Ethyl acetate	13.6	0.19	98
3-Methylbutyl acetate	1.31	0.01	99
Ethyl butanoate	0.04	<0.01	100
Ethyl hexanoate	0.17	<0.01	100
Ethyl octanoate	0.60	<0.01	100
Total esters	15.7	0.20	99
Aldehydes			
Acetaldehyde	56.6	3.78	93
2-Methylbutanal	0.02	0.003	87
3-Methylbutanal	0.15	0.009	93
Hexanal	0.002	0.009	
Phenylacetaldehyde	0.13	0.016	87
Furfural	0.32	<0.001	100
Methional	0.06	<0.001	100
(*E*)-2-Nonenal	<0.001	<0.001	100
Total aldehydes	57.2	3.82	93
Ketones			
2,3-Butanedione	24.7	n.d.	100
2,3-Pentanedione	23.9	n.d.	100
Total ketones	48.5		100

maintain the transfer of ethanol from the beer towards the dialysate (Leskošek & Mitrović, 1994). Like other membrane separation processes, dialysis removes key aroma compounds from the beer together with the ethanol. These losses can be as high as 85% for esters, 85% for higher alcohols and 70% for low chain fatty acids (up to C_{12}) (Stein, 1993). The dialysing liquid (or "stripping solution") is usually water, which can be

recirculated and reused after removal of the ethanol and other components retained (Stein, 1993). Tilgner & Schmitz proposed a dealcoholisation method for alcoholic beverages using fruit or vegetable juices on the dialysate side of the membrane (US 4,664,918, 1987). The main drawback to this dialysis was the transfer of flavour compounds from the juice into the beer, that affected its quality negatively.

1.7.2. Biological methods

The most common way to produce alcohol free beers is the application of brewing strategies derived from modifications of the traditional beer brewing process. These methods do not require any special equipment, as post-fermentation separation techniques (physical methods) do, involving lower investment cost for breweries, and a lower carbon footprint. During the last few decades, several approaches have been used to control the final concentration of ethanol in beer: modification of the microorganisms (use of special yeasts or other microorganisms), modification of the wort to be fermented (low fermentable wort), or modification of the fermentation conditions (arrested fermentation, cold contact fermentation).

1.7.2.1. Use of special yeasts or other microorganisms

One strategy to produce alcohol-free beers is based on wort fermentation using different yeast species to the traditionally used *Saccharomyces cerevisiae*. These alternative microorganisms usually present a low or no capacity to ferment sugars in wort, while desirable esters and higher alcohols are biosynthesised. Beer researchers have tried different types of microorganisms, from special strains of *Saccharomyces cerevisiae*, to yeasts from other genera and even bacteria.

Successful results have been obtained by fermenting wort with mutant strains of *Saccharomyces cerevisiae* deficient in the tricarboxylic acid cycle (Navrátil, Dömény, Šturdík, Šmogrovičová, & Gemeiner, 2002). After an average of 85 hours fermentation at 15 °C, the authors reported alcohol contents between 0.09 and 0.31% w/w. Furthermore, the concentration of esters (ethyl acetate, 3-methylbutyl acetate, 2-methylpropyl acetate, ethyl hexanoate, and others) and higher alcohols (2-methylpropanol, butanol, 2- and 3-methylbutanol, 2-phenylethanol), were higher than the control (where a regular strain employed in brewing), whereas the formation of diacetyl (2,3-butanedione) reached concentrations higher than in the reference strain. The authors explained that the limited production of ethanol by these yeast strains might be due to several factors, such as the higher production of acids. This affects the activities of alcohol dehydrogenase and pyruvate decarboxylase, both with optima at higher pH values. The authors reported that the organoleptic quality of these beers was comparable to commercial beers, but no formal sensory evaluation was carried out.

Other microorganisms have been tried for the fermentation of wort due to their ability to produce flavour-active volatiles considered as desirable in beer. Spontaneous mutants of *Saccharomyces pastorianus* have been isolated and used in alcohol-free beer production due to their outstanding capacity to produce 3-methylbutyl acetate and 3-methylbutanol while the ethanol concentration was as low as 13.7 g/L (1.74% ABV) (Strejc, Siříšťová, Karabín, Almeida e Silva, & Brányik, 2013). These beers, brewed by arrested fermentation (See section 1.7.2.3), exhibited a fruity, banana-like aroma and were rated as “good” by a sensory panel. *S. pastorianus* has also been used in continuous alcohol-free beer fermentation. In a continuous fermentation, the immobilisation of yeast is a common option for easing the separation of the microorganisms from the liquid. Mota

et al. (2011) produced alcohol-free beers fermented with *S. pastorianus* and a strain of *S. cerevisiae* with disruption in the *KGD2* gene. This strain, deficient in fumarase and α-ketoglutarate dehydrogenase, has been proven to produce alcohol-free beers with an alcohol content below 0.5% ABV (Selecký, Šmogrovičová, & Sulo, 2008). However, the concentration of aroma compounds in the final beer was lower than those fermented by *S. cerevisiae ΔKGD2* (Mota et al., 2011).

The use of microorganisms other than yeasts from the genus *Saccharomyces* has been tried for the fermentation of worts. The most successful yeast used in the industrial production of alcohol-free beer is *Saccharomycodes ludwigii*, the main reason being its inability to use maltose and maltotriose as a nutrient (Brányik et al., 2012). Strains of *S. ludwigii* have been proven to produce beers with alcohol contents below 0.75% ABV (Table 1.4) whilst they were able to synthesise fruity esters and higher alcohols (De Francesco, Turchetti, Sileoni, Marconi, & Perretti, 2015). The researchers also utilised different strains from *Zygosaccharomyces rouxii*, with higher production of volatiles but also varying amounts of ethanol (0.93 to 3.32% ABV, depending on the strain). *Mrakia gelida*, a psychrophilic yeast, has been also tried in the production of low-alcohol beers, with alcohol contents below 1.40% ABV (De Francesco et al., 2018). The beers were subjected to sensory evaluation with successful results comparable to beers fermented by *S. ludwigii*, used as control in this study, and with higher scores for the attribute "fruity/estery". Bellut et al. (2019) utilised different species of the genus *Cyberlindnera*. All the beers produced contained low levels of ethanol, with a maximum of 0.67% ABV. Moreover, the aroma of most of these beers was described as fruity and banana-like attributed to high concentrations of 3-methylbutyl acetate and 3-methylbutanol. Nonetheless, one of the samples, fermented by *Cyberlindnera misumaiensis*, presented a

Table 1.4 Ethanol content and flavour compounds in low-alcohol beers fermented by different yeast species.

Compounds	*S. ludwigii*[1]	*Z. rouxii*[1]	*M. gelida*[2]	*S. ludwigii*[2]	*C. misumaiensis 837A*[3]	*C. fabianii NT Cyb*[3]	*C. jadinii L1*[3]	*C. subsufficiens C6.1*[3]	*C. mrakii CBS1707*[3]	*C. subsufficiens CBS5763*[3]	*P. kluivery A*[4]	*P. kluivery B*[4]
Ethanol, % ABV	0.51 - 0.88 - 1.36	0.93 - 2.02 - 3.32	1.16	1.23	0.55	0.63	0.66	0.63	0.54	0.67	0.1	0.2
Esters, mg/L												
Ethyl acetate	1.17 - 4.33 - 14.9	2.12 - 23.7 - 70.9	0.6	9.3	65.70	22.55	9.27	4.90	8.10	5.17	-	-
3-Methylbutyl acetate	0.008 - 0.011 - 0.022	0.008 - 0.064 - 0.21	n.d.	0.03	0.90	n.d.	0.15	1.60	1.67	1.03	1.96	4.94
Ethyl hexanoate	0.014 - 0.016 - 0.020	0.017 - 0.024 - 0.045	0.009	0.011	-	-	-	-	-	-	0.03	0.07
Ethyl octanoate	0.010 - 0.012 - 0.013	0.011 - 0.016 - 0.020	0.006	0.009	-	-	-	-	-	-	0.12	0.13
Higher alcohols, mg/L												
1-Propanol	3.13 - 4.01 - 5.57	5.64 - 14.5 - 33.6	5.7	2.6	4.03	3.73	4.40	3.27	2.93	3.33	-	-
2-Methylpropanol	5.27 - 9.90 - 15.3	14.6 - 39.6 - 68.9	9.8	13.0	7.57	7.70	8.27	8.03	5.33	7.20	-	-
3-Methylbutanol	14.1 - 18.2 - 28.9	16.4 - 46.1 - 75.8	6.0	14.3	11.2	16.4	23.2	11.7	11.9	10.5	2.00	2.00
2-Methylbutanol	3.72 - 5.22 - 6.98	6.76 - 13.3 - 20.4	1.4	5.5	-	-	-	-	-	-	-	-
2-Phenylethanol	13.7 - 15.4 - 20.0	9.80 - 18.8 - 34.5	2.6	6.8	-	-	-	-	-	-	-	-
Carbonyl compounds, µg/L												
Acetaldehyde	1850 - 2870 - 4420	5580 - 7260 - 8150	2300	918	9700	8050	2600	3370	3830	2570	-	-
2-Methylbutanal	7.95 - 24.8 - 79.1	7.73 - 29.6 - 46.8	1.3	1.8	-	-	-	-	-	-	-	-
3-Methylbutanal	31.9 - 69.9 - 119	17.1 - 68.4 - 143	7.2	6.2	-	-	-	-	-	-	-	-
Hexanal	1.06 - 1.57 - 2.13	0.91 - 1.77 - 2.34	1.0	0.7	-	-	-	-	-	-	-	-
Furfural	19.2 - 24.9 - 28.6	8.06 - 10.0 - 13.4	7.3	10.3	-	-	-	-	-	-	-	-
Methional	7.31 - 11.4 - 17.2	9.60 - 17.5 - 39.9	7.8	5.6	-	-	-	-	-	-	-	-
Phenylacetaldehyde	22.6 - 38.7 - 76.9	13.3 - 23.5 - 36.7	9.2	10.3	-	-	-	-	-	-	-	-
2,3-Butanedione	5.41 - 8.31 - 15.8	234 - 460 - 851	7.8	8.0	-	-	-	-	-	-	-	-

Data rounded up to three significant figures. n.d.- not detected. [1] De Francesco et al. (2015) (data shown as minimum value – average – maximum value); [2] De Francesco et al. (2018); [3] Bellut et al. (2019); [4] Patent WO 2014/135673 A2 (2014).

strong solventy aroma at an unacceptable level, this being related to a high concentration of ethyl acetate. The authors concluded that, although the concentration of esters was high and well-above the threshold, their profile was unbalanced and so was the overall aroma of the beer.

Saerens and Swiegers used two strains of *Pichia kluyveri* (strain A and strain B) for the fermentation of alcohol-free beers (WO 2014/135673 A2, 2014). The authors claimed that the beers produced by this microorganism had a flavour very close to that of commercial alcoholic beers. These beers contained between 0.1% (strain A) and 0.2% ABV (strain B) and high concentrations of esters and 3-methylbutanol (Table 1.4). These yeasts also produced less 2,3-butanedione and organic acids, like hexanoic, octanoic and decanoic acid, than *S. ludwigii*. *P. kluyveri* was found to be an interesting yeast species for the fermentation of alcohol-free beer because of its ability to consume only glucose and not other sugars. Another yeast of the *Pichia* genus, *P. farinosa*, has also been used for the fermentation of alcohol-free beers. A German patent claimed that a beer with less than 0.5% ABV can be produced by *P. farinosa* with a fruity flavour (DD 288619 A5, 1983).

1.7.2.2. Production of low fermentable wort

Mashing is the first stage in preparation of wort, and it consists of mixing ground malted cereals and water. This mixture is heated following a specified temperature schedule that differs depending on the final beer desired. These temperatures are commonly chosen to be within the range of optimal activity for the major starch-digesting enzymes present in wort: α- and β-amylases. The degradation of starch into maltose (4-O-α-D-glucopyranosyl-D-glucose), a fermentable sugar, is carried out by β-amylase at an optimal temperature range of 60-65 °C, whilst α-amylase converts starch into fermentable and non-fermentable glucose-polysaccharides (glucose, maltose,

maltotriose, and higher dextrins) at a temperature optimum of 65-70 °C (Montanari, Floridi, Marconi, Tironzelli, & Fantozzi, 2005). At the end of mashing, temperature is raised to 76-78 °C for enzyme deactivation. By modifying the mashing temperature regime, Sieben & Zastrow reduced the fermentability of the mash by about 60%, reaching final ethanol concentrations of less than 2% (US 5,021,246, 1991). Part of the mash was kept at around 49 °C, while a portion of it was transferred into a cooker and boiled for 20 min for the deactivation of the amylolytic enzymes. The authors claimed that, by following this procedure, a low-alcohol beer having the same taste, aroma and mouthfeel as a regular alcoholic beer, but no formal sensory evaluation was reported. Other authors reject such a fact and support that the strategy of controlling or restricting amylolysis is rarely successful for the production of alcohol-free beers and it usually requires combining this method with others, such as the control of the fermentation (Briggs, Boulton, Brookes, & Stevens, 2004).

1.7.2.3. Arrested fermentation

Also known as “stopped”, “limited” or “restricted” fermentation, arrested fermentation’s principle is stopping yeast activity at the right time for a desired concentration of ethanol. The biological process can be stopped by temperature inactivation of yeasts (most commonly used is rapid cooling to 0 °C or pasteurization) or by removing cells from the liquid (by employing centrifugation or filtration) (Brányik et al., 2012). Arrested fermentation has been applied to beers fermented either in batch or continuous reactor. Lehnert et al. (2009) compared the characteristics of beers brewed by both methods. The fermentation in batch was stopped once 0.4% ABV was reached, and in both systems the temperature was kept constant at 8 °C. The aroma of the beers brewed in the continuous reactor was described as worty, estery or medicinal, depending

on the strain of *S. pastorianus* used. On the other hand, the beers brewed in batch reactor presented an aroma described as "normal", with a yeasty off-flavour.

The extract content has been also used as a criterion to stop a fermentation for alcohol-free beer. In brewing, the extract content is defined as the concentration of dissolved solids in a wort or beer. It is measured in degrees Plato (°P), which are equivalent to grams of extract per 100 g of wort. Jiang et al. (2017) combined arrested fermentation with vacuum distillation for brewing an alcohol-free beer with no off-flavours. *S ludwigii* was used for a batch fermentation maintained at 18 °C until the extract content was lower than 1.5 °P. This condition was reached after about 15 days. Then the beer was kept at 0 °C for two days aiming to obtain a "young beer" with less than 1% ABV. Next, the beer was submitted to vacuum distillation in a continuous vacuum evaporator (0.05-0.06 MPa, 64-68 °C). Following this method, the authors claimed that an alcohol-free beer, with an ethanol content below 0.5% ABV and "rich, smooth and round" flavour could be obtained, even though no formal sensory evaluation was reported.

1.7.2.4. Cold contact fermentation

Cold contact fermentation (CCF) has gained popularity over the last few decades as a process for the industrial production of alcohol-free beer. The purpose of this kind of processes is to reduce yeast activity by applying a very low temperature during fermentation, thus reducing or even avoiding the production of ethanol. CCF was first patented in Switzerland in 1982 by Fritz Schur (CH 646844 A5, 1982). The inventor reported a method for the preparation of alcohol-free beverages with a yeasty aroma, consisting of putting the wort in contact with the yeast at a temperature below 3 °C, preferably as close as possible to the freezing point, at -0.5 to -0.4 °C (US 4,661,355,

1987). After fermentation for 48 h, an alcohol-free beer with an ethanol content below 0.05% ABV was prepared following this method. However, these beers are characterised by an aroma reminiscent of malt or wort, lacking the fruity character of alcoholic beers. The formation of esters and higher alcohols, responsible for the fruity aroma, has been found to be positively dependent on temperature (Landaud, Latrille, & Corrieu, 2001). Moreover, researchers have shown that the malty, worty character of beers brewed by CCF is caused by Strecker aldehydes, especially 2-methylbutanal (malty aroma), 3-methylbutanal (malty) and methional (cooked potato) (Perpète & Collin, 1999a). Unlike their correspondent alcohols and acetate esters, the perception thresholds of these Strecker aldehydes are extremely low, below 1 μg/L for 3-methylbutanal and methional (Figure 1.3). Therefore, even small concentrations of them can impart a strong malty, worty flavour to the beer.

1.8. Concluding remarks and future perspective

In the present review paper, the topic of alcohol-free beer has been covered comprehensively from the perspective of flavour and sensory aspects. The literature evidences the increase in popularity of these beverages across the world, accompanied by a decrease in the consumption of alcoholic drinks. Surprisingly, this behaviour is observed at all age ranges, even for young people. The reasons for this change in trend have been associated with a greater awareness of the consumers of the risks to health of alcohol consumption, along with other motivations grounds on ethical or religious reasons. This interest of the consumers towards non-alcoholic drinks has been reflected in an explosion of new products in the market worldwide and the improvement of the ones already available.

The production of an alcohol-free beer with similar organoleptic characteristics as an alcoholic beer is a complex task and a scientific and technological challenge. The contribution of ethanol to the sensory perception of alcohol-free beers has often been neglected or ignored. However, its role in body or fullness in mouth, release of volatile flavour compounds and bitterness is clear. Amongst the variety of production strategies and methods, none of them fulfils all the requirements of an acceptable, full-bodied, fruity beer, and thus a combination of them or a post-fermentation treatment, such as flavour reconstitution, is required. Physical methods affect the flavour chemistry of these beers in a completely different way to biological methods, and therefore the improvement strategy must be addressed in a different way. Whereas in physical methods the reduction of losses of flavour compounds is the target, off-flavours from biological methods, like Strecker aldehydes, are intended to be avoided or formed to very low concentration, removed, or transformed into pleasant, fruity higher alcohols and esters. The optimisation of distillation processes and semi-permeable membranes to be more selective to ethanol is necessary for physical methods, as well as the comprehensive understanding of the factors involved in the formation of flavour compounds in biological methods.

In conclusion, the quality of alcohol-free beers has improved considerably over the last few decades, although there is still a gap of knowledge regarding the behaviour of flavour compounds throughout the brewing process. Despite the studies carried out in flavour-active compounds in alcohol-free beers, a comprehensive flavour chemistry study is not available in the literature thus far. In the case of beers brewed by biological methods, there is no further awareness of the contribution of other flavour compounds than Strecker aldehydes. Since researchers have reported the presence of dozens of

flavour active compounds in Bavarian wheat beers (Langos, Granvogl, & Schieberle, 2013) and 40 in Bavarian Pilsner-type beers (Fritsch & Schieberle, 2005), a similar approach could be considered for alcohol-free beers following the sensomics approach. Additionally, the literature reviewed shows that, in general, there is an important lack of knowledge regarding the effect of process technology on the organoleptic properties of these beers. Formal sensory evaluations carried out by trained panellists are necessary in order to produce beers with better quality and broader consumers' acceptance.

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Conflicts of Interest

The authors declare no conflicts of interest.

Chapter 2.

Chapter 2. Elucidating the role of aroma compounds in alcohol-free beer and their contribution to the worty flavour - A sensomics study

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Abstract

Alcohol-free beers (AFB) brewed by cold contact fermentation process exhibit a flavour reminiscent of wort. The aims of this study were to identify and quantify the odour active compounds in AFB, as well as to elucidate the contribution of these to the overall aroma and worty character of the beer. The sensomics approach was used for this purpose. Twenty-eight odour-active aroma compounds were perceived by GC-Olfactometry, one of them remaining unidentified. The most odour-active compound was methional (boiled potato-like aroma), followed by 3-methylbutanal (cocoa-like), (*E*)-β-damascenone (apple, jam-like), 5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone (curry, spicy-like), and phenylacetaldehyde (floral, honey-like). The contribution of these flavour compounds to the worty aroma of AFB has been decoded by the sensory assessment of recombinate and omission tests. The outcomes from this study may be of great relevance for the brewing industry in order to design strategies for the reduction of the wortiness of AFB.

Keywords: alcohol-free beer, aroma extract dilution analysis, solvent-assisted flavour evaporation, aroma recombination, omission test

Highlights:

- The Sensomic approach has been applied to alcohol-free beer for the first time.
- Twenty-seven aroma compounds have been identified and quantified in alcohol-free beer.
- Five compounds were found to be key odorants in these beers.
- Sensory attributes for describing “worty aroma” in beer are reported.

2.1. Introduction

Consumption of alcohol-free beer (AFB) has experienced unprecedented growth over the last few years. This is mainly associated with restrictions in alcohol consumption for various reasons, such as medical advice (during pregnancy, those with cardiovascular or hepatic diseases, sport professionals), driving legislation, religious grounds or health awareness (Andrés-Iglesias, Blanco, et al., 2016). As reported by The Brewers of Europe (2016), the trend for the next few years in most European countries is an increase in the consumption of non-alcoholic beverages. Brewing companies are aware of this and they are investing in the development of new non-alcoholic products and the improvement of the ones currently on the market.

There is a variety of methods for the production of AFBs, usually classified into two categories: physical and biological methods (Brányik et al., 2012). Physical methods, such as vacuum distillation (Andrés-Iglesias, Blanco, et al., 2016) or membrane separation (Catarino et al., 2006), are based on the dealcoholisation of a regular alcoholic beer, hence they require special equipment for this purpose. On the other hand, in the biological approach the fermentation process is modified aiming to limit the formation of ethanol by using either non-traditional yeasts or genetically modified microorganisms (Strejc et al., 2013) or adapting the process conditions, such as fermentation temperature and time. Biological methods present the advantage of generally not requiring any special equipment, thus reducing considerably the initial investment from the brewer and the carbon footprint.

Related to the different biological methods, the development of new strains of microorganisms for the brewing industry might raise concerns for both producers and consumers because of the uncertainty that this can generate from the consumers'

perspective. Therefore, cold contact fermentation (CCF) is a more commonly used method to produce AFB (US 4,661,355, 1987). This is based on short fermentation time at low temperature, just above 0 °C, aiming to limit yeast metabolism and thus the formation of ethanol. Unfortunately, the formation of desired flavour compounds such as esters (e.g. 3-methylbutyl acetate, 2-phenylethyl acetate, ethyl acetate) and higher alcohols (e.g. 3-methyl-1-butanol, 2-phenylethanol), and the reduction of carbonyl compounds, is very limited too (Perpète & Collin, 1999b; Pires et al., 2014). Consequently, these beers are characterised by a lack of the appreciated fruity flavour present in Lager and other alcoholic beers, and their flavour is commonly described as malty and reminiscent of wort. The literature shows that some Strecker aldehydes, particularly 2-methylbutanal, 3-methylbutanal and methional, have an important role in the negative attributes associated with the malty, worty flavour of these AFBs (Perpète & Collin, 1999a). These aldehydes have exceptionally low odour thresholds and impart potent worty, malty and cocoa-like aromas even at low concentrations (Piornos et al., 2019).

Despite the findings of previous works carried out on AFB (Perpète & Collin, 1999a), there is no further information about the contribution of other odour-active compounds to the overall aroma of AFB. Following the sensomics approach, over 30 odour-active compounds were identified in two commercial Bavarian wheat beers (Langos et al., 2013) and 40 in Bavarian Pilsner-type beers (Fritsch & Schieberle, 2005). Therefore, our hypothesis was that, in addition to the Strecker aldehydes already identified, there are other flavour compounds contributing to the worty flavour of AFBs. Thus, the aim of this work was to determine the contribution of the odour-active compounds in AFB to the overall aroma by means of quantitative chemical and sensory analyses.

2.2. Materials and methods

2.2.1. Alcohol-free beer

An AFB (less than 0.05% ABV) was brewed, bottled and pasteurised in Heineken's pilot brewery (Zoeterwoude, The Netherlands) in January 2016 following a standard cold-contact fermentation procedure (brewing conditions not specified). No external flavourings were added. Samples of the wort used to produce this beer were also collected, bottled and pasteurised.

2.2.2. Chemicals

Diethyl ether, dansyl chloride and saturated alkane standards were purchased from Sigma-Aldrich (Gillingham, Dorset, UK). O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride (PFBHA) was purchased from Fluka™ (Loughborough, UK). The following food-grade aroma compounds were purchased from Sigma-Aldrich (purity in parenthesis): acetaldehyde (≥ 99%), acetic acid (≥ 99.5%), 2,3-butanedione (97%), butanoic acid (≥ 99%), (*E*)-β-damascenone (≥ 98%), dimethyl sulfide (≥ 99%), 5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone (97%), 5(or 2)-ethyl-4-hydroxy-2(or 5)-methyl-3(*2H*)-furanone (96%), (*Z*)-4-heptenal (≥ 98%), 3-hydroxy-4,5-dimethyl-2(*5H*)-furanone (10% in propylene glycol), 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (≥ 98%), methional (≥ 97%), 2-methoxy-4-methylphenol (≥ 98%), 2-methoxyphenol (≥ 99%), 2-methoxy-4-vinylphenol (≥ 98%), 2-methylbutanal (≥ 95%), 3-methylbutanal (≥ 97%), 3-methylbutanoic acid (99%), 3-methyl-1-butanol (≥ 98%), 2-methylpropanal (≥ 98%), 2,3-pentanedione (≥ 96%), phenylacetaldehyde (10% in ethanol), 2-phenylacetic acid (≥ 99%), 2-phenylethanol (≥ 99%), vanillin (≥ 97%), 4-vinylphenol (10% in propylene

glycol). Encapsulated 3-methyl-2-butene-1-thiol flavour standard (0.02-4 ng/g) was purchased from FlavorActiV™ (Aston Rowant, UK).

2.2.3. Isolation of volatile fractions

For the isolation of volatiles from the AFB and the wort, the procedure described by Langos, Granvogl, & Schieberle (2013) was employed with slight modifications. Briefly, the AFB sample (1 kg) was extracted with redistilled diethyl ether (250 mL × 4). The organic phases were combined, dried over anhydrous Na_2SO_4 and filtered before concentration using a Vigreux distillation column (60 cm, 1 cm i.d.) at 40 °C until a final volume of approximately 100 mL was reached. In order to separate the non-volatile materials from the extract, this concentrated extract was submitted to a high-vacuum distillation process known as solvent-assisted flavour evaporation (SAFE) (Engel, Bahr, & Schieberle, 1999) technique at 25 °C and 10^{-5} Pa. The distillate was fractioned into an acidic and a basic/neutral fraction using $NaHCO_3$ 0.5 M solution (60 mL × 3). After washing with 30 mL of a saturated NaCl solution three times, the organic layer was kept for further treatment (basic/neutral organic extract). In parallel, the aqueous phase was acidified to pH 2.25 ± 0.10 by adding HCl solution (10 M or 1 M), extracted using redistilled diethyl ether (60 mL × 3) and the extracts combined (acidic organic extract). Both basic/neutral and acidic organic extracts were concentrated using a Kuderna-Danish concentrator at 45 °C (final volume ~400 µL for each extract) and stored at -80 °C until use.

2.2.4. Gas chromatography analyses of concentrated aroma extracts

In order to identify odour-active compounds in the concentrated aroma extracts, these were analysed by GC-Olfactometry (GC-O) using a 5890 Series II gas chromatograph

from Hewlett Packard (Waldbronn, Germany) fitted with an FID detector held at 250 °C. The organic extracts (2 µL) were injected and two capillaries with different polarities were employed: Rxi®-5 Sil MS capillary (30 m, 0.25 mm i.d., 1.0 µm df) non-polar column and a Stabilwax®-DA (30 m, 0.25 mm i.d., 0.25 µm df) polar column, both from Restek (Bellefonte, PA, USA). The temperature gradients were set as follows: 40 °C for 2 min, then a rise of 5 °C/min up to 200 °C and 15 °C/min from 200 °C to 300 °C, and then held for 19 min for the non-polar column; 40 °C for 2 min, then rise of 4 °C/min up to 200 °C, then from 200 °C up to 250 °C at 15 °C/min, and then held for 15 min for the polar column. Helium was used as carrier gas (2 mL/min). The sample was split 1:1 at the end of the column, followed by two untreated silica-fused capillaries of the same dimensions (1 m, 0.32 mm i.d.). An ODO II sniffing port from SGE (Ringwood, Victoria, Australia), where the flow was diluted with a moist make up gas, was utilised. Every sample was sniffed by at least 3 experienced assessors in duplicate.

The concentrated aroma extracts were also analysed by GC-MS using equivalent capillaries and chromatographic conditions as used for the GC-O analyses. The instrument employed in this case was a gas chromatograph model 7890A coupled to a 5975C inert XL EI/CI MSD triple axis mass spectroscopy detector and a 7683B Series autosampler, all from Agilent Technologies (Santa Clara, CA, USA). The carrier gas was helium at a constant flow rate of 1 mL/min. Mass spectra were recorded in the EI mode at an ionisation voltage of 70 eV and source temperature of 200 °C.

Due to their low-quality signal in the MS chromatograms, some compounds were identified by GC- Accurate-Mass Time of Flight-Mass Spectrometry (GC-ToF-MS), which is a more sensitive detection technique. The organic extracts (1 µL) were injected manually in either splitless or split (1:10) mode. The oven temperature in the 6890 gas

chromatograph (Agilent) was initially set at 40 °C for 2 min, then 5 °C/min to 300 °C, and held final temperature for 15 min. The carrier gas employed was helium at a flowrate 0.8 mL/min. A Micromass GCT TOF mass spectrometer from Waters (Milford, MA, USA) was used as detector. Mass Spectra were recorded in the EI mode at an ionisation voltage of 70 eV and a source temperature of 180 °C. The samples were analysed using two columns of different polarities (DB-5 and FFAP).

2.2.5. HS-SPME-GC-O

The method described by Lignou, Parker, Oruña-Concha, & Mottram (2013) was used, with modifications. AFB (100 g) was weighed into a 500-mL screw-capped Erlenmeyer flask. The flask was placed in a water bath at 45 °C and, after equilibration for 10 min, a divinylbenzene/ Carboxen®/ polydimethylsiloxane (DVB/CAR/PDMS) and a Carboxen®/polydimethylsiloxane (CAR/PDMS) fibres (Supelco, Bellefonte, PA, USA) were exposed simultaneously to the headspace for 30 min (Elmore, Mottram, & Hierro, 2001). A pre-holed septum was used to hold and expose the fibres. After extraction, the fibres were desorbed into the injection port of the GC-O-FID as described in Section 2.2.4: first, the DVB/CAR/PDMS fibre and then the CAR/PDMS fibre, for 2 min each. Volatiles were cryo-focussed in the GC column by dry ice during desorption, as reported previously (Lignou et al., 2013). For some of these GC analyses the initial temperature of the GC run was kept at 27 °C for 10 min in order improve the separation of the highly volatile compounds.

2.2.6. Aroma extract dilution analysis

Basic/neutral and acidic extracts of the AFB were diluted stepwise 1:1 using redistilled diethyl ether and assessed by GC-O using the non-polar column as described

in Section 2.2.4. The flavour dilution (FD) factor for a specific odour region is defined as the highest dilution at which the odour can still be perceived at the sniffing port of the GC-O.

2.2.7. Identification of odour-active compounds

Mass spectral libraries, such as NIST 2011 and Inramass (INRA, France), were used for primary identification using ChemStation software (Agilent). Then, linear retention indices (LRI) were calculated from retention time of n-alkanes obtained by analyses performed using the same conditions as for sample analyses. Authentic compounds were analysed using the same chromatographic method to confirm their identity by LRI comparison and odour quality. Where identification of GC-MS data from the AFB sample was difficult, the GC-O and GC-MS data from the wort samples were referred to. Once a candidate compound was found in the wort, its identity was confirmed in the AFB by applying the criteria above.

2.2.8. Quantification of 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, phenylacetaldehyde, (*Z*)-4-heptenal and methional

A previously reported derivatisation technique was used, with slight modifications (Saison et al., 2009). Samples were prepared under CO_2 atmosphere (0.075% O_2) to prevent the degradation of aldehydes. Beer samples (30.0 g) were weighed and 30 µl of internal standard solution (benzaldehyde-D_6, 2-methylbutanal-D_{10}, and 4-methylthiobutanal; 10.0 mg/L of each in absolute ethanol) were added using a gas-tight syringe. Aliquots (4.0 g) of sample were transferred to 10-mL SPME vials and then placed in an MPS autosampler (Gerstel GmbH, Mülheim an der Ruhr, Germany) provided with an SPME fibre (65 µm, PDMS/DVB, Supelco). First, the SPME fibre was exposed to the

headspace of the vial containing 14 g of PFBHA solution (200 mg/L), for 10 min at 30 °C, and then exposed to the headspace of the vial containing the sample (30 min, at 30 °C). The derivatised volatiles were desorbed at the injection port (held at 250 °C) of an Agilent 7890A gas chromatograph, equipped with a VF-17MS column (30 m, 0.25 mm, 0.25 µm df) from Agilent. A split ratio of 5 was applied and helium at 1 mL/min was used as carrier gas. The initial temperature in the oven was held at 50 °C for 2 min, then raising up to 100 °C at 5 °C/min and from 100 to 260 °C at 10 °C/min, maintaining final temperature for 2 min. The Agilent 5975C inert XL EI/CI mass spectrometer with Triple Axis Detector was set up for negative chemical ionisation (NCI) using methane as reagent gas (1.5 mL/min, ionised at 230 eV). The ionisation energy was 70 eV and the source temperature 230 °C. For all aldehydes targeted in this method, a suitable ion fragment was chosen. As most PFBHA-aldehyde derivative compounds, i.e. pentafluorobenzyl oximes, consist of two peaks (syn- and antisomers), the peak areas were summed. Calibration curves for aldehydes quantitation were prepared by standard addition to the AFB.

2.2.9. Quantification of 4-vinylphenol

After degassing an aliquot of AFB by using an ultrasound water bath and filtering it (0.22 µm pore size), the sample (10 µL) was injected and analysed using an Acquity UPLC® from Waters (Milford, MA, USA) coupled to an Acquity UPLC® FLR fluorescence detector. The separation was performed at 40 °C using an Acquity UPLC® BEH C18 column (1.7 µm particle size, 2.1 i.d. × 150 mm). The chromatographic signal was recorded at 257 nm for excitation and 334 nm for emission. An aqueous solution of NaH_2PO_4 (pH 2.7) was used as mobile phase A and acetonitrile as mobile phase B. The flow rate was kept constant at 0.250 mL/min and the following gradient was applied:

95% A for 2 min, decrease to 10% A to 19 min and then kept constant to 23 min, increase to 95% A to 23.5 min and kept constant to 26 min. The calibration curve was built by standard addition using the AFB matrix.

2.2.10. Quantification of carboxylic acids, alcohols and (*E*)-β-damascenone

Butanoic acid, 3-methylbutanoic acid, 3-methyl-1-butanol, 2-phenylethanol, 2-phenylacetic acid, and (*E*)-β-damascenone were analysed by HS-SPME-GC-MS. A mixture of internal standards (3-methyl-1-pentanol for 3-methyl-1-butanol, 2-ethylbutanoic acid for butanoic and 3-methylbutanoic acids, β-ionone for (*E*)-β-damascenone, and benzoic acid for 2-phenylethanol and 2-phenylacetic acid; 25 µL at 1000 mg/L in absolute ethanol) was added to 25 mL of AFB. An aliquot (3 g) was poured into a 20-mL screw-capped SPME vial and 2.75 g $(NH_4)_2SO_4$ and 0.75 g $NaH_2PO_4{\cdot}H_2O$ were added in order to increase the release of volatiles (Fiorini, Pacetti, Gabbianelli, Gabrielli, & Ballini, 2015). The vials were incubated at 35 °C for 15 min before exposure to the SPME fibre (DVB/CAR/PDMS) for 30 min. The fibre was then desorbed into the GC inlet port at 250 °C for 20 min. A Stabilwax®-DA (See Section 2.2.4) polar column was used, keeping it at 40 °C for 2 min, then 10 °C/min to 120 °C for 1 min, then 4 °C/min to 250 °C for 5 min. Helium was used as carrier gas. The chromatograph and the MS detection conditions were the same as in Section 2.2.4. Calibration curves were prepared using the AFB matrix.

2.2.11. Quantification of dimethyl sulfide and acetic acid

The method described above (Section 2.2.10) was modified for the quantification of these compounds. In both cases, the addition of salts was omitted. For dimethyl sulfide, the beer sample (20 mL) was spiked with 1 µL 1,4-dichlorobenzene (100 mg/L in diethyl ether) as internal standard and 5-mL aliquots were analysed. For acetic acid, the aliquot

(5 mL) was acidified with 250 µL HCl (6 M) and 50 µL of acetic acid-d_3 (1000 mg/L) was used as internal standard. In both cases, an Agilent J&W HP-5MS column (30 m, 0.25 mm, 0.25 µm df) was employed and the following gradient was applied: 40 °C for 2 min, then 5 °C/min to 100 °C, then 12 °C/min to 300 °C, keeping this for 10 min.

2.2.12. Quantification of furanones and vanillin

After the AFB sample was degassed and filtered as described in Section 2.2.9, 1 µL was injected into an Acquity UPLC® chromatograph, coupled to a Xevo® TQ-S tandem triple quadrupole MS detector from Waters. The same column as in Section 2.2.9 was employed for these analyses (column temperature 50 °C). The mobile phase was composed of water (0.1% formic acid) as solvent A and acetonitrile (0.1% formic acid) as solvent B. The flow rate was set at 0.25 mL/min and the following gradient was used: 95% to 70% A for 7 min, further decrease to 5% A after 3 min, kept for 2 min, and then increase to 95% A in 1 min, then held for 3 min (total run time 16 min). The following settings were applied at the detector: source temperature 150 °C, cone gas flow 150 L/h, collision gas flow 0.21 mL/h, desolvation temperature 600 °C, desolvation gas flow 1000 L/h. Chromatograms were acquired using the Multiple Reaction Monitoring (MRM) in the positive mode, with different settings for every compound: 3-hydroxy-4,5-dimethyl-2(*5H*)-furanone (retention time 4.13 min, MS/MS transition 129.13→82.18), 5-ethyl-4-hydroxy-2-methyl-3(*2H*)-furanone (5.33 min, 143.13→69.07), vanillin (5.71 min, 153.10→93.10), 5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone (5.90 min, 143.13→69.07). The cone voltage and collision energy were kept at 20 V and 10 V, respectively, for all the compounds.

2.2.13. Quantification of 2,3-butanedione, 2,3-pentanedione and acetaldehyde

First, the ethanol content of the samples was adjusted to 5% v/v by adding absolute ethanol. The sample (250 mL, kept at 0-10 °C) was mixed with 2.0 mL of cold 2,3-hexanedione internal standard solution and 2.0 mL and left to stand for 5 min. For the vicinal diketones, an aliquot (5 mL) of the sample was transferred into a 10-mL vial and the headspace analysed by gas chromatography using an electron capture detector (ECD). 2,3-Hexanedione was used as internal standard for the vicinal diketones. This was prepared by diluting 150 µL of 90% 2,3-hexanedione in 100 mL ethanol. Then, 10.0 mL of this stock solution were diluted further with 50 mL ethanol and topped up to 1000 mL with water.

In the case of acetaldehyde, a gas chromatograph fitted with a DBWaxETR (60 m, 0.32 mm, 1 µm df) capillary column and a flame ionisation detector (FID) was used for the analysis of the headspace. 1-Butanol was utilised as internal standard.

2.2.14. Quantification of 2-methoxy-4-vinylphenol

AFB samples (20 µL) were injected in an HPLC system fitted with a Supelcosil Abz+ (250 × 4.6 mm) column and a 2-cm long column guard with similar characteristics. The column oven was kept at 25 °C and the flowrate constant at 1.0 mL/min. The eluents used were methanol/citrate buffer (0.05 M, pH 5.40) 1:1 (mobile phase A) and methanol (mobile phase B). The samples were eluted at 100% A for the first 20 min, then decreased to 50% in 0.1 min, kept for 9.9 min, increased to 100% in 0.1 min and kept for 7.9 min. The total run time was 38 min. Detection was carried out by using a UV detector at 260 nm.

2.2.15. Quantification of 2-methoxyphenol, 2-methoxy-4-methylphenol and 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone

For the quantification of 2-methoxyphenol and 2-methoxy-4-methylphenol, the method published by Beaudry, Ross, & Vachon (2007) was applied, with modifications. Acetone (500 µL) was added to 100 µL of beer, vortexed for 1 min and centrifuged at 13,700 × g for 10 min. Then, the supernatant was transferred to a new vial and mixed with 200 µL of dansyl chloride solution (1 mg/mL in acetone) and 40 µL NaOH 0.1 M. The dansylated sample (5 µL) was injected into a LC-ESI-MS/MS system consisting of a 1260 Infinity HPLC coupled to a 6410 Triple Quad LC/MS detector, all from Agilent (Santa Clara, CA, USA). An Agilent Zorbax® SB-18 (2.1 × 100 mm, 1.8 µm) column was utilised and a solution containing acetonitrile, water and formic acid (65/35/0.1, in volume) was used as mobile phase (constant flow rate 0.35 mL/min). The detector was set for positive mode and the signal was recorded using dynamic MRM under these conditions: 2-methoxyphenol (fragmentor voltage 150 V, quantitative transition 358.1→171.1 (collision energy 20 V), qualitative transition 358.1→156.1 (43 V)), 2-methoxy-4-methylphenol (170 V, 372.1→171.1 (23 V), 372.1→156.1(45 V)). For 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone, the derivatisation reaction was not required and thus omitted. The MRM settings for this compound were: 80 V, 129.1→43.1 (10 V), positive mode.

2.2.16. Aroma recombination and omission sensory tests

Aroma recombinates were prepared using an AFB-model comprising a mixture of sugars (7.2 g/L glucose, 2.1 g/L fructose, 0.6 g/L sucrose, 26.9 g/L maltose, and 3.6 g/L maltotriose) in carbonated water, as described by Piornos et al. (2019). The aroma compounds were dissolved in propylene glycol at a concentration 10^4 times higher than in the actual AFB. For 3-methyl-2-butene-1-thiol, the encapsulated flavour standard

(60 mg) was dissolved in 500 μL of a mixture of 30% ethanol and 70% propylene glycol and added to the recombinate at 150 μL/100 mL (0.0036-0.72 ng/L final concentration as specified by the supplier). Along with the aroma recombinate containing all the compounds identified and quantified, nine additional recombinates were prepared with one or two compounds missing for the omission tests (omitted recombinates). These samples (5 mL), together with the AFB and its wort (diluted 1:1 with filtered tap water in order to match the overall intensity of its aroma with the AFB's aroma) were poured into 27-mL screw-capped clear glass vials (height 72 mm, internal diameter 23 mm). The samples were prepared two hours prior to the sensory evaluation to allow headspace equilibration and presented at a temperature between 9 and 14 °C.

Ten screened and trained sensory panellists from the Sensory Science Centre of the University of Reading, with a minimum of six months experience in sensory evaluation of flavour, participated in quantitative descriptive analysis (QDA®) tests. In the initial vocabulary session, the panellists were asked to describe the aroma of the samples, followed by a discussion in order to work towards a consensus vocabulary. In subsequent sessions, the consensus vocabulary was confirmed by standardising aroma descriptors against various references (Table 2.1). In the following scoring sessions, the panellists were asked scales (0 to 100). The sensory assessments were carried out in duplicate on different to open the vials, sniff the samples and score against each descriptor on unstructured line days, in individual sensory booths under red light at a room temperature of 20 °C. The samples were split into two groups (maximum 13 per day), and presented monadically, in a balanced order and coded with three-digit numbers. In the first group, the AFB, the diluted wort, the full recombinate and five omitted recombinates were assessed, whereas in the second group the samples were the full

Table 2.1 References for the aroma attributes in AFB, its recombinate and diluted wort.

Aroma attribute	Reference	Brand and supplier
Malt, cereal	Light dried malt extract dissolved in warm water (25 g/L)	Ritchies (Ritchie Products Limited, Burton-on-Trend, UK)
Potato	Solution of methional in water (85 µg/L)	Sigma-Aldrich (Gillingham, UK)
Hay (green tea)	Green tea loose leaves	Local supplier
Honey (hot)	Wildflower honey, dissolved in hot water	Rowse® (Wallingford, UK)
Floral	Geraniol (98%) diluted in water (50 µL/L)	Sigma-Aldrich (Gillingham, UK)
Prunes	Dried pitted prunes	Morrisons Savers (Wm Morrison Supermarkets PLC., Bradford, UK)
Dark brown sugar	Dark brown soft sugar	Billington's (The Silver Spoon Company, Peterborough, UK)
Apple (stewed)	Bramley apples, peeled, cut and cooked for 30 min	Local supplier
Yeast	Dried easy bake yeast, 3.5 g dissolved in 200 mL of warm water	Allinson's (Allinson Flour, Peterborough, UK)
Curry, fenugreek	Ground fenugreek	Schwartz™ (McCormick & Company, Inc., Hunt Valley, MD, USA)

recombinate and four omitted recombinates. Between samples, a time delay (30 s) was applied and the panellists were supplied with filtered water for refreshment if needed. The project was designed, presented and data captured using Compusense Cloud (Compusense Inc., Guelph, ON, Canada). The QDA® data were analysed in SenPAQ 5.01 (Qi Statistics, Reading, UK) using two-way ANOVA, with sample fitted as a fixed effects and panellist as a random effect, and both treatment effects tested against sample by assessor interaction. Significant differences between sample pairs were tested using Fisher's LSD multiple comparison test at $p = 0.05$.

2.3. Results and discussion.

2.3.1. Identification of the key aroma compounds in AFB

For the present study, a non-commercial AFB was chosen because of its characteristic worty aroma. For the extraction of volatile compounds, two different methodologies were applied for the extraction of volatiles: SAFE and SPME. After sniffing both acidic and basic/neutral SAFE extracts by GC-O, some common highly volatile aroma compounds had not been detected in our beer samples, so two-fibre SPME-GC-O was applied in order to trap a greater amount and variety of these highly volatile aroma compounds. The aroma extract dilution analysis was used as a criterion to reject the least important compounds. **Error! Reference source not found.** shows the most odour-active c ompounds (those with an FD factor ≥ 16) found in the SAFE extracts, as well as those from the SPME experiments. The chromatograms and olfactograms of SAFE extracts from the wort were used as a guide since, in most cases, the same odour regions were found in both products and the wort provided a more concentrated extract. Moreover, flavour compounds at the same LRI were usually found at higher intensity in the wort. Thus, these compounds were primarily identified in the wort and then their presence was confirmed in the AFB. The full olfactograms for both the AFB and the wort are presented in Supplementary Table S 1 to S 4 (Appendix 1). Twenty-eight odour-active regions perceived at the sniffing port were present at high FD factor. Twenty-six odour-active compounds were identified by considering their mass spectra, odour quality at the sniffing port and LRI. Since 3-methyl-2-butene-1-thiol did not produce any chromatographic peak or mass spectrum, this was

Table 2.2 Aroma compounds (FD ≥ 16) identified by GC-Olfactometry in SAFE and SPME extracts

No	Compound	Odour description[b]	Fraction[c]	LRI[a]		FD factor[d]	Ref.
				Rxi-5	Stabil-wax		
1	acetaldehyde	green apple	spme	500	718	na	1
2	dimethyl sulfide	sweetcorn	spme	530	768	na	1,2
3	2-methylpropanal	cocoa, ripen melon	spme	569	806	na	3
4	2,3-butanedione	creamy, butter	b	587	1001	512	4,5
5	acetic acid	vinegar	a	589	1460	128	6
6	3-methylbutanal	cocoa	a, spme	642	930	32	1,3,5,7
7	2-methylbutanal	cocoa	spme	651	924	na	3,5,7
8	2,3-pentanedione	creamy, butter	spme	706	1045	na	5
9	3-methyl-1-butanol	beer, malt	a, b	730	1215	16	1,4,5,6,8
10	butanoic acid	cheese	a	795	1642	256	1,4,6
11	3-methyl-2-butene-1-thiol[e]	sulfur, cannabis	spme	822	1100	na	9
12	3-methylbutanoic acid	cheese	a	861	1684	128	1,4,5,6
13	(*Z*)-4-heptenal	fishy	spme	894	1228	na	10[f]
14	methional	boiled potato	a, b	922	1468	512	2,3,4,5,6,7
15	4-hydroxy-2,5-dimethyl-3(*2H*)-furanone	candy floss, caramel	a	1046	2047	32	1,4,5,6
16	phenylacetaldehyde	floral, honey	a, b	1054	1667	16	3,5
17	2-methoxyphenol	smoky, roasted	a, b	1096	1881	128	1,4,5,6
18	3-hydroxy-4,5-dimethyl-2(*5H*)-furanone	curry, spicy	a, b	1112	2208	256	1,5,6,11
19	2-phenylethanol	rose, honey	a, b	1126	1934	64	1,4,5,6,8
20	5-ethyl-4-hydroxy-2-methyl-3(*2H*)-furanone	candy floss, caramel	a	1149	2096	32	1,5

No	Compound	Odour description[b]	Fraction[c]	LRI[a]		FD factor[d]	Ref.
				Rxi-5	Stabil-wax		
21	5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone	curry, spicy	a, b	1188	2378	1024	5,11
22	2-methoxy-4-methylphenol	smoky, spicy	a, b	1192	1976	512	
23	4-vinylphenol	smoky, leather	a, b	1213	2400	512	5,6
24	2-phenylacetic acid	floral, urine	a	1247	2631	256	1,6
25	2-methoxy-4-vinylphenol	smoky, cloves	b	1309	2211	16	1,4,5,6,8
26	(*E*)-β-damascenone	apple, jam	b	1378	1849	64	1,4,5,6
27	vanillin	vanilla	a	1404	2604	512	4,6
28	unknown	plastic, rubber	a	1420	---	128	

[a] Linear Retention Index. [b] Most frequent odour descriptors used at the sniffing port. [c] Compounds perceived in acidic (a), neutral/basic (b) fractions and/or by HS-SPME-GC-O (spme). [d] Flavour dilution factor; na for not applicable. [e] Compound identified considering odour quality and linear retention indices. [f] Compound previously identified in barley malt. References: [1]Fritsch & Schieberle (2005), [2]Anderson & Howard (1974), [3]Vesely, Lusk, Basarova, Seabrooks, & Ryder (2003), [4]Kishimoto, Wanikawa, Kono, & Shibata (2006), [5]Kishimoto, Noba, Yako, Kobayashi, & Watanabe (2018), [6]Langos, Granvogl, & Schieberle (2013), [7]Perpète & Collin (1999), [8]Andrés-Iglesias, Blanco, García-Serna, Pando, & Montero (2016), [9]Vermeulen, Lejeune, Tran, & Collin (2006), [10]Fickert & Schieberle (1998), [11]Scholtes, Nizet, & Collin (2012)

identified based on its characteristic aroma, LRI on two columns and its relevance to beer flavour (Lusk, Murakami, Nielsen, Kay, & Ryder, 2009). Unfortunately, one compound (Compound no. **28** in **Error! Reference source not found.**: $LRI_{Rxi\text{-}5}$ 1420, plastic, rubber a roma) remained unidentified. The LRIs for the compounds identified were calculated from two columns of different polarity and confirmed with reference standards. The compounds with highest FD factors in the basic/neutral and acidic fractions were 5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone (**21**) (FD factor 1024), followed by 2,3-butanedione (**4**), methional (**14**), 2-methoxy-4-methylphenol (**22**), and 4-vinylphenol (**23**), all with FD factors of 512 (**Error! Reference source not found.**). However, it is noted that the 2,3-b utanedione was only perceived in the basic/neutral fraction, and vanillin only in the acidic fraction. There were other important compounds that were only detected by SPME-GC-O. These were acetaldehyde (**1**), dimethyl sulfide (**2**), 2-methylpropanal (**3**), 2,3-pentanedione (**8**) and (*Z*)-4-heptenal (**13**), previously reported in beer (Anderson & Howard, 1974; Fritsch & Schieberle, 2005; Kishimoto et al., 2018; Vesely et al., 2003) and barley malt (Fickert & Schieberle, 1998). Using the low temperature programme for SPME-GC-O described in Section 2.2.5, 2-methylbutanal (**7**) could be separated from its isomer 3-methylbutanal (**6**) and thus differentiated at the sniffing port.

The twenty-six compounds identified were quantified by different analytical methods (Table 2.3). The compounds with highest concentrations were 2-phenylethanol (20,700 µg/L), acetic acid (13,500 µg/L), 2-phenylacetic acid (1,930 µg/L), and acetaldehyde (1,200 µg/L). On the other hand, the ones with the lowest concentrations were (*Z*)-4-heptenal (0.063 µg/L), 2-methoxy-4-methyphenol (1.15 µg/L), and 2,3-pentanedione (4.1 µg/L). 3-Methyl-2-butene-1-thiol was not successfully quantified in AFB. As concentrations are not a direct measurement of the potency of aroma

Table 2.3 Concentrations and odour activity values (OAV) of aroma compounds in AFB.

Compound	Concentration[a], µg/L	Detection threshold[b], µg/L	OAV
methional	85.4 ± 1.22	0.47	181
3-methylbutanal	38.4 ± 0.45	0.61	62
(*E*)-β-damascenone	10.4 ± 0.87	0.23	45
5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone	42.3 ± 2.02	1.17	36
phenylacetaldehyde	160 ± 7.34	5.42	29
acetaldehyde	1,200 ± 55	45.8	26
2-phenylethanol	20,700 ± 1,540	1,880	11
2-methylpropanal	24.0 ± 0.46	4.3	6
(*Z*)-4-heptenal	0.063 ± 0.0043	0.016	4
3-methyl-1-butanol	233 ± 2.59	77	3
5-ethyl-4-hydroxy-2-methyl-3(*2H*)-furanone	309 ± 1.88	102	3
2,3-butanedione	14.2 ± 0.63	5.2	2.5
2-methoxy-4-vinylphenol	180 ± 7.3	81	2.2
2-methoxyphenol	3.56 ± 0.24	2.1	1.7
4-hydroxy-2,5-dimethyl-3(*2H*)-furanone	113 ± 2.56	141	< 1
2-methylbutanal	16.5 ± 0.19	23	< 1
3-methylbutanoic acid	213 ± 48.7	377	< 1
2-phenylacetic acid	1,930 ± 177	5,160	< 1
dimethyl sulfide	16.0 ± 0.20	48	< 1
2,3-pentanedione	4.1 ± 0.13	13	< 1
vanillin	163 ± 5.76	1490	< 1
3-hydroxy-4,5-dimethyl-2(*5H*)-furanone	2.18 ± 0.04	25	< 1
acetic acid	13,500 ± 1,270	353,000	< 1
2-methoxy-4-methylphenol	1.15 ± 0.008	36.8	< 1
butanoic acid	21.7 ± 3.20	2,080	< 1
4-vinylphenol	10.5 ± 0.85	2,750	< 1
3-methyl-2-butene-1-thiol[c]	n.d.	n.d.	n.d.

[a] Concentrations expressed as the average of three replicates ± standard deviation. [b] Orthonasal detection thresholds in AFB matrix retrieved from Piornos et al. (2019), 0. [c] Concentration below the limit of quantification. n.d.= values could not be determined

compounds, this was standardised by calculating odour activity values (OAV), i.e. the ratio of concentrations and odour detection thresholds (Table 2.3). Fourteen compounds showed OAVs higher than 1, indicating that their concentration was higher than the detection threshold, and that they were likely to contribute the aroma of AFB. The compounds with the highest OAV was methional (OAV 181), followed by 3-methylbutanal

(OAV 62), (*E*)-β-damascenone (OAV 45), 5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone (OAV 36), and phenylacetaldehyde (OAV 29).

It is interesting that the rankings of odour-active compounds according to their FD factor and OAV do not match. Compounds with high FD factors, such as 2-methoxy-4-methylphenol (**22**), 4-vinylphenol (**23**) or vanillin (**27**), were on the bottom half of the OAV ranking, all of them below 1 (Table 2.3). The opposite was observed for 3-methylbutanal, with a concentration 62 times higher than its threshold, although its FD factor was only 32. It must be taken into consideration that FD factors give an approximate idea of the importance of aroma compounds present in the solvent extract prepared from the AFB, and not in the AFB itself. This was mainly attributed to different factors, such as different extraction yields for the different compounds from diethyl ether, differences in the loss rate during concentration steps, etc. Another reason would be the different release of flavour compounds from the foodstuff due to the interaction with the other constituents of the matrix (Wagner et al., 2016).

2.3.2. Aroma recombination and individual contributions by omitted recombinates

After the odour-active compounds had been identified and quantified, a recombinate was prepared by mixing them, at the concentrations that were present in the AFB (Table 2.3), into the artificial AFB-like matrix used in our previous study (Piornos et al., 2019). Although some of the compounds were present at concentrations below their thresholds, they were included in the recombinate because of the demonstrated effect of sub-threshold compounds on the overall aroma of a foodstuff (Kishimoto et al., 2018). 3-Methyl-2-butene-1-thiol was also added to the recombinate even though its concentration could not be determined analytically. Recombinates containing different

concentrations of this compound (data not shown) were presented to the panellists and they chose the one where the aroma was closest to the AFB of reference.

Along with the full recombinate, nine omitted recombinates were assessed in two groups by the sensory panel. Table 2.4 shows the QDA® scores for aroma descriptors in AFB, diluted wort (50% in filtered tap water) and different recombinates. The panellists provided a useful vocabulary for breaking down the concept of "worty aroma" or "wortiness" into single descriptors, since the 10 descriptors were all used to score the wort. Moreover, the AFB was a good example of a worty beer as there was no significant difference in scores for any of the attributes between the AFB and the 50% wort sample ($p>0.05$). The full recombinate was also found to have no significant differences compared to the AFB, indicating that the identification and quantification of the aroma compounds had been good enough to prepare a recombinate which reproduced the aroma of the AFB.

Considering all the omission samples, there were significant differences ($p<0.05$) between the sample for the attribute "floral" for the first group of samples and "honey (hot)" for the second group. Other attributes were significantly different at a higher probability value ($p<0.1$), such as "floral", "curry, fenugreek" and "hay (green tea)" in group 2. QDA® was chosen as the most appropriate sensory method for assessing the samples so that the effect that individual flavour compounds had on single aroma notes could be observed. In order to verify whether samples significantly differed in aroma

Table 2.4 QDA® scores for aroma attributes in AFB, wort, and different recombinates of the AFB.

Samples	Curry, fenugreek	Honey (hot)	Floral	Malt, cereal	Hay (green tea)	Yeast	Potato	Prunes	Dark brown sugar	Apple (stewed)
Group 1										
AFB	4.8^{b}	17.8^{a}	3.6^{bc}	37.4^{ab}	12.9^{a}	16.8^{ab}	12.7^{a}	14.7^{a}	13.7^{a}	7.0^{a}
Diluted wort	3.7^{b}	15.8^{a}	2.5^{c}	41.6^{a}	11.6^{a}	20.4^{a}	14.0^{a}	14.9^{a}	14.3^{a}	6.5^{a}
Full recombinate	5.2^{b}	22.4^{a}	8.9^{abc}	31.2^{ab}	11.8^{a}	18.8^{a}	6.2^{a}	15.6^{a}	11.6^{a}	6.1^{a}
Omitted recombinates:*										
(*Z*)-4-heptenal	6.9^{ab}	20.8^{a}	9.8^{ab}	33.2^{ab}	15.5^{a}	17.7^{ab}	6.5^{a}	19.9^{a}	14.7^{a}	8.2^{a}
methional	10.2^{a}	21.4^{a}	10.5^{a}	28.0^{b}	13.7^{a}	11.9^{b}	6.1^{a}	12.3^{a}	13.9^{a}	4.3^{a}
2-methylbutanal and 2-methylpropanal	6.4^{ab}	24.6^{a}	8.7^{abc}	33.0^{ab}	12.6^{a}	17.7^{ab}	8.2^{a}	14.5^{a}	8.9^{a}	7.5^{a}
3-methyl-2-butene-1-thiol	4.5^{b}	22.3^{a}	9.6^{ab}	33.3^{ab}	10.0^{a}	19.2^{a}	8.3^{a}	15.5^{a}	11.1^{a}	8.9^{a}
2-phenylethanol	4.8^{b}	21.2^{a}	4.5^{abc}	35.4^{ab}	10.6^{a}	17.3^{ab}	8.8^{a}	15.9^{a}	15.9^{a}	8.4^{a}
Significance of sample (p value)	*0.159*	*0.739*	*0.092*	*0.387*	*0.666*	*0.235*	*0.443*	*0.803*	*0.555*	*0.799*
Group 2										
Full recombinate	5.0^{b}	25.6^{a}	6.9^{ab}	35.0^{ab}	9.7^{ab}	20.8^{a}	5.9^{ab}	15.5^{a}	13.5^{a}	7.3^{a}
Omitted recombinates:*										
5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone	3.7^{b}	18.8^{b}	9.6^{a}	31.9^{ab}	9.1^{ab}	20.2^{a}	9.4^{a}	14.0^{a}	11.8^{a}	5.8^{a}
(*E*)-β-damascenone	8.9^{ab}	15.7^{b}	4.1^{b}	37.2^{a}	12.2^{a}	19.3^{a}	7.1^{ab}	17.6^{a}	13.0^{a}	4.3^{a}
3-methylbutanal	6.5^{ab}	20.3^{ab}	8.2^{ab}	29.3^{b}	10.4^{ab}	20.5^{a}	9.4^{a}	20.0^{a}	17.5^{a}	4.6^{a}
phenylacetaldehyde	10.8^{a}	19.6^{b}	5.3^{ab}	35.7^{ab}	7.5^{b}	20.7^{a}	5.0^{b}	15.3^{a}	17.8^{a}	7.7^{a}
Significance of sample (p value)	*0.061*	*0.034*	*0.099*	*0.159*	*0.092*	*0.972*	*0.129*	*0.436*	*0.379*	*0.403*

(*) In these recombinates, the compound named has been omitted from the mixture. Samples with the same superscript letters (within a column and a group) indicates that the samples were not found to differ significantly for the specified attribute ($p < 0.05$).

overall, then sensory discrimination tests (like triangle test or 3-AFC) could be carried out, but this would not have clarified the effects on individual aroma attributes The absence of methional, 5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone, (*E*)-β-damascenone, 3-methylbutanal and phenylacetaldehyde produced the greatest differences in QDA® scores for certain attributes compared to the full recombinate (Table 2.4). These five compounds were the ones with highest OAV, which suggests that this parameter was a good estimation of the potency of aroma compounds in a food. (*E*)-β-Damascenone, 5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone, phenylacetaldehyde and 3-methylbutanal, contributed to the "hot honey" character of the AFB, since a significantly lower score was observed when these compounds were absent. In the case of 3-methybutanal, the difference was significant at a higher probability value ($p = 0.081$), according to the probability values from Fisher's test (data not shown). The first three have a sweet character to them and are likely to be positively influencing the hot honey note. On the other hand, 3-methylbutanal has a malty grainy note, and its role in the hot honey note is less clear. The "yeast" note was caused by methional, but omission of methional also led to an increase in the perception of the "curry, fenugreek" note. 3-Methylbutanal and methional were the main contributors to "malt, cereal"; their absence produced the lowest score for this attribute. However, these differences to the full recombinate were not significant, even when using a higher threshold p-value ($p>0.10$).

The absence of 2-methylbutanal together with 2-methylpropanal did not produce any significant differences to the full recombinate, and thus these two compounds were regarded as making little contribution to the overall aroma of the AFB. This is different to the findings of Perpète & Collin (1999b), in which 2-methylbutanal was considered a key compound to the worty flavour of AFB resulting from cold contact fermentations. In

certain cases, the absence of a compound boosted the scores of other attributes. This can be explained because of the masking effect of the absent compound for that attribute, i.e. the presence of that compound can reduce the impression of another aroma note to which it is not contributing directly. For instance, the absence of methional and phenylacetaldehyde increased the perception of the "curry, fenugreek" note, which means that these compounds were covering this note when present.

2.3.3. Origins of the key aroma compounds

Among the compounds with higher OAV, most of them were Strecker aldehydes, such as methional, 3-methylbutanal, phenylacetaldehyde, and 2-methylpropanal. These aldehydes derive from the amino acids methionine, leucine, phenylalanine and valine, respectively (Perpète & Collin, 1999b). These compounds are usually reduced by yeast, so are present in Lager beers at much lower concentrations than in AFB brewed by cold-contact fermentation. Kishimoto et al. (2018) reported the concentrations of 2-methylbutanal, 3-methylbutanal, methional, and phenylacetaldehyde in a Pilsner beer to be 1.7, 4.9, 1.2, and 4.5 μg/L, respectively, whereas in our AFB these were 16.5, 38.4, 85.4, and 160 μg/L. The contribution of these compounds to the overall aroma of Pilsner beer was almost insignificant, while in Bavarian wheat beers the only Strecker aldehyde found at a concentration higher than its threshold was 3-methylbutanal (Langos et al., 2013). These Strecker aldehydes are already formed during malt kilning (Beal & Mottram, 1994; Fickert & Schieberle, 1998) but also during mashing and wort boiling through the Maillard reaction in both cases (Wietstock, Baldus, Öhlschläger, & Methner, 2017).

(*E*)-β-Damascenone has been found in commercial Belgian beers (Chevance, Guyot-Declerck, Dupont, & Collin, 2002; Schieberle, 1991), wheat beers (Langos et al., 2013), unhopped wort (De Schutter et al., 2008a), and barley malt (Fickert & Schieberle, 1998).

Langos et al. (2013) speculated that this aroma compound might be liberated from a glycosidic precursor. Chevance et al. (2002) demonstrated, by treating a beer sample with β-glycosidase, that the origin of (*E*)-β-damascenone in beer was partially attributed to hydrolysis of glycosides, but this alleged glycoside was not identified. The authors also suggested an alternative source of this norisoprenoid from the degradation of the carotenoid neoxanthin. According to Kollmannsberger, Biendl, & Nitz (2006), (*E*)-β-damascenone can also originate from the β-D-glucoside of 3-hydroxy-β-damascone present in hops. On the other hand, 5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone, also known as "abhexon" or "maple furanone", has been found in Pilsner beer (Kishimoto et al., 2018), Gueuze beer (Scholtes et al., 2012), barley malt (Fickert & Schieberle, 1998), as well as other foodstuffs such as coffee (Blank, Sen, & Grosch, 1992). Several formation pathways have been proposed for this compound. Sulser, De Pizzol, & Büchi (1967) proposed that 5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone can be formed from the aldol condensation of two molecules of α-ketobutyric acid originated from the degradation of threonine. Another possible formation mechanism involves the condensation of α-ketobutyric acid and propanal (Collin, Nizet, Bouuaert, & Despatures, 2012).

2.4. Conclusions

In the present study, the odour-active volatile compounds in an AFB have been identified and quantified. A vocabulary for the description of "worty aroma" has been developed. The aroma of AFB was reproduced by a recombinate containing 27 aroma compounds at the concentrations present in the beer. Five of these were found to be key aroma compounds: 5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone, (*E*)-β-damascenone, methional, 3-methylbutanal and phenylacetaldehyde, which confirmed our initial hypothesis of this piece of research, that compounds other than Strecker aldehydes make

an important contribution to the aroma of wort. The three latter derive from the Strecker degradation of amino acids. The formation of 5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone was also attributed to the Maillard reaction, whereas the origin of (*E*)-β-damascenone in beer is not clear yet. The role of these compounds was confirmed through the sensory evaluation of omitted recombinates. The findings from this study can be of great interest to the brewing industry in order to design strategies to reduce the levels of these compounds responsible of the worty character of AFB, and thus improve their quality and consumer acceptability.

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Conflicts of interest

The authors declare no conflicts of interest.

Chapter 3.

Chapter 3. Orthonasal and retronasal detection thresholds of 27 aroma compounds in a model alcohol-free beer: Effect of threshold calculation method

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Abstract

Detection thresholds are used routinely to determine the odour-active compounds in foods. The composition of a food matrix, such as hydrophobicity or solids content, has an impact on the release of flavour compounds, and thus on thresholds. In the case of beer, thresholds determined in alcoholic beer may not be the same for alcohol-free beer (AFB). Therefore, the aim of this study was to determine detection thresholds for aroma compounds typically found in beer, within a model AFB. The model was designed to match the sugar concentration and pH of an AFB brewed by a cold contact process. Thresholds were measured using a 3-AFC procedure and calculated using either Best Estimate Threshold (BET) method or by logistic regression. Moreover, an algorithm for the removal of false positives was applied to adjust the assessors' raw responses. Retronasal thresholds were generally lower than orthonasal. Those calculated by BET were significantly higher ($p<0.05$) than those from logistic regression, and removal of false positives also produced significantly higher thresholds than those from raw data. The use of logistic regression has the advantage of providing the mathematical model describing the behaviour of the group. The results from this study can be used to better understand the role of flavour compounds in AFB and the effect of the calculation method to prevent under- or overestimated results.

Keywords: alcohol-free beer, orthonasal threshold, retronasal threshold, best estimate threshold, logistic regression, method comparison

Highlights

- Alcohol-free beers from cold contact process contain more sugars than Lagers.
- Sugars might have induced higher thresholds for the most polar compounds.
- Thresholds from literature were comparable to ours but extremely varied.
- Best Estimate Thresholds were significantly higher than logistic regression.
- Results and confidence intervals were affected by the presence of false positives.

3.1. Introduction

Detection thresholds are commonly used in flavour chemistry and sensory science as a measure of the potency of flavour compounds. They are defined as the minimum concentration of a flavour compound at which its presence can be detected in a food or beverage, but this concept has also been applied to other research fields, such as air pollution (Leonardos, Kendall, & Barnard, 1969). Flavour compounds can be ranked according to their odour activity by comparing their concentration in a food and their detection threshold. This is of great importance because odour activity values are widely used as a criterion to select which compounds are the main contributors to the aroma of a food, although it is known that flavour compounds may contribute to the overall aroma of a food even at subthreshold concentrations due to synergistic effects with other odorants (Kishimoto et al., 2018). Odour activity values are a very useful tool in flavour research and have been used to identify key odorants in a wide variety of foods, including virgin olive oil (Guth & Grosch, 1993), rape honey (Ruisinger & Schieberle, 2012), and wheat beer (Langos et al., 2013).

Aroma detection thresholds depend on many variables and are difficult to predict, if not impossible. Apart from the natural differences in sensitivity of humans to different flavour compounds (Schranz, Lorber, Klos, Kerschbaumer, & Buettner, 2017), other factors affect perception too. One source of difference relates to the way that individuals are exposed to the odorant, either orthonasally or retronasally. When sniffing a food, flavour molecules have to be released from the food matrix to the air and then travel through the nasal cavity to reach the olfactory mucosa (Espinosa Díaz, 2004). This corresponds to orthonasal perception of the odorant, whereas in the case of retronasal

perception the flavours are released in the mouth and cross the nasopharynx via the posterior nares before reaching the nasal cavity and olfactory mucosa.

The release of the flavour compounds from the food matrix is the starting point for both orthonasal and retronasal sensory experiences. Along with other factors, such as temperature, the composition of the food matrix plays a key role in the release of volatiles compounds (Hansson, Anderson, & Leufvén, 2001). For example, the orthonasal detection threshold for the sweaty, cheesy flavour compound 3-methylbutanoic acid in water has been reported to be 490 μg/L (Czerny et al., 2008), whereas in sunflower oil the reported threshold was only 22 μg/L (Reiners & Grosch, 1998). Other food components, such as sugars or ethanol, also have a significant effect on the release of volatiles from the food to the air phase. Perry & Hayes (2016)concluded that thresholds determined in one food matrix should not be translated to a different food system. Such assumptions can lead to under- or overestimation of the real potency of flavour chemicals in foods when comparing their concentration with inappropriate threshold values.

Alcoholic and alcohol-free beers are a good example of two similar food matrices where different composition may affect volatile release. Lager beers usually contain 5% alcohol by volume (ABV) and low remaining fermentable sugars, i.e. glucose, fructose, sucrose, maltose and maltotriose. There are studies in the literature reporting detection thresholds of flavour compounds in Lager beers (Meilgaard, 1975b; Saison et al., 2009). However, thresholds determined in this alcohol-containing matrix may not be applicable to alcohol-free beers (AFB). In the case of AFB, the absence of alcohol (below 0.05% ABV), and the presence of non-fermented sugars from wort in beers brewed by cold contact fermentation, are likely to make the release of flavour compounds from this matrix different from alcoholic Lager beers.

The sensory method most commonly employed in determining thresholds is the three-alternative forced choice (3-AFC) discrimination method. However, even where this sensory method is applied consistently across studies, another source of variation in published threshold values is due to the calculation method used. The most commonly used calculation method is Best Estimate Threshold (BET) (Czerny et al., 2008; Plotto, Margaría, Goodner, & Baldwin, 2008; Plotto, Margaría, Goodner, Goodrich, & Baldwin, 2004). According to ISO 13301 (2002), this method consists of calculating the geometrical mean of "*the highest concentration missed and the next higher concentration*". This is done for every assessor's response and the average of the group is then calculated, this being the final threshold value. This ISO standard discloses some of the disadvantages of this method, such as the calculation of thresholds out of the range of concentrations assessed when an assessor's threshold falls above or below the range evaluated. Moreover, BET values do not give any further information about the behaviour of the group for concentrations of the odorant other than the calculated threshold. In recent years, authors have started using an alternative calculation approach by means of psychometric sigmoid functions. These functions consider the probability of perceiving the presence of the flavour compound (i.e. the probability of identifying the correct sample during the experiment) against compound concentration. When using this approach, the threshold is often defined as the concentration at which there is a 50% probability of detecting the flavour compound (Lawless, 2010). Several mathematical models have been used for this purpose, such as Weibull distribution, logistic function (Hough, Methven, & Lawless, 2013) or the Hill equation, often used in biochemistry (Perry & Hayes, 2016). By using this modelling approach, concentrations other than 50% probability can be easily calculated, and these may be useful in certain cases, for instance, to avoid detection of off-notes in foods by very sensitive consumers (Lawless, 2010). By

comparing thresholds calculated using BET and fitting the data to the Hill equation, Perry & Hayes (2016) observed differences between both methods, BET values being lower than detection thresholds (DTs) calculated from the Hill equation in most of the experiments reported. The authors did not discuss the differences between both algorithms that led to the different threshold values. Furthermore, false positives, i.e. correct answers given by chance, could have an effect in the final threshold values. Hough et al. (2013) proposed a threshold calculation method by logistic regression using different functions, which included the application of an algorithm for the adjustment of false positives. The weight of these false positive responses was not evaluated nor their impact on the threshold value. Certainly, the false positives are expected to influence the final threshold values.

It is reasonable to consider that the release of flavour compounds from AFBs brewed by cold contact fermentation is not comparable to water or Lager beer-like systems (usually 5% ethanol in water). Considering the impact of alcohol on flavour release, it was hypothesised that orthonasal and retronasal DTs from the AFB would be different to those previously published in alcoholic beers. Furthermore, the second hypothesis of this study was that the threshold calculation method had a significant effect on the final value, as well as the presence of false positives. Hence, the aim of this study was to determine orthonasal and retronasal detection thresholds in a model AFB of aroma compounds typically found in beer. The effect of the calculation method (BET and logistic regression) and the impact of false positives on the final threshold values were tested too.

3.2. Materials and methods

3.2.1. Materials

Carbonated water (Sparkling spring water, Aldi Stores Ltd., UK), sucrose (> 90%, Silver Spoon, UK), fructose (> 90%, Tate & Lyle, UK), and glucose powder (> 90%, Thornton & Ross Ltd., UK) were purchased at a local store. C☆Sweet™ glucose syrup (composition in dry base: 5% w/w glucose, 75% w/w maltose, 10% w/w maltotriose, 10% w/w unspecified components) was donated by Cargill (Manchester, UK).

3.2.2. Aroma compounds

The following aroma compounds were purchased from Sigma-Aldrich (purity in parenthesis): acetaldehyde (≥99%), acetic acid (≥99.5%), 2,3-butanedione (97%), butanoic acid (≥99%), (*E*)-β-damascenone (≥ 98%), dimethyl sulfide (≥99%), 5 (or 2)-ethyl-4-hydroxy-2 (or 5)-methyl-3(*2H*)-furanone (homofuraneol, 96%), (*Z*)-4-heptenal (≥98%), 3-hydroxy-4,5-dimethyl-2(*5H*)-furanone (sotolone, 10% in propylene glycol), 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (furaneol, ≥98%), methional (≥97%), 2'-methoxyacetophenone (99%), 2-methoxy-4-methylphenol (≥98%), 2-methoxyphenol (≥99%), 2-methoxy-4-vinylphenol (≥98%), 2-methylbutanal (≥95%), 3-methylbutanal (≥97%), 3-methylbutanoic acid (99%), 3-methyl-1-butanol (≥98%), 2-methylpropanal (≥98%), 2-methylthiophene (98%), 2,3-pentanedione (≥96%), phenylacetaldehyde (10% in ethanol), 2-phenylacetic acid (≥99%), 2-phenylethanol (≥99%), vanillin (≥97%), 4-vinylphenol (10% in propylene glycol). All were food grade except 2'-methoxyacetophenone and 2-methylthiophene.

3.2.3. Preparation of the model alcohol-free beer

A model beer was prepared to match the sugar content of an alcohol-free beer brewed following a cold contact fermentation procedure (brewing conditions not specified). This alcohol-free beer was brewed, bottled and pasteurised in Heineken's pilot brewery (Zoeterwoude, The Netherlands). First, a five-fold concentrated solution of sugars was prepared in tap water. Then, one part of the sugar solution was diluted into four parts of carbonated water, reaching the final concentration of sugars: 7.2 g/L glucose, 2.1 g/L fructose, 0.6 g/L sucrose, 26.9 g/L maltose and 3.6 g/L maltotriose. In parallel, a stock solution of odorants was prepared in absolute ethanol (Sigma-Aldrich, UK). Then, 400 µL stock solutions containing the odorant (absolute ethanol for blanks) were added to one litre of model beer. The final pH of the model was 4.50 and the final ethanol content was 0.04%.

3.2.4. Sensory methodology

For each compound, the aim was to collect threshold data from 24 trained and experienced sensory assessors. To achieve this, allowing for absences, there was a pool of 33 assessors (8 men, 25 women, ages 25 to 60). The assessors were recruited from the flavour chemistry group of The University of Reading, all of them having experience in describing aroma of chemicals. Besides, 9-12 trained panellists from a professional sensory panel were considered for our study. Preliminary sensory experiments were carried out in order to establish the range of concentrations for the threshold experiments, as well as for familiarising the panellists with the aroma chemicals. For 7 out of 26 compounds for orthonasal assessment and 2 for retronasal assessment, only 12 assessors were available. The experiments were designed following a three-alternative forced choice (3-AFC) methodology (ISO 13301, 2002). Each sample (10 mL) was

presented in a screw-capped 27-mL clear glass vial (height 72 mm, internal diameter 23 mm) at a temperature between 9 and 14 °C. Six concentrations of each compound were presented in ascending order, each being 3 times more concentrated than the previous sample. Each concentration was presented along with two blank samples per level. Within each set of three, the order of blanks and the sample was balanced and randomised (AAB, ABA, or BAA) across the panellists, and all samples were coded with 3-digit random numbers. During each one-hour sensory session, three compounds were presented to the panel. After sniffing all the samples for orthonasal perception, the samples were presented for a second time, in a random but balanced order, and the panellists were asked to taste them for retronasal perception. The vials were presented uncapped to avoid interference with aroma from the headspace when assessing the samples for retronasal perception. Compusense Cloud (Compusense Inc., Guelph, ON, Canada) was used to guide panellists during the study as well as to collect responses. The experiments were carried out in individual sensory booths (controlled temperature 18-20 °C) at the Sensory Science Centre of The University of Reading.

3.2.5. Data analysis

3.2.5.1. Adjustment of assessors' responses by chance

In order to remove false positives, i.e. positive responses given by chance, the methodology published by (Hough et al., 2013) was followed. Responses were classified into four different cases exemplified in Table 3.1:

Case 1: Negative response. If the panellist could not identify the sample containing the aroma compounds, this remained as "no" in all cases.

Table 3.1 Example of an assessor's response showing the different cases according to the algorithm for the removal of false positives.

Concentration, µg/L	1	3	9	27	81	273
Assessor's response	no	yes	no	yes	yes	yes
Case	1	2	1	2	3	4

Case 2: "Yes before or next to no". This applies to all positive responses before a negative answer, and also those just after a negative response (i.e. those first in a row of correct answers). In these cases, first, the proportion of discriminators (P_d) was calculated (Lawless, 2010) (Eq. 3.1):

$$P_d = \frac{P_{corr} - P_{chance}}{1 - P_{chance}} \qquad \text{Eq. 3.1}$$

Where P_{corr} is the proportion of correct answers at a concentration level and P_{chance} is the probability of getting a correct answer by chance (in 3-AFC tests, this is 1/3). Then, the ratio P_d/P_{corr} was calculated and compared with a random number X from 0.000 to 1.0000 generated using the function "RAND". If $P_d/P_{corr} < X$, the original positive response was corrected and replaced by a negative answer.

Case 3: "Second yes after last no". In this case, the same procedure as in case 2 was followed, although the P_{chance} used in this case was 1/9. This was because this positive response is the second in a row, so the chance of getting two correct answers is (1/3) × (1/3).

Case 4: "Third and further yes after no". The probability of choosing a third correct answer by chance is (1/3) × (1/3) × (1/3). This is below 5%, so it was assumed that these were real positives and consequently kept as positives.

The different steps and criteria were implemented into an Excel spreadsheet (Microsoft Office 365 ProPlus).

3.2.5.2. Best estimated threshold (BET)

BETs were calculated from raw and adjusted data according to the procedure reported in ISO 13301 (2002). BETs for each assessor and compound were calculated as the geometric mean of the highest concentration for a negative response and the next concentration. In the case where an assessor's response was either negative or positive for all the concentrations presented, the BET was calculated as the geometrical mean using the next concentration in the series (up or down, respectively) which had not been tested.

3.2.5.3. Logistic regression

The raw and adjusted data were fitted to the logistic function (Eq. 3.2) using XLSTAT 2012:

$$P_c(\ln C) = \frac{1}{1+e^{-(\alpha+\beta \ln C)}} \qquad \text{Eq. 3.2}$$

Where P_c is the probability of a correct answer, α is the factor that sets the displacement of the curve along the abscissa axis, and β is the steepness factor. The detection threshold was considered as the concentration at which the probability of correct answer was 0.50.

3.2.6. Statistical analysis

Thresholds calculated by BET and logistic regression, from raw data and after removal of false positives (adjusted data), were compared aiming to determine

significant differences between these four different methods. T-test for paired samples ($\alpha = 0.05$) was applied to the logarithms of the threshold values grouped into methods, i.e. not distinguishing between orthonasal and retronasal thresholds for this purpose.

3.3. Results

3.3.1. Orthonasal and retronasal thresholds in a model AFB

Table 3.2 shows the orthonasal detection thresholds for 26 aroma compounds in a model alcohol-free beer, calculated by the four different methods. The overall range of values obtained for different compounds was noticeably broad, from below 1 µg/L to more than 100,000 µg/L. The highest orthonasal DTs, (those over 1,000 µg/L, i.e. 1 ppm), were found for acetic acid (131,000-391,000 µg/L), 2-methylthiophene (1,732-11,800 µg/L), and 2-phenylacetic acid (1,174-5,830 µg/L). On the other hand, the lowest values (those below 1 µg/L, i.e. 1 ppb) were found for (*Z*)-4-heptenal (0.0035-0.022 µg/L), methional (0.19-0.68 µg/L), and 3-methylbutanal (0.31-0.64 µg/L). A similar scenario was observed for these compounds when assessed for retronasal perception. Table 3.3 shows the results for retronasal detection thresholds for 20 aroma compounds. The compounds with the highest retronasal detection thresholds were acetic acid (22,100-104,000 µg/L), 4-vinylphenol (90.0-4,210 µg/L), and 2-phenylacetic acid (12.6-1,690 µg/L). As for orthonasal perception, methional (0.040-1.78 µg/L) and 3-methylbutanal (0.22-0.74 µg/L) exhibited the lowest retronasal threshold values. Orthonasal threshold values were higher than retronasal for most of the compounds evaluated. The only exceptions were dimethyl sulfide and 3-methyl-1-butanol, for which retronasal detection thresholds were higher than orthonasal. For other compounds (methional, 3-methylbutanal, and 4-vinylphenol), the difference between orthonasal and retronasal thresholds was less apparent as it was dependent on the method used to calculate the threshold.

3.3.1. Comparison of calculation methods

In this study, two different threshold calculation methods were used, as well as an algorithm for the removal of false positives. As shown in Table 3.2 and Table 3.3, both orthonasal and retronasal detection thresholds were affected by the calculation method (BET or logistic regression) and the removal of false positives (raw and adjusted data). Figure 3.1 shows the comparison plots for the different calculation approaches, where orthonasal and retronasal thresholds from each method are plotted against each other. Thresholds calculated from adjusted data were higher than those from raw data, independently of the compound assessed, this increase being higher in the case of the logistic regression than the BET. This can be observed when comparing the trendline equations (Figure 3.1a and 1b), where, although the slopes were very close to one, the lines do not pass through zero and there is a significant intercept. The interpretation of these trendline equations and the meaning of this intercept is complicated by the fact that the thresholds are plotted on a log log plot. The trendline equations were expressed in the following terms: $\ln DT_1 = a \cdot \ln DT_2 + \ln (b)$ where $a \approx 1$ and the intercept is $\ln (b)$. Using the standard rules of logarithms, $DT_1 = DT_2 \times b$, so b represents the constant ratio between the methods. The intercept from the graph gives ln (b), so the constant ratio is the exponential of the intercept, or exp (b).

In the case of the adjustment of false positives, the intercept in Figure 3.1a (+1.4698) was higher than in Figure 3.1b (+0.3792). This means that the values from logistic regression and adjusted data were, on average, 4.3 times (i.e. exp (+1.4698)) higher than those from raw data, whereas this difference was only 1.5 times (exp (+0.3792)) in the

Table 3.2 Orthonasal detection thresholds for 26 aroma compounds in an alcohol-free beer model system, calculated by four different methods.

No.	Compound	Odour quality	Orthonasal detection threshold, µg/L				
			Logistic regression		BET		Threshold range in literature, µg/L
			Raw	Adjusted	Raw	Adjusted	
1	acetaldehyde*	fruity, solvent	14.5	45.8	37.5	49.3	11.7[a] – 900[b]
2	acetic acid	vinegar	131,000	355,000	297,000	391,000	100[c] – 522,000[d]
3	2,3-butanedione	caramel, raw meat, butter	1.25	5.19	4.28	6.18	1[e] – 15[f, g]
4	butanoic acid	cheese, sour, vomit	907	2,080	1,390	2,190	1[c] – 4,752[d]
5	(*E*)-β-damascenone	apple, jam	0.065	0.23	0.12	0.20	
6	dimethyl sulfide*	vegetables, garlic, savoury	13.4	48.4	47.2	89.5	0.24[h] – 5[b]
7	5-ethyl-4-hydroxy-2-methyl-3(*2H*)-furanone (homofuraneol)	candy floss, caramel	35.3	102	83.2	131	1.15[i]
8	(*Z*)-4-heptenal*	lamb fat, rancid oil, fish, rubber	0.0035	0.016	0.014	0.022	0.0087[e]
9	3-hydroxy-4,5-dimethyl-2(*5H*)-furanone (sotolone)*	curry, cooked sugar	8.68	28.3	22.9	27.5	0.3[g, j] – 20[i]
10	4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (furaneol)	candy floss, strawberry	49.4	148	87.3	158	1[c] – 1,000[c]
11	methional	boiled potato, metallic	0.19	0.47	0.47	0.68	0.2[g, k, l] – 1.8[e, j]
12	2'-methoxyacetophenone	plastic, chemical, petrol	688	2,260	2,880	3,300	
13	2-methoxy-4-methylphenol	smoky, bacon, vanilla	20.7	37.2	27.7	34.8	21[e]
14	2-methoxyphenol	smoky, chemical	0.67	2.10	1.59	2.51	0.84[e] – 3.39[a]
15	2-methoxy-4-vinylphenol	cloves, medicinal, bacon	33.1	81.5	79.5	99.9	3[m] – 100[j]
16	2-methylbutanal	fruity, sweet	1.88	23.4	37.0	50.9	1.5[e] – 5.6[d]
17	3-methylbutanal	malty, cheese	0.31	0.61	0.47	0.64	0.15[n] – 8[b]
18	3-methylbutanoic acid*	cheese, fruity, sour	89.4	376	360	624	132[o] – 2,754[d]
19	3-methyl-1-butanol	banana, nail polish remover	23.3	89.0	96.5	127	203[h, p] – 4,750[q]
20	2-methylpropanal	nutty, chemical	1.01	4.32	3.44	5.69	0.49[e] – 43.5[o]
21	2-methylthiophene*	vegetable stock, onion, solvent	1,732	7,970	9,000	11,800	
22	2,3-pentanedione*	butter, caramel	3.06	12.9	13.7	18.0	30[f] – 500,000[b]
23	phenylacetaldehyde	rose, floral	1.63	5.42	4.38	6.04	4[k, l] – 9[b]

No.	Compound	Odour quality	Orthonasal detection threshold, µg/L				Threshold range in literature, µg/L
			Logistic regression		BET		
			Raw	Adjusted	Raw	Adjusted	
24	2-phenylacetic acid	floral	1,174	5,150	3,860	5,830	68[r] – 6,100[e]
25	2-phenylethanol	floral, rose, bread dough	569	1,880	1,580	3,000	140[e] – 1,122[a, h]
26	vanillin	vanilla, caramel	396	1,490	1,040	1,880	4.9[j] – 53[e, s]
27	4-vinylphenol	leather, chemical, plastic	665	2,980	2,540	4,020	10.4[a] – 78[j]

*Compounds assessed by 12 panellists, remaining compounds by 24 panellists. [a]Buttery, Turnbaugh, & Ling (1988), [b]Rothe et al. (1972), [c]Larsen & Poll, (1992), [d]Schnabel, Belitz, & von Ranson (1988), [e]Czerny et al. (2008), [f]Blank, Sen, & Grosch (1991), [g]Guth & Grosch (1994), [h]Buttery, Teranishi, Flath, & Ling (1990), [i]Semmelroch, Laskawy, Blank, & Grosch (1995), [j]Langos et al. (2013), [k]Buttery, Seifert, Guadagni, & Ling (1971), [l]Guadagni, Buttery, & Turnbaugh (1972), [m]Buttery, Guadagni, Ling, Seifert, & Lipton (1976), [n]Guadagni et al. (1963), [o]Amoore, Venstrom, & Davis (1968), [p]Baldwin, Scott, Shewmaker, & Schuch (2000), [q]Karahadian, Josephson, & Lindsay (1985), [r]Wagner, Granvogl, & Schieberle (2016), [s]Sellami, Mall, & Schieberle (2018). Full references in 0.

Table 3.3 Retronasal detection thresholds for 20 aroma compounds in an alcohol-free beer model system, calculated by four different methods.

No.	Compound	Odour quality	Retronasal detection threshold, µg/L					
			Logistic regression		BET		Threshold range in literature, µg/L	
			Raw	Adjusted	Raw	Adjusted	In water	In beer
2	acetic acid	vinegar	22,100	60,000	68,600	104,000	54,000[a]	175,000[h]
3	2,3-butanedione	butter, dairy	0.19	0.74	1.30	1.64	0.2[b] – 5[c]	17[i] – 150[h]
4	butanoic acid	cheese	255	575	462	666	6,800[a]	2,200[h]
5	(*E*)-β-damascenone	apple, jam	0.011	0.042	0.026	0.043		
6	dimethyl sulfide*	sweet, vegetable, savoury	39.3	74.8	56.7	81.7		50[h]
8	5-ethyl-4-hydroxy-2-methyl-3(*2H*)-furanone (homofuraneol)	candy floss, caramel	27.9	134	131	238		
9	3-hydroxy-4,5-dimethyl-2(*5H*)-furanone (sotolone)*	curry, molasses	1.24	3.59	4.41	5.80		
10	4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (furaneol)	candy floss, strawberry	81.5	270	190	300	30[d]	
12	methional	boiled potato, metallic	0.040	0.73	1.12	1.78	0.04[c, e]	4.2[i] – 250[h]
13	2-methoxy-4-methylphenol	smoky, bacon, vanilla	0.079	1.86	4.65	5.85		
14	2-methoxyphenol	vanilla, smoky	0.42	0.99	1.21	1.91	0.75[e]	
15	2-methoxy-4-vinylphenol	cloves, medicinal, bacon	1.90	8.33	24.2	30.4		300[h]
16	2-methylbutanal	fruity, sweet, cheesy	1.57	8.99	15.5	22.3	0.03[b] – 40[f]	45[i] – 1,250[h]
18	3-methylbutanal	nutty, cheesy	0.22	0.44	0.56	0.74	0.04[b] – 60[f]	600[h]
19	3-methyl-1-butanol	banana, cheese, fermented	128	262	220	303	4,750[f]	70,000[h]
22	2-methylpropanal	chocolate	0.16	0.86	1.65	2.17	0.006[b] – 180[f]	1,000[h]
23	phenylacetaldehyde	rose, floral, green	0.10	0.68	1.33	2.11	40[f]	105[i] – 1,600[h]
24	2-phenylacetic acid	floral, metallic, musty	12.6	218	1,290	1,690		2,500[h]

No.	Compound	Odour quality	Retronasal detection threshold, µg/L					
			Logistic regression		BET		Threshold range in literature, µg/L	
			Raw	Adjusted	Raw	Adjusted	In water	In beer
25	2-phenylethanol	floral, beer, rose	110	278	579	874	240[f] – 750[g]	40,000[j] – 125,000[h]
26	vanillin	vanilla	45.9	448	754	1,040		
27	4-vinylphenol	chemical, medicinal	90.0	2,340	2,540	4,210		

Compounds 1, 7, 11, 17, 20, and 21 in Table 2 were not assessed for retronasal perception. *Compounds assessed by 12 panellists; remaining compounds by 24 panellists. [a]Patton (1964), [b]Rothe & Thomas (1962), [c]Milo & Grosch (1993), [d]Pittet, Rittersbacher, & Muralidhara (1970), [e]Cerny & Grosch (1993), [f]Sheldon, Lindsay, Libbey, & Morgan (1971), [g]Ohloff (1978), [h]Meilgaard (1975), [i]Saison et al. (2009), [j]Engan (1972). Full references in 0.

case of BET. Differences were also found between BET and logistic regression methodologies from the same sets of data (raw and adjusted data) (Figure 3.1c and d). In both cases, BET produced higher threshold values than logistic regression and the difference was greater for raw data (intercept +1.5825, ratio 4.9) than adjusted data (intercept +0.4804, ratio 1.6). In order to identify significant differences between methods, t-tests for paired samples were applied. P-values from these tests showed significant differences ($p<0.05$) between the results from BET and logistic regression ($p=1.4\times10^{-14}$ for BET raw vs. logistic regression raw; $p=1.2\times10^{-9}$ for BET adjusted vs. logistic regression adjusted), as well as for those calculated from raw and adjusted data for both methods ($p=7.2\times10^{-27}$ for BET raw vs. BET adjusted; $p=7.1\times10^{-21}$ for logistic regression raw vs. logistic regression adjusted). Surprisingly, thresholds from logistic regression from adjusted data and standard BET from raw data were not significantly different ($p=0.31$).

3.3.1. Logistic regression for the calculation of thresholds

Appendix 3 shows the parameters that define the logistic models for the probability of a correct answer (i.e. correct identification of the aroma compound) against the logarithm of the concentration of the compound. The logistic model used here is defined by two parameters: α sets the displacement along the x-axis, and β is the steepness factor. According to Eq. 3.2, a lower value of α is translated in a higher value for the inflexion point of the sigmoidal curve, whereas higher values of β give steeper curves. For both orthonasal and retronasal studies, the adjustment of the data for the removal of false positives produced a decrease in the α parameter, which resulted in a displacement of the curve towards the right and, thus, higher thresholds. The steepness factor β was also

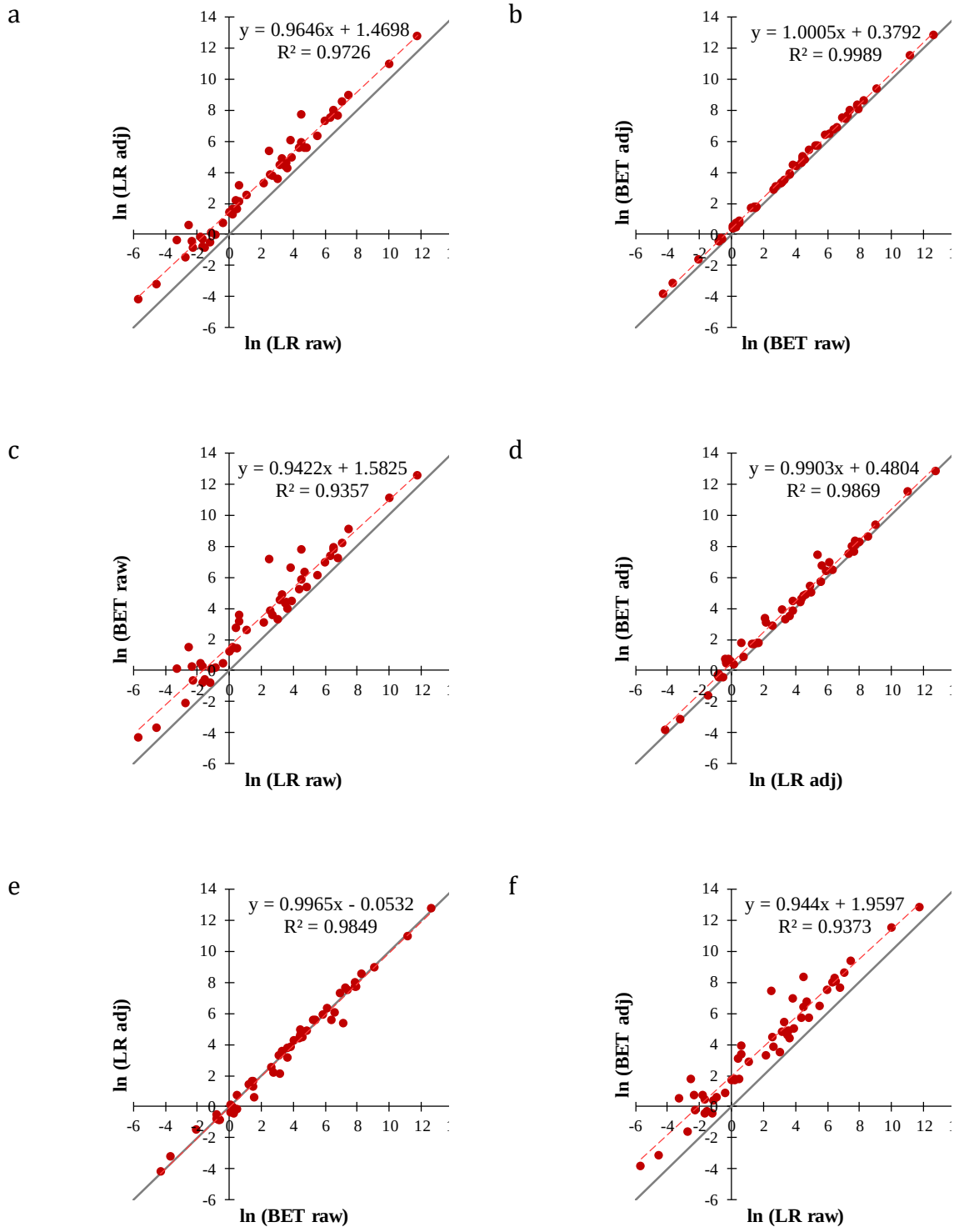

Figure 3.1 Comparison of methods. Natural logarithms of orthonasal and retronasal thresholds (in µg/L) calculated by the different methodologies have been plotted, as well as the linear trend line (red) and the identity line (grey). BET raw: Best Estimate Threshold from raw data; BET adj: BET from adjusted data (i.e. with false positives removed); LR raw: Logistic regression from raw data; LR adj: Logistic regression with adjusted data.

affected by the adjustment of the data because the β-values from adjusted data were higher than those from raw data. An exception to this trend was the orthonasal model for (*Z*)-4-heptenal, for which the α-factor was higher after the removal of false positives. Despite this, the orthonasal detection thresholds for these compounds were still higher because the effect of the α-factor was compensated for by a higher β-factor.

The removal of false positives also affected the goodness of fit of the logistic model. The adjustment of the data produced an increase of the pseudo-R^2 values in all cases, for both orthonasal and retronasal models (Appendix 3). Furthermore, the confidence interval for the thresholds calculated using this method were considerably narrower after the removal of false positives (Figure 3.2). For example, the error bar for the retronasal detection threshold of vanillin was reduced from three orders of magnitude to only one (Figure 3.2b). For a few compounds (2-methylbutanal and 3-methyl-1-butanol for orthonasal, and methional, 2-methoxy-4-methylphenol, 2-phenylacetic acid, and 4-vinylphenol for retronasal detection thresholds) confidence intervals could not be calculated properly when using raw data because the calculation method could not converge to a solution after 100 iterations. This issue was resolved after the removal of false positives, when confidence intervals could be calculated in all cases.

3.4. Discussion

In the present study, orthonasal and retronasal detection thresholds for 26 and 20 aroma compounds, respectively, in a model alcohol-free beer are reported. Assessors were asked to both sniff and taste the samples and their responses were collected in order to calculate orthonasal and retronasal detection thresholds. The matrix was designed to mimic an alcohol-free beer (AFB) brewed by cold contact fermentation, which contained considerably higher amount of sugars (40 g/L) than alcoholic beers or

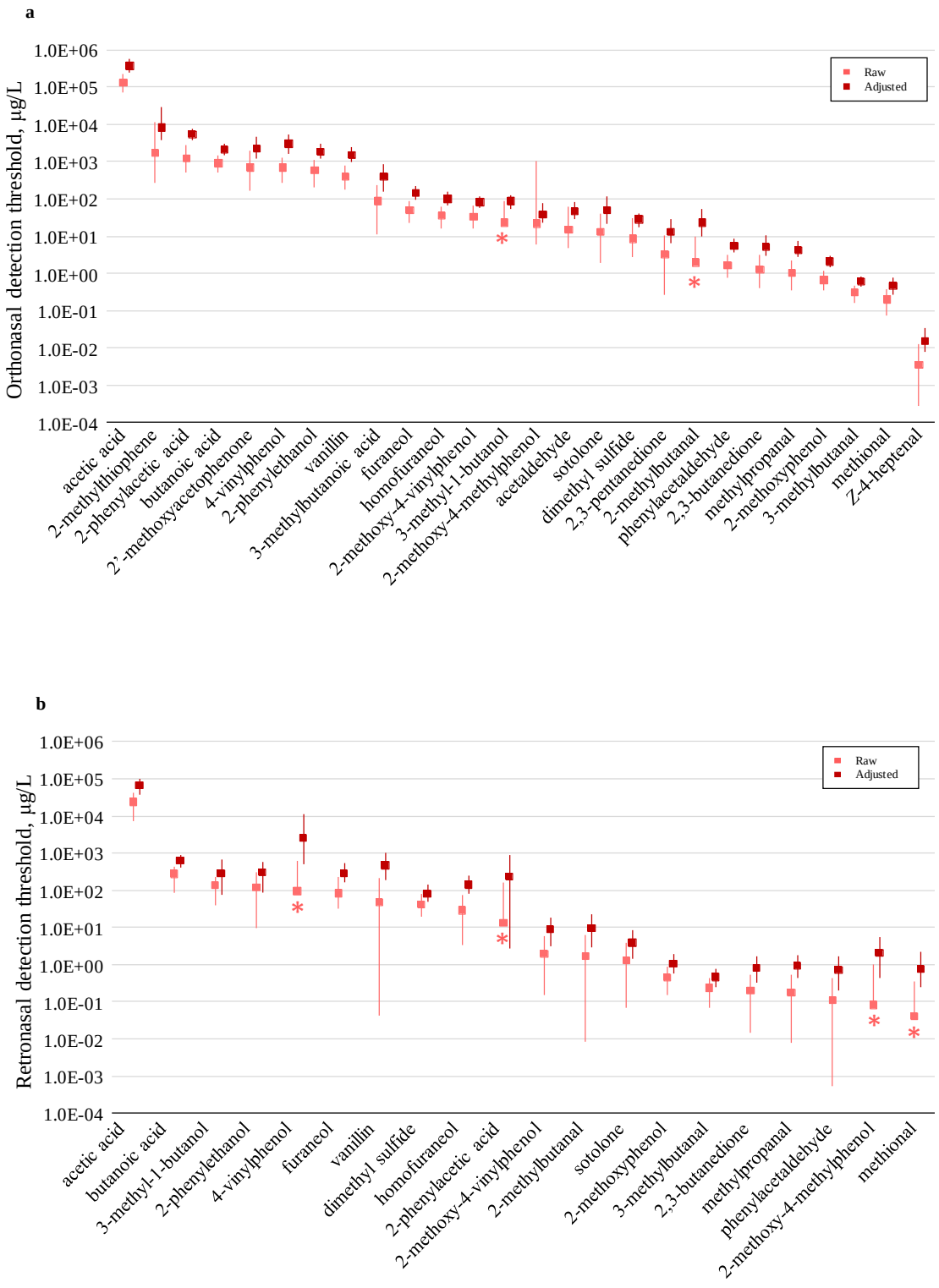

Figure 3.2 Detection thresholds calculated by logistic regression showing confidence intervals (α = 95%) for orthonasal (a) and retronasal (b) perceptions, *Confidence interval not available.

AFBs produced through a dealcoholisation process. For this purpose, sugars in a reference AFB brewed by cold contact fermentation were quantified (data not shown). Amongst the aroma compounds assessed, some of them stood out for their extremely low orthonasal and retronasal detection thresholds. These potent odour compounds are contributors to the aromas of different foodstuffs, for instance, (*Z*)-4-heptenal for cod and other fish (Josephson & Lindsay, 1987), methional for alcohol-free beers (Perpète & Collin, 1999a), and 3-methylbutanal for barley malt (Beal & Mottram, 1994).

In the literature, perception thresholds are available for different aroma compounds determined in a variety of matrices, e.g. water (Czerny et al., 2008), air (Schranz et al., 2017), and beer (Langstaff et al., 1991b; Meilgaard, 1975a, 1982). In Figure 3.3, those found in the literature (diamonds) for water, 9.4% ethanol or beer are compared to those from the present study (horizontal bars) for both orthonasal (Figure 3.3a) and retronasal (Figure 3.3b) perception. Further details of these threshold values from the literature can be found in 0. Before plotting them, all the thresholds units were converted into µg/L in order to ease the comparison.

3.4.1. Orthonasal thresholds

The impact of ethanol on aroma release was demonstrated instrumentally by Perpète & Collin (2000), who observed higher retention of 2-methylbutanal and 3-methylbutanal when increasing the concentration of ethanol from 0 to 5% in an aqueous solution. This was explained by the 'cosolvent' effect of ethanol in water, thus increasing the solubility of these aldehydes and reducing their partition coefficients between the water/ethanol solution and the air (Tsachaki et al., 2008). However, Figure 3.3a shows that the literature detection thresholds which had been determined in 9.4% ethanol fell within the same range as those determined in water in 4 out of 5 cases.

Perpète & Collin (2000) demonstrated the effect of sugars on the release of 2- and 3-methylbutanal. The presence of sugars produced an increase in the release of these aldehydes, with a maximum sugar concentration of 40 g/L. At higher concentrations of sugars, the salting-out effect decreased, and the aldehydes were retained in the sugar solution. Bredie, Mottram, & Birch (1994) showed an increase in volatility with added glucose (200 g/L) for hydrophobic compounds such as menthol and limonene, but no effect with the more polar compounds (isoamyl acetate and 2,3-butanedione). This is in accord with our data, which covers a range of more polar compounds, rather than the terpenes and longer chain aldehydes which showed the biggest effects in these literature studies. On average the more polar compounds in our study (**6:** homofuraneol, **8:** sotolone, **9:** furaneol, **12:** 2-methoxy-4-methylphenol, **14:** 2-methoxy-4-vinylphenol, **25:** vanillin, and **26:** 4-vinylphenol) presented higher orthonasal thresholds than those from the literature (Figure 3.3a). This may be due to the interaction between the sugars and these more polar volatiles. The effect of carbonation was studied by Saint-Eve et al. (2009), who looked at the effect of adding 10 g/L sucrose on aroma release of carbonated beverages. Carbonation had by far the bigger effect and increased volatile release, but added sucrose had no impact on aroma release in the carbonated samples. Our results did not show a corresponding decrease in aroma threshold with carbonation, but surface activity, bubble size and bubble frequency are important parameters which we could not readily control.

3.4.1. Retronasal thresholds

Retronasal thresholds were much scarcer in the literature though, most of them being comparable to our results (Figure 3.3b). In this study, retronasal thresholds for 2,3-butanedione (**3**), butanoic acid (**4**), 2-methoxy-2-vinylphenol (**14**), 3-methyl-1-butanol

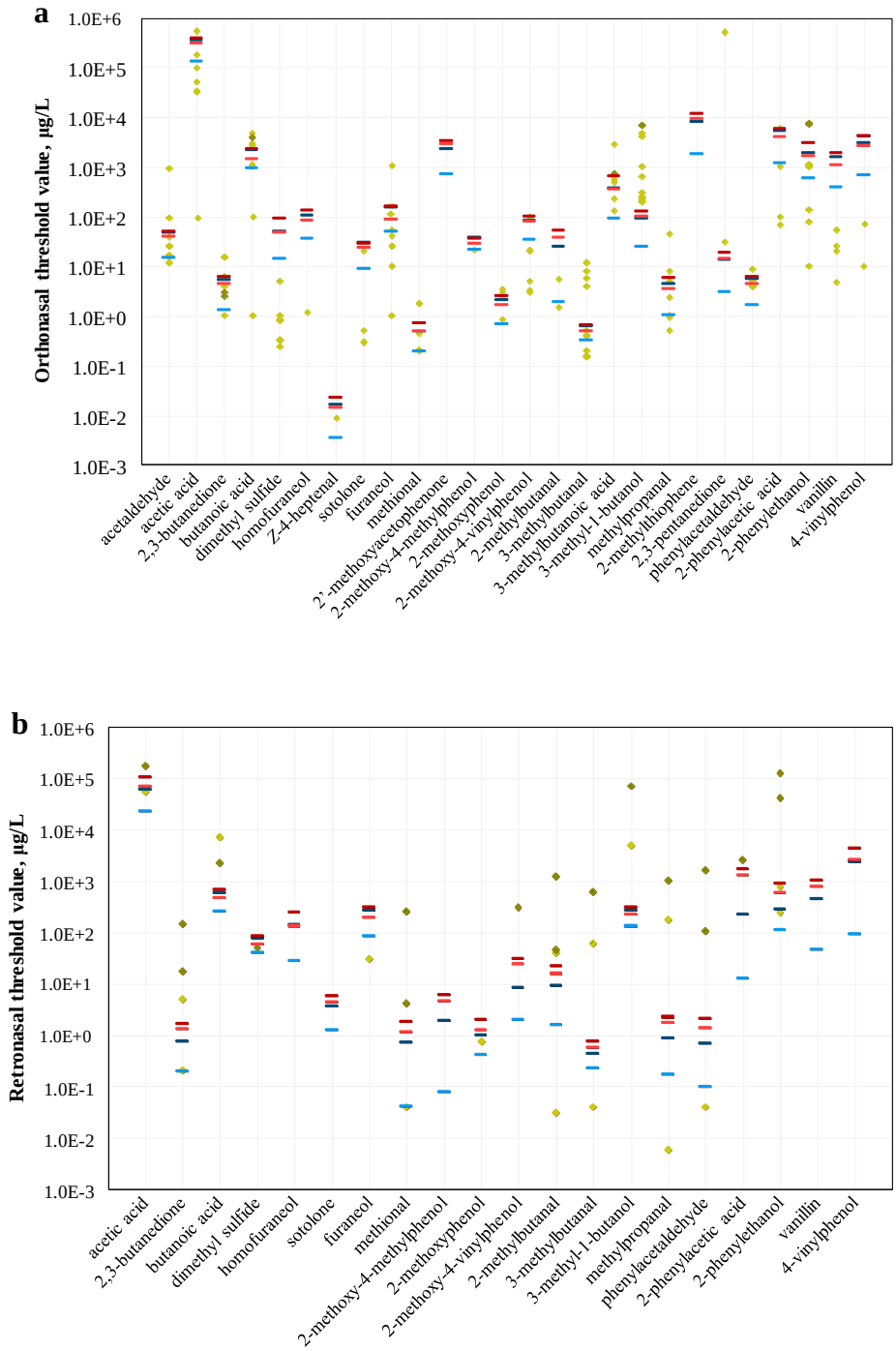

Figure 3.3 Comparison of orthonasal (a) and retronasal (b) detection thresholds determined in this study and those found in the literature (0). Legend: Thresholds calculated by (—) BET from raw data, (—) BET from adjusted data, (—) logistic regression from raw data, (—) logistic regression from adjusted data; thresholds from the literature: (♦) in water and (♦) other matrices (9.4% ethanol in Figure 3.3a or beer in Figure 3.3b).

(**18**) and 2-phenylacetic acid (**23**) were lower than those from the literature, whereas furaneol (**9**) showed a higher threshold in the AFB model. Apart from the matrix effect, the differences between thresholds from the literature and our results could be due to the diversity of methodologies employed in the last years. This includes differences in calculation method (BET, interpolation using probability vs. concentration graphs), number of panellists and sample presentation (triangle, 3-AFC, duo-trio test, sets of samples presented in either ascending or descending concentrations) (Guadagni, Buttery, & Okano, 1963; Langos et al., 2013; Rothe, Wölm, Tunger, & Siebert, 1972). Moreover, it is not rare in threshold studies that the authors do not specify some of the details of their studies, this fact making comparisons less valid. This was demonstrated in a comprehensive literature search and summarised in 0, which shows thresholds in the literature and the main characteristics of the sensory study.

Regarding the results for orthonasal and retronasal perception, retronasal DTs tended to be lower than orthonasal for most of the compounds assessed, independently of the data treatment (Table 3.2 and Table 3.3). The reason behind this does not seem to be very clear. Retronasal perception is a more complex process which also involves changes in temperature of the foodstuff, dilution with saliva, binding to mucous membranes in mouth and tongue, increase of air/food surface area and the mixing effect of swallowing (Taylor & Roozen, 1996). Due to the higher complexity of the retronasal pathway, Espinosa Díaz, (2004) hypothesised a higher efficiency of the orthonasal pathway, thus requiring lower concentrations of odorants for the same odour intensity as the retronasal pathway. On the other hand, the opposite behaviour was observed by Voirol & Daget (1986) for vanillin and citral, which was related to a higher concentration of these odorants in the vapor phase when put in the mouth, as well as the influence of other non-chemical interactions. From the results of the current study it appears that

most of the compounds studied corresponded with the latter theory as their retronasal thresholds were lower. For the compounds that were the exceptions to this, there is no clear reason why they were all detected at lower levels orthonasally. Dimethyl sulfide is a highly volatile compound and hence it is perhaps unsurprising that its orthonasal DT would be lower. However, this was not the case for the other three compounds (homofuraneol, furaneol, and 3-methyl-1-butanol). The relatively low hydrophobicity of these four compounds did not seem to be reason behind this behaviour either. Predicted log p values for dimethyl sulfide, homofuraneol, furaneol and 3-methyl-1-butanol were 0.89, 1.31, 0.82 and 1.26, respectively. Other compounds had similar log p values but their orthonasal DTs were not lower than the corresponding retronasal DTs. Examples of this were 2-methylpropanal (log p = 0.74), 2-methoxyphenol (1.34) and 2-methylbutanal (1.25). The predicted log p values were retrieved from the ChemSpider database of the Royal Society of Chemistry.

3.4.2. Threshold calculation method

Regarding the two different calculation methods used in this study, thresholds calculated by BET and logistic regression were found to be significantly different ($p < 0.05$) for both orthonasal and retronasal data. Logistic regression generated lower threshold values from both raw and adjusted data. Psychometric functions take into consideration all the positive responses along the entire range of concentrations. On the other hand, BET only considers positive answers that are not followed by negative answers. This makes logistic curves displaced towards the left to lower concentrations, resulting in lower threshold values. Previous studies have compared the standardised BET method with logistic regression. Perry & Hayes (2016) found that thresholds from BET were lower than those calculated by using logistic regression. These results, which

may seem to be contradictory to those from the present study, might be explained by the fact that these authors used an equation model that it is restricted from 33% to 100% probabilities on the ordinate axis. In our study, we did not use a restricted model, as shown in Eq. 2, so the probability of correct answer can vary from 0% to 100%. In our opinion, the use of an algorithm for the removal of false positives already discards the correct answers given by chance, so the restriction at 33% chance should not be necessary anymore. When using restricted models, it is common to define the threshold at 66.6% chance as the middle point of the curve (between 33.3% and 100%). This might be another reason why these authors obtained higher threshold values with logistic regression. Lawless (2010) also used 66.6% probability as the corrected 50% detection level following a similar reasoning.

The effect of the removal or correction of false positives was also covered in the present study. As shown above, threshold values increased significantly after the application of this algorithm. In previous studies, differences between BET raw and logistic regression adjusted thresholds were observed. Hough et al. (2013) reported that the BET method using raw data produced lower thresholds than logistic regression from adjusted data. This was associated with the fact that in logistic regression the adjustment of the responses pushed the threshold upwards, whereas this data treatment was not applied when using BET. In our study, there was not a clear trend when comparing these two sets of thresholds. Not all BET raw thresholds were lower than the corresponding logistic regression adjusted threshold (Figure 3.1e) and on average the results from these methodologies were not significantly different ($p = 0.31$). This demonstrated that logistic regression along with the removal of false positives is a methodology comparable to the standardised BET, with the advantage of providing further information such as the

mathematical model describing the response of the group at different concentrations of an aroma compound.

3.5. Conclusions

Orthonasal and retronasal detection thresholds of 27 and 21 aroma compounds, respectively, are reported in a model AFB for the first time. The determination of perception thresholds in the correct matrix is crucial for estimating the potency of flavour compounds in conditions closer to the real beverage. After a comprehensive literature research, it has been proven that our results were comparable to those for most of the compounds studied. However, a group of polar compounds (mainly furanones and phenols) consistently showed higher orthonasal detection thresholds than in water (literature values), this being tentatively attributed to the interaction of these and the sugars in the model AFB. Nonetheless, comparison of threshold values from different studies may be very risky (or even not valid) due to the lack of consistency of the methods for threshold determination. Besides, four different methodologies for threshold calculation were applied and compared, elucidating the role of the calculation procedure in the final threshold value. Threshold values were found to be method-dependent (BET and logistic regression), as well as affected by the presence of false positives or correct answers given by chance. Although BET is a standardised commonly used threshold calculation method, logistic regression is recommended for the amount of information extracted from the data. Using this method, the probability of correct answer and its confidence intervals can be calculated at different concentrations, making it more versatile as it is not restricted to the calculation of thresholds following its usual definition (i.e. at 50% probability). This information related to the response of the group cannot be obtained if using the BET methodology. Additionally, data treatment for the

removal of false positives is strongly recommended in order to obtain a more realistic mathematical model. Consequently, it is strongly recommended to check the experimental setup, matrix in which the odourant was presented and threshold calculation method before using a value from the literature. The results reported in the present study can be of great importance for the brewing industry when studying the aroma composition of alcohol-free beers brewed by cold contact fermentation. The market for alcohol-free beers is currently undergoing huge growth worldwide, and the determination of perception thresholds is essential to understand the role of flavours compounds and their contribution to the overall aroma.

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Conflicts of interest

The authors declare no conflicts of interest.

Chapter 4.

Chapter 4. Effect of kilning temperature on the levels of (*E*)-β-damascenone in malt, mashing and wort boiling in the brewing process

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Abstract

(*E*)-β-Damascenone (bDam) is one of the most important aroma compounds in foods and in both regular and alcohol-free beers. In the present study, the effect of the curing temperature during kilning on the level of bDam in malt was monitored, as well as during mashing and wort boiling. Two different varieties of malt (spring and winter) were compared. The results showed different trends during malt kilning, with an increase in the levels of the aroma compound at 78 and 90 °C. During mashing and wort boiling, bDam was formed following different trends, this associated to a balance between formation and evaporation. Moreover, malts and worts from winter barley contained more bDam than spring barley. Further research is required in order to identify and monitor the precursors of this potent aroma compound.

Keywords: damascenone, malting, wort boiling, brewing.

4.1. Introduction

bDam is widely known in flavour science because of its extremely low orthonasal detection threshold of 0.004 μg/L in water (Langos et al., 2013). Other researchers reported even lower orthonasal detection thresholds of 0.00075 μg/L in water (Semmelroch, Laskawy, Blank, & Grosch, 1995). Even being present at very low concentrations, it has been demonstrated that it plays a key role in the aroma of several foods, as varied as rape honey (Ruisinger & Schieberle, 2012), Syrah wine (Zhao, Gao, Qian, & Li, 2017) and blackberries (Klesk & Qian, 2003), where this compound imparts its characteristic pleasant, fruity aroma. In rape honey, its concentration was just 7.6 μg/kg, but this represented an odour activity value (OAV) of 760, i.e. 760 times more concentrated than the reported orthonasal detection threshold.

In beers, their fruity aroma has been traditionally related to the presence of esters and alcohols. However, the role of bDam must not be underestimated. In Bavarian wheat beers, this compound was reported to have the highest OAV besides ethanol (Langos et al., 2013). In pale lager and Pilsner, its concentration was 1.6 μg/L (Schieberle, 1991) and 2.3 μg/L (Fritsch & Schieberle, 2005), respectively. bDam has been previously reported in unhopped wort (De Schutter et al., 2008a). Hops has been described as one possible source of bDam, where the presence of its precursor β-D-glucoside of 3-hydroxy-β-damascone has been previously identified (Kollmannsberger et al., 2006). However, bDam has also been found in barley malt (Farley & Nursten, 1980; Fickert & Schieberle, 1998).

The concentration of bDam in wort was detected at extremely high level (450 μg/kg wort) (Chevance et al., 2002). Nonetheless, its concentration decreased remarkably after fermentation (7 days at 20 °C), this way leading to a non-detectable final concentration in fresh beer. However, in the case of alcohol-free beers produced following a cold contact fermentation

(CCF) procedure, the low fermentation temperature (close to 0 °C) not only limited the formation of ethanol (Perpète & Collin, 1999a), but also the reduction on the level of bDam. In our previous study, the concentration of bDam in an alcohol-free beer brewed by CCF was 10.4 µg/L beer, well above its orthonasal detection threshold 0.23 µg/L (0).

Because of the high contribution of this compound to the overall aroma of beer, it is essential to understand the factors affecting its formation through the brewing process. Since it was demonstrated that bDam was formed during thermal processes (Kumazawa & Masuda, 2001), the hypothesis of this study was that the concentration of bDam in malt is affected by the kilning temperature, and that kilning at different temperature has an impact on the worts prepared from these malts. Consequently, the objectives of the present research were to study the formation of this compound during the curing stage of malt kilning from two varieties of barley, as well as to determine its levels at different stages of mashing and wort boiling prepared with malts cured at different temperatures.

4.2. Materials and methods

4.2.1. Chemicals

bDam (≥98%) was purchased from Sigma-Aldrich (Gillingham, UK); α-ionone was acquired from IFF (New York, NY, USA) and absolute ethanol was from VWR (Lutterworth, UK).

4.2.2. Preparation of malt samples

The malt samples used for this study were the same as in our kinetic study (0). Briefly, the green malt from two different varieties (spring variety “Planet” and winter “Etincel”) were kilned in a pilot scale micro-malting equipment from Nordon & Cie. (Nancy, France). The samples (650 g) were kilned following a temperature gradient

based on the one used at industrial scale: initial temperature of 25 °C, raised to 55 °C in 10 min, then increased to 64 °C in 45 min, kept for 4 hours and 50 min and then increased to 65 °C in 3 hours and 15 min. After this stage (drying stage), the malt was cured isothermally at 65, 78 or 90 °C for 8.4 h. Samples were collected at the beginning of the curing stage, after 4.8 h and at the end of curing. After collection from the micro-malting equipment, the rootlets of the grains were removed by manual rubbing, sieved off and the malt stored at -30 °C.

4.2.3. Mashing and wort boiling experiments

The final malt samples, i.e. those cured for 8.4 h, were coarsely ground using a coffee grinder and sieved through a 355-µm mesh size sieve in order to remove undesired fine particles. The ground malt (40 g) was added to 120 mL deionised water at 55 °C (mash). The mash was kept at this temperature for 15 min with constant magnetic stirring. Then, the temperature was increased to 63 °C, kept for 40 min, then raised to 72 °C and kept for another 15 min, and a final step of 78 °C for 20 min. The wort obtained this way was filtered through a 1-mm mesh sieve and then boiled for 30 min in an open beaker. Samples were taken at the beginning of the 63 °C-step, at the end of the 72 °C-step and after wort boiling (Figure 4.1).

4.2.4. Quantification of (*E*)-β-damascenone

Malt samples (5 g) were extracted with 25 mL of deionised water at room temperature for 60 min under stirring. The malt extracts, as well as the mash and wort from Section 4.2.3, were centrifuged at 5500×g for 15 min at 4 °C. The pH was adjusted to 3.0 using 1 M HCl. Aliquots (5 mL) were poured into a 20 ml SPME vials containing

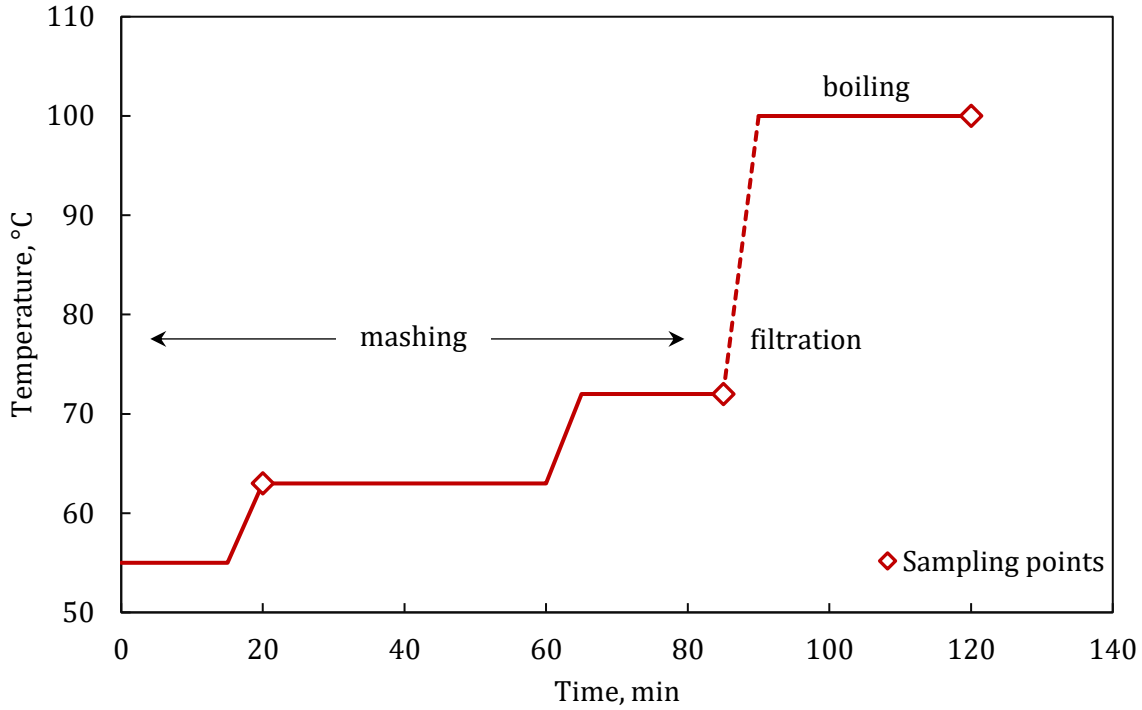

Figure 4.1 Temperature gradient during mashing and wort boiling experiments.

1.3 g NaCl. α-Ionone (5 µL at 5 mg/L in ethanol) was used as internal standard. The vials were incubated at 60 °C for 10 min, and then a DVB/Carboxen®/PDMS SPME fibre (65 µm, 2 cm) from Supelco (Bellefonte, PA, USA) was exposed for 45 min. The fibre was desorbed in the injection port of the GC in splitless mode at 250 °C for 20 min. The instrument used was a 7890A gas chromatograph coupled to a 5975C inert XL EI/CI MSD triple axis mass spectroscopy detector from Agilent Technologies (Santa Clara, CA, USA). The carrier gas was helium at 3 mL/min. A non-polar ZB-5MSi column (30 m, 0.25 mm i.d., 1.0 µm df) from Phenomenex (Torrance, CA, USA) was used. Data acquisition was performed in SIM mode, using the ions (first ion for quantification) 121 and 190 for bDam, and 121 and 192 for α-ionone. Mass spectra were recorded in the EI mode at an ionisation voltage of 70 eV and source temperature of 230 °C. The experiments were

performed in duplicate and the results were expressed in relation to the concentration of the internal standard, assuming a response factor of 1 between bDam and α-ionone.

4.2.5. Statistical analysis

Duncan's test for multiple comparisons has been applied at a significance level α=0.95 by using the software InfoStat 2017, developed by the National University of Córdoba (Córdoba, Argentina).

4.3. Results

The effect of curing time on the level of bDam in barley malt at different kilning temperatures (isothermally) was studied. Figure 4.2 shows the concentration of this compound in terms of the ratio of the peak areas of bDam and the internal standard. The results show an increase in the amount of bDam in malts kilned at 78 and 90 °C. At 78 °C the maximum level was reached after 8.4 h curing, whereas at 90 °C the levels stabilised at the maximum after 4.8 h, this being not significantly different to the samples kilned for longer time (8.4 h). The results showed a certain degree of variability, especially those samples kilned at 65 °C. The reason behind this might be the variability associated with real food matrices. The green malt samples were collected from the industrial germination boxes and kilned using a pilot-scale oven. Heating in this kind of pilot-scale ovens is not completely homogenous and also the hot air employed to process the malt may not be spread equally across the samples. The processed malts were placed in aeriated boxes in the oven in different positions and this possibly led to uneven air flow.

The malts prepared for the first part of this study were used for mashing and the preparation of wort. The mixture of malt grains steeped in water is known as mash,

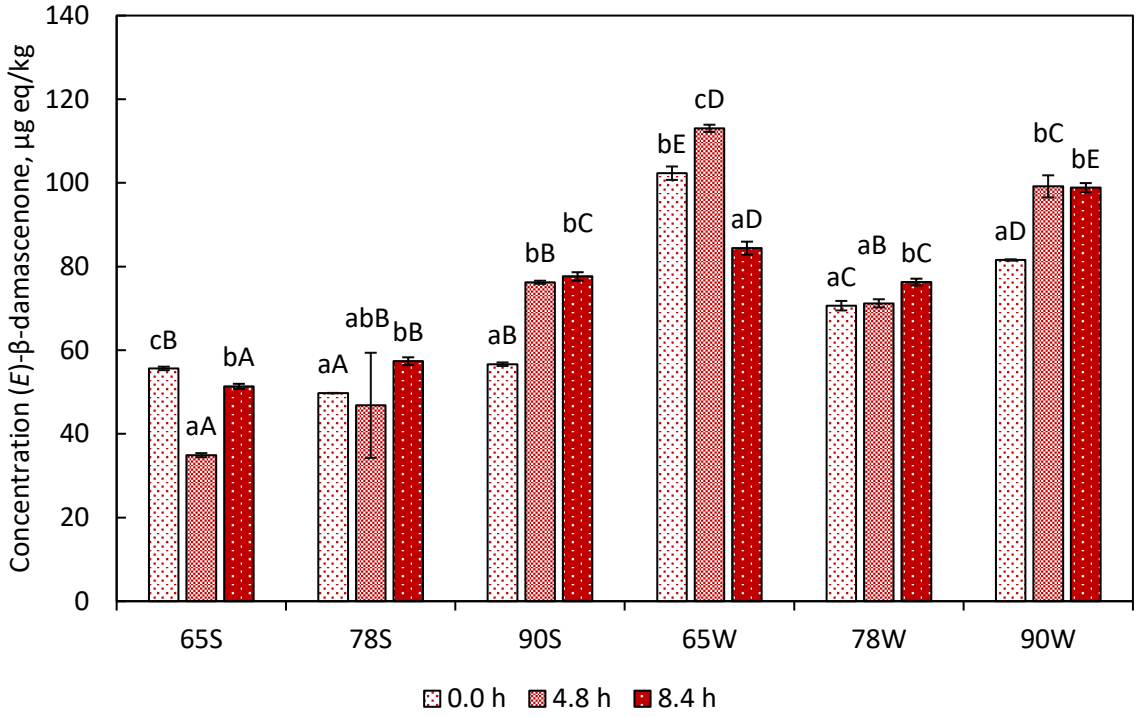

Figure 4.2 Effect of curing time and temperature (65, 78 and 90 °C) on the formation of (*E*)-β-damascenone during malt kilning for spring (S) and winter (W) barley. Results expressed as µg equivalent of α-ionone per kg malt. Significant differences ($p<0.05$) between curing time within experiments and between experiments at the same curing time are represented by lower and uppercase letters, respectively.

whereas the wort is the liquid extract after filtration. In order to understand the behaviour of bDam during mashing and wort boiling, different samples were collected at the beginning of the mashing process, i.e. before the step at 63 °C, end of mashing and end of wort boiling. Figure 4.3 shows the levels of bDam throughout the mashing and wort boiling processes. The data showed different trends dependent on the variety of barley used. Spring barley malts kilned at 65 and 78 °C showed an increasing trend during mashing and wort boiling. However, bDam decreased slightly during mashing and increased after wort boiling. Similar behaviour was observed in malts from winter barley, but the differences were larger. For these malts, the concentration after mashing decreased significantly and increased to their highest values after wort boiling. These

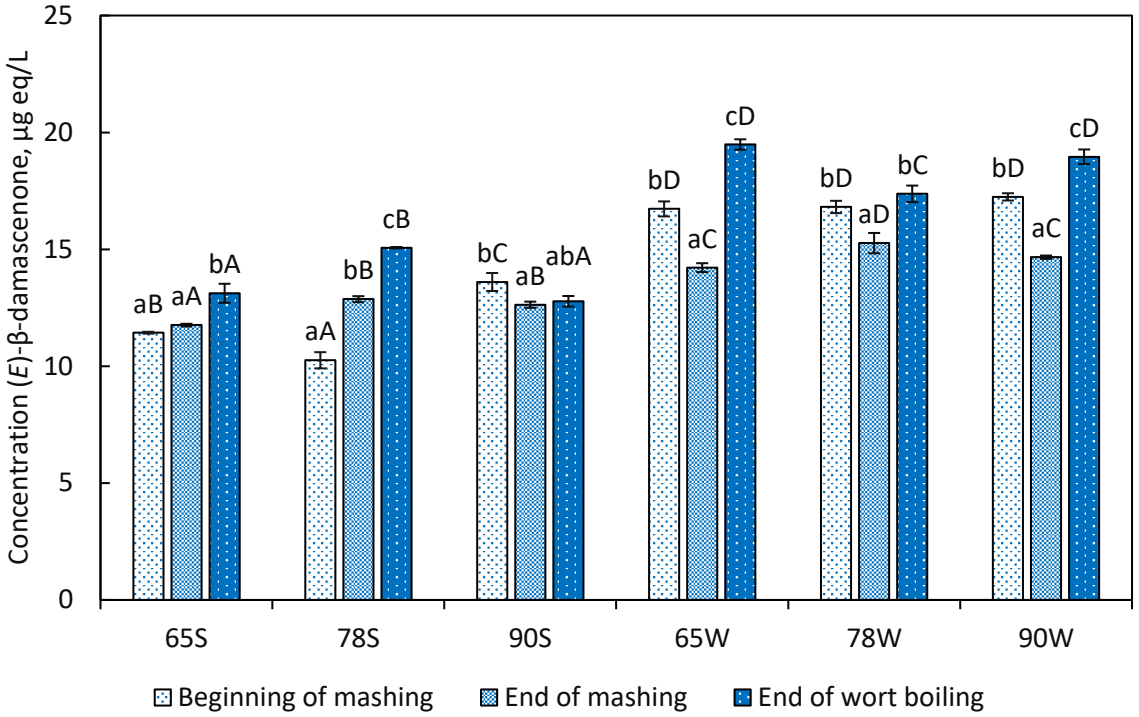

Figure 4.3 Levels of (*E*)-β-damascenone during mashing and wort boiling for spring (S) and winter (W) malts kilned at different temperatures (65, 78 and 90 °C). Results expressed as µg equivalent of α-ionone per L wort. Significant differences ($p<0.05$) between samples within experiments and between experiments at the same sampling stage are represented by lower and uppercase letters, respectively.

results suggested a balance of loss and formation of bDam: this compound was lost during mashing due to evaporation and its formation was not enough to result in an overall accumulation. However, the higher temperature applied during wort boiling might lead to a formation rate higher than the loss, and also the evaporation of water during boiling could have contributed to an increase in the final concentration of bDam. When considering the concentration of bDam in malts at the end of kilning, the theoretical concentration of bDam at the beginning of mashing was 42% higher, in average (data not shown), than the values determined empirically. This was associated to the extraction method in both cases, with much longer extraction time for the malt (60 min) because in this case the aim was to quantify the total amount of bDam.

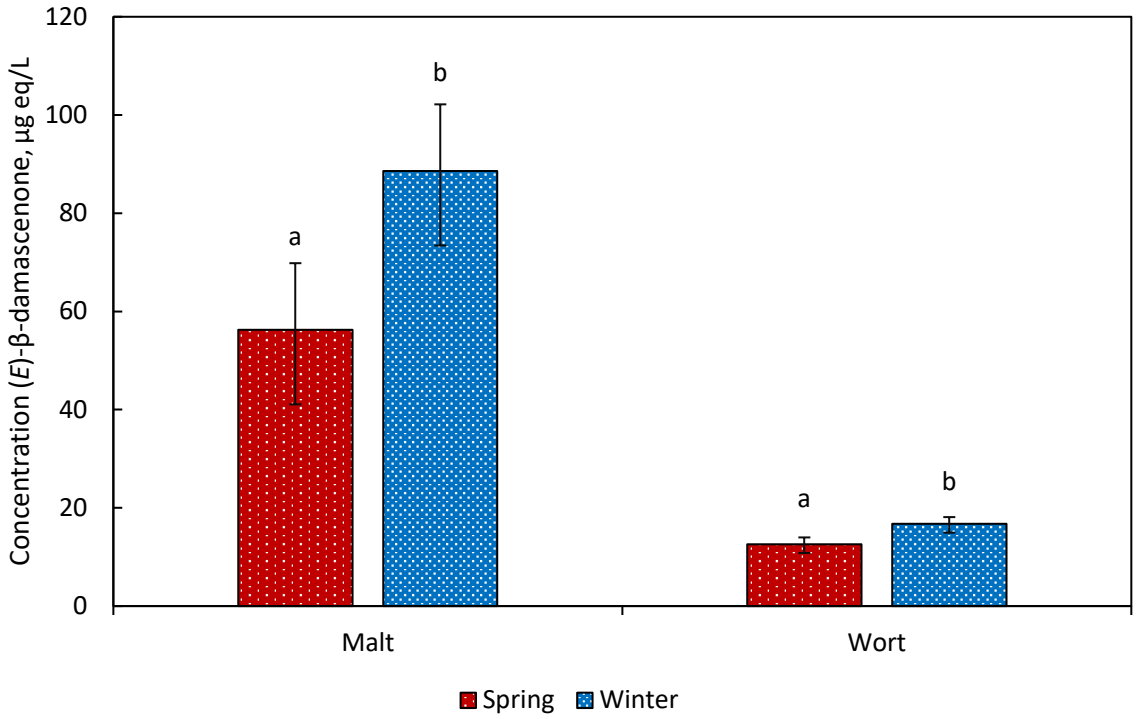

Figure 4.4 Average levels of (E)-β-damascenone in malts from two different varieties and wort produced from them. Different letters mean significant differences ($p<0.05$) within the varieties.

With respect to the varieties of barley used in this study, a higher average amount of bDam was found in winter barley than in the spring variety (Figure 4.4). The differences were significant ($p<0.05$) for both the malts, as well as in the worts prepared from those malts. Malts from barley winter contained 57% more bDam than spring barley, in average. In the case of the wort, the difference was smaller, around 33% higher.

4.4. Discussion

bDam is an aroma compound present in a huge variety of foods, mainly of plant origin. It is widely accepted that this compound can be formed from the hydrolytic degradation of the carotenoid neoxanthin (Isoe, Katsumura, & Sakan, 1973; Puglisi, Elsey, Prager, Skouroumounis, & Sefton, 2001). These authors proposed a formation mechanism via the so-called "grasshopper ketone" (Figure 4.5a). However, this formation

route in barley has not been confirmed yet, despite neoxanthin was found in barley grains (Sreenivasulu et al., 2010). Little is known regarding the role of cultivar on the content of carotenoids in barley grain. Other external (or abiotic) factors, such as water stress (drought), affected negatively the concentration of carotenoids in barley (Jaleel et al., 2009), but it was not proven that this had any impact on the formation of flavours during heat treatment. Carotenoid content in wheat was found to be lower in spring varieties, but this was not proven in barley (Konopka, Czaplicki, & Rotkiewicz, 2006). Other studies

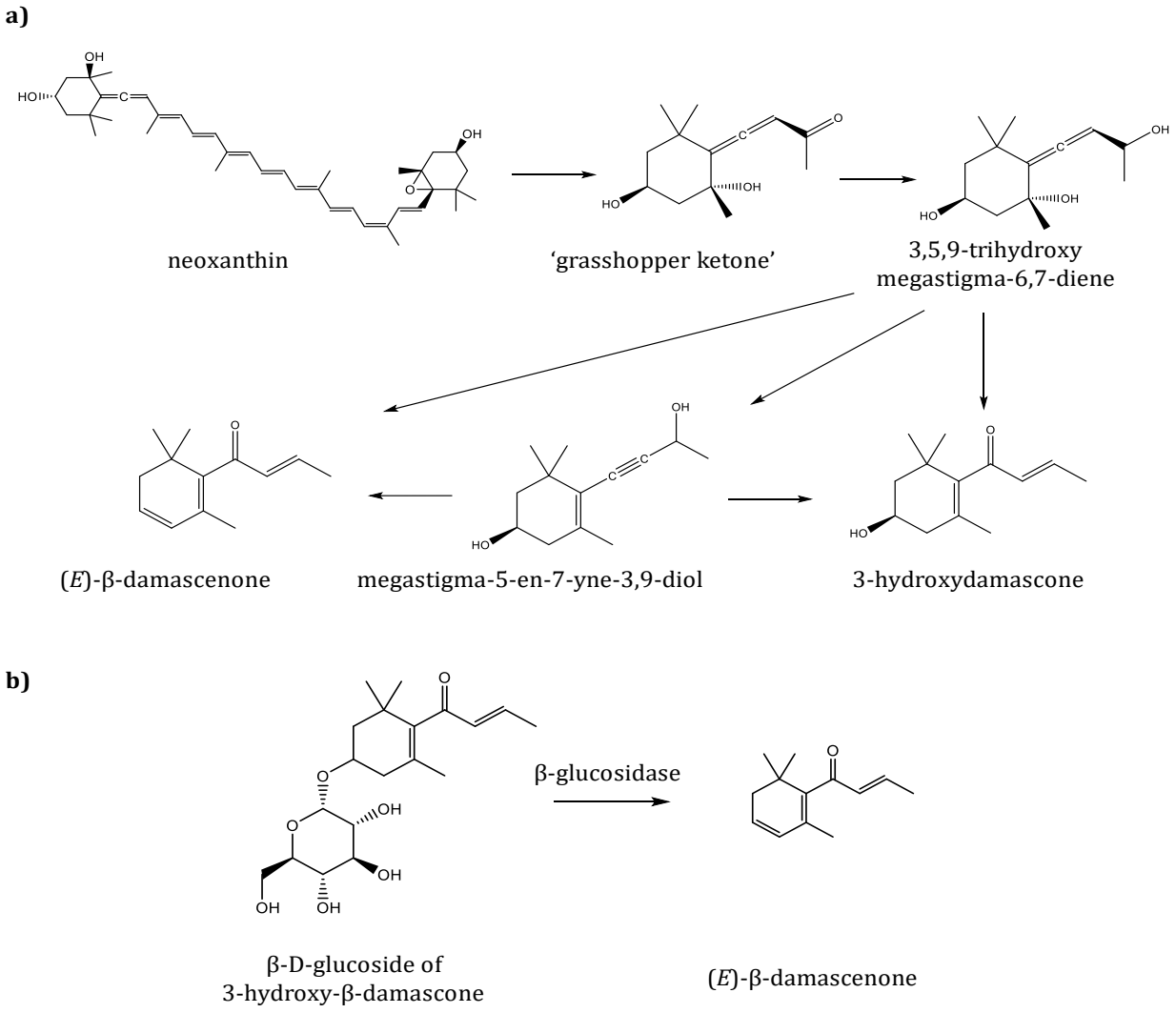

Figure 4.5 Mechanism for the formation of (E)-β-damascenone from neoxanthin (a) and from the glucoside precursor (b). Adapted from Puglisi et al. (2001) and Kollmannsberger et al. (2006).

proved that this compound can be formed from the enzymatic hydrolysis of the β-D-glucosides of 5-megastigmen-7-yne-3,9-diol and 3-hydroxy-β-damascone (Skouroumounis, Massy-Westropp, Sefton, & Williams, 1992). Figure 4.5b shows the structure of the β-D-glucosides of 3-hydroxy-β-damascone. These precursors have been identified in fruit juice but not in barley. Nonetheless, the theory of a glycoside precursors has been supported by results showing an increase in the concentration of bDam in beers after the addition of β-glucosidase (Chevance et al., 2002).

The formation of bDam in malt and wort was favoured at higher temperatures. This has been observed in other foods after heat treatment. For instance, a considerable increase in the concentration of bDam (13.5 times higher) has been reported in black tea infusions after sterilisation by heat treatment (121 °C for 10 min) (Kumazawa & Masuda, 2001). An increase in the concentration of bDam was observed after heating mandarin juice (Araki & Sakakibara, 1991). It is of great importance to understand how process conditions, like temperature, affect the formation of bDam in malt and wort. This is even more critical when brewing alcohol-free beers by fermentation at very low temperature (such is the case of CCF). The formation of bDam was proven to happen from the thermal degradation of 9'-cis-neoxanthin in a model system containing peroxyacetic acid at temperatures from 60 to 90 °C (Bezman et al., 2005). The present piece of research must be regarded as a preliminary study and further experiments could provide more insight on the formation of bDam in malts and worts, such as the absolute quantification of bDam. Moreover, identification of precursors and monitoring of their behaviour throughout processing are required in order to elucidate the chemical mechanism and the factors that affect its rate of formation. This would help control the amount of bDam in malts and beers by knowing the behaviour of its precursors from the raw materials.

4.5. Conclusions

bDam was monitored during the curing stage of malt kilning as well as during mashing and wort boiling. During curing in kilning, the level of bDam increased over time at the highest curing temperatures (78 and 90 °C), with maxima reached after 8.4 h curing at 90 °C. These results demonstrated the great importance of temperature and time during malt kilning. Regarding mashing and wort boiling using malts cured at different temperatures, this factor did have a significant impact. Greater differences were found between winter and spring barleys, mostly related to the trend of the levels of bDam. On average, malts and worts from winter barley presented significantly higher amounts of bDam than those from spring barley ($p<0.05$). Further research could provide better understanding of the differences related to variety, and thus be able to better control the concentration of this powerful aroma compound in the beer.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

Chapter 5.

Chapter 5. Multi-response kinetic modelling of the formation of five Strecker aldehydes during kilning of barley malt

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Abstract

Strecker aldehydes are responsible for the characteristic aroma of malts, but also important aroma compounds in beer. In malt, they are formed during the curing stage of kilning. The formation of five Strecker aldehydes (2-methylpropanal, 2-methylbutanal, 3-methybutanal, phenylacetaldehyde and methional) during this process was studied at pilot scale. Green malts were dried and cured at different temperatures (65, 78 and 90 °C) isothermally for 8.4 h. Multi-response kinetic modelling was used to develop a mathematical model based on precursors concentration, Amadori rearrangement products (ARP), Strecker aldehydes, temperature and time. This model demonstrated that the formation of Strecker aldehydes in malt was controlled from the formation of two intermediates: ARP and short chain dicarbonyls (SCDC). The kinetic model proposed in this study will help maltsters and researchers understand and manipulate the formation of these compounds in malt and the organoleptic quality of the beers brewed from them.

Keywords: Multi-response kinetic modelling, malting, Strecker aldehydes, flavour precursors

5.1. Introduction

Barley (*Hordeum vulgare* L.) is the fourth most important cereal crop in the world, after wheat, corn and rice. Although the majority of the 160 million tons produced yearly are destined to feeding cattle, its second most common application, around 30%, is the production of barley malt, 90% of which is used by the brewing industry (Akar, Avci, & Dusunceli, 2004). Malting consists of three process steps: (1) steeping, to hydrate the seed kernels from around 12% to at least 40% moisture; (2) germination, to activate the production of hydrolytic enzymes (proteases, amylases and others) and cell wall breakdown; and (3) kilning, to dry off the kernels, and generate flavour compounds and colour (Gupta, Abu-Ghannam, & Gallaghar, 2010). The bigger changes in barley occur during germination, when the cell walls of starch granules is hydrolysed through a process called cytolysis, thus releasing both starch and other endosperm breakdown products (sugars, amino acids, etc.) (Briggs et al., 2004).

The enzymatic activity initiated by germination affects mainly the carbohydrates and the proteins in the kernels. Hydrolysis of starch, the main component of barley grain (51-64%, average 59%) (Holtekjølen, Uhlen, Bråthen, Sahlstrøm, & Knutsen, 2006), is triggered during germination. Although β-amylases are already present in the barley grains (Georg-Kraemer, Mundstock, & Cavalli-Molina, 2001), these and α-amylases are expressed mostly during germination, this way increasing their hydrolytic activity and the amount of free sugars (Piendl, 1971). Fructose, glucose, sucrose and maltose are the most abundant free sugars in barley malt (Yichao Huang, Carragher, & Cozzolino, 2016). These fermentable sugars are responsible for the sweet taste of beer, whereas longer chain dextrins (e.g. maltotetraose, maltopentose, maltohexose, etc.) are reported to provide body and mouthfeel (Vriesekoop, Rathband, MacKinlay, & Bryce, 2010).

Germination also promotes the formation of protein degrading enzymes, such as endoproteases and carboxypeptidases, which hydrolyse seed storage proteins into soluble proteins, peptides and free amino acids (Steiner, Gastl, & Becker, 2011; Zhang & Jones, 1995). Alkali-soluble glutelins and prolamin, called 'hordein' in barley, are the main proteins in barley grains, constituting approximately 30% and 37% of total proteins, respectively (Steiner et al., 2011). According to Robbins & Pomeranz (1971), the most abundant amino acids in barley proteins are glutamic acid, proline and aspartic acid. Proteins in barley, however, are present in a much lower proportion (10 to 17%) than carbohydrates (Steiner et al., 2011) but these and their nitrogen-containing degradation products are essential for yeast metabolism during wort fermentation (Lekkas, Stewart, Hill, Taidi, & Hodgson, 2005).

Besides their role as nutrients for yeast, sugars and amino acids are of key importance for the formation of volatile compounds through the Maillard reaction, as well as the development of the characteristic colour of the different types of malts. Amongst the volatile compounds found in barley malt, 3-methylbutanal and 2-methylbutanal were identified as responsible for the characteristic aroma of this cereal product (Beal & Mottram, 1994). These Strecker aldehydes, along with methional and 2-methylpropanal, were also reported as key odourants in alcohol-free beers and impart characteristic malty and worty flavours (Perpète & Collin, 2000a). These compounds are formed through the Strecker degradation of amino acids, comprising the α-deamination and decarboxylation of the amino acid by reaction with a reducing sugar, generally, or their α-dicarbonyl products (Rizzi, 2008). During the Maillard reaction, reducing sugars form the intermediates deoxyosones, which are degradation products of 1-amino-1-deoxy-ketoses, also called Amadori rearrangement products (ARP), if the initial sugar is an aldose, or 2-amino-2-deoxy-aldoses (also known as Heyns rearrangement products)

from ketoses (Kocadağlı & Gökmen, 2016). ARP have been identified in barley malt (Meitinger, Hartmann, & Schieberle, 2014) and different types of beer, with concentrations of fructosyl lysine ranging from 6.8 to 27.0 mg/L (Hellwig, Witte, & Henle, 2016). Some of these compounds, such as fructosyl glutamic acid, fructosyl valine and fructosyl methionine, have been described as umami enhancer compounds (Kaneko, Kumazawa, & Nishimura, 2011; Robert et al., 2003). Deoxyosones undergo further degradation reactions, leading to the formation of more reactive SCDC, such as glyoxal, 2-methylglyoxal and 2,3-butanedione (Smuda & Glomb, 2013). SCDC are more reactive than the longer chain deoxyosones and hence regarded as the main contributors to the formation of Strecker aldehydes.

Consequently, the nature of the amino acid determines the Strecker aldehyde that will be formed as a product of the reaction with the dicarbonyls. Thus, methionine, valine, leucine, isoleucine, and phenylalanine produce the aroma compounds methional (potato-like aroma), 2-methylpropanal (malt-like), 3-methylbutanal (malt-like), 2-methylbutanal (malt-like), and phenylacetaldehyde (honey-like), respectively (Pripis-Nicolau, De Revel, Bertrand, & Maujean, 2000). On the other hand, the type of sugar involved in the reaction generally influences the reaction rate, so hexoses like glucose and fructose are more reactive than disaccharides like maltose or trisaccharides like maltotriose (Reineccius, 2006).

5.1.1. Kinetic modelling

The distinct aroma of the different types of malts is developed during kilning through the Maillard reaction and it is strongly dependent on the processing conditions: time, temperature and moisture content. The effect of these variables has been widely studied using mathematical models in order to better understand their role in the product (e.g., concentration of a certain compound or sensory characteristics). Two different types of models can be distinguished: empirical and mechanistic models. Empirical models relate dependent variables to independent variables through mathematical functions, and thus, the intrinsic connectivity between them is not required to be known (Thakur, 1991). Consequently, the relation between the variables is only based on the observations used for building up the model. Thakur (1991) described these models as 'retroactive' and 'locally predictive' because they rely strongly on the empirical observations and consequently extrapolative predictions are not recommended. On the other hand, mechanistic models are based on the knowledge of the behaviour of the system through equations that describe the relationship between the variables. Therefore, a basic understanding of the connection of the different factors involved is necessary to develop this kind of mathematical models.

The response surface methodology (RSM) is usually followed in order to build empirical models, but it also helps optimise processes for maximum or minimum outcomes. Kim et al. (1993) reported an empirical model for the prediction of the overall acceptability and roasted flavour of malts where the independent variables were germination time and kilning temperature. Second-order models were proposed, with no significant interaction between the independent variables (i.e. no X_1X_2 factor) and suggested 4 days of germination and 77 °C drying temperature to achieve optimal

acceptability and roasted flavour. Other authors focused on the formation of aroma compounds during malt kilning. A mathematical model (based on RSM) for the formation of methional and phenylacetaldehyde in Australian malted barley showed that both kilning time and temperature had a positive effect on the concentration of those compounds (S. Huang et al., 2016). In this case, second order models were reported, with significant second-order interactions between the independent variables. Herrmann, Gastl, Thiele, & Back (2007) followed a similar approach to study the influence of several malting parameters on the formation of flavour compounds during kilning. For instance, the authors associated lower amounts of Strecker aldehydes for longer germination times with the higher growth of the grain rootlets under such conditions. However, this was only a speculation because the authors did not present strong evidence for this relationship. This type of models only relates the independent variables with an outcome and do not represent the chemical reaction pathways or the presence of intermediate steps in the reaction. Besides, none of these studies considered the role of the different precursors to the formation of these compounds.

Empirical models can also be used for calculating certain kinetic parameters, such as the activation energy. Cremer & Eichner (2000) studied the formation of Strecker aldehydes in low moisture model systems containing glucose (1.0 mol/kg) and amino acids (Ala, Val, Leu, Ile, all at 50 mmol/kg) and heated at temperatures from 80 to 110 °C. By using the Arrhenius equation, the authors reported that the formation of these compounds followed a zero-order reaction and their activation energies were in the range of 115-124 kJ/mol. Another study showed that the formation of different aldehydes during pressure-assisted thermal treatment of milk followed zero-, first- or second-order reaction models, depending on the compound (Vázquez-Landaverde, Qian, & Torres, 2007). The authors also reported that the activation energies were dependent on the

pressure, decreasing at higher pressure for linear aldehydes and increasing for 2-methylpropanal and 2,3-butanedione, and thus a clear effect of pressure on the aroma of thermal-treated milk was demonstrated.

Mechanistic models are much more complex to approach. In the case of kinetic studies, models usually represent a simplified version of the chemical reactions occurring in the system (Parker, 2013), this version though reflecting the kinetic mechanism of a reaction which usually consists of a limited number of kinetic control points. Unlike empirical models, mechanistic models take into account the behaviour of both the precursors and the products, as well as intermediate species. This is translated into a system of differential equations which describe the variation of the different chemical species along time, where the kinetic rate constants are the unknown parameters. These, in turn, are functions of the activation energy and other parameters, depending on the shape of the Arrhenius equation used. Due to the complexity of the problem, an analytical solution to the differential equations is nearly impossible to achieve in most cases, and hence iterative numerical analysis is required as a tool for providing good estimates of the unknown parameters. For this purpose, multi-response kinetic modelling has been widely used to generate, analyse and evaluate mechanistic models (Balagiannis, 2015). This mathematical method uses the information from various responses simultaneously, so that better parameters can be estimated and get better model fits (Martins & Van Boekel, 2003). Moreover, multi-response kinetic models provide information regarding the presence of intermediate species and their relative stability, which can also be used in order to identify rate-limiting reaction steps.

Modelling of the Maillard reaction has mainly focused on the formation of colour species. Mundt & Wedzicha (2003) postulated a model for the reaction of glucose and

fructose with glycine. The results showed that both reducing sugars reacted with the amino acid to form a common intermediate which degraded into melanoidins. The authors also observed the inhibition of the formation of melanoidins by metabisulfite ions through one rate-determining step to produce a sulphohexosone. The degradation pathways of fructosyl glycine, a relatively stable Amadori intermediate of the condensation of glucose and glycine, were dependent mostly on the pH. A kinetic model showed that at lower pH (5.5) 1,2-enolisation was favoured, whereas higher pH (6.8) followed 2,3-enolisation (Martins & Van Boekel, 2003). Fructosyl glycine, or its rearrangement products (named E_1 and E_2 in this study), degraded into reactive 1- and 3-deoxyosones and a pool of unidentified carbohydrate fragments (potentially carbonyl compounds).

Multi-response kinetic modelling has been also used for volatile aroma compounds. Yarong Huang, Tippmann & Becker (2016) proposed a kinetic model for the formation of 2- and 3-methylbutanal from sugar (glucose or maltose) and amino acid (leucine or isoleucine) in a model reaction medium consisting of an aqueous phosphate buffer (0.1 mol/L, pH 5.2). This kinetic model consisted of a three-step consecutive reaction. Balagiannis et al. (2009) proposed a kinetic model for the formation of 2- and 3-methylbutanal in a heated liver extract, via the reaction of an intermediate with isoleucine and leucine, respectively. This intermediate was thought to be a group of reactive dicarbonyl compounds formed from the degradation of glucose. Furthermore, the degradation of Strecker aldehydes through the Maillard reaction was found to be relevant to the system. In a further study, the researchers added ribose to the model and applied it to a beef muscle extract (Balagiannis, Howard, Parker, Desforges, & Mottram, 2010). The results showed that ribose supplementation (10-fold the original concentration) increased the concentration of 2-methylbutanal, but in the case of 3-

methylbutanal the increase was apparently much smaller, this being attributed to the degradation at a greater degree.

In our previous study (0), Strecker aldehydes were described as key contributors to the undesired malty and worty character of alcohol-free beers. Strecker aldehydes are formed in malt during kilning and potentially following a similar chemical reaction pathway as in other food matrices. From a preliminary study (unpublished), it was observed that little increments on the curing temperature had a great impact on the concentration of methional in malt. Consequently, understanding how processing conditions affect the formation of these flavour compounds is of great importance to manipulate the process and control their levels in malt, and potentially in the beer. Thus, the hypothesis of the present research was that the formation of Strecker aldehydes during malt kilning was dependent on the processing conditions (temperature and time) and this takes place via the degradation of relatively reactive sugars (glucose and fructose) and amino acids, and the formation of several intermediates of different stability and reactivity (such as ARP and SCDC). Also, the formation of the flavour compounds was considered to happen only during the curing stage of malt kilning, and not during the mild temperature drying stage. Therefore, the aim of this study was to develop a kinetic model for the formation of five Strecker aldehydes (2-methylpropanal, 2- and 3-methylbutanal, phenylacetaldehyde and methional) during the curing stage of kilning for the range of temperatures used for Lager malts.

5.2. Materials and methods

5.2.1. Preparation of malt samples: micro-malting

Barley was received at Mouterij Albert (Puurs, Belgium), steeped in water for one day and germinated for five days. Two different varieties of barley were used in this study: the two-row spring variety 'RGT Planet' and six-row winter variety 'Etincel'. The germinated grains, i.e. the green malt, were collected from the germination box at the industry's premises. Green malt samples were kilned using pilot-scale micro-malting equipment from Nordon & Cie. (Nancy, France). The green malt was placed in cubic shape stainless-steel baskets (150 mm side), split diagonally into two parts by a piece of steel (650 ± 2 g green malt in each side). The micro-malting equipment was provided with eight baskets with grilled bottom to allow ventilation throughout (Figure 5.1).

The kilning programmes (Figure 5.2) were based on the ones used at industrial scale. They had an initial drying process, starting at 25 °C and reaching 55 °C within 10 min, then increasing to 64 °C within 45 min. The temperature was then kept constant for 4 h and 50 min and raised to 65 °C within 3.25 h. After the drying process, the temperature

Figure 5.1 Baskets for pilot-scale kilning experiments and micro-malting equipment

was increased to the curing temperature (65°C, 78°C or 90 °C) in 10 min and kept constant for 8.4 h. The baskets were taken out randomly from the different positions in the oven and the empty space replaced by an empty basket with a lid. The total duration of the malting experiments was 16 h and 52 min, the samples being taken every 72 min only during the curing stage. After kilning, the rootlets were removed by manual rubbing, separated by sieving through 1.8×23 mm mesh and stored in a freezer at -30 ± 1 °C to limit thermal reactions. The kilning experiments were performed in duplicate from two different batches of barley for each variety in different days, except for the experiments at 65 °C, where the duplicates were from the same batch due to availability at the industrial premises on the day of collection

5.2.2. Chemicals

2-Methylpropanal (≥ 98%), 2-methylbutanal (≥ 95%), 3-methylbutanal (≥ 97%), methional (≥ 97%), phenylacetaldehyde (≥ 95%), 2-methylbenzaldehyde (97%), a standard of 18 L-amino acids (product code AAS18-5ML) and DL-norvaline (≥ 98%) were

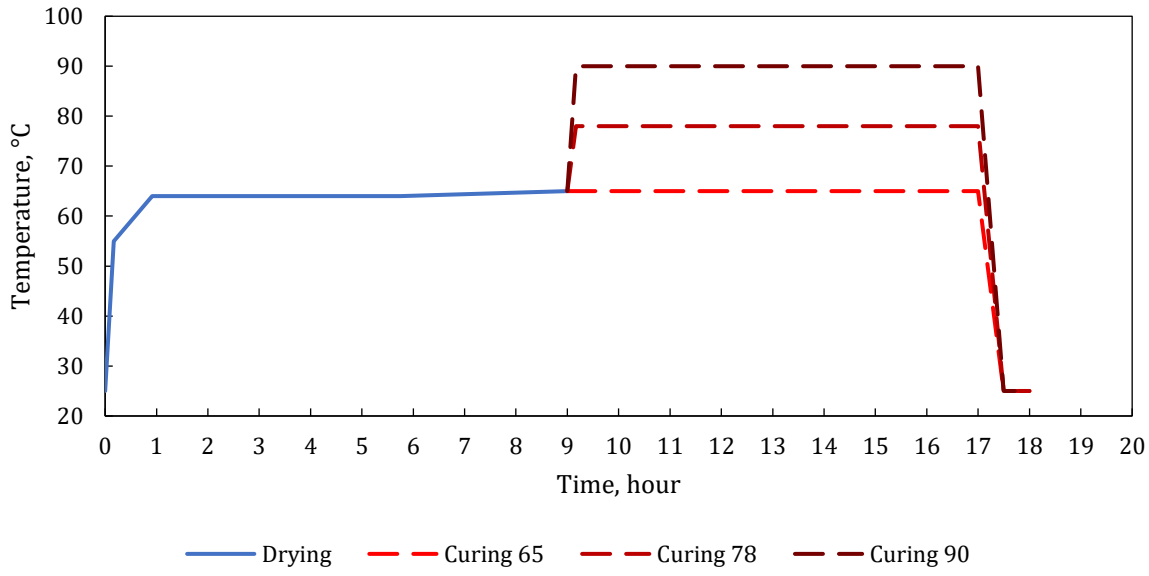

Figure 5.2 Kilning programmes with constant drying stage and isothermal curing at different temperatures.

purchased from Sigma-Aldrich (Gillingham, UK). D(+)-glucose anhydrous (99.5%) and D(-)-fructose (≥ 99%) were from Fluka (Loughborough, UK) and 2-methylpentanal (≥ 95%) and D(+)-trehalose dihydrate (≥ 98%) from TCI (Oxford, UK). LC/MS grade formic acid (≥ 99%) and ammonium formate were from Fisher Scientific (Loughborough, UK). Acetonitrile (≥ 99%, LC/MS grade) was supplied by VWR (Lutterworth, UK). Fructosyl valine (FruVal, CAS no. 10003-64-2, 95%), fructosyl leucine (FruLeu, 34393-18-5, 95%), fructosyl isoleucine (FruIle, 87304-79-8, 96%), fructosyl phenylalanine (FruPhe, 31105-03-0, 95%), fructosyl alanine (FruAla, 16124-24-6, 95%), fructosyl glycine (FruGly, 60644-20-4, 95%), fructosyl proline (FruPro, 29118-61-4, 97%) were acquired from Toronto Research Chemicals (Toronto, ON, Canada).

5.2.3. Extraction and quantification of non-volatile compounds

The malt samples were ground to a fine powder using a mill provided with a size 40 mesh. The ground sample (1.0 g) was extracted using 10 mL of ultrapure water (18.2 MΩ) containing 1.25 mM of L-norvaline and 15 µM of trehalose as internal standards for amino acids and sugars, respectively. The samples were stirred for 15 min at approximately 1700 rpm using a MultiReax shaker from Heidolph (Schwabach, Germany). After centrifugation at 5500×g for 15 min at 4 °C, the aqueous layer was separated, and the pellet was reextracted using 5 mL×2 water containing L-norvaline and trehalose. The three extracts were combined and stored at -18 °C until analysis. The extractions were performed in duplicate.

5.2.3.1. Quantification of free amino acids

For each malt extract, an aliquot (250 µL) was diluted with 1000 µL acetonitrile, centrifuged at 500×g for 3 min, and filtered through a 0.2-µm syringe filter. The samples

(5 µL) were injected in a 1260 Infinity HPLC coupled to a 6410 Triple Quad LC/MS detector, all from Agilent Technologies (Santa Clara, CA, USA). A Synchronis™ HILIC column (150×4.6 mm i.d., 3 µm) with a Synchronis™ HILIC precolumn (10×4.6 mm i.d., 3 µm) from Thermo Fisher (Waltham, MA, USA) was used, kept at 20 °C. Mobile phase A was water containing 5 mM ammonium formate and 0.5% formic acid and mobile phase B was water/acetonitrile (9:1, v/v) with 5 mM ammonium formate and 0.5% formic acid. The flow rate was 1 mL/min, and the eluent gradient was as follows: start at 10% A and increase to 40% A in 8 min, then decrease to 10% A in 1 min, and kept to 10% A for 4 min. The electrospray ionisation (ESI) source settings were: gas temperature 330 °C, gas flow 13 L/min, nebuliser pressure 40 psi, capillary voltage 4000 V. Chromatograms were recorded in the positive ionisation mode, with cell acceleration voltage 7 V, using the dynamic MRM (multiple reaction monitoring) scan mode under the conditions showed in Appendix 4 for every compound analysed. Calibration standards (0-2.5 mM) of 18 amino acids were prepared in acetonitrile/water (1:4, v/v) and norvaline was used as internal standard.

5.2.3.2. Quantification of free sugars

The samples prepared in Section 5.2.3.1 were used for the quantification of free sugars in the same LC-MS/MS instrument described in Section 5.2.3.1. The column employed was a LUNA® Omega SUGAR 100 Å (150×2.1 mm, particle size 3 µm) from Phenomenex (Torrance, CA, USA) provided with a column guard with the same characteristics, and kept at35 °C. A constant flowrate of 0.5 mL/min was used and the eluents were ultrapure water (eluent A) and LC-MS/MS grade acetonitrile (eluent B). The following eluent gradient was applied: 10% A for 5 min, then increasing to 40% A in 5 min and kept for 6 min, before decreasing to 10% A in 4 min. The total run time was 20 min

with a 12-min postrun. Detection parameters were set as follows: gas temperature 275 °C, gas flow 12 L/min, nebuliser pressure 50 psi, capillary voltage 6000 V. Chromatograms were recorded in the negative ionisation mode and MRM settings for every compound are shown in Appendix 4. Calibration standards (0-1 mM) of glucose and fructose were prepared in water/acetonitrile (2:8). Trehalose was used as internal standard.

5.2.3.3. Quantification of ARP

The malt extracts were subjected to a 50-fold dilution with ultrapure water, filtered through a 0.2-µm syringe filter and analysed with the LC-MS/MS system described above. A Discovery HS F5-3 (150×2.1 mm i.d., 3 µm) column, fitted with a column guard of similar characteristics, from Supelco (St. Louis, MO, USA), was used. The column was kept at 55 °C throughout the analysis. Water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) were used as mobile phases at a constant flowrate of 0.3 mL/min, following the next gradient: 98% A for the first 5 min, then decreasing to 0% A within 3.5 min, then kept for 6 min at 0% A and increased back to 98% A within 0.5 min. The total runtime was 15 min with additional 15 min post run for equilibration. The MS/MS detector was set at the following conditions: gas temperature 265 °C, gas flow 13 L/min, nebuliser 40 psi, capillary voltage 4000 V, positive mode, cell accelerator voltage 4 V. MRM settings for all the ARP quantified can be found in Appendix 4. Calibration standards (0-1000 µg/L) were prepared in ultrapure water and no internal standard was used for ARP.

5.2.4. Quantification of Strecker aldehydes

Ground malt samples (Section 5.2.3) (1.0 g for experiments at 65 and 78 °C, 0.5 g for 90°C) were weighed in SPME vials, together with 5 mL of saturated NaCl aqueous solution and 5 µL of internal standard solution (100 mg/L of 2-methylpentanal and 100 mg/L of 2-methylbenzaldehyde in absolute ethanol). 2-Methylpentanal was used as internal standard for 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, and methional; and 2-methylbenzaldehyde for phenylacetaldehyde. The samples were incubated at 50 °C for 10 min and then a PDMS/DVB/Carboxen® SPME fibre was exposed to the headspace of the vial for 20 min. The fibre was desorbed into the injection port at 250 °C for 20 min. A 7890A gas chromatography system was used, coupled to a 5975C mass spectrometry detector from Agilent. The chromatographic separation was achieved with a Zebron™ ZB-5MSi column (30 m, 0.25 mm, 1 µm) from Phenomenex®. The following temperature gradient was used: 50 °C for 2 min, then 6 °C/min up to 300 °C and kept at this temperature for 15 min. The single ion mode (SIM) was applied with a dwell time of 250 ms for every ion. The following ions were monitored, the first of them being used for quantification: 41 and 72 for 2-methylpropanal, 41 and 57 for 2-methylbutanal and 3-methylbutanal, 58 and 71 for 2-methylpentanal, 48 and 104 for methional, and 91 and 120 for phenylacetaldehyde and 2-methylbenzaldehyde. The standards for calibration (0-1000 µg/L) were spiked in freeze-dried green malt in order to account for the matrix effect on the release of the volatiles to the headspace of the samples. The analyses were performed in duplicate.

5.2.5. Kinetic modelling

The kinetic rate constants, k, were expressed in the form of the re-parametrised Arrhenius equation:

$$k = k' \cdot e^{\frac{Ea}{Rg}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)} \qquad \text{(Eq. 5.1)}$$

where T_{ref} is a reference temperature (set at 343.15 K in this study, i.e. 70.00 °C), T is the experimental temperature (K), Ea is the activation energy (J/mol), Rg is the universal gas constant (8.314 J/mol K), and k' is the rate constant at $T=T_{ref}$. Multi-response parameter estimation and model simulations were performed using the Athena Visual Studio programme (version 14.2) (AthenaVISUAL Inc., Naperville, IL, USA). Bayesian estimation for multiple responses (diagonal covariance) was chosen to estimate the parameters k' and Ea.

For discriminating between models, the Akaike information criterion (AIC) was employed (Martins & Van Boekel, 2003). AIC was calculated as follows:

$$AIC = n \ln\left(\frac{SS}{n}\right) + 2(p+1) \qquad \text{(Eq. 5.2)}$$

Where n is the number of data points, SS is the sum of squared residuals, and p is the number of estimated parameters. AIC values were calculated for different models, and the ones with higher AIC discarded.

5.2.6. Standard methods for the characterisation of malts

Friability, partly unmodified grains (PUG), whole unmodified grains (WUG), moisture, colour, density, viscosity, soluble protein and free amino nitrogen were

measured in the finished malts (i.e. after 8.4 h curing) following the specifications of the standard Analytica-EBC (1987).

5.2.6.1. Friability and unmodified grains

Friability is a standard measurement of the degree of physical transformation of barley into malt. The malt (50 g) was placed into the friabilimeter drum from Pfeuffer GmbH (Kitzingen, Germany) and processed for 8 min. Then, the content was weighed, and the friability was expressed as the percentage of malt which was milled and thus did not remain in the drum. The content of the drum was sieved through a mesh with rectangular slots (2.2×23 mm). Partially unmodified grains (PUG) was calculated as the percentage of malt bigger than 2.2 mm, so retained on the sieve. The whole unmodified grains (WUG) were picked, weighed and the percentage of these was calculated.

5.2.6.2. Moisture

The malt was ground using a DLFU disc mill from Bühler-Miag (Beilngries, Germany) with a 0.2-mm disc. The ground sample (20 g) was dried at 105-107 °C for 3.5 h and the moisture was expressed as the weight percentage lost after drying.

5.2.6.3. Preparation of Congress wort

The ground malt (50.0 g) was placed into a mash beaker and 200 mL of distilled water at 46 °C were added. A LB Electronic mashing device from Lochner Labor und Technik GmbH (Berching, Germany) was utilised. The mash beakers were then put into the water bath and kept at 45 °C for 30 min, then raised at 1 °C/min to 70 °C. At this point, a further 100 mL of distilled water at 70 °C was added and the mash maintained for 1 hour, then cooled down to room temperature in 10-15 min. The mash was stirred during the entire mashing process. Finally, the wort was filtered through a paper filter, returning

the first 100 mL of filtrate to the funnel. Worts prepared following this method were used for measurements of extract yield, viscosity, density, colour, free amino nitrogen, and soluble protein.

5.2.6.4. Viscosity, density and pH

A DMA 4500 density meter coupled to an AMVn automated micro viscometer and an SPV sample changer, all from Anton Paar GmbH (Graz, Austria), was used for density and viscosity measurements in the Congress wort prepared in Section 5.2.6.3. pH was measured using a lab pH-meter.

5.2.6.5. Extract yield

The extract content of the wort (E_2) was calculated according to the following formulae:

$$E_1 = \frac{P(M+800)}{100-P} \quad \text{(Eq. 5.3)}$$

$$E_2 = \frac{E_1 \cdot 100}{100-M} \quad \text{(Eq. 5.4)}$$

Where E_1 (% w/w) is the extract content in the sample, P (g/100 g) is the density of the wort, M (% w/w) is the moisture content of the malt, 800 is the amount of distilled water added to the mash to 100 g of malt, and E_2 (% w/w) is the extract content on dry malt.

5.2.6.6. Soluble and total nitrogen

A San++ continuous flow analyser from Skalar Analytical B.V. (Breda, The Netherlands) was used for the quantification of soluble nitrogen. The determination of total nitrogen in malt was carried out according to the Kjeldahl method.

5.2.6.7. Colour

The colour of the wort was compared visually with a standardised colour scale (EBC-colour scale) divided in 0.5 units, using a colour comparator. Typical values for pale beers are between 3.5 and 4.5 EBC units.

5.2.7. Statistical analysis

Analysis of variance (ANOVA) and Duncan test (α=0.95) was applied to several parameters of the finished malts, i.e. those collected at the end of kilning, by employing the statistical software InfoStat 2017, developed by the National University of Córdoba (Córdoba, Argentina). Two-way ANOVA (multivariate linear analysis) was carried out with SPSS® Statistics Version 22 from IBM® (Armonk, NY, USA).

5.3. Results

5.3.1. General observations

In the present study, the formation of five Strecker aldehydes (3-methylbutanal, 2-methylbutanal, 2-methylpropanal, phenylacetaldehyde and methional) was monitored in barley malt during the curing stage of kilning. The amino acids from which these aldehydes derive (leucine, isoleucine, valine, phenylalanine and methionine) were quantified too, together with two reducing sugars (glucose and fructose) that also participate in the Maillard reaction. Additionally, stable intermediates ARP (FruAla, FruGly, FruIle, FruLeu, FruPhe, FruPro, FruVal) (glucose-amino acids conjugates) were determined analytically and used for the kinetic model. Other amino acids not directly involved in the formation of the target aldehydes were quantified too. These amino acids were divided into two groups: AAi (Ala, Gly and Pro) and AAj (Glu, Arg, His, Lys, Ser, Thr, and Tyr), depending on whether their ARP were also quantified (AAi) or not (AAj). Hence,

analytical data of the ARP from AAi (i.e. FruAAi) were available and included in the kinetic model. However, fructosyl methionine (FruMet) and the fructosyl derivatives of AAj were not quantified. Moisture of kilned malts was measured too (Figure 5.3). Malts kilned at 65 °C reached final moistures of around 3.5% w, whereas at 78 °C the final values were of around 1.5% w and below 1.0% w at 90 °C. Figure 5.4 shows the concentration of ARP

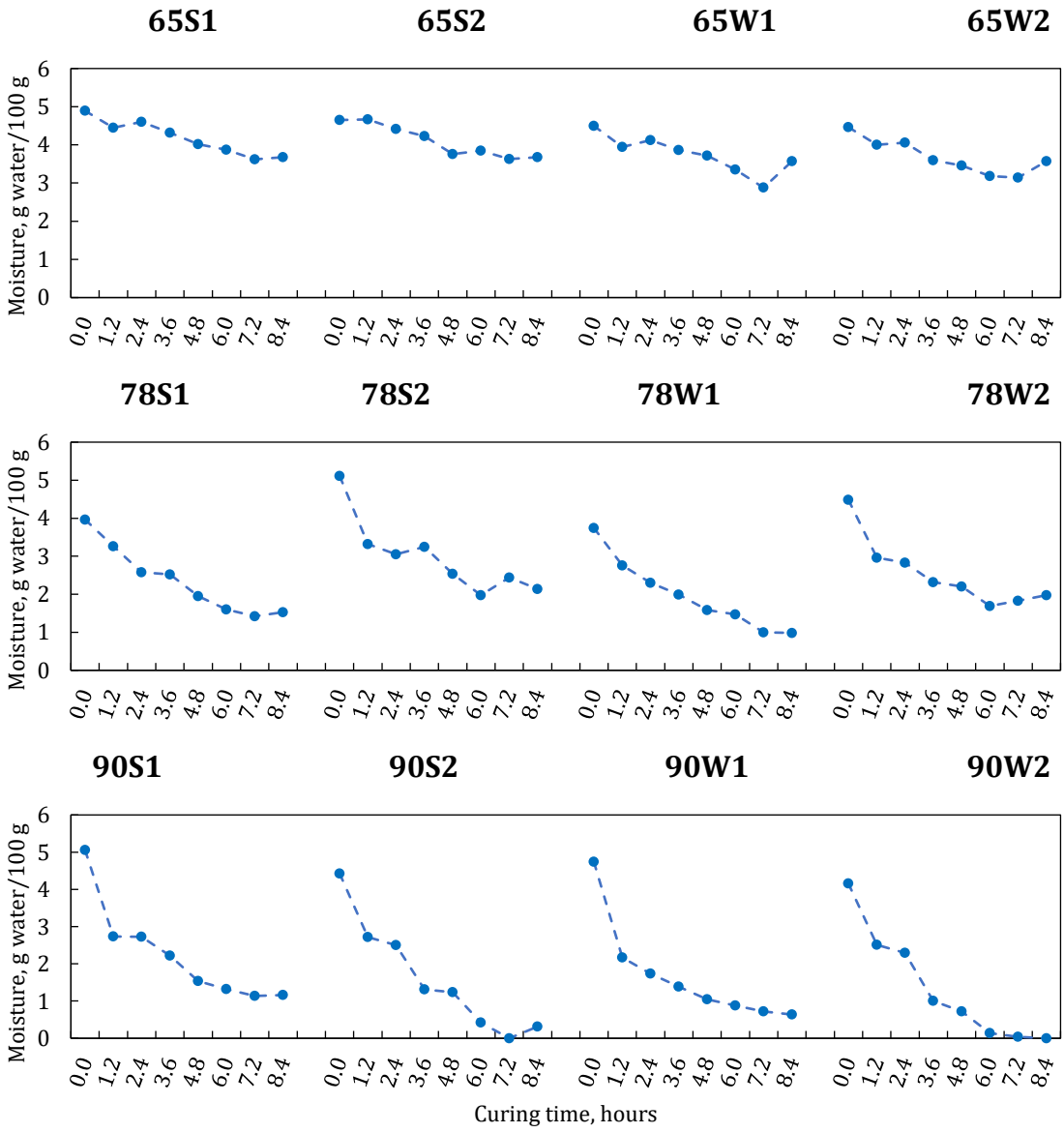

Figure 5.3 Changes in moisture content of malts kilned at different temperatures. S: spring barley; W: winter.

and Strecker aldehydes for barley malt (spring variety) cured at three different temperatures. The full graphs for sugars, amino acids, ARP and Strecker aldehydes for spring and winter barleys in duplicate are shown in Appendix 5. The temperatures used in this research were chosen in order to cover the normal range of curing temperatures for pale malts (Yahya et al., 2014). Sampling was done during the curing stage, i.e. the step where the increase in temperature leads to the formation of flavour and colour in malt. Thus, the previous drying stage was kept constant for all the experiments (Figure 5.2). This hypothesis was confirmed by the results from Strecker aldehydes that showed very low concentrations after the drying stage (Figure 5.4 and Appendix 5), which proves that the formation of these flavour compounds occurred in the later curing stage. The results showed that the formation of Strecker aldehydes was dependent on the curing temperature. For instance, at 65 °C the levels of 3-methylbutanal were very low after 8.4 hours curing, around 0.0071 mmol/kg in average, whereas much higher concentrations were found at 78 and 90 °C, of approximately 0.14 and 0.47 mmol/kg in average, respectively. In average, spring barley malt contained higher levels of Strecker aldehydes in malts kilned at 90 °C at the end of the curing stage, but the opposite was observed at 78 °C, with higher averaged amounts for winter barley malts. At 65 °C the difference was minimal. However, ANOVA showed that these differences between seasons (varieties) were not significant ($p>0.05$) for any of the aldehydes (0).

The data from ARP followed a trend typical of a reactive intermediate, increasing at early times and then decreasing. The concentration of aldehydes was always increasing during the processing time on this study. It is very likely that the concentration of aldehydes would have decreased for longer processing times due to degradation into other compounds or loss by evaporation, but this hypothesis could not be proven from our results. Sugars and amino acids, however, showed a less clear trend due to a greater

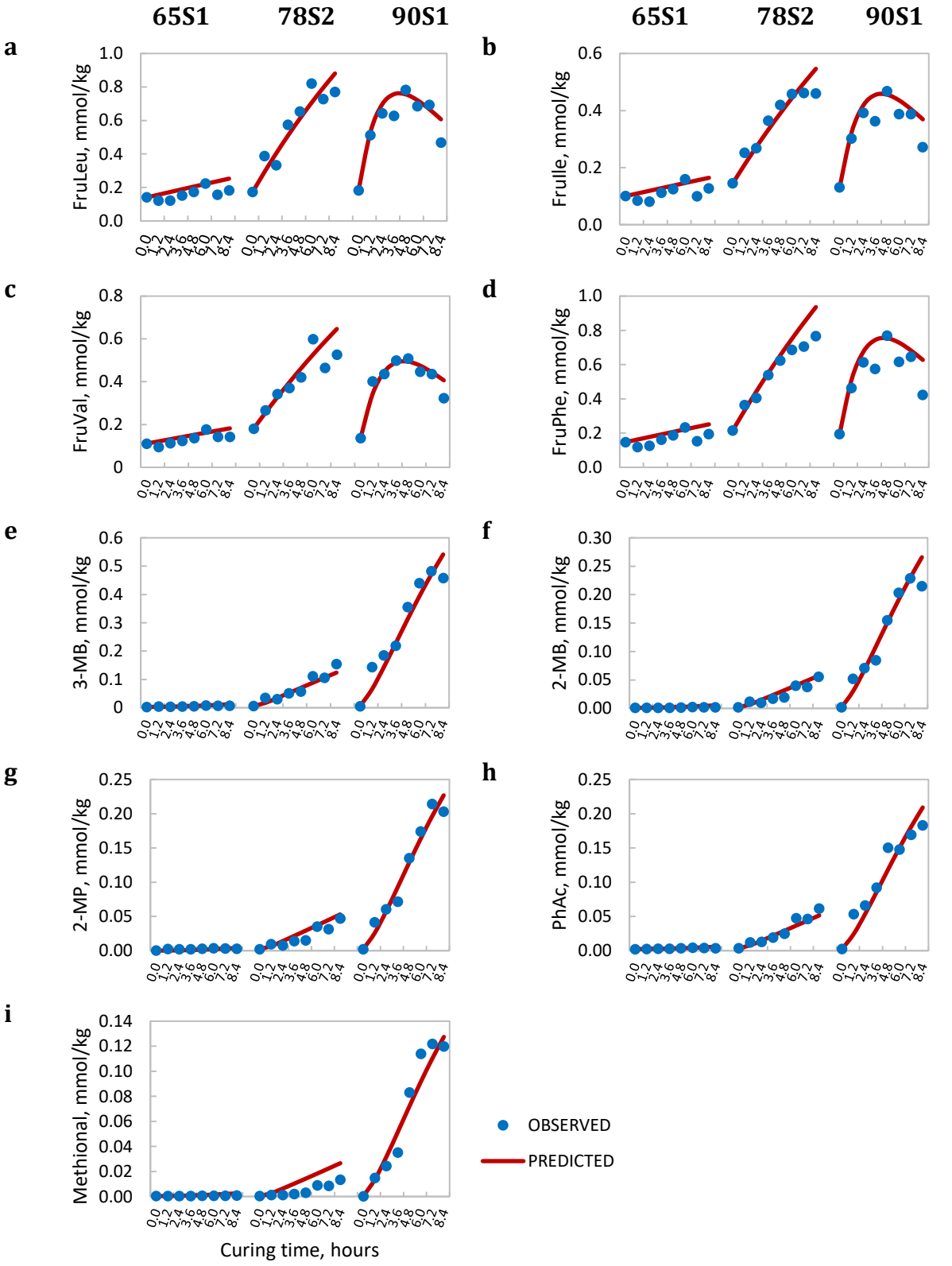

Figure 5.4 Concentration of ARP and Strecker aldehydes during the curing stage of kilning at different temperatures (65, 78 or 90 °C). The whole set of data can be found in Supplementary Figure S 1 (Appendix 5).

scatter of the data (Appendix 5). In the case of glucose and fructose, the high variability of the results was associated with the fact that the raw materials (i.e. the green malts) used for this study were collected from industrial-scale germination boxes. The heterogeneity of the sample and other less well controlled factors, such as the degree of transformation of the starch into fermentable sugars in the germinating grain, were indicated as possible reasons behind the variability of sugars and amino acids. Besides, the green malts were kilned in a micro-malting equipment fitted with eight compartments. The samples were taken randomly from these compartment or boxes, and the different positions in the oven probably contributed to the variability of the results due to a non-homogeneous temperature of the air flowrate through the boxes. Furthermore, real food matrices often present the disadvantage of having much higher variability in their measured responses than model systems due to unaccountable chemical and/or physical interactions. Despite the variation of sugars and amino acids, the results for ARP and Strecker aldehydes showed much clearer trends and the experimental replicates were similar.

5.3.2. Development of the kinetic model

The chemistry of the Maillard reaction is very complex, with reactions in series and in parallel, multiple precursors and intermediates of different stability, and a wide variety of products. Furthermore, if the aim is to understand the kinetics of these reactions, the task is even harder. For this purpose, multi-response kinetic modelling has been employed in order to solve the system of differential equations derived from a kinetic mechanism comprising several kinetic parameters. The kinetic mechanism is structured in such a way so that it would represent the rate limiting steps in the chemical reactions

under investigation. Multi-response modelling uses all the model's responses simultaneously, thus providing a more reliable estimation of the unknown parameters.

The development of the kinetic model was based on previous studies from our research group. Balagiannis et al. (2009) proposed a kinetic model which involved the formation of 3-methylbutanal and 2-methylbutanal in a meat model system heated at 120 °C, 130 °C and 140 °C. Additionally, Parker et al. (2012) and (Balagiannis et al., 2019) developed and proposed some comprehensive kinetic models on the formation of acrylamide in French fries. Although acrylamide is not a volatile aroma compound, its formation followed a pathway similar to that of Strecker aldehydes, with the presence of highly reactive dicarbonyl intermediates, and the formation of ARP, among others. Based on the aforementioned studies, a simplified kinetic model was chosen as a starting point for the present study. The process of developing a kinetic model usually involves checking many variations of a basic mechanism, evaluating the integrity of the estimated parameters and tracking the fit of the model to the analytical data and other quality factors, like the normality on the distribution of the residuals. Although it is based on a trial and error strategy, the choice of reactions assessed is logically developed based on the underlying chemistry of the Maillard reaction. In our case, a basic reaction route backbone was chosen, consisting of the reaction of glucose and an amino acid, resulting in the formation of an ARP, then an intermediate (Int1) with the release of that amino acid, and the further reaction of another amino acid with Int1 to produce a Strecker aldehyde. Amongst the variations applied to this route, the most significant ones were the addition of a reaction for the formation of Int1 from the degradation of fructose, and the formation of Maillard reaction products (MRP) from Int1 and amino acids. The degradation of fructose into a reactive intermediate, like 1-deoxyglucosone, has been reported in a kinetic mechanism for the formation of α-dicarbonyl compounds in a

glucose/wheat model systems heated at 160-200 °C (Kocadağlı & Gökmen, 2016). In addition, Mundt & Wedzicha (2003) studied the kinetics of the browning in a glucose (1.0 M)/fructose (1.0 M)/glycine (0.5 M) mixture (pH 5.5, 0.2 M acetate buffer) heated at 55 °C. They reported that fructose was converted to a key reactive intermediate via a single kinetic step, while glucose via a two-step process. The addition of the one-step degradation of fructose improved the fit of the model in terms of lower sum of squared residuals. This value was used as a guide to judge the goodness of fit of the models, usually in a qualitative manner in order to verify whether the model improved or worsened after a modification.

Other modifications to the model did not produce any reductions in the sum of squared residuals, and thus discarded. One of these was the addition of a route for the direct formation of Strecker aldehydes and sugar degradation product (SDP) from the ARP. This route has been proposed by several researchers as one of several possible mechanisms for the formation of Strecker aldehydes from ARP (Cremer, Vollenbroeker, & Eichner, 2000; Yaylayan, 2003). However, the presence of this alternative pathway for the formation of Strecker aldehydes altered the main route via Int1, the parameters associated with the main route dropping down to zero for most of the aldehydes (data not shown). This meant that the formation of aldehydes was happening only via the direct route from ARP, and not through the main route via Int1. The direct route, without the main route, was tested in a kinetic model, but the sum of the squared residuals was higher than through the main route alone. Besides, according to the Akaike criterion, this model (AIC=672) was discarded in favour of the one via Int1 (AIC=639). AIC is widely employed for the discrimination between kinetic models and it takes into account both the accuracy of the models (sum of squared residuals) as well as its complexity (number of parameters). The main route via an intermediate (Int1 in our case) is more widely

recognised and generally accepted as the principal for the formation of Strecker aldehydes (Parker, 2015). Some reactions that were dismissed too were: the degradation of ARP, Int1 or the aldehydes into unknown or non-quantified species, the presence of another intermediate before Int1, or the formation of MRP from amino acids only. These routes did either not produce any relevant improvement of the model's quality or the parameters related to them were null. The use of individual kinetic rate constants for each reaction has been checked too, but the results showed that the estimated parameters gained in quality (narrower error bars) when they were combined and used in several reactions.

5.3.3. Postulated kinetic model

After several iterations on various models, we concluded to the kinetic mechanism in for the formation of Strecker aldehydes the Maillard reaction during the curing stage of malt kilning. According to our kinetic mechanism, glucose reacted with amino acids to form the corresponding fructosyl amino acid derivative (ARP). Then, the ARPs degraded into a common Int1 with concurrent regeneration of the amino acids. The amino acids that did not participate directly in the formation of Strecker aldehydes (AAi and AAj), did take part during the first stages of the Maillard reaction by reacting with glucose and forming the reactive intermediates Int1. Also, these amino acids reacted with Int1 for the formation of other Maillard reaction products (MRP).

This intermediate compound Int1 did not reach concentrations as high as other species in this system, with a maximal predicted level of around 0.17 mmol/kg (Supplementary Figure S 2 in Appendix 5). Its trend showed an equilibrium between

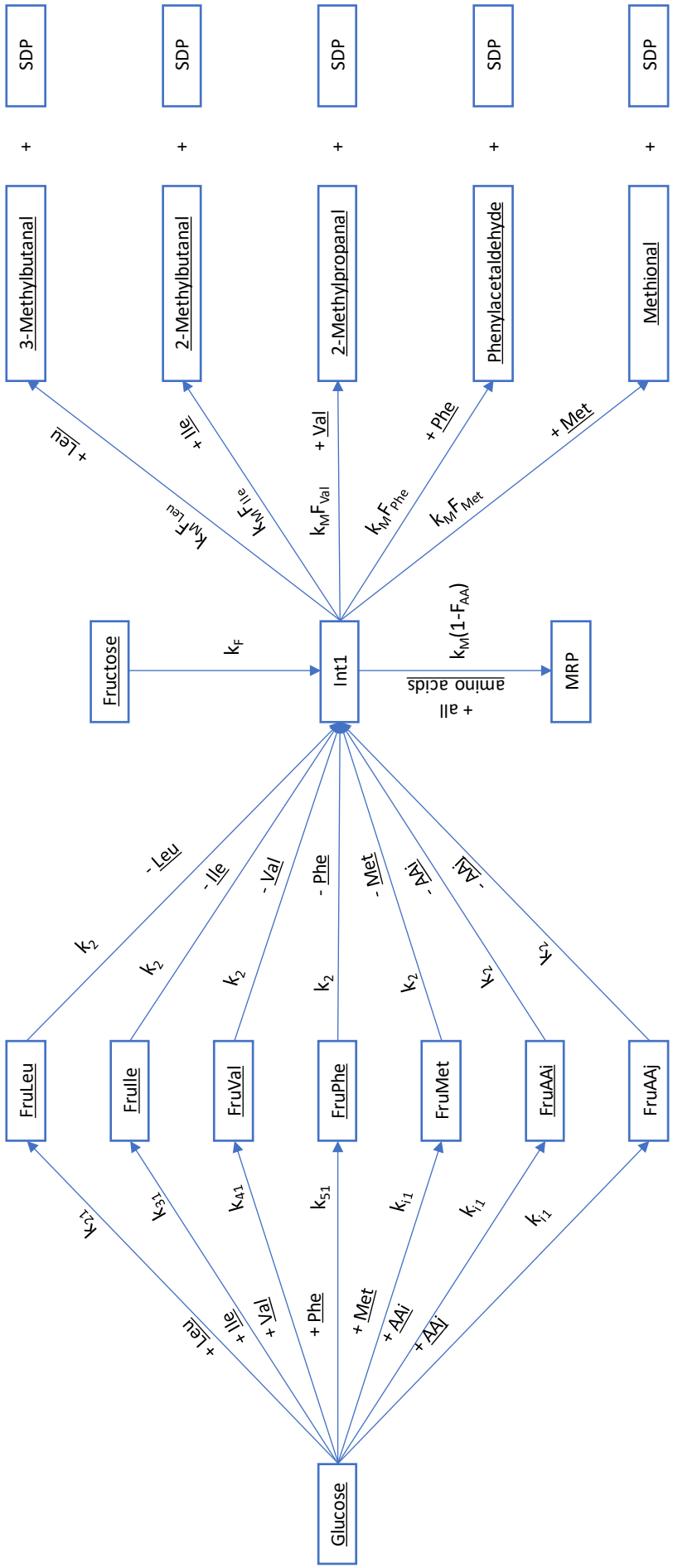

Figure 5.5 Postulated kinetic mechanism for the formation of five Strecker aldehydes from glucose, fructose and amino acids. The species underlined were determined analytically. k represents the kinetic rate constant involved in each reaction

formation and degradation, the degradation having more weight at higher temperatures, suggesting that these compounds were relatively stable at lower temperatures. The degradation of fructose was found to be a relevant source of Int1. This reactive intermediate was not quantified in this study, but we assumed that it corresponded to a pool of SCDC, such as glyoxal and methylglyoxal. Lastly, Int1 further reacted with free amino acids leucine, isoleucine, valine, phenylalanine and methionine in order to form the Strecker aldehydes 3- and 2-methylbutanal, 2-methylpropanal, phenylacetaldehyde and methional, respectively. Additionally, sugar degradation products (SDP) were formed from the backbone of Int1. In parallel, Int1 reacted with all the amino acids and formed a group of unidentified Maillard compounds (MRP). This kinetic mechanism is translated into the following system of differential equations:

$$\frac{d[Gluc]}{dt} = -[Gluc] \cdot \left(k_{21}[Leu] + k_{31}[Ile] + k_{41}[Val] + k_{51}[Phe] + k_{i1}([Met] + [AAi] + [AAj])\right)$$

$$\frac{d[Fruc]}{dt} = -k_F[Fruc]$$

$$\frac{d[Leu]}{dt} = -k_{21}[Gluc][Leu] + k_{22}[FruLeu] - k_M[Int1][Leu]$$

$$\frac{d[Ile]}{dt} = -k_{31}[Gluc][Ile] + k_{32}[FruIle] - k_M[Int1][Ile]$$

$$\frac{d[Val]}{dt} = -k_{41}[Gluc][Val] + k_{42}[FruVal] - k_M[Int1][Val]$$

$$\frac{d[Phe]}{dt} = -k_{51}[Gluc][Phe] + k_{52}[FruPhe] - k_M[Int1][Phe]$$

$$\frac{d[Met]}{dt} = -k_{i1}[Gluc][Met] + k_{i2}[FruMet] - k_M[Int1][Met]$$

$$\frac{d[AAi]}{dt} = -k_{i1}[Gluc][AAi] + k_{i2}[FruAAi] - k_M[Int1][AAi]$$

$$\frac{d[AAj]}{dt} = -k_{i1}[Gluc][AAj] + k_{i2}[FruAAj] - k_M[Int1][AAj]$$

$$\frac{d[FruLeu]}{dt} = k_{21}[Gluc][Leu] - k_{22}[FruLeu]$$

$$\frac{d[FruIle]}{dt} = k_{31}[Gluc][Ile] - k_{32}[FruIle]$$

$$\frac{d[FruVal]}{dt} = k_{41}[Gluc][Val] - k_{42}[FruVal]$$

$$\frac{d[FruPhe]}{dt} = k_{51}[Gluc][Phe] - k_{52}[FruPhe]$$

$$\frac{d[FruMet]}{dt} = k_{i1}[Gluc][Met] - k_{i2}[FruMet]$$

$$\frac{d[FruAAi]}{dt} = k_{i1}[Gluc][AAi] - k_{i2}[FruAAi]$$

$$\frac{d[FruAAj]}{dt} = k_{i1}[Gluc][AAj] - k_{i2}[FruAAj]$$

$$\frac{d[Int1]}{dt} = k_F[Fruc] + k_{22}[FruLeu] + k_{32}[FruIle] + k_{42}[FruVal] + k_{52}[FruPhe] + k_{i2} \cdot ([FruMet] + [FruAAi] + [FruAAj]) - k_M[Int1] \cdot ([Leu] + [Ile] + [Val] + [Phe] + [Met] + [AAi] + [AAj])$$

$$\frac{d[3MB]}{dt} = k_M F_{Leu}[Int1][Leu]$$

$$\frac{d[2MB]}{dt} = k_M F_{Ile}[Int1][Ile]$$

$$\frac{d[2MP]}{dt} = k_M F_{Val}[Int1][Val]$$

$$\frac{d[PhAc]}{dt} = k_M F_{Phe}[Int1][Phe]$$

$$\frac{d[Meth]}{dt} = k_M F_{Met}[Int1][Met]$$

$$\frac{d[MRP]}{dt} = k_M[Int1] \cdot \{(1 - F_{Leu})[Leu] + (1 - F_{Ile})[Ile] + (1 - F_{Val})[Val] + (1 - F_{Phe})[Phe] + (1 - F_{Met})[Met] + [AAi] + [AAj]\}$$

$$\frac{d[SDP]}{dt} = k_M[Int1] \cdot (F_{Leu}[Leu] + F_{Ile}[Ile] + F_{Val}[Val] + F_{Phe}[Phe] + F_{Met}[Met])$$

Some of the reactions in this kinetic model shared the same kinetic rate constant. Reducing the number of parameters was found to improve the quality of the parameter estimates by shrinking the confidence interval without worsening the overall fit of the model. Individual kinetic rate constants were used for the reaction between glucose and Leu, Ile, Val and Phe, whereas a common k_{i1} was used for Met, AAj and AAi. For all ARP, the kinetic rate constants for the degradation reaction were the same (k_2), all depending on the same parameters k'_2 and Ea_2. Besides, k_M was used for all reactions between Int1 and the amino acids. In the case of the amino acids forming Strecker aldehydes, k_M was multiplied by a factor F_{AA} which represented the proportion of amino acid that was used to form the aldehyde; the rest ($1-F_{AA}$) followed the route for the formation of MRP. In the differential equations for the amino acids and Int1, this factor was not used explicitly because the consumption of these compounds did not distinguish between the products (either Strecker aldehydes or MRP).

The parameters k' and Ea (Eq. 5.1) were optimised by Bayesian estimation for multiple responses in order to minimise the sum of squared residuals of the predicted versus observed values. The Bayesian approach uses the information from the data and an iteration for the calculation of the next iteration in order to provide more accurate and realistic estimates (Balagiannis, 2015). The estimation was performed in several runs,

Table 5.1 Parameter estimates for the postulated kinetic model shown in .

Parameter	Rate constant function in which the parameter was used	Optimal estimate ± 95% HPD* (% error)
k'_{21} (kg mmol^{-1} h^{-1})	k_{21}	$1.05 \cdot 10^{-4} \pm 7.09 \cdot 10^{-6}$ (6.8%)
k'_{31} (kg mmol^{-1} h^{-1})	k_{31}	$6.05 \cdot 10^{-5} \pm 4.29 \cdot 10^{-6}$ (7.1%)
k'_{41} (kg mmol^{-1} h^{-1})	k_{41}	$2.57 \cdot 10^{-5} \pm 1.47 \cdot 10^{-6}$ (5.7%)
k'_{51} (kg mmol^{-1} h^{-1})	k_{51}	$5.08 \cdot 10^{-5} \pm 3.57 \cdot 10^{-6}$ (7.0%)
k'_{i1} (kg mmol^{-1} h^{-1})	k_{i1}	$1.16 \cdot 10^{-5} \pm 6.77 \cdot 10^{-7}$ (6.0%)
Ea_1 (kJ/mol)	k_{21}, k_{31}, k_{41}, k_{51}, k_{i1}	175 (fixed)
k'_2 (h^{-1})	k_2	$7.30 \cdot 10^{-4} \pm 3.34 \cdot 10^{-5}$ (4.6%)
Ea_2 (kJ/mol)	k_2	315 (fixed)
F_{Leu}		1.000 (upper bound)
F_{Ile}		0.518 ± 0.0307 (5.9%)
F_{Val}		0.172 ± 0.0105 (6.1%)
F_{Phe}		0.207 ± 0.0109 (5.3%)
F_{Met}		0.269 ± 0.0244 (9.1%)
k'_M (kg mmol^{-1} h^{-1})	k_M	$5.19 \cdot 10^{-4} \pm 4.77 \cdot 10^{-4}$ (92%)
Ea_M (kJ/mol)	k_M	255 (fixed)
k'_F (h^{-1})	k_F	$1.82 \cdot 10^{-2} \pm 3.27 \cdot 10^{-3}$ (18%)
Ea_F (kJ/mol)	k_F	121 (fixed)

* Highest posterior density

alternatively keeping one group of parameters, i.e. k' or Ea, fixed and estimating the rest. The optimisation was achieved with the determinant criterion (Box & Draper, 1965) which is ideal for multi-response calculations since it avoids some statistical restrains/implications that need to be addressed in the usual least square minimisation (i.e. normality of residuals and error independence for each response) (Balagiannis, 2015). The residual plots (not shown) were checked and they were randomly scattered, which is an extra indication of the good quality of the model. Table 5.1 shows the optimal values for the estimated parameters. The quality of most of the parameters estimated was very good when looking at their confidence intervals (highest posterior density, HPD, for Bayesian statistics), these being equal or below 10% of the actual estimated value for 10 out of 13 parameters. Two parameters, k'_F and k'_M, showed greater errors of 18 and 92%, respectively, and F_{Leu} reached the upper limit for the F factors, i.e. 1.0. This meant that

100% of leucine that reacted with Int1 generated 3-methylbutanal, and not MRP. For the formation of 2-methylbutanal, F_{Ile} was 51.8%, whereas lower values (around 20%) were estimated for 2-methylpropanal, phenylacetaldehyde and methional. The high uncertainty of k'_M was associated to the fact that it was involved in the degradation of Int1 and its concentration was not determined analytically.

The activation energies, Ea, were kept fixed at the last calculation run because some of them were giving undetermined variability status, and in consequence no confidence interval is reported. Activation energies were found to be in the range between 121 and 315 kJ/mol (Table 5.1), considerably higher than similar kinetic studies. In our study, the activation energy related to the formation of Strecker aldehydes, Ea_M, was 255 kJ/mol, but Ea_2, for the cleavage of ARP, was even higher (315 kJ/mol). Huang et al. (2016) studied the formation of 2- and 3-methybutanal using maltose or glucose as reducing sugars in a wort-like model system. They reported activation energies in the range of 83-121·kJ/mol, depending on the amino acid and sugar. Balagiannis et al. (2009) reported an activation energy of 48.7 kJ/mol for the formation of these two aldehydes in a heated extract of beef liver. Higher values were found for the formation of acetaldehyde, 2-methylpropanal, 2-methylbutanal and 3-methylbutanal in a low moisture model system, between 115 and 124 kJ/mol (Cremer & Eichner, 2000). Other authors reported higher Ea (237 kJ/mol) for the degradation of glucose into formic and acetic acids (Martins & Van Boekel, 2005b). Ea values determine how sensitive the kinetic rate constants are to temperature changes. Figure 5.6 shows the effect of temperature on the different kinetic rate constants used in our model. In logarithmic scale, the slope of the curves depended on the activation energy. For higher Ea, the variation of the kinetic rate constant with temperature was larger, which in the case of Ea_2 suggested that ARP were relatively

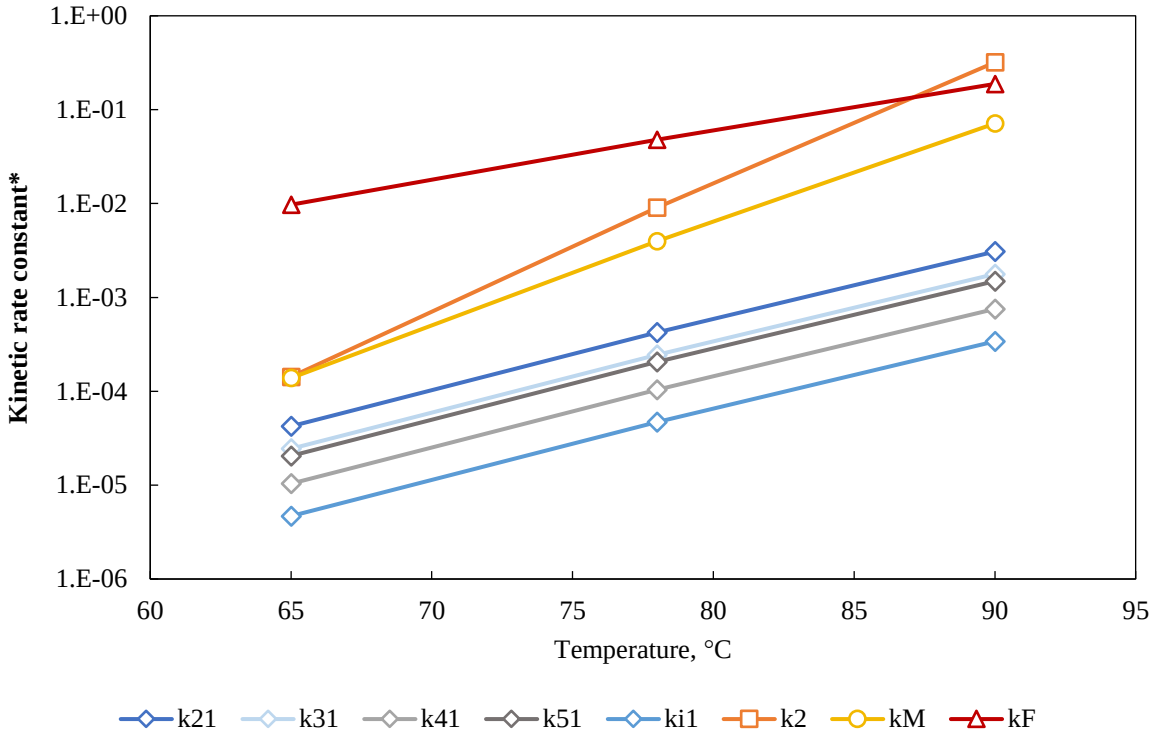

Figure 5.6 Effect of temperature on the kinetic rate constants. (*) k_{21}, k_{31}, k_{41}, k_{51}, k_{i1} and k_M in kg mmol^{-1} h^{-1}; k_2 and k_F in h^{-1}.

stable at lower temperatures, but the temperature rise accelerated their degradation. An increase of temperature from 65 to 78 °C resulted in a 10.8-fold increase of Strecker aldehydes, while increase from 78 to 90 °C resulted in an increase of 4.2 times. Hence, relatively small temperature increases had a great impact on a high increase in Strecker compounds concentration. The intermediate steps (k_2, k_M) showed higher Eas than the first steps (k_1, k_F). This meant that as processing temperature increased, the intermediate compounds degradation rate increased to a larger extend than the degradation rate of the precursors. For the same amount of degraded initial precursors, a higher amount of Streckers was formed in relation to lower temperatures. Consequently, by keeping the curing temperature low, the formation of Streckers was suppressed considerably, as it was evident from our results (Supplementary Figure S 1 in Appendix 5).

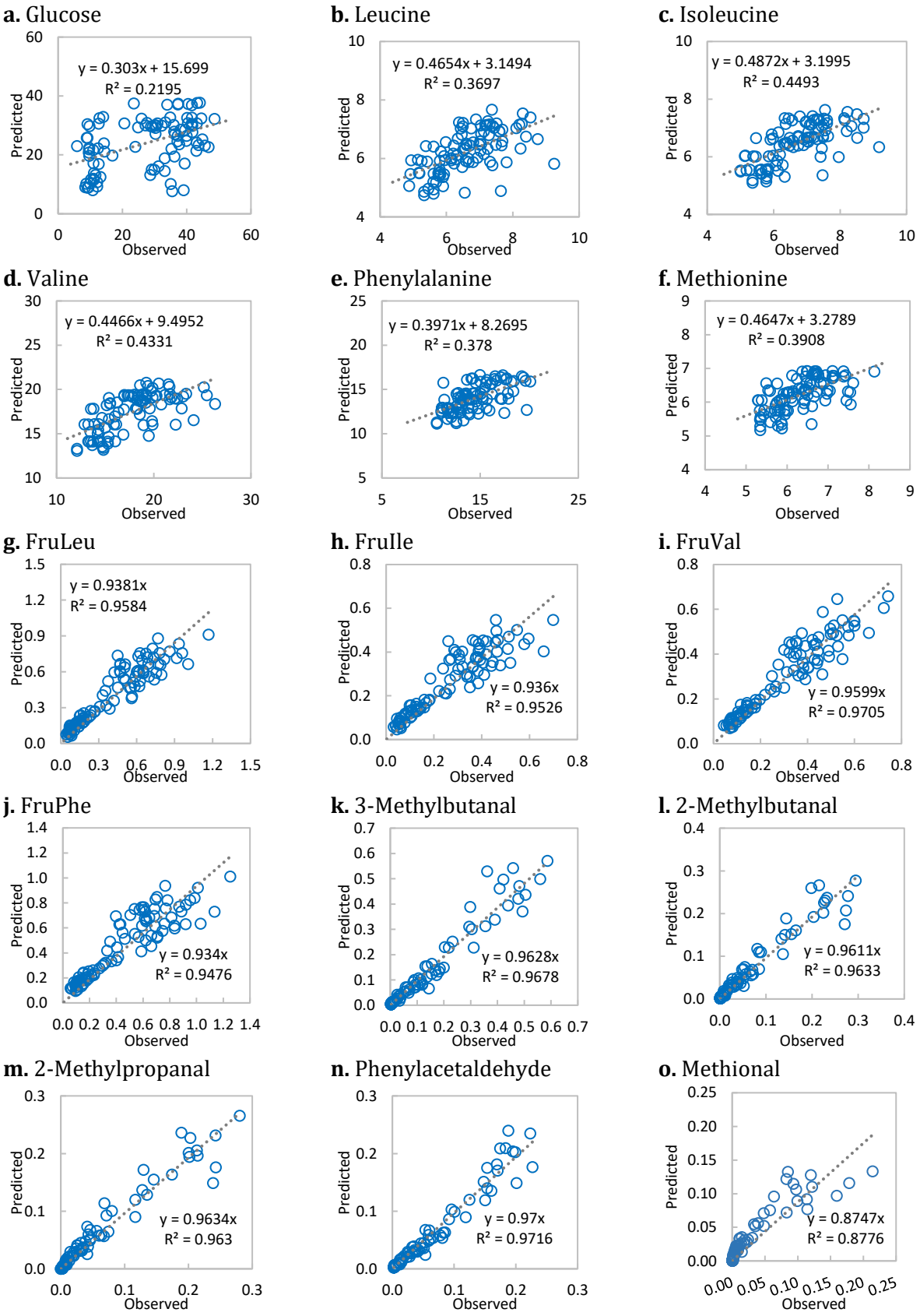

Figure 5.7 Predicted against observed values (all in mmol/kg) from the kinetic model.

Figure 5.7 shows the predicted vs. observed plots for the compounds quantified. Generally, the quality of the fit of the model was good for most of the compounds. Predicted vs. observed data showed R^2 close to or higher than 0.80 for ARP and around 0.95 for all Strecker aldehydes apart from methional (0.81). Besides, the slopes were close to 1 for these compounds, meaning that the predicted data were close to the observed. On the other hand, the linear regression for sugars and amino acids did not show good correlation between predicted and observed data. The parameters of the linear regression curve were far from ideal too, because the slopes were not close to 1 and the intercepts to 0. This was a consequence of the scatter of the observed data for the reasons explained in Section 5.3.1.

5.3.4. Discussion of the chemical mechanism

The building of the kinetic model was always based on the known chemistry of the Maillard reaction and the Strecker degradation. Figure 5.8 shows the postulated chemical mechanism for the formation of Strecker aldehydes from reducing sugars on which the kinetic model has been based. As described by Hodge (1953), reducing sugars react with amino acids to form ARP via the formation of a Schiff base. Then, the amino acid fragment of the ARP is regenerated, and the sugar backbone breaks down to form a pool of reactive intermediates, dicarbonyls included. Dicarbonyls have been identified in other systems as SCDC like glyoxal, methylglyoxal, 2,3-butanedione, and others (Kocadağlı & Gökmen, 2016). Previous studies have proven the presence of other dicarbonyls with the same number of carbon atoms as the original sugar prior to breaking down to shorter chain dicarbonyls. Martins & Van Boekel (2005a) identified and monitored the formation of 1- and 3-deoxyglucosone from the reaction between glucose and glycine at high

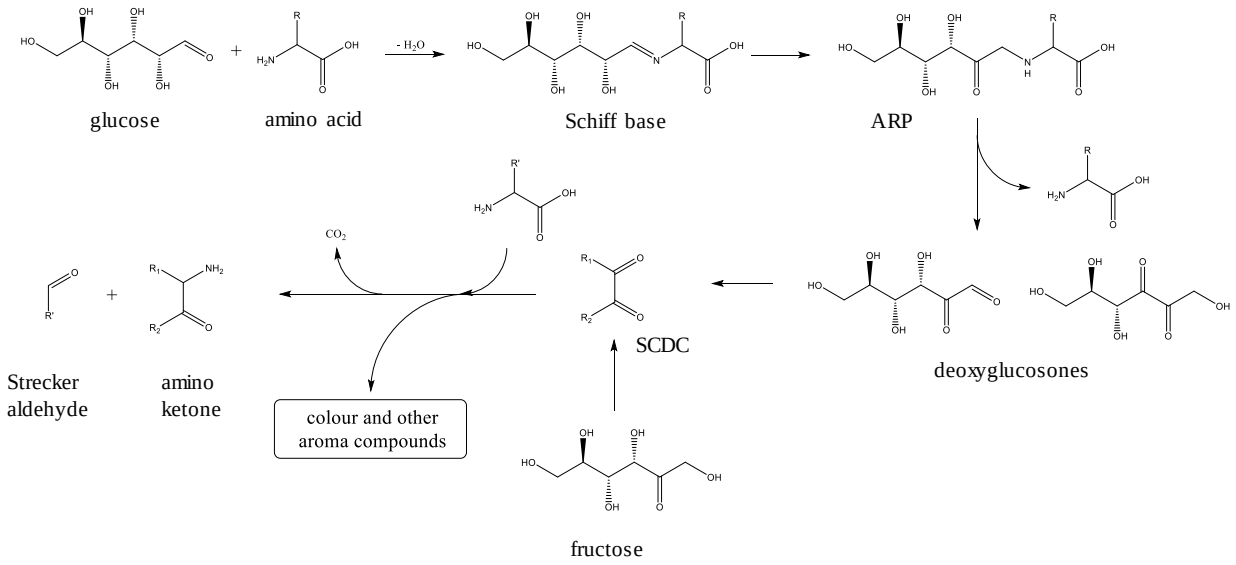

Figure 5.8 Postulated chemical mechanism for the formation of Strecker aldehydes from glucose and fructose and amino acids. ARP: Amadori rearrangement product, SCDC: short chain dicarbonyls.

temperature (80-120 °C). 3-Deoxyglucosone has been already identified in barley malt and formed during malt kilning (Nobis et al., 2019). Kocadağlı & Gökmen (2016) quantified several dicarbonyl compounds in model biscuits baked at 200 °C. Deoxyglucosones were formed at much higher concentrations (6.7 to 130.7 mg/kg) than SCDC, with amounts from 1.8 to 19.7 mg/kg for both glyoxal and methylglyoxal. These reactive compounds, SCDC, further react with amino acids in order to form another Schiff base. Then, the carboxyl group of the amino acid part is released as CO_2 generating the Strecker aldehyde, whereas the sugar part forms an amino ketone (Parker, 2015). In our kinetic model, these amino ketones can be identified as SDP, which also covered any further products from these compounds.

Kinetic models represent a simplification of the real chemical mechanism that only include rate limiting reactions. In most cases, the presence of unstable species is translated into fast reactions from the point of view of kinetics. These species are formed and are quickly degraded into other compounds; thus, although these reactions are

considered for chemical mechanisms, they are omitted in kinetic models. Huang et al. (2016) postulated a kinetic model for the formation of 2- and 3-methylbutanal from reducing sugars and isoleucine and leucine, respectively. The proposed model consisted of three reactions in series, with no parallel reactions. The formation of these aldehydes in a meat extract was explained by a kinetic model where amino acids react with a cluster of very reactive intermediates from the degradation of glucose (Balagiannis et al., 2009). Moreover, kinetic mechanisms with a similar structure have been used for modelling the formation of acrylamide in foods. Acrylamide is formed from the reaction of asparagine with reducing sugars or reactive intermediates, such as dicarbonyls (Balagiannis et al., 2019).

5.3.5. Characterisation of finished malts

Several routine parameters for the assessment of the quality of malts were determined in the finished malts. Table 5.2 shows the results from both spring and winter malts kilned at different temperatures for a total time of 16 h and 52 min. Extract yield is a measurement of the solids from the malt dissolved in the wort, and it is directly related to the sugar content and fermentability. Regarding the extract yield, this was not affected by temperature, but spring barley provided significantly higher ($p<0.0001$) extract yields than winter barley. Besides variety, extract yield has been reported to be affected by agronomical factors, mainly related to climate conditions (Molina-Cano, Rubio, Igartua, Gracia, & Montoya, 2000). The protein content of the malts was in the range from 10.0 to 11.4% dm, with no significant differences between the varieties ($p=0.128$). Winter barley showed higher soluble protein values than spring barley, with no significant effect

Table 5.2 Quality parameters of finished malts.

Malt	Extract yield,% dm	Protein,% dm	Soluble protein,% dm	FAN, mg/L	Friability,%	PUG,%	WUG,%	Viscosity, mPa·s	Colour EBC	pH
65S	84.0 ± 0.3^{a}	10.4 ± 0.2^{ab}	3.7 ± 0.2^{a}	107 ± 5.7^{a}	86.0 ± 0.8^{a}	2.3 ± 0.9^{a}	1.2 ± 0.92^{a}	1.61 ± 0.03^{a}	3.5 ± 0.00^{a}	6.18 ± 0.03^{c}
78S	83.9 ± 0.4^{a}	10.5 ± 0.2^{ab}	3.9 ± 0.4^{ab}	127 ± 17.7^{ab}	87.2 ± 1.7^{a}	2.0 ± 1.7^{a}	0.9 ± 0.71^{a}	1.61 ± 0.09^{a}	4.3 ± 0.35^{b}	6.17 ± 0.03^{c}
90S	84.2 ± 0.1^{a}	10.1 ± 0.0^{a}	3.9 ± 0.3^{ab}	135 ± 9.2^{ab}	91.8 ± 3.1^{b}	0.6 ± 0.3^{a}	0.2 ± 0.07^{a}	1.54 ± 0.05^{a}	4.3 ± 0.35^{b}	6.19 ± 0.01^{c}
65W	79.7 ± 0.8^{b}	10.0 ± 0.1^{a}	4.1 ± 0.7^{ab}	113 ± 17.7^{a}	85.7 ± 0.7^{a}	1.7 ± 0.4^{a}	0.4 ± 0.21^{a}	1.57 ± 0.04^{a}	3.5 ± 0.00^{a}	6.16 ± 0.06^{c}
78W	79.7 ± 0.0^{b}	11.4 ± 0.0^{c}	4.9 ± 0.2^{b}	152 ± 11.3^{b}	88.8 ± 0.8^{ab}	0.8 ± 0.0^{a}	0.3 ± 0.07^{a}	1.54 ± 0.01^{a}	5.3 ± 0.35^{c}	5.95 ± 0.03^{a}
90W	80.0 ± 0.3^{b}	10.8 ± 0.6^{bc}	4.4 ± 0.3^{ab}	138 ± 2.1^{ab}	89.1 ± 1.6^{ab}	0.6 ± 0.2^{a}	0.2 ± 0.07^{a}	1.52 ± 0.04^{a}	4.8 ± 0.35^{bc}	6.06 ± 0.06^{b}
Significance of season (p-value)	<0.0001	0.128	0.019	0.144	0.667	0.212	0.113	0.133	0.040	0.008

Results expressed as the average ± standard deviation of the malts kilned at different temperatures (65, 78 and 90 °C) and season (S for spring, W for winter) in duplicate. Superscript letters mean significant differences ($p<0.05$) within columns.

of kilning temperature according to the results of the Duncan's test. Molina-Cano et al. (2000) stated that extract yield and protein content were negatively correlated, what probably could be applied to soluble protein too. The trend of free amino nitrogen (FAN) was similar to that of soluble protein, with higher values at higher temperatures, although these differences were generally not significant. Another parameter for the quality of malts is friability. This measures the degree of transformation of the barley grain into malt by quantifying the percentage of grains that break under pressure. During germination, the starchy endosperm softens due to degradation of the cell walls, in contrast with hard, unmodified barley (Briggs et al., 2004). Hence, malted barley breaks under pressure in a crumbly, floury manner. Higher kilning temperature increased significantly the friability of the malt, with maxima of around 90%, independently of the variety. This might be due to the lower moisture of the malts kilned at higher temperature (Figure 5.3). The grains that did not break during the processing in the friabilimeter were classified into partially and whole unmodified grains (PUG and WUG). The average PUG value was 1.32%, whereas WUG was 0.49%, with no significant differences ($p>0.05$) for any of the samples. The viscosity of the worts was 1.56 mPa·s in average and no significant differences were found between the samples. Regarding colour, higher kilning temperature led to an increase in colour. The effect of thermal processing was also reflected in the formation of colour. The drying stage did not produce any apparent change in colour in comparison to the green malt, as well as curing at 65 °C. Both spring and winter malts kilned at 65 °C presented 3.5 EBC colour units, whereas malts cured at 78 and 90 °C presented a darker brown colour with 4.3 to 5.3 averaged units in the standard colour scale. The sample with the highest colour value, 78W, also showed the highest protein content and FAN and the lowest pH in the wort. Higher formation of colour and the decrease in pH are usual outcomes of the Maillard reaction (Hodge, 1953).

ARP were higher for the batch 78W2 than the rest of malts kilned at 78 °C, which meant a higher extent of the Maillard reaction. However, this was not translated into a higher concentration of Strecker aldehydes in the finished malts. The content of precursors was not higher in this batch either, what did not explain the values from the characterisation parameters.

In this study, two different barley cultivars were used, a two-row spring and a six-row winter variety. Traditionally, two groups of varieties of barley cultivars (and wheat) are distinguished: winter and spring varieties. Winter barleys are sown in late autumn and harvested in late spring or the beginning of summer, whereas spring varieties are sown in late winter or early spring and harvested in summer (Igartua Arregui, Ciudad Bautista, Gracia Gimeno, & Casas Cendoya, 2015). Winter varieties are usually sown in countries with milder climates, like France and Germany; spring varieties are almost the only option for colder countries, like the Scandinavian countries, because of the harsh conditions in winter (Garstang et al., 2011). Spring varieties are usually considered to have higher protein contents than winter barleys. According to Brennan et al. (1997), the winter variety "Halcyon" contained considerably lower amount of protein (1.54%) than the spring varieties "Chariot" (1.92%), "Britannia" (2.23%) and "Hart" (2.01%). However, from our results, no significant differences in the protein content were observed between "RGT Planet" and "Etincel" cultivars (Table 5.2). Many agronomical factors affect the protein content of the grain, the most important ones being soil type, legume-non legume rotation, fertiliser application and yield potential (Garstang et al., 2011). The latter depends more on the cultivar and date of seeding, among other parameters. Therefore, extrapolating the conclusions from this study with regard to spring/winter differences was not recommended since we only employed two barley cultivars. Significant

differences were reported even between different spring cultivars in terms of protein content, sugar concentration in wort and other parameters (Gunkel, Voetz, & Rath, 2002).

5.4. Conclusions

The formation of Strecker aldehydes (2-methylpropanal, 2- and 3-methybutanal, phenylacetaldehyde and methional) during malt kilning at pilot scale has been studied in the present piece of research. The results demonstrated the clear dependence of the concentration of Strecker aldehydes in malt with temperature during the curing stage of kilning. The highest levels of these compounds were reached at 90 °C. Multi-response kinetic modelling has been used as a tool to construct a kinetic model in which the most important rate limiting reactions have been identified. This mathematical model was based on the known chemistry of the Maillard reaction and Strecker degradation. After having estimated the kinetic parameters of the models, the effect of temperature and time on the formation of Strecker aldehydes was elucidated. Also, Strecker aldehydes are key aroma compounds in alcohol-free beers brewed by cold contact fermentation, as well as precursors for the formation of fruity alcohols and esters in alcoholic beers. Thus, it is possible to control and manipulate the kilning process in order to predict the final amount of these important flavour compounds in malt, and in consequence the organoleptic quality of the malt and the beers brewed from them.

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Conflicts of interest

The authors declare no conflicts of interest.

Chapter 6.

Chapter 6. Concluding remarks and future perspective

6.1. General discussion

This PhD thesis provides a comprehensive study on the compounds responsible to the characteristic worty flavour of AFB brewed by cold contact fermentation. The main aims of this project were to identify the odour-active compounds in these beers, their role in the overall aroma and their formation during malt kilning.

After an extensive revision of the literature (**0.**), the information published showed a clear difference between AFB brewed by physical and biological methods in terms of flavour. Physical methods refer to those post-treatment methods for the removal of alcohol from an alcoholic beer: distillation and membrane processes. On the other hand, the biological methods are based on limiting the formation of ethanol during fermentation by using either especial microorganisms or modifications of the fermentation (mostly by reducing time and/or temperature). These have the advantage of not requiring any specific equipment, apart from that used for regular beer, this way reducing the initial investment for the brewer. Unfortunately, AFB brewed using biological methods usually exhibits a characteristic worty aroma that is considered as an off-flavour.

Following the sensomic approach, twenty-seven odour-active compounds were identified in AFB (**0**). The aroma compounds were identified by crossing the results from GC-Olfactometry with GC-MS chromatograms. Amongst them, the ones showing the highest OAV, i.e. the highest ratio concentration/threshold, were methional, 3-methylbutanal, (*E*)-β-damascenone, 5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone and phenylacetaldehyde. In addition to three Strecker aldehydes, the contribution of other

two compounds ((*E*)-β-damascenone and 5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone) to the worty flavour of AFB is reported for the first time.

The sensory evaluation of the original AFB provided a vocabulary for describing the worty aroma of these beers. After the sensory assessment of several recombinates each prepared with one of the most important compounds omitted, the role of these to the overall aroma was elucidated. (*E*)-β-Damascenone, 3-methylbutanal, phenylacetaldehyde and 5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone contributed to the hot honey character of the AFB, whereas methional was responsible to the yeast attribute. These results make an important contribution to knowledge of AFB, since this is the first time that a comprehensive, systematic study of the flavour compounds in these beers has been reported.

One of the criteria used to rank the compounds according to their aroma potency was the odour activity value. This is calculated as the ratio of the concentration of the compound and the perception threshold. The perception threshold is affected by the composition of the matrix in which the compound is assessed. In **0**, the orthonasal and retronasal detection thresholds of 26 and 20 of the compounds identified in the AFB, respectively, were determined. In the literature, there are available a large amount of threshold values in different matrices, mostly water or ethanol/water mixtures. Our study provides a set of thresholds determined in an AFB-like matrix composed by a mixture of sugars in carbonated water in order to mimic the composition of AFB. The results showed that thresholds for the different flavour compounds were spread in a very broad range of values, from below 0.01 μg/L for (*Z*)-4-heptenal to higher than 100 mg/L for acetic acid. Furthermore, knowing the threshold value of a compound in the right matrix is essential to understand its potency because it affects directly the OAV.

Thresholds from the literature showed were spread in a broad range of values, with the ones obtained in this study falling outside this range for some compounds. The most important aroma compounds in AFB showed extremely low detection thresholds, from 0.47 µg/L for methional to 5.42 µg/L for phenylacetaldehyde. This implied that these key character compounds could be perceivable even at very low concentrations. These results suggested that the control of their formation during the brewing process might be a good strategy to limit their concentration in the beer. Their elimination in a post-treatment could lead to the presence of perceivable traces due to the extremely low detection thresholds. In other words, it is important to remove even the smallest trace, since it may still be above threshold. For instance, methional is present at 181 times above threshold, and post-treatment would have to be 99.4% efficient to get it below threshold.

The calculation method had a great impact on the threshold value for both ortho- and retronasal perception. BET produced higher threshold values than logistic regression in all cases; the removal of false positives also increased the threshold values calculated by either of the methods. We recommended to use logistic regression with false positives removed for a more accurate calculation, which additionally provided confidence intervals of the estimation. In conclusion, we have proven that many factors affect threshold values, such as the matrix in which they were determined, the calculation methods and the presence of false positive responses. Thus, when using threshold values from secondary sources, it is crucial to know all the details of the study in order to evaluate whether they are suitable or not for the intended application.

In the last two chapters of this thesis, the formation of key aroma compounds was studied at different steps of the brewing process. (*E*)-β-Damascenone (bDam) is a very

potent aroma compound, which played a key role as a contributor to the worty character of AFB. The formation of bDam has been reported during heat treatment of foods. Since it was present in malts and unhopped wort, its formation was thought to be dependent on the processing temperature. The results from our preliminary study (**0**) showed that the amounts of (*E*)-β-damascenone in malt increased during the curing stage of kilning, whereas during mashing and wort boiling the trend was more variable. The increase of bDam during malting was significant ($p<0.05$) in malts kilned at 78 °C and 90 °C, but the trend at 65 °C was not clear. Regarding mashing and wort boiling, the levels of bDam were different for both varieties. In the winter cultivar used in this study, the concentration after mashing decreased significantly and increased to their highest values after wort boiling, whereas the spring variety used showed increasing trends at 65 and 78 °C but decreasing at 90 °C. Malts and worts from winter barley presented significantly higher amounts ($p<0.05$) of bDam than spring barley. The lack of knowledge with respect to the precursors of this compound in barley made difficult understanding these differences. This short study demonstrated the importance of processing conditions, mainly temperature, and variety on the formation of such a potent aroma compound.

In the last research chapter of this PhD thesis (**0**), the formation of the five most important Strecker aldehydes in AFB was monitored during malt kilning. Strecker aldehydes have been found as contributors to the aroma of malts, but also to the malty, worty character of AFB, as it has been proven in this thesis. According to the literature, they were responsible to the aroma of malts too, and it was confirmed that they formed during thermal processing of malts via the Maillard reaction and Strecker degradation. Based on the known chemistry of the Maillard reaction, the hypothesis of this study was that the formation of Strecker aldehydes in malt during kilning involved the reaction of a

reducing sugar and amino acids via the formation of intermediates, such as Amadori rearrangement product and reactive dicarbonyl. The results showed that these compounds were only formed during the curing stage of kilning, since their concentrations were relatively very low and constant during the drying stage. From the kinetic model developed in this study, we concluded that the formation of these aldehydes in malt occurred through three rate limiting reactions and two intermediates of different stability. The first intermediate corresponded to the Amadori rearrangement products (ARP) from the condensation of glucose and amino acids, whereas the second one was potentially a pool of short chain dicarbonyls, like glyoxal and methylglyoxal. This second intermediate was also formed directly from the thermal degradation of fructose. ARP were very sensitive to temperature changes due to the high activation energy for their degradation. This has a great impact of the formation of SCDC and the subsequent Strecker aldehydes. The concentration of the aroma compounds can be predicted from the concentration of precursors and the processing conditions by means of the mathematical modelling of such a complex network of reactions that is the Maillard reaction. Thus, the malt kilning process can be controlled and manipulated in order to produce malts with the desired organoleptic characteristics.

6.2. Contribution to knowledge

The main contributions to scientific knowledge are summarised in this section:

1. Twenty-seven odour-active volatile compounds have been identified in AFB following the sensomic approach. Five of them were key contributors to the worty character of these beers: methional (boiled potato-like aroma), followed by 3-methylbutanal (cocoa-like), (*E*)-β-damascenone (apple, jam-like), 5-ethyl-3-

hydroxy-4-methyl-2(*5H*)-furanone (curry, spicy-like), and phenylacetaldehyde (floral, honey-like).

2. A vocabulary for breaking down the concept of "worty aroma" was developed, composed by the following attributes: "curry, fenugreek", "honey (hot)", "floral", "malt, cereal", "hay (green tea)", "yeast", "potato", "prunes", "dark brown sugar", and "apple (stewed)".
3. Orthonasal and retronasal detection thresholds of the odour-active compounds identified were determined in an AFB-like matrix. These values were compared with the ones already available in the literature, this proving the importance of the matrix and calculation method in threshold studies.
4. Threshold calculation method (BET or logistic regression) had a great impact on the final threshold value, as well as the presence of false positives.
5. The amount of (*E*)-β-damascenone in malt increased for higher kilning temperature, whilst the trend during mashing and wort boiling seemed to be a balance between formation and evaporation/degradation.
6. During malt kilning, Strecker aldehydes were formed during the curing stage, along with colour. These compounds were formed in a three-step reaction with the formation of two intermediates: ARP (glucose-amino acid conjugates) and short chain dicarbonyls.
7. A mathematical model representing the kinetic mechanism of the formation of Strecker aldehydes was developed. Kinetic parameters of the re-parametrised Arrhenius equation (activation energies and k' parameters) were estimated by multi-response modelling.
8. The degradation of ARP was very sensitive to temperature changes due to its high activation energy that provoked a great increase of its kinetic rate constant.

6.3. Limitations of this research

Along the development of this PhD project, several interesting findings could not be fully addressed mainly because of the limited time frame and scope of the study. The limitations identified are explained as follows:

- The alcohol-free beer used in this study was chosen for its characteristic worty aroma amongst several prototypes brewed at Heineken's pilot brewery in Zoeterwoude. It would have been interesting to compare the beer chosen with others from the market in terms of their sensory characteristics and concentration of key aroma compounds.
- Although 3-methyl-2-butene-1-thiol was identified in the AFB by its odour quality and LRI values, its concentration could not be determined analytically because it was below the limit of detection of the method used. For this reason, further concentration steps should be applied or more sensitive analytical techniques. Moreover, its detection threshold was not determined because of unavailability of the standard compound at the time of the study. On the other hand, one of the odour-active compounds remained unidentified, but even without it, there were no significant differences between the recombinate and the AFB. These results suggested that its contribution to the overall aroma was irrelevant.
- For some of the odour-active compounds, only orthonasal detection thresholds were determined, and not retronasal too, mainly due to limited availability of the sensory panel to evaluate the samples.
- In the study of the formation of (*E*)-β-damascenone in malts and worts, the experiments were carried out in one replicate, although the analyses were done in duplicate. This was due to technical issues with the SPME-GC-MS, which shortened

considerably the time intended for the study. In consequence, the results were regarded as preliminary.

- The malt samples used for the formation kinetics of Strecker aldehydes were kilned at the range of temperature typical for pale malts. Thus, the extrapolation of the kinetic model to much higher temperatures is not recommended because other reactions not considered in this model might show up, such as the degradation of the aldehydes.
- Besides, this model was developed from data from only two barley cultivars (six-row winter "Etincel" and two-row spring "RGT Planet"), so conclusions regarding differences between spring/winter cultivars or two-row/six-row cultivars were not possible to provide.

6.4. Future work

Considering the limitations of the outcomes of this project, as well as some interesting findings which diverted from the main scope, the following possible future works were identified for the continuation of this line of research:

- Further studies on the recombination of flavour compounds could provide further information about the role of flavour compounds in AFB. It would be interesting to determine the lowest concentration at which the key aroma compounds in AFB contribute to the worty character or they become unperceivable. The presence of synergistic effects between flavour compounds has not been investigated either.
- Ethanol had a great effect on the release of volatile flavour compounds from the liquid into the air. Little research is available in the literature relating to this phenomenon and further research on the physico-chemistry and thermodynamics behind this

behaviour would be very relevant, not only for the perception of flavour compounds, but also for the implication in dealcoholisation processes. Since the concentration of ethanol alters the equilibrium liquid/air, this affects the dealcoholisation processes based on this principle, such as vacuum distillation.

- Due to time limitations, the formation of 5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone (abhexon) during malt kilning was not done, which should be covered in future work. The identification of its precursors in malt and the formation mechanism would be very relevant for the control of the concentration in malts.
- As noticed from the literature, precursors for (*E*)-β-damascenone are not identified in barley yet. The identification of its precursors would be helpful for understanding the effect of processing conditions on the formation of this compound. Also, the differences found between the cultivars used in this study could be explained based on the concentration of precursors.
- In the kinetic model, the intermediate Int1 was not identified or quantified, even though it was associated with short chain dicarbonyls, potentially glyoxal and methylglyoxal. Identifying and monitoring these compounds would improve the kinetic model. Moreover, other reducing sugars, like maltose, could be added to the model and its contribution to the formation of Strecker aldehydes.
- Although the fit of the kinetic model to the experimental data is of good quality, the validation is required for testing its suitability for predicting the formation of Strecker aldehydes under other conditions, such as temperatures out of the range already tested or different concentrations of precursors.
- Strecker aldehydes are formed during other operations in the brewing process involving heat treatment. These are mashing and wort boiling. Extending the current kinetic study to these processes would provide a comprehensive overview of the

formation of these compounds during the brewing process. Also, the relative contribution of each process to the total amount could be known and thus, better strategies for the mitigation of these off-flavours in AFB could be designed and optimised.

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Appendices

Appendix 1. Odour-active volatiles in SAFE extracts of AFB and wort.

Supplementary Table S 1. Odour regions found in AFB (basic/neutral fraction)

No.	Descriptor[a]	Compound	FD[b] (Rxi-5)	LRI[c] in Rxi-5		LRI[c] in Stabilwax	
				Exp.	Auth.	Exp.	Auth.
1	Fruity	Acetaldehyde	1	<500	500	<700	709
2	Alcohol, fruity	Unk.	2	<500	-	<700	-
3	Cream, butter	2,3-Butanedione	512	579	587	961	1001
4	Strecker	3-Methylbutanal	1	648	642	952	930
5	Strecker cocoa	2-Methylbutanal	1	654	651	952	924
6	Beer, malt, bread	3-Methyl-1-butanol	8	725	730	1225	1215
7	Fruity, soapy	Unk.	1	787	-	-	-
8	Cocoa, chocolate	Unk.	1	866	-	-	-
9	Boiled potatoes	Methional	64	925	922	1470	1468
10	Roasty, rice, pyrazine	Unk.	8	992	-	1354	-
11	Candy, fruity	Unk.	2	1014	-	-	-
12	Spicy, roasty	Unk.	8	1043	-	1666	-
13	Chocolate cake	Unk.	1	1047	-	-	-
14	Rose, honey	Phenylacetaldehyde	16	1059	1054	1649	1667
15	Green, soil, grass	Unk.	1	1090	-	-	-
16	Vanilla, chocolate	Guaiacol	1	1092	1096	1872	1881
17	Curry, meaty, roasted	Sotolone	256	1102	1112	2223	2208
18	Nail polish, floral, wort	Unk.	4	1114	-	-	-
19	Rose, honey, beer	2-Phenylethanol	64	1127	1126	1930	1934
20	Shoe store, odd honey	Unk.	1	1149	-	-	-
21	Spicy, smoky, meaty	Abhexon	256	1162	1188	2380	2378
22	Wort, bread dough, vegetables	Terpinen-4-ol	8	1170	1189	-	-
23	Shoes, hot honey, chemical	Unk.	4	1172	-	-	-

No.	Descriptor[a]	Compound	FD[b] (Rxi-5)	LRI[c] in Rxi-5		LRI[c] in Stabilwax	
				Exp.	Auth.	Exp.	Auth.
24	Spicy, smoky, burnt	4-Methylguaiacol	512	1180	1192	1983	1976
25	Meaty, burnt	4-Vinylphenol	8	1210	1213	2410	2400
26	Lemon	Geraniol	1	1248	-	-	-
27	Minty, fresh, vegetables	2-Phenyl-2-butenal	2	1273	-	-	-
28	Burnt, plastic	4-Ethylguaiacol	4	1281	1284	-	2017
29	Lemon, minty	Unk.	2	1296	-	-	-
30	Smokey, cloves, woody	4-Vinylguaiacol	16	1330	1309	2223	2211
31	Shoes, hot honey, chemical	2'-Methoxyacetophenone	8	1341	-	-	-
32	Apple, apricot, jam	(*E*)-β-Damascenone	64	1389	1378	1835	1849
33	Floral, plastic, fatty, green	Unk.	1	1453	-	-	-
34	Candle smoke, burnt	Unk.	4	1479	-	-	-
35	Nutmeg, clove, spicy	Unk.	8	1535	-	-	-
36	Candle smoke, spicy	Unk.	4	1568	-	-	-
37	Candle smoke, cloves	Unk.	4	1584	-	-	-
38	Floral, green, grassy	Unk.	1	1641	-	-	-

[a] Most frequent odour descriptors used at the sniffing port. [b] Flavour dilution factor from GC-Olfactometry in Rxi-5 column. [c] Linear Retention Index in two different columns. Exp.: Experimental LRI, Auth.: LRI for authentic compound. Unk.: Unknown or unidentified compound.

Supplementary Table S 2 Odour regions found in AFB (acidic fraction)

No.	Descriptor[a]	Compound	FD[b] (Rxi-5)	LRI[c] in Rxi-5		LRI[c] in Stabilwax	
				Exp.	Auth.	Exp.	Auth.
1	Butter, creamy	2,3-Butanedione	512	571	587	998	1001
2	Acetic	Acetic acid	128	620	589	1429	1460
3	Cocoa, Strecker	2- and 3-Methylbutanal	32	648	642	948	930
4	Bready, potato, Strecker	3-Methyl-1-butanol	16	755	730	1207	1215
5	Beer, wort, floral	Unk.	2	830	-	-	-
6	Cheesy	Butanoic acid	256	825	795	1609	1642
7	Cheesy	Unk.	1	856	-	-	-
8	Cheese, feety	Unk.	2	870	-	-	-
9	Feet, hoover, vomit, Strecker-like	Unk.	2	880	-	-	-
10	Cheese, feety, rancid	3-Methylbutanoic acid	128	886	861	1646	1684
11	Potato	Methional	512	917	922	1448	1468
12	Cheese, vomit	Pentanoic acid	2	950	926	-	1718
13	Mushroom, vegetables, earthy	1-Octen-3-one	1	982	978	1296	1302
14	Phenolic, hospital, plastic	Phenol	8	998	989	1999	1997
15	Candy floss, caramel	Furaneol	32	1030	1046	2015	2047
16	Honey, floral, rose	Phenylacetaldehyde	4	1060	1054	1631	1667
17	Green, plastic, chemical, mushroom	Unk.	4	1063	-	-	-
18	Phenolic, hospital, plastic	2-methylphenol?	8	1084	-	1954	-
19	Smokey, roasted	Guaiacol	128	1103	1096	1849	1881
20	Smokey, spicy	Sotolone	128	1109	1112	2213	2208
21	Honey, floral, rose	2-Phenylethanol	32	1124	1126	1927	1934
22	Candy floss, caramel	Homofuraneol	32	1135	1149	2074	2096
23	Curry, smoky, spicy	Abhexon	1024	1154	1188	2383	2378
24	Rubber, shoes, phenolic, kids paint	4-Methylguaiacol	8	1173	1192	-	1976
25	Smokey, burnt plastic	4-Vinylphenol	512	1206	1213	2410	2400
26	Phenol, plastic, medicinal	Unk.	8	1229	-	-	-
27	Urine	Unk.	8	1246	-	-	-

No.	Descriptor[a]	Compound	FD[b] (Rxi-5)	LRI[c] in Rxi-5		LRI[c] in Stabilwax	
				Exp.	Auth.	Exp.	Auth.
28	Nutty, smoky, caramel	Unk.	8	1262	-	-	-
29	Phenol, chemical, antiseptic	Unk.	8	1278	-	-	-
30	Honey, floral, urine	2-Phenylacetic acid	256	1293	1247	2640	2631
31	Urine, floral	Unk.	2	1328	-	-	-
32	Floral, lilies	Unk.	8	1344	-	-	-
33	Green, grass, plastic	Unk.	8	1352	-	-	-
34	Floral	Unk.	2	1356	-	-	-
35	Floral	Unk.	2	1385	-	-	-
36	Vanilla, creamy	Vanillin	512	1412	1404	2594	2604
37	Plastic, rubber, phenolic	Unk.	128	1420	-	-	-

[a] Most frequent odour descriptors used at the sniffing port. [b] Flavour dilution factor from GC-Olfactometry in Rxi-5 column. [c] Linear Retention Index in two different columns. Exp.: Experimental LRI, Auth.: LRI for authentic compound. Unk.: Unknown or unidentified compound.

Supplementary Table S 3 Odour regions found in wort (basic/neutral fraction)

No.	Descriptor[a]	Compound	LRI[b] in Rxi-5		LRI[b] in Stabilwax	
			Exp.	Auth.	Exp.	Auth.
1	Solvent, fruity	Acetaldehyde	<500	500	736	718
2	Cream, butter	2,3-Butanedione	563	587	988	1001
3	Cocoa	3-Methylbutanal	638	642	956	930
4	Cocoa	2-Methylbutanal	663	651	927	924
5	Butter	2,3-Pentanedione	710	706	1076	1045
6	Cheese	Unk.	749	-	-	-
7	Bread-like	3-Methyl-2-butenal	786	781	-	-
8	Fresh, green	Hexanal	808	802	1098	1091
9	Green	1-Hexanol	838	867	1310	1340
10	Cheese	3-Methylbutanoic acid	886	861	1676	1684
11	Rancid, linseed	(*Z*)-4-heptenal	909	894	1245	1228
12	Boiled potatoes	Methional	921	922	1466	1468
13	Smokey	5-Methylfurfural	945	968	-	1574
14	Leeks, boiled onion	Methionol	962	980	1729	1718
15	Pickled onions, garlic	Dimethyl trisulfide	973	984	1385	1390
16	Mushroom	1-Octen-3-ol	986	983	1413	1444
17	Green	Unk.	991		-	
18	Sweet	6-Methyl-5-hepten-2-one	1013	987	-	1341
19	Roasty, spicy	Unk.	1027	-	-	-
20	Smokey, popcorn	Unk.	1041	-	-	-
21	Curry, spicy	2-Acetylthiazole	1050	1027	1660	1650
22	Rose, honey	Phenylacetaldehyde	1054	1054	1658	1667
23	Rose	Unk.	1060	-	-	-
24	Mushroom, cardboard	Unk.	1085	-	-	-
25	Coffee, smoke	Unk.	1081	-	-	-
26	Baked bread	Unk.	1089	-	-	-
27	Smokey, medicinal	Guaiacol	1093	1096	1873	1881

No.	Descriptor[a]	Compound	LRI[b] in Rxi-5		LRI[b] in Stabilwax	
			Exp.	Auth.	Exp.	Auth.
28	Candy floss	Unk.	1096	-	-	-
29	Urinous	Unk.	1107	-	-	-
30	Popcorn	Unk.	1111	-	-	-
31	Soil	Unk.	1117	-	-	-
32	Honey, floral	2-Phenylethanol	1130	1126	1922	1934
33	Curry, spicy	Unk.	1145	-	-	-
34	Fatty, creamy, waxy	Unk.	1157	-	-	-
35	Violets	Unk.	1166	-	-	-
36	Cucumber, new shoes	Unk.	1164	-	-	-
37	Bready, honey	4-Oxoisophorone?	1169	1142	-	1677
38	Hot honey, new shoes	Unk.	1186	-	-	-
39	Sour, pickle, smoky, meaty	Unk.	1200	-	-	-
40	Roasty, spicy	Unk.	1219	-	-	-
41	Hospital, medicines	4-Vinylphenol	1221	1213	-	2400
42	Fries	(*E*, *E*)-2,4-Nonadienal	1235	1232	1721	1698
43	Lemon	Geraniol	1251	1255	-	1845
44	Minty, citrus	Unk.	1266	-	-	-
45	Minty, citrus	Unk.	1278	-	-	-
46	Wood, smoke	4-Ethylguaiacol	1294	1284	-	2017
47	Tea leaves	Unk.	1306	-	-	-
48	Smokey, sweet	Unk.	1316	-	-	-
49	Rose	Unk.	1320	-	-	-
50	Cloves	4-Vinylguaiacol	1324	1309	2225	2211
51	Phenolic, leather, wood	Unk.	1337	-	-	-
52	Rose	Unk.	1346	-	-	-
53	Hot honey, new shoes	Unk.	1352	-	-	-
54	Creamy, fruity, floral	γ-Nonalactone	1374	1372	-	2036
55	Peach, rose	(*E*)-β-Damascenone	1394	1378	1830	1849
56	Cheese, dairy, butter	Unk.	1411	-	-	-

No.	Descriptor[a]	Compound	LRI[b] in Rxi-5		LRI[b] in Stabilwax	
			Exp.	Auth.	Exp.	Auth.
57	Creamy, vanilla	Vanillin	1439	1404	-	2604
58	Cloves, match	Unk.	1458	-	-	-
59	Sweet, perfumy	Unk.	1481	-	-	-
60	Smokey	Unk.	1509	-	-	-
61	Clove, spicy, smoke	Unk.	1514	-	-	-
62	Incense	Unk.	1583	-	-	-

[a] Most frequent odour descriptors used at the sniffing port. [b] Linear Retention Index in two different columns. Exp.: Experimental LRI, Auth.: LRI for authentic compound. Unk.: Unknown or unidentified compound.

Supplementary Table S 4 Odour regions found in wort (acidic fraction)

No.	Descriptor[a]	Compound	LRI[b] in Rxi-5		LRI[b] in Stabilwax	
			Exp.	Auth.	Exp.	Auth.
1	Fruity, alcoholic	Unk.	<500	-	-	-
2	Creamy	Butanedione	566	587	981	1001
3	Cocoa	2- and 3-Methylbutanal	636	642	-	930
4	Vinegar	Acetic acid	660	589	1435	1460
5	Green	Unk.	788	-	-	-
6	Beer-like	Unk.	828	-	-	-
7	Cheese	Butanoic acid	782	795	1630	1642
8	Cheese	3-Methylbutanoic acid?	890	886	1665	1648
9	Cheese	2-methylbutanoic acid?	907	-	-	-
10	Boiled potatoes	Methional	924	922	1469	1468
11	Cheese	Unk.	929	-	-	-
12	Cheese	Pentanoic acid	942	950	-	1718
13	Cheese	Unk.	958	-	-	-
14	Piney, hospital	Phenol	1010	989	1988	1997
15	Spicy, curry	Unk.	1031	-	-	-
16	Rose, honey	Phenylacetaldehyde	1060	1054	1660	1667
17	Antiseptic	2-methylphenol?	1084	1066	-	1969
18	Smokey	Guaiacol	1103	1096	1868	1881
19	Candy floss	Homofuraneol	1119	1149	2096	2096
20	Honey	2-Phenylethanol	1123	1126	1924	1934
21	Meaty, burnt, savoury	Unk.	1128	-	-	-
22	Curry, celery, maple syrup	Abhexon	1148	1188	2341	2378
23	Cleaning agent	Unk.	1196	-	-	-
24	Band aid, plastic	4-Vinylphenol	1220	1213	-	2400
25	Curry	Unk.	1226	-	-	-
26	Cleaning agent	Unk.	1280	-	-	-
27	Honey, urinous	Unk.	1309	-	-	-
28	Floral, lilies	2-Phenylacetic acid	1325	1247	-	2631

No.	Descriptor[a]	Compound	LRI[b] in Rxi-5		LRI[b] in Stabilwax	
			Exp.	Auth.	Exp.	Auth.
29	Honey, spicy	Unk.	1347	-	-	-
30	Band aid, plastic	Unk.	1398	-	-	-
31	Vanilla	Vanillin	1402	1404	-	2604
32	Cloves	Unk.	1478	-	-	-

[a] Most frequent odour descriptors used at the sniffing port. [b] Linear Retention Index in two different columns. Exp.: Experimental LRI, Auth.: LRI for authentic compound. Unk.: Unknown or unidentified compound.

Appendix 2. Compilation of literature thresholds

This spreadsheet file can be found as "**Appendix B**" in the electronic version of this chapter, published in the Food Research International journal.

Click on this link to access it.

Appendix 3. Logistic model parameters for the detection responses against concentration

Supplementary Table S 5. Logistic model parameters for orthonasal detection response.

No.	Compound	Raw data			Adjusted data		
		α	β	Pseudo-R^2 (Nagelkerke)	α	β	Pseudo-R^2 (Nagelkerke)
1	acetaldehyde	-1.386	0.518	0.327	-6.556	1.714	0.718
2	acetic acid	-8.866	0.752	0.441	-35.395	2.769	0.865
3	2,3-butanedione	-0.089	0.396	0.214	-1.338	0.812	0.457
4	butanoic acid	-6.517	0.957	0.504	-11.859	1.552	0.729
5	(E)-β-damascenone	0.641	0.235	0.123	1.182	0.807	0.394
6	dimethyl sulfide	-1.279	0.493	0.306	-3.009	0.775	0.486
7	5-ethyl-4-hydroxy-2-methyl-3(*2H*)-furanone (homofuraneol)	-2.733	0.767	0.407	-5.700	1.232	0.645
8	(*Z*)-4-heptenal	2.324	0.410	0.257	3.720	0.897	0.543
9	3-hydroxy-4,5-dimethyl-2(*5H*)-furanone (sotolone)	-1.129	0.522	0.331	-5.754	1.782	0.766
10	4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (furaneol)	-2.465	0.643	0.371	-5.579	1.128	0.616
11	methional	1.015	0.618	0.337	0.721	0.943	0.534
12	2'-methoxyacetophenone	-2.295	0.351	0.186	-4.877	0.631	0.365
13	2-methoxy-4-methylphenol	-0.825	0.272	0.140	-3.719	1.029	0.480
14	2-methoxyphenol	0.286	0.716	0.413	-1.165	1.573	0.742
15	2-methoxy-4-vinylphenol	-2.007	0.580	0.335	-6.349	1.444	0.685
16	2-methylbutanal	-0.139	0.220	0.115	-1.453	0.461	0.256
17	3-methylbutanal	1.338	1.131	0.516	0.944	1.886	0.743
18	3-methylbutanoic acid	-2.579	0.574	0.342	-4.318	0.728	0.458
19	3-methyl-1-butanol	-2.706	0.839	0.318	-8.658	1.929	0.605
20	methylpropanal	-0.004	0.475	0.261	-1.355	0.926	0.529
21	2-methylthiophene	-2.805	0.376	0.238	-6.803	0.757	0.445
22	2,3-pentanedione	-0.477	0.427	0.266	-2.335	0.913	0.554
23	phenylacetaldehyde	-0.284	0.582	0.334	-1.951	1.154	0.619
24	2-phenylacetic acid	-3.257	0.461	0.256	-15.078	1.764	0.723
25	2-phenylethanol	-3.502	0.552	0.304	-7.191	0.954	0.548
26	vanillin	-3.479	0.581	0.331	-7.109	0.973	0.550
27	4-vinylphenol	-3.414	0.528	0.279	-6.582	0.831	0.479

Supplementary Table S 6. Logistic model parameters for retronasal detection response.

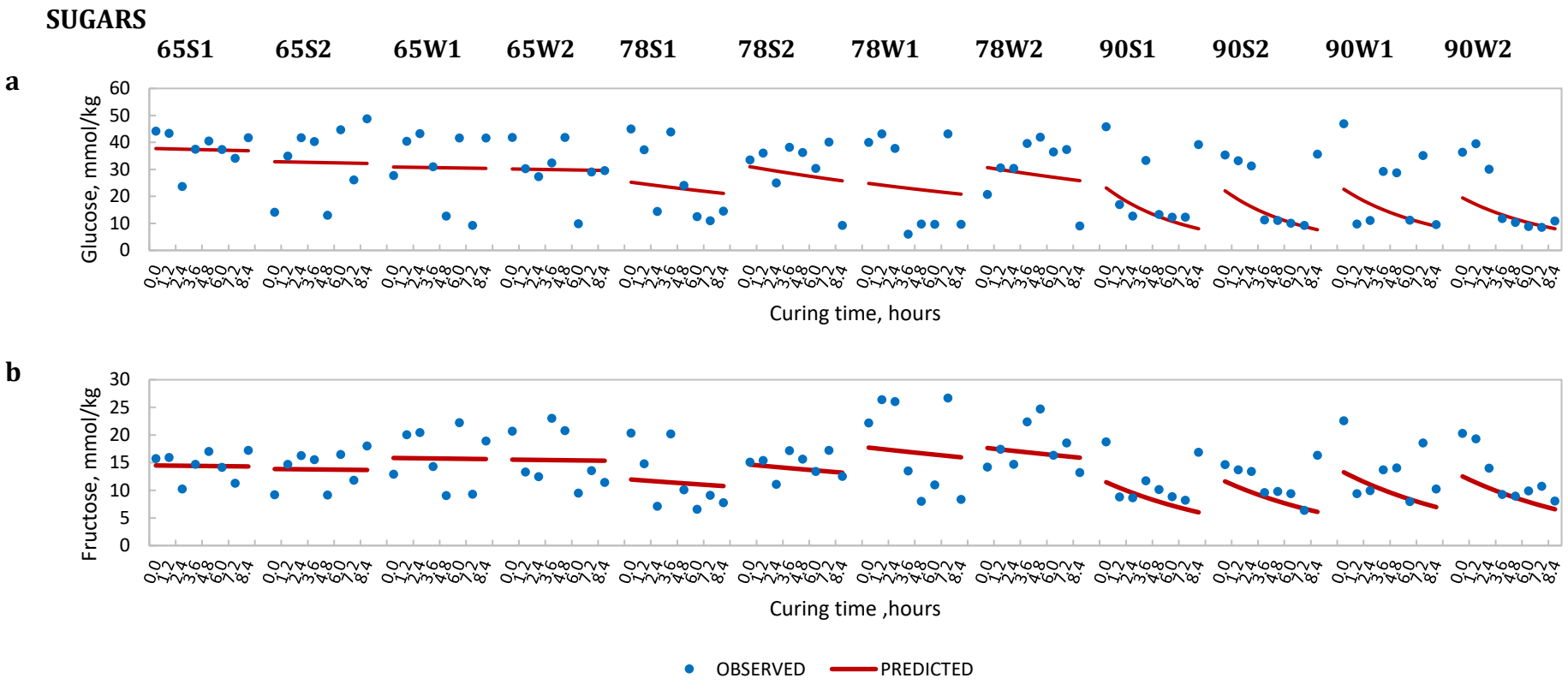
		Raw data			Adjusted data		
No.	**Compound**	**α**	**β**	**Pseudo-R^2 (Nagelkerke)**	**α**	**β**	**Pseudo-R^2 (Nagelkerke)**
2	acetic acid	-6.536	0.653	0.341	-10.533	0.957	0.528
3	2,3-butanedione	0.630	0.378	0.192	0.161	0.525	0.294
4	butanoic acid	-5.764	1.040	0.433	-10.127	1.594	0.642
5	(E)-β-damascenone	2.040	0.449	0.246	1.946	0.613	0.353
6	dimethyl sulfide	-3.434	0.935	0.568	-7.006	1.624	0.764
8	5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone (homofuraneol)	1.148	0.515	0.269	0.649	0.813	0.471
9	3-hydroxy-4,5-dimethyl-2(*5H*)-furanone (sotolone)	-0.106	0.484	0.291	-0.935	0.732	0.454
10	4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (furaneol)	-1.81	0.405	0.220	-4.264	0.769	0.435
12	methional	0.558	0.172	0.096	0.117	0.369	0.197
13	2-methoxy-4-methylphenol	0.410	0.161	0.092	-0.220	0.354	0.187
14	2-methoxyphenol	0.461	0.526	0.289	0.007	0.724	0.424
15	2-methoxy-4-vinylphenol	-0.291	0.416	0.208	-1.071	0.502	0.276
16	2-methylbutanal	-0.134	0.296	0.148	-0.964	0.439	0.238
18	3-methylbutanal	1.158	0.767	0.371	0.785	0.948	0.483
19	3-methyl-1-butanol	-4.713	0.983	0.463	-8.245	1.488	0.668
22	methylpropanal	0.700	0.389	0.193	0.093	0.628	0.355
23	phenylacetaldehyde	0.774	0.336	0.163	0.179	0.461	0.248
24	2-phenylacetic acid	-0.484	0.191	0.101	-1.412	0.262	0.135
25	2-phenylethanol	-2.388	0.508	0.245	-3.425	0.609	0.318
26	vanillin	-1.049	0.274	0.137	-2.980	0.488	0.272
27	4-vinylphenol	-0.819	0.185	0.101	-2.180	0.289	0.151

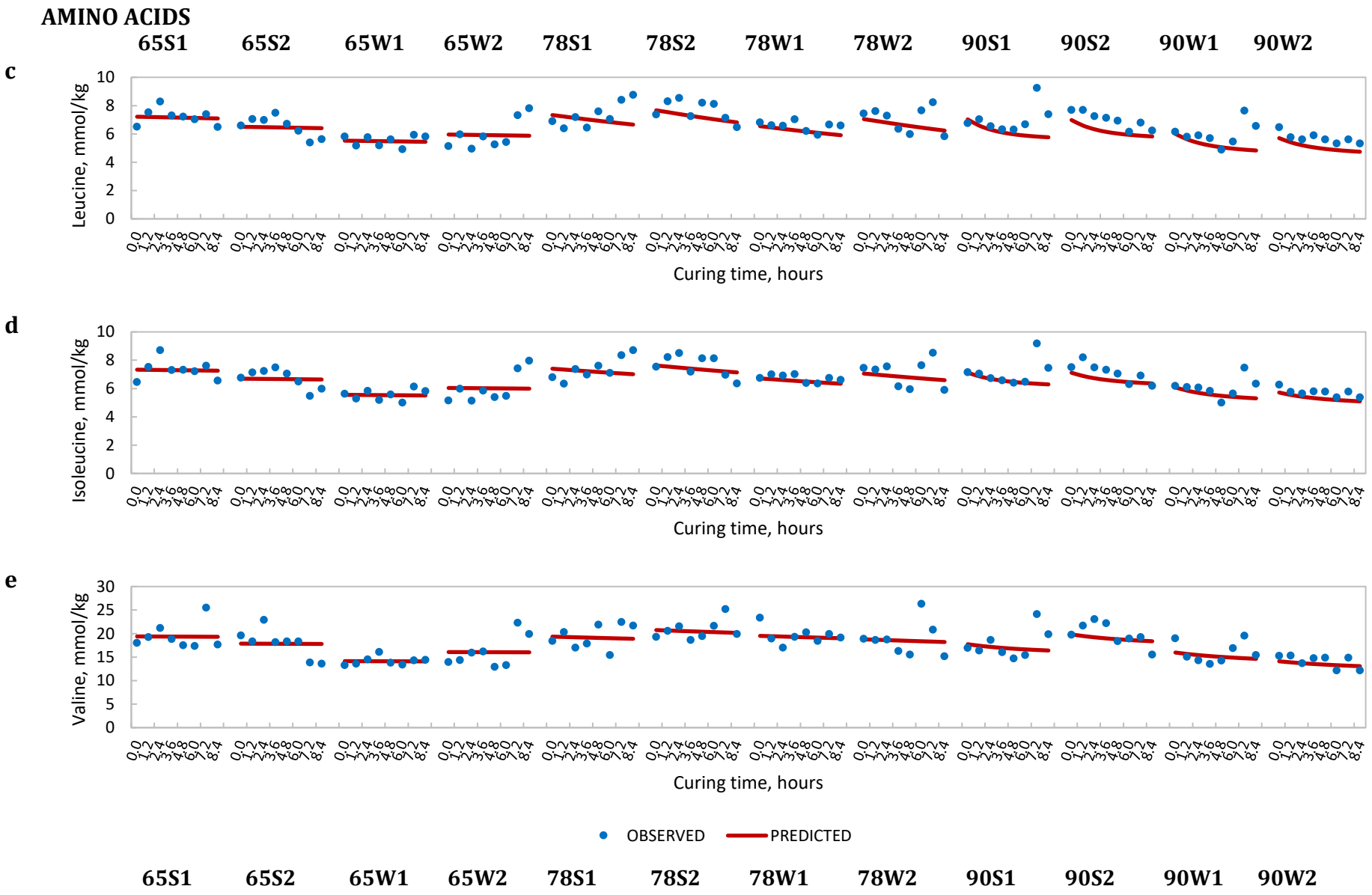
Appendix 4. LC-MS/MS detection parameters for non-volatile components of malt

Supplementary Table S 7. MRM conditions for amino acids, sugars and ARP.

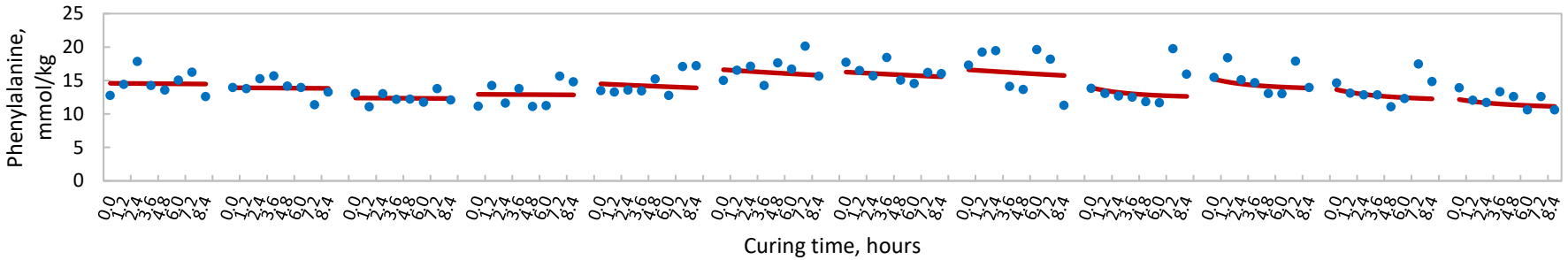
Compound	Cell Accelerator voltage, V	Fragmentor voltage, V	MS/MS transition (Collision energy, V)	
			Quantitative	Qualitative
Amino acids				
Phe	7	64	166.1 → 120.1 (8)	166.1 → 103.1 (20)
Trp	7	78	205.1 → 188.1 (4)	205.1 → 146.1 (20)
Ile	7	60	132.1 → 69.1 (16)	132.1 → 44.1 (20)
Leu	7	60	132.1 → 86.1 (4)	132.1 → 44.1 (20)
Met	7	64	150.0 → 133.0 (4)	150.0 → 56.1 (16)
Tyr	7	50	182.1 → 165.0 (4)	182.1 → 136.1 (8)
Val	7	64	118.1 → 72.1 (4)	118.1 → 55.2 (20)
Pro	7	64	116.0 → 70.1 (16)	
Glu	7	50	148.0 → 130.0 (4)	148.0 → 84.1 (12)
Thr	7	50	120.0 → 74.0 (8)	120.0 → 56.1 (16)
Ala	7	64	90.1 → 44.2 (8)	
Gly	7	50	76.0 → 30.0 (4)	
Gln	7	64	147.1 → 130.1 (4)	147.1 → 84.1 (12)
Asn	7	78	133.1 → 87.1 (4)	133.1 → 74.1 (12)
Asp	7	64	134.0 → 88.1 (4)	134.0 → 74.0 (8)
Ser	7	64	106.0 → 60.1 (8)	106.0 → 42.1 (20)
His	7	92	156.1 → 110.1 (8)	156.1 → 83.1 (20)
Arg	7	92	175.1 → 70.1 (24)	175.1 → 60.1 (12)
Lys	7	92	147.1 → 130.0 (4)	147.1 → 84.1 (16)
Norvaline	7	40	118.1 → 72.1 (4)	118.1 → 30.2 (16)
Sugars				
Glucose	1	60	179.1 → 58.9 (12)	179.1 → 89.1 (2)
Fructose	1	60	179.1 → 89.1 (2)	179.1 → 58.9 (12)
Trehalose	0	170	341.1 → 119.1 (12)	341.1 → 89.1 (14)
ARP				
FruVal	4	66	280.3 → 262.1 (4)	280.3 → 216.1 (16)
FruIle	4	52	294.2 → 144.0 (26)	294.2 → 230.1 (16)
FruLeu	4	66	294.2 → 276.1 (4)	294.2 → 88.0 (24)
FruPhe	4	66	328.1 → 310.1 (4)	328.1 → 132.1 (28)
FruAla	4	66	252.3 → 216.1 (12)	252.3 → 56.1 (40)
FruGly	4	66	238.1 → 220.1 (4)	238.1 → 202.1 (8)
FruPro	4	112	278.4 → 260.1 (8)	278.4 → 242.1 (16)

Appendix 5. Analytical and predicted concentrations of the precursors, intermediate species and products for the formation of Strecker aldehydes.

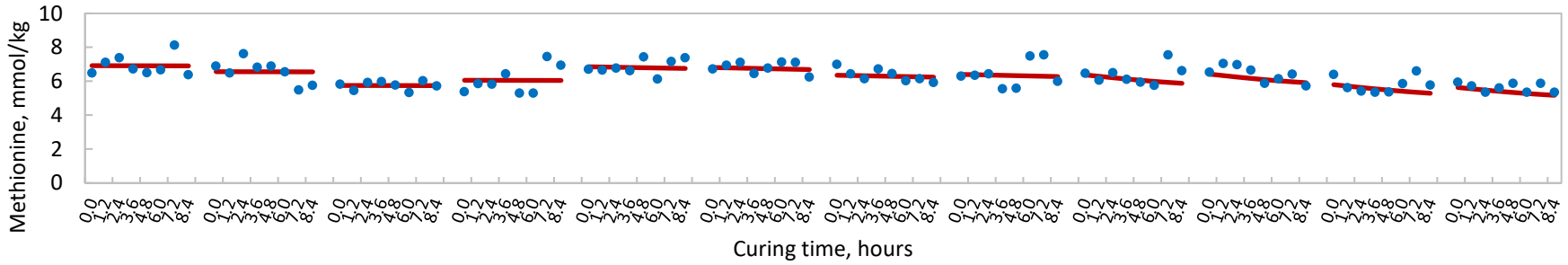

SUGARS
65S1
65S2
65W1
65W2
78S1
78S2
78W1
78W2
90S1
90S2
90W1
90W2
a
Glucose, mmol/kg
Curing time, hours
b
Fructose, mmol/kg
Curing time ,hours
OBSERVED
PREDICTED


AMINO ACIDS
65S1
65S2
65W1
65W2
78S1
78S2
78W1
78W2
90S1
90S2
90W1
90W2
c
Leucine, mmol/kg
d
Isoleucine, mmol/kg
e
Valine, mmol/kg
Curing time, hours
OBSERVED
PREDICTED

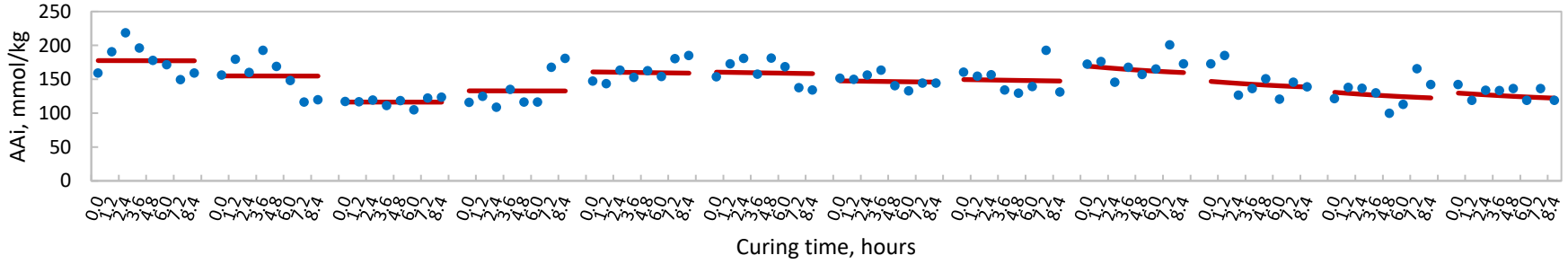
f


Phenylalanine, mmol/kg
25
20
15
10
5
0
Curing time, hours

g

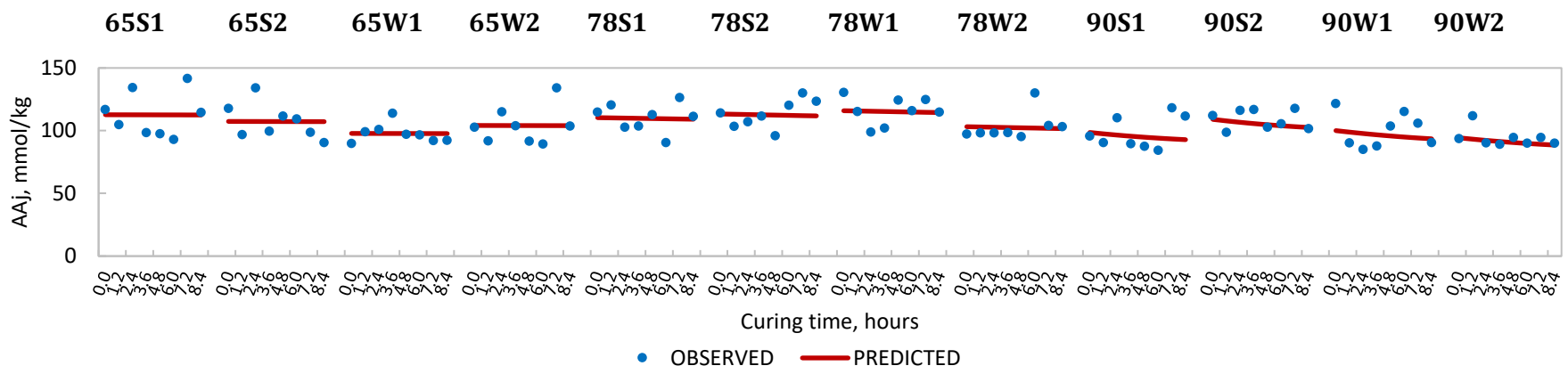

Methionine, mmol/kg
10
8
6
4
2
0
Curing time, hours

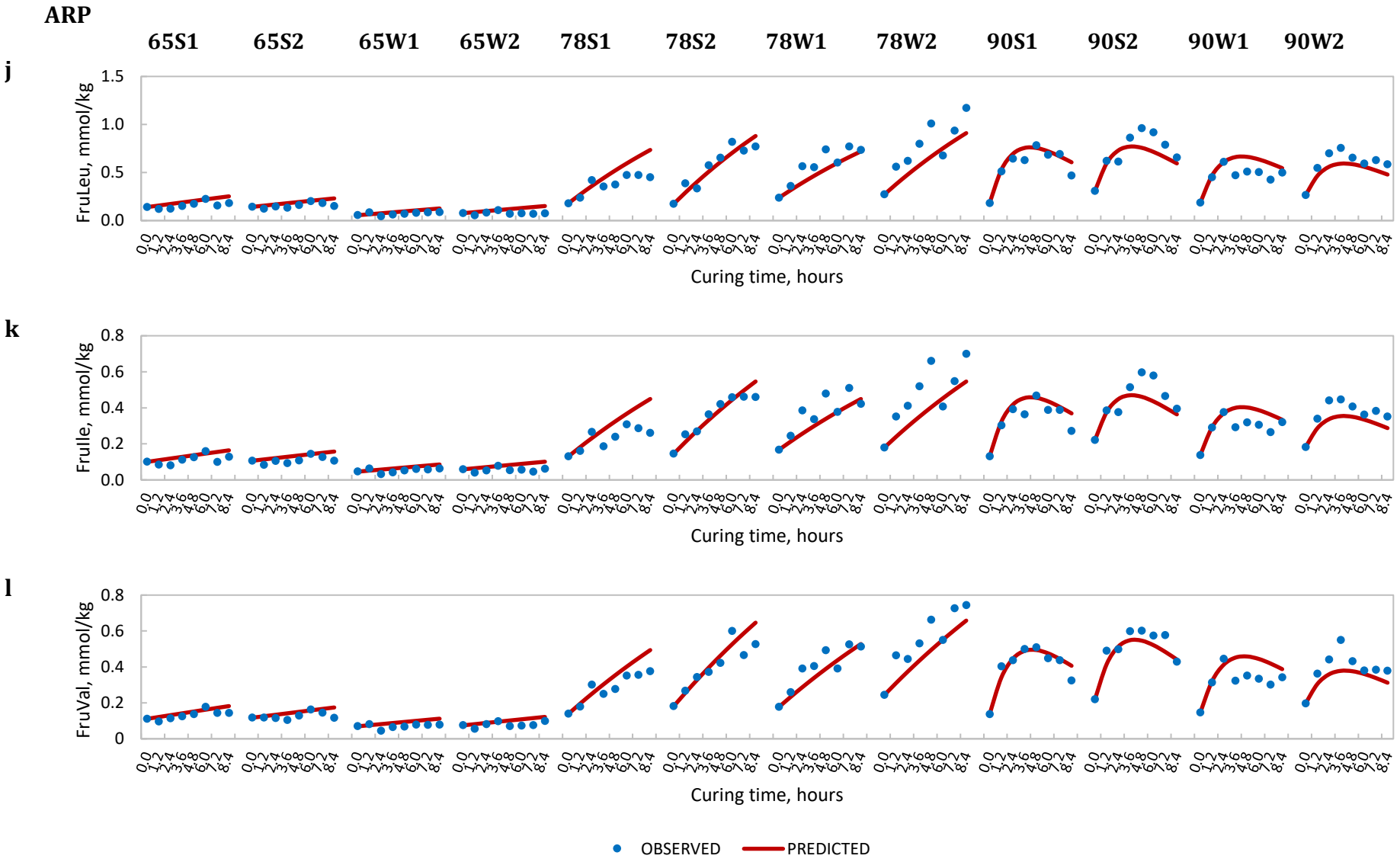
h


AAi, mmol/kg
250
200
150
100
50
0
Curing time, hours

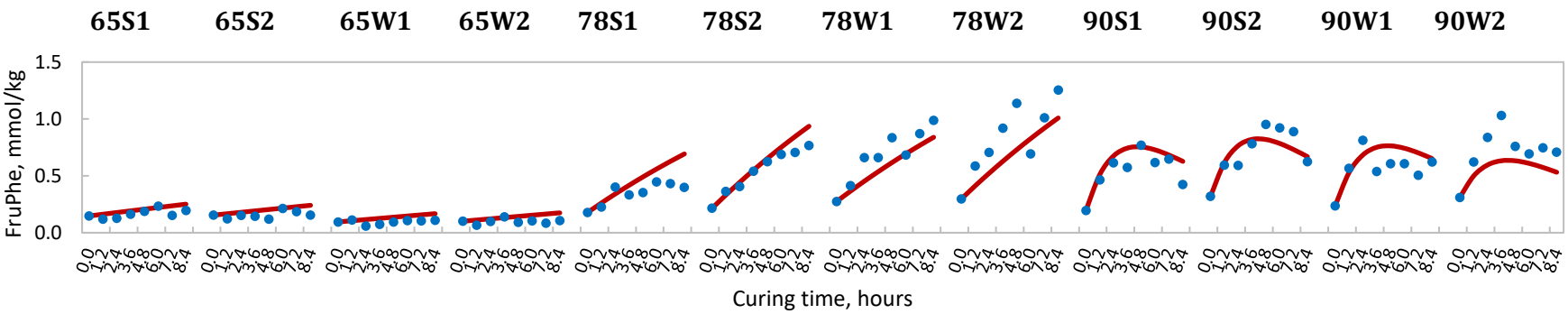

OBSERVED
PREDICTED

i

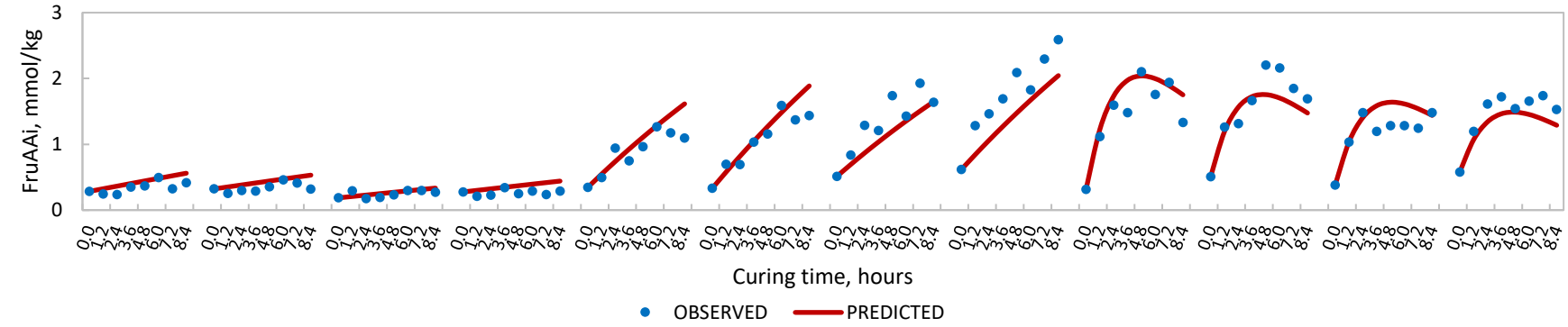

65S1
65S2
65W1
65W2
78S1
78S2
78W1
78W2
90S1
90S2
90W1
90W2
AAj, mmol/kg
150
100
50
0
Curing time, hours
OBSERVED
PREDICTED

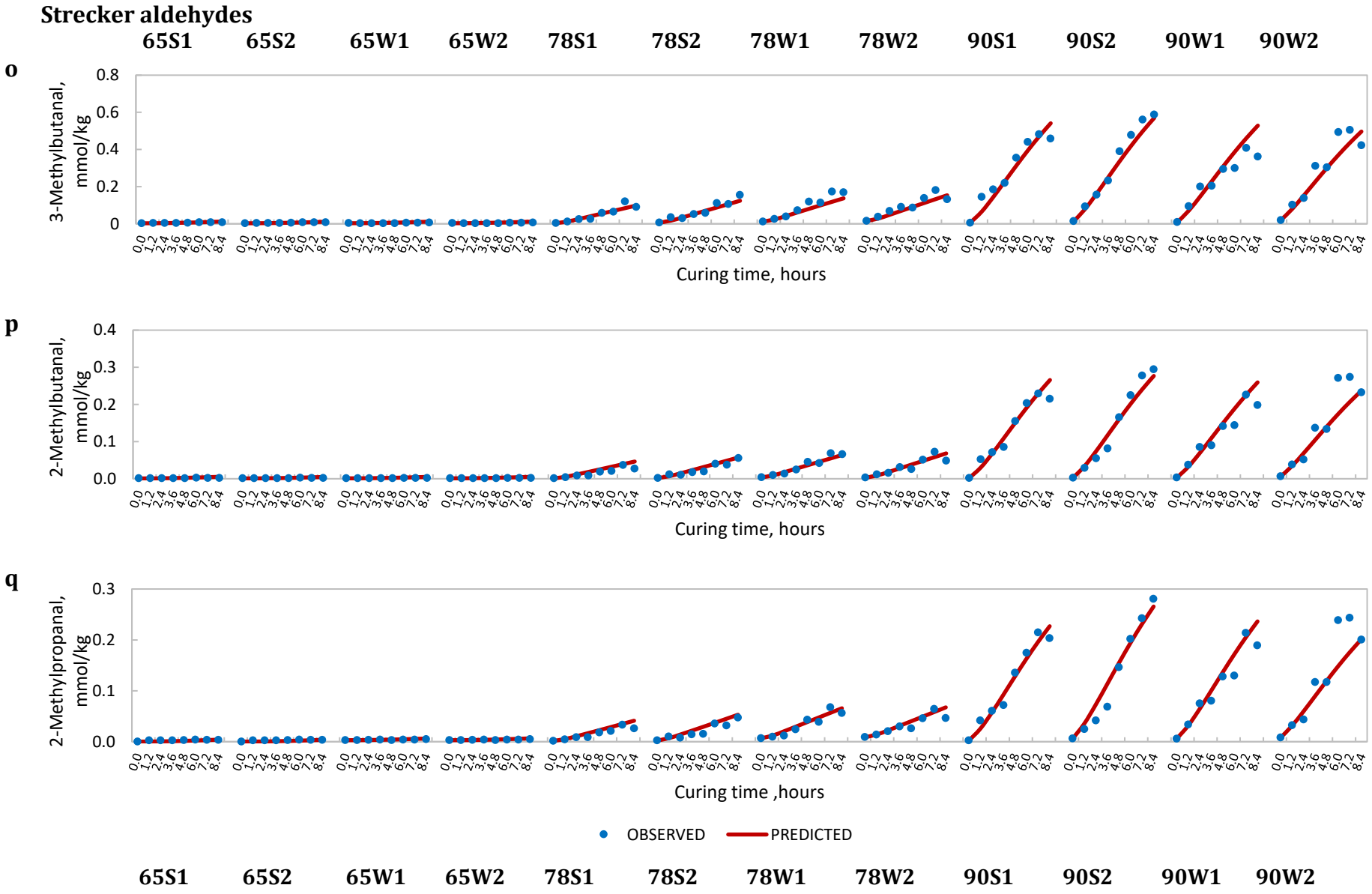

ARP
65S1
65S2
65W1
65W2
78S1
78S2
78W1
78W2
90S1
90S2
90W1
90W2
j
FruLeu, mmol/kg
k
FruIle, mmol/kg
l
FruVal, mmol/kg
Curing time, hours
OBSERVED
PREDICTED

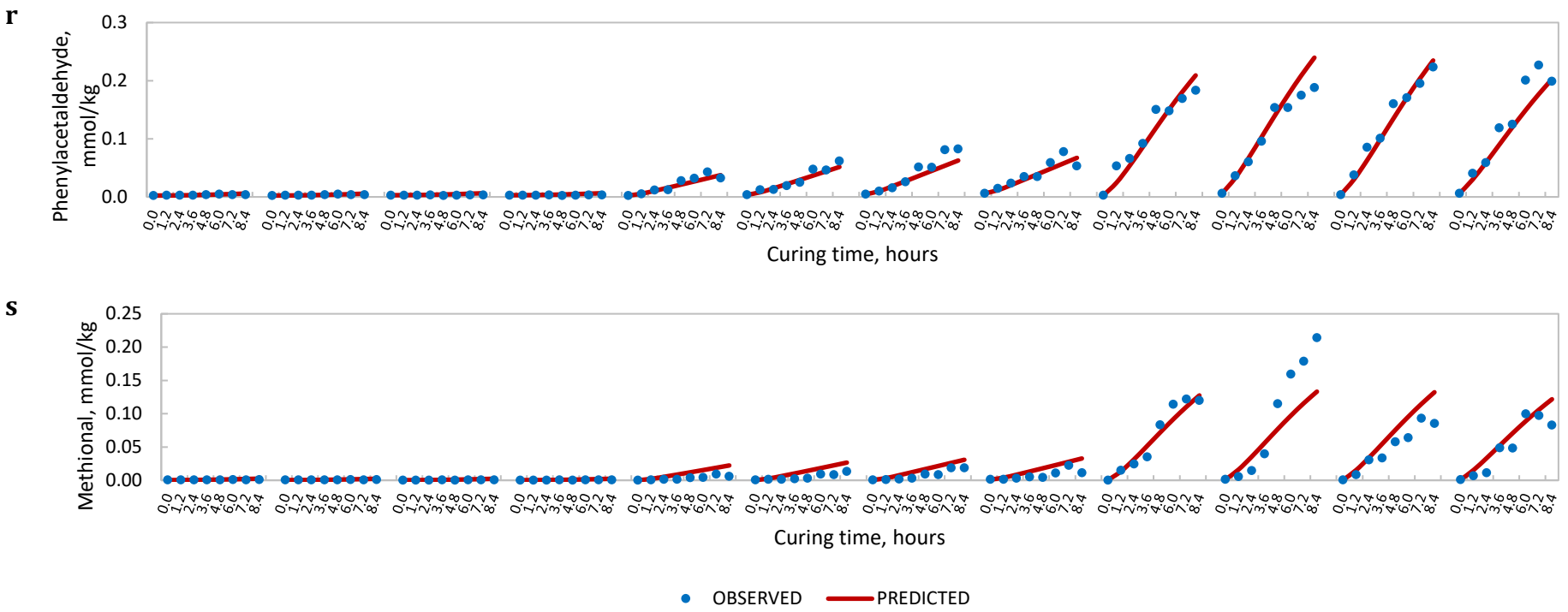
m


65S1
65S2
65W1
65W2
78S1
78S2
78W1
78W2
90S1
90S2
90W1
90W2
FruPhe, mmol/kg
1.5
1.0
0.5
0.0
Curing time, hours

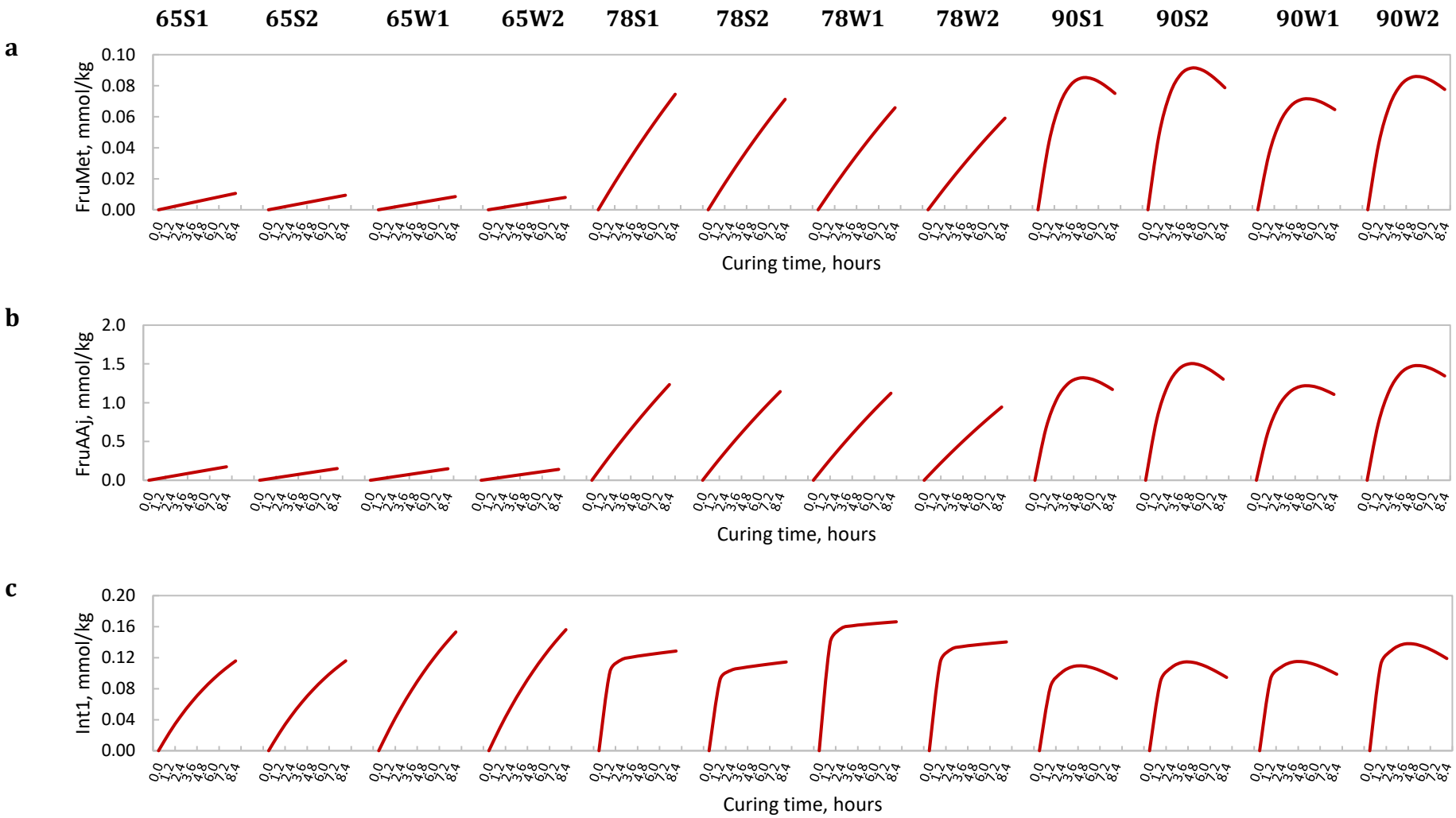
n

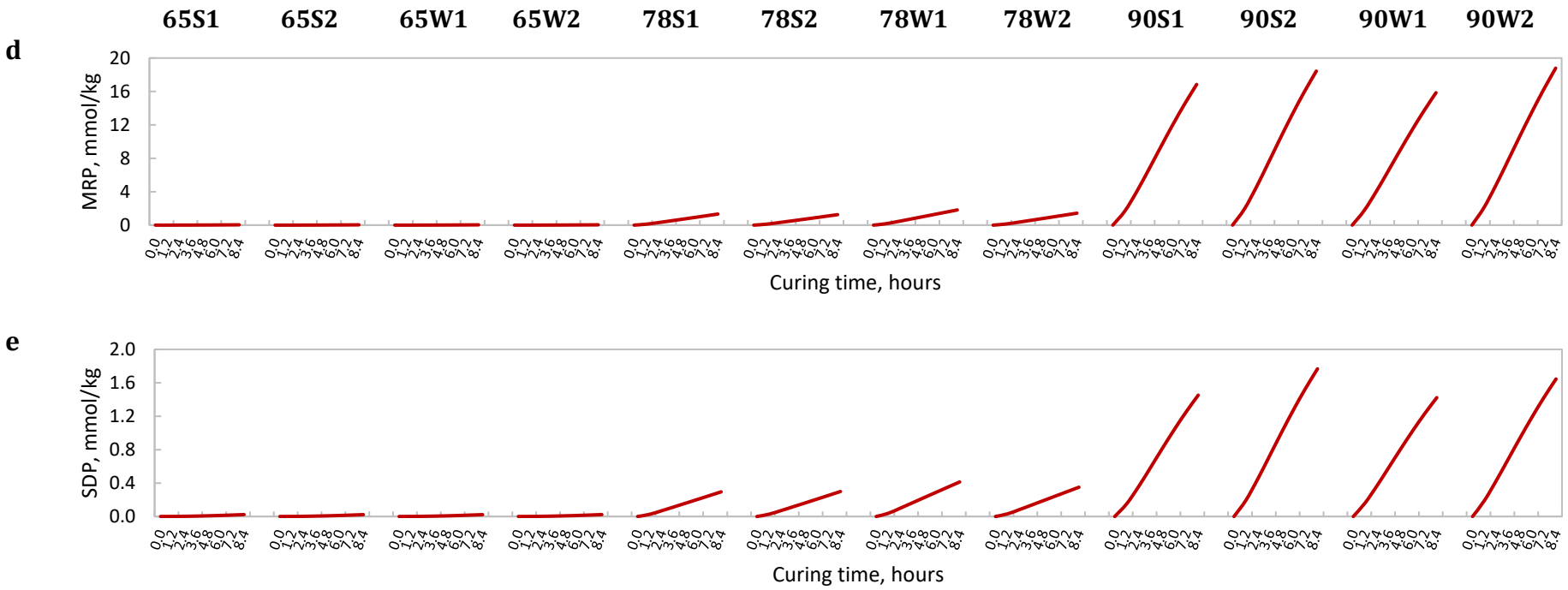

FruAAi, mmol/kg
3
2
1
0
Curing time, hours
OBSERVED
PREDICTED


Strecker aldehydes
65S1
65S2
65W1
65W2
78S1
78S2
78W1
78W2
90S1
90S2
90W1
90W2
o
3-Methylbutanal, mmol/kg
0.8
0.6
0.4
0.2
0
Curing time, hours
p
2-Methylbutanal, mmol/kg
0.4
0.3
0.2
0.1
0.0
Curing time, hours
q
2-Methylpropanal, mmol/kg
0.3
0.2
0.1
0.0
Curing time ,hours
OBSERVED
PREDICTED



Supplementary Figure S 1 Concentration of sugars (a-b), amino acids (c-i), ARP (j-n) and Strecker aldehydes (o-s) as a function of time during the curing stage of kilning at different temperatures (65, 78 or 90 °C) for two different varieties of barley (S for spring, W for winter) and two replicates.


65S1
65S2
65W1
65W2
78S1
78S2
78W1
78W2
90S1
90S2
90W1
90W2
a
FruMet, mmol/kg
0.10
0.08
0.06
0.04
0.02
0.00
Curing time, hours
b
FruAAj, mmol/kg
2.0
1.5
1.0
0.5
0.0
Curing time, hours
c
Int1, mmol/kg
0.20
0.16
0.12
0.08
0.04
0.00
Curing time, hours



Supplementary Figure S 2 Predicted concentration of the compounds non quantified analytically. FruMet: fructosyl methionine; FruAAj: fructosyl derivatives from amino acids AAj; Int1: intermediate 1; MRP: Maillard reaction products; SDP: sugar degradation products.

Appendix 6. Two-way ANOVA for Strecker aldehydes in finished malts.

Supplementary Table S 8. Results from two-way analysis of variance for Strecker aldehydes at α=0.05.

Source		Sum of squares	DF	Mean Square	F	Sig.
Corrected Model	3MB	0.448	5	0.090	41.595	<0.001
	2MB	0.123	5	0.025	34.437	<0.001
	2MP	0.106	5	0.021	38.503	<0.001
	PhAc	0.082	5	0.016	85.010	<0.001
	Meth	0.045	5	0.009	11.973	0.004
Intercept	3MB	0.480	1	0.480	222.479	<0.001
	2MB	0.109	1	0.109	152.469	<0.001
	2MP	0.094	1	0.094	170.623	<0.001
	PhAc	0.090	1	0.090	465.029	<0.001
	Meth	0.026	1	0.026	34.031	0.001
Temperature	3MB	0.430	2	0.215	99.836	<0.001
	2MB	0.121	2	0.061	84.838	<0.001
	2MP	0.104	2	0.052	94.039	<0.001
	PhAc	0.081	2	0.040	209.680	<0.001
	Meth	0.038	2	0.019	25.313	0.001
Variety	3MB	0.004	1	0.004	1.678	0.243
	2MB	0.000	1	0.000	0.265	0.625
	2MP	0.000	1	0.000	0.587	0.473
	PhAc	0.001	1	0.001	3.687	0.103
	Meth	0.002	1	0.002	2.697	0.152
*Temperature * Variety*	3MB	0.014	2	0.007	3.314	0.107
	2MB	0.002	2	0.001	1.121	0.386
	2MP	0.002	2	0.001	1.926	0.226
	PhAc	0.000	2	0.000	1.001	0.421
	Meth	0.005	2	0.002	3.270	0.110
Error	3MB	0.013	6	0.002		
	2MB	0.004	6	0.001		
	2MP	0.003	6	0.001		
	PhAc	0.001	6	0.000		
	Meth	0.005	6	0.001		
Total	3MB	0.941	12			
	2MB	0.237	12			
	2MP	0.204	12			
	PhAc	0.173	12			
	Meth	0.075	12			

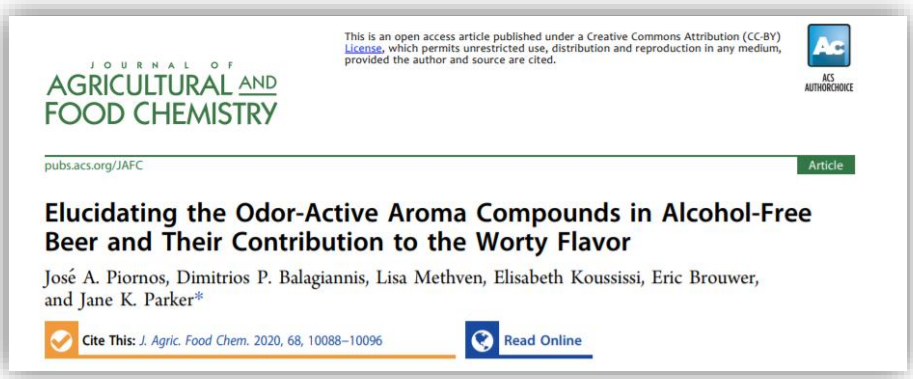
Abbreviations: 3MB: 3-methylbutanal, 2MB: 2-methylbutanal, 2MP: 2-methylpropanal, PhAc: phenylacetaldehyde, Meth: methional, DF: degrees of freedom.

About the author

José Antonio Piornos Martínez was born in Murcia (Spain) in 1989. He graduated from the degree in Chemical Engineering at the University of Murcia in March 2013, with a research placement at the Department of Microbiology of the University of Barcelona. Short after graduating, he moved to Chile where he worked as a research assistant at CGNA from January 2014 to March 2016 in the southern city of Temuco. This was when he developed his passion for research in Food Science and also was able to publish several papers about the functional properties of protein isolates from yellow lupin. In parallel to his job, he studied an MSc in Engineering Sciences, with specialisation in Biotechnology, at the University of La Frontera. During this period, he had the opportunity to do a research placement at INRA and the *École Nationale Vétérinaire, Agro-Alimentaire et de l'Alimentation* in Nantes (France). After three years living in Chile, in April 2016 he moved to the United Kingdom to do his PhD at the University of Reading. During the last four years, he has performed several research placements in the R&D labs of Heineken in Zoeterwoude (The Netherlands) and in Mouterij Albert in Ruisbroek (Belgium), as well as presented his work at the most important conferences for flavour and sensory science in Europe.

Publications

- **Piornos, J. A.**, Balagiannis, D. P., Methven, L., Koussissi, E., Brouwer, E., Parker, J. K. (2020). Elucidating the odor-active aroma compounds in alcohol-free beer and their contribution to the worty flavor. *Journal of Agricultural and Food Chemistry*, **68(37)**, 10088-10096. https://doi.org/10.1021/acs.jafc.0c03902



- **Piornos, J. A.**, Delgado, A., de La Burgade, R. C. J., Methven, L., Balagiannis, D. P., Koussissi, E., Brouwer, E., Parker, J. K. (2019). Orthonasal and retronasal detection thresholds of 26 aroma compounds in a model alcohol-free beer: Effect of threshold calculation method. *Food Research International*, **123**, 317–326. https://doi.org/10.1016/J.FOODRES.2019.04.034

- **Piornos, J.A.**, Balagiannis, D.P., Koussissi, E., Brouwer, E., & Parker, J.K. (2018). Characterisation of the key aroma compounds in alcohol- free beer base by gas chromatography-olfactometry. In B. Siegmund, & E. Leitner (Eds.). Flavour Science – Proceedings of the XV Weurman Flavour Research Symposium (pp. 343-346). Graz: Verlag der Technischen Universität Graz.

Characterisation of the key aroma compounds in alcohol- free beer base by gas chromatography-olfactometry

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Abstract

The pleasant fruity flavour of lager beers is one of the most appreciated features of these beverages, whereas alcohol-free beers (AFB) also exhibit a flavour reminiscent of wort. Even though several studies have been carried out to characterise the key odorants in different alcoholic beers, there are no similar works for AFB. Hence, the aim of this research is to identify the compounds contributing to the characteristic aroma of AFB. In this work, the volatile fraction of an AFB-base (without added flavourings) was isolated using solvent assisted flavour evaporation (SAFE) and analysed by GC-MS and GC-Olfactometry. Twenty-three odour regions showed odour activity in GC-O experiments, amongst which the most potent were methional, phenylacetaldehyde, 2-methoxyphenol, β-damascenone, 2-phenylacetic acid, 2-phenylethanol, and 5-ethyl-3-hydroxy-4-methyl-2(5*H*)-furanone. The presence of these compounds plays a crucial role in AFB aroma.

Introduction

AFB consumption has increased over the last few years, mainly in response to strict drink driving legislation, medical recommendation or religious grounds, but also due to a growth in health awareness. According to current UK legislation, the description "alcohol-free" may be applied to products containing "an alcoholic strength by volume of not more than 0.05 per cent".

These beers usually exhibit a flavour reminiscent of wort. Recent literature shows that Strecker aldehydes, particularly 2-methylbutanal, 3-methylbutanal and methional, are responsible for the negative attributes associated with AFB flavour [1], and these compounds are also present in barley malt [2]. These aldehydes have exceptionally low odour thresholds (1.25 μg/L, 0.6 μg/L and 0.25 μg/L for 2-methylbutanal, 3-methylbutanal and methional, respectively [3]) and impart potent worty, malty aromas even at very low concentrations.

Although worty aroma of AFB has been related to Strecker aldehydes [1], there is no information in literature about the possible contribution of other odour-active compounds to the overall aroma of

AFB. Sensomic methodology has been employed to identify the key odorants in different beers, such as pale lager [4] and wheat beers [5]. In the latter example, the authors found more than 30 odorants contributing to the characteristic aroma of wheat beer, Strecker aldehydes being amongst them. The aim of this study was to identify a more complete set of odour-active volatile compounds present in AFB by means of Sensomic methodology.

Experimental

Materials

An alcohol-free beer-base (AFB-base), without any external flavour added, was brewed, bottled and pasteurised in Heineken's pilot brewery (Zoeterwoude, The Netherlands) in January 2016 following a standard cold-contact fermentation procedure (brewing conditions not specified). Diethyl ether and saturated alkane standards were purchased from Sigma (Dorset, UK).

Isolation of the volatile fraction

For the isolation of volatiles from the AFB-base, the procedure described by Langos et al. was employed with slight modifications [5]. Briefly, 1 kg of sample was extracted with redistilled diethyl ether (250 mL × 4). The organic phase was dried over anhydrous Na_2SO_4 and filtered before concentration using a Vigreux distillation column (60 cm, 1 cm i.d.) at 40 °C until a final volume of approximately 100 mL was reached. To separate the non-volatile materials from the extract, this was submitted to a high-vacuum distillation process known as solvent assisted flavour evaporation (SAFE) technique (evaporation at 25 °C and 10^{-5} Pa). The distillate was fractioned into an acidic and a basic/neutral fraction using $NaHCO_3$ 0.5 M solution (60 mL × 3). After washing with 30 mL of a saturated NaCl solution three times, the organic layer was kept for further treatment (basic organic extract). In parallel, the basic aqueous phase was acidified to pH 2.25±0.10 by adding HCl solution (10 M or 1 M) and extracted using redistilled diethyl ether (60 mL × 3) and the extracts combined (acidic organic extract). Both basic and acidic organic extracts were concentrated using a Kuderna-Danish concentrator at 45 °C (final volume ~400 µL for each extract). The concentrated aroma extracts were kept at -80 °C until use.

Gas chromatography analyses of concentrated aroma extracts

In order to identify odour-active compounds in the concentrated aroma extracts, these were analysed by GC-Olfactometry (GC-O) using a 5890 Series II gas chromatograph (Hewlett Packard, Waldbronn, Germany) provided with an FID detector held at 250 °C. A sample (2 µL) was injected and two capillaries with different polarities were employed: Rxi®-5 Sil MS capillary (30 m, 0.25 mm i.d., 1.0 µm df) non-polar column and a Stabilwax®-DA (30 m, 0.25 mm i.d., 0.25 µm df) polar column, both from Restek (Bellefonte, Pennsylvania, USA). The temperature gradients were set as follows:

40 °C for 2 min, then a rise of 5 °C/min up to 200 °C and 15 °C/min from 200 °C to 300 °C, and then held for 19 min for the non-polar column; 40 °C for 2 min, then rise of 4 °C/min up to 200 °C, then from 200 °C up to 250 °C at 15 °C/min, and then held for 15 min for the polar column. Helium was used as a carrier gas (2 mL/min). The sample was split 1:1 at the end of the column, followed by two untreated silica-fused capillaries of the same dimensions (1 m, 0.32 mm i.d.). An ODO II sniffing port (SGE, Ringwood, Victoria, Australia), where the flow was diluted with a moist make up gas, was utilised. Every sample was analysed by at least 3 assessors in duplicate. The assessors scored the intensity of the aromas perceived on a scale from 1 ("very weak") to 10 ("very strong"). These results were reported as the modified frequency, defined as $MF(\%)=[F(\%)\cdot I(\%)]^{1/2}$, where F(%) is the detection frequency and I(%) is the average intensity expressed as the percentage of the maximum intensity [6].

The concentrated aroma extracts were also analysed by GC-MS using equivalent capillaries and chromatographic conditions as used for the GC-O analyses. The instrument employed for these analyses was a gas chromatograph model 7890A coupled to a 5975C inert XL EI/CI MSD triple axis mass spectroscopy detector and a 7683B Series autosampler (Agilent Technologies, Santa Clara, CA, USA). The carrier gas was helium at a flow rate of 1mL/min. Mass spectra were recorded in the electron-impact mode at an ionisation voltage of 70 eV and source temperature of 200 °C. For this reason, concentrated aroma extracts (basic and acidic fractions) were prepared from AFB-base using the methodology described previously [5].

Results and discussion

The sensomic approach was applied for the identification of key odorants in alcohol-free beer. Recently, this methodology has been applied to identify key flavour compounds in a wide variety of foodstuff and beverages, such as hazelnuts [7] and rapeseed oil [8]. These extracts from the AFB-base were sniffed by GC-O on columns of different polarity and mass spectra were obtained from GC-MS analyses. Twenty-three odour regions were found in total in both basic and acidic fractions from the AFB-base. Table 1 shows the most active odour regions found in the SAFE extracts. Amongst them, the highest MF values corresponded to 2-methoxyphenol, β-damascenone, 5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone, 2-phenylacetic acid, 2-phenylethanol and the Strecker aldehydes methional and phenylacetaldehyde. The presence of these compounds might explain the honey-like, worty aroma of alcohol-free beers brewed by cold contact fermentation. Moreover, two Strecker aldehydes were found to be important: 2-methylbutanal and 3-methylbutanal. These two, along with methional, have been previously reported as contributors to malty and worty aromas in alcohol-free beers [1, 2].

Similar work has been carried out in other beers, such as wheat beer [5] and pale lager beer [4], where higher alcohols and esters were found to be main contributors to the overall aroma. Examples of these are ethyl hexanoate, ethyl butanoate, 3-methylbutyl acetate, and 3-methyl-1-butanol. In our case, no fruity esters were detected by GC-O. This was associated with the mild conditions for cold contact

fermentation process, where yeast was not active enough to synthesise esters throughout the Ehrlich pathway [9]. Butanedione, also found in this study, has been reported as an off-flavour in lager beers [10].

Moreover, in this study we used an alcohol-free beer "base" which was prepared without the addition of external flavours which provide the desirable fruity note which is not generated during cold

Table 1: Odour regions and attributed compounds found by GC-Olfactometry (n=3 in duplicate) in acidic and/or basic fractions of a SAFE extract of an alcohol-free beer-base

LRI					
Rxi-5	**Stabil wax**	**Odour quality[a]**	**Odorant[b]**	**Fn[c]**	**%MF[d]**
579	1000	cream, butter	butanedione	b	80
648	950	malty, cocoa	3-methylbutanal	a	65
664	1429	vinegar	acetic acid	a	76
680	950	cocoa	2-methylbutanal	b	60
725	1225	banana, alcoholic	3-methyl-1-butanol	b,a	44
845	1609	cheese	butanoic acid	a	76
886	1646	cheese, rancid	3-methylbutanoic acid	a	83
917	1470	boiled potato	methional	b,a	91
992	1354	cooked rice	2-acetyl-1-pyrroline *	b	31
1059	1649	rose, honey	phenylacetaldehyde	b,a	95
1103	1872	smoky	2-methoxyphenol	b,a	92
1109	2188	smoky, spicy	3-hydroxy-4,5-dimethyl-2(5*H*)-furanone	a	56
1125	2074	candy floss	5-ethyl-4-hydroxy-2-methyl-3(2*H*)-furanone	a	48
1127	1930	rose, honey	2-phenylethanol	b,a	86
1130	2223	cloves, woody	2-methoxy-4-vinylphenol	b	67
1154	2223	curry, spicy	5-ethyl-3-hydroxy-4-methyl-2(5*H*)-furanone	b,a	89
1180	1983	spicy, smoky	2-methoxy-4-methylphenol	b,a	55
1206	2380	leather	4-vinylphenol	b,a	68
1293	2540	honey, floral	2-phenylacetic acid	a	87
1382	2022	honey, rubber	2'-methoxyacetophenone	b	73
1389	1835	apple, apricot	β-damascenone	b	87
1400	-	hospital, phenolic	unknown.	a	56
1472	2556	vanilla	vanillin	a	73

[a]Odour perceived at the sniffing port of the GC-O.

[b]Compounds were identified by comparison of their mass spectrum and LRI on two columns with those of authentic standards, and confirmed by detection in the extract by GC-MS

[c]Fraction where the compound was found: basic/neutral (b) or acidic (a).

[d]$MF(\%)=[F(\%)\cdot I(\%)]^{1/2}$, where F(%) is the detection frequency and I(%) is the average intensity expressed as the percentage of the maximum intensity

*Tentative identification based on odour description and LRI.

contact fermentation. The addition of external flavours to commercial alcohol-free beers is common practice of brewers to improve the flavour of AFB.

We conclude that the information generated from this study will help in the identification of the less desirable worty notes in alcohol-free beers. Further quantitative and sensory analysis will elucidate the actual role of the key odorants in the overall aroma of these beverages.

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Presentations at international conferences

- **Piornos, J.A.**, Balagiannis, D.P., Koussissi, E., Brouwer, E., & Parker, J.K. Characterisation of the key aroma compounds in alcohol-free beer base by aroma extract dilution analysis. Poster presentation at the XV Weurman Flavour Research Symposium. 18th-22nd September **2017**; Graz, Austria. Best poster award.
- **Piornos, J.A.**, Delgado, A., De la Burgade, R., Methven, L., Balagiannis, D.P., Koussissi, E., Brouwer, E., & Parker, J.K. Determination of orthonasal and retronasal detection thresholds in a model alcohol-free beer: Comparison of calculation methods. Poster presentation at the Eighth European Conference on Sensory and Consumer Research 'Eurosense 2018'. 2nd-5th September **2018**; Verona, Italy.
- **Piornos, J.A**., Methven, L., Balagiannis, D.P., Koussissi, E., Brouwer, E., & Parker, J.K. Quantification of odour active compounds and calculation of their orthonasal and retronasal detection thresholds in alcohol-free beer. Flash poster presentation at the 12th Wartburg Symposium on Flavor Chemistry and Biology. 21st-24th May **2019**; Eisenach, Germany.

Characterisation of the key aroma compounds in alcohol free beer base by aroma extract dilution analysis

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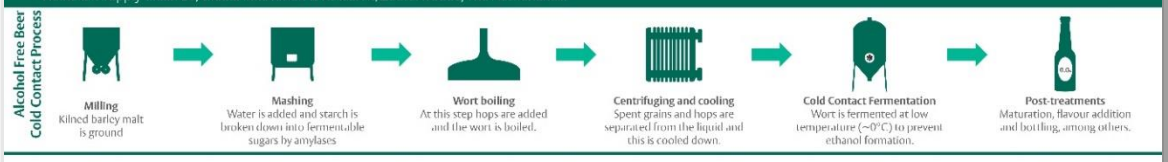


Introduction

Alcohol-free beers brewed by cold contact process usually exhibit a flavour reminiscent of wort and lack fruitiness [1]. Although the addition of flavourings after fermentation is a common practice, the typical "worty" aroma cannot easily be masked. The aim of this research is to identify the compounds contributing to the characteristic aroma of these beers by means of the Sensomics approach.

Materials and methods

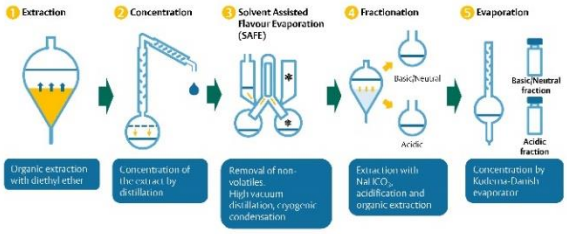
Base beer sample

- Brewed by Cold Contact Process
- Alcohol free product (<0.05%)
- NO external flavours added to "Base" beer
- Pasteurised and bottled

Two fibres-HS-SPME

Volatile compounds were adsorbed/absorbed onto the SPME fibre.
Simultaneous extraction of two fibres :
- **DVB/Carboxen®/PDMS:** wide range of compounds
- **Carboxen®/PDMS:** highly volatile compounds

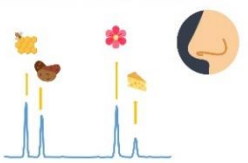
Isolation of volatile compound fractions by SAFE[2]



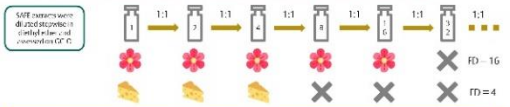
Identification of odour-active compounds

GC-MS and GC-Olfactometry were performed on SAFE and SPME extracts.

Criteria for identification:
- LRI on polar (FFAP) and non-polar (DB-5) columns
- Odour description
- Bibliography
- Agreement with analyses of authentic compounds

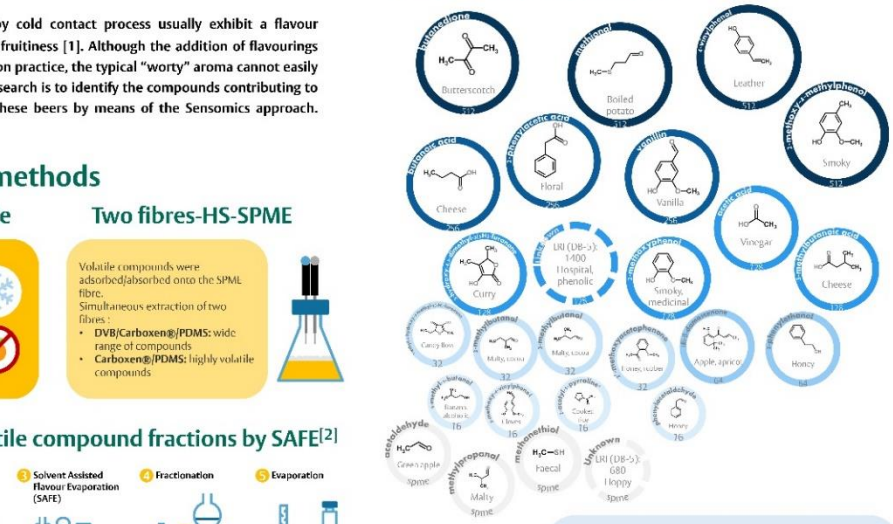


Aroma extract dilution analysis (AEDA)



FD factor for a specific odour region is defined as the highest dilution at which the odour can still be perceived at the sniffing port of the GC-O.

Results and discussion



- 22 odour-active compounds had **FD ≥ 16**; with methional, butanedione, 4-vinylphenol, and 2-methoxy-4-methylphenol amongst the highest.
- 4 additional compounds were found in **SPME-GC-O** experiments.
- **No fruity ester**, typically found in lager and other beers **[2]**, was among these compounds, explaining the lack of fruitiness.
- **Strecker aldehydes** (methylpropanal, 2-methylbutanal, 3-methylbutanal, phenylacetaldehyde, methional) have been reported as contributors to the malty, worty aroma of alcohol free beers [1].
- Identification of 2-acetyl-1-pyrroline is not conclusive.
- Furthermore, 2 compounds remain unidentified.

Conclusions

- The lack of fruity flavour was associated with the absence of odour-active fruity esters.
- Methional was a potential contributor to the "worty" aroma of the alcohol free base beer studied.
- Quantification of these compounds is of great importance to understand their role in the aroma of the product.

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Acknowledgements

- This project is sponsored by Heineken Supply Chain, Global Innovation & Research.
- Icons retrieved from **www.flaticon.com**, authored by Freepik (snowflake), peach, flower, honeycomb), Smashicons (nose) and Madebyoliver (cheese, potatoes).

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Determination of orthonasal and retronasal detection thresholds in a model alcohol free beer: Comparison of calculation methods

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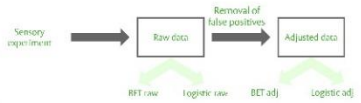
Introduction

The composition of a food matrix, such as ethanol or sugar content, has an impact on the release of flavour compounds, and thus on detection thresholds (DT) [1]. Hence, DTs determined in water or ethanol solutions might not be suitable for alcohol-free beers (AFB).

The aim of this study is to determine detection thresholds of aroma compounds in an artificial AFB-like matrix, as well as to compare the effect of the calculation method on the final threshold value.
To do so, thresholds were calculated using two different methods (Best Estimated Threshold and Logistic regression), from both raw data and adjusted data for the removal of false positives.



Materials and methods

Experimental design:
Six concentrations in ascending order (3 bracketing)

The matrix was composed of a mixture of sugars (40.4 g/L) in carbonated water:
- Glucose (7.2 g/L), fructose (2.1 g/L), maltose (2.50 g/L), sucrose (0.06 g/L), maltotriose (3.6 g/L)

3-AFC (alternative forced choice): 1 sample and 2 blanks per level of concentration.

24 experienced panellists were asked to both sniff and taste the samples.

The panellists' responses were collected using Compusense sensory analysis software.

Calculation methods and data analysis

Best Estimated Threshold (BET)

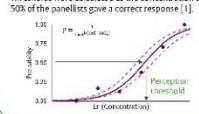
Concentration	1 mg/L	3 mg/L	9 mg/L	27 mg/L
Chosen correctly?	No	No	Yes	Yes

$$\text{BET} = \sqrt[2]{C_n \cdot C_{n+1}} = \sqrt{3 \cdot 9} = 5.19 \text{ mg/L}$$

BET is calculated as the geometrical mean of the concentrations for the higher negative response and the next positive one [2].

Logistic regression

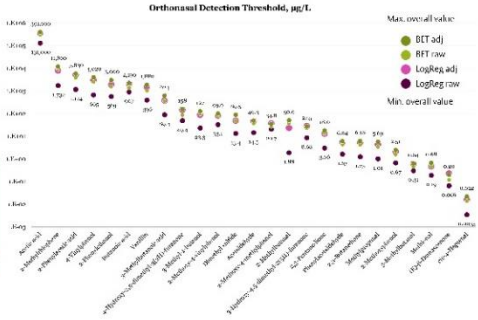
Thresholds were calculated as the concentration at which 50% of the panellists gave a correct response [1].



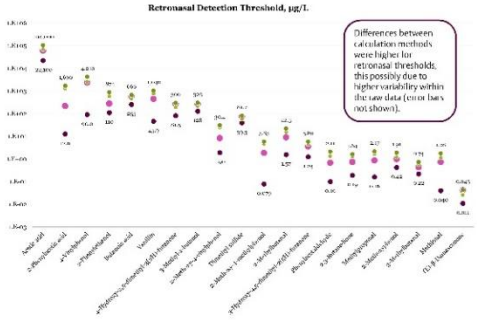
Data adjustment: Removal of False Positives

False positives are those positive responses given by chance and not related to real differences, Hough et al. (2013) reported an algorithm for the removal of these false responses by comparing them with the rest of the panel [3].

Results



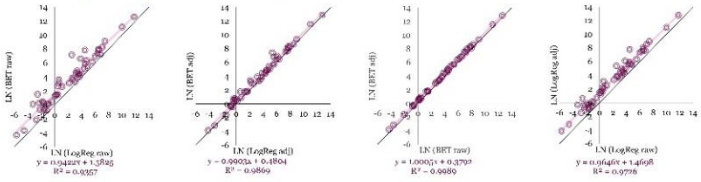
Both orthonasal and retronasal detection thresholds were spread along a 10^6 unit-broad range of values, from hundreds of ng/L for acetic acid to less than 0.2 ng/L for (*E*) β damascenone and cis-4-heptenal.



Differences between calculation methods were higher for retronasal thresholds, this possibly due to higher variability within the raw data (error bars not shown).

Comparison of calculation methods: BET vs. Logistic regression; raw vs. adjusted data

The results from each calculation method (both ortho- and retronasal) were plotted and compared using t-test for paired samples in order to find significant differences between methods.



Using unadjusted data, higher threshold values were obtained from BET than logistic regression (Intercept +1.38).

The same scenario was observed when using adjusted data, although the average difference was lower (Intercept +0.43).

For both BET and Logistic regression, the removal of false positives generated significantly higher threshold values ($p < 0.05$).

Conclusions

- Threshold values were dependent on the calculation method chosen, as well as on the treatment of the data for the removal of false positives.
- Threshold values calculated by BET were higher than those from Logistic Regression, as well as the removal of false positives also increased the final results with respect to the raw data.
- Significant differences were found between both methods (BET or Logistic regression) and data treatment (raw or adjusted data).
- The results from this study will help understand the effect of the calculation method in the final threshold and thus prevent under- or overestimating the potency of aroma compounds.

Contact Information
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References
1. Perry & Hayes, *Foods*, **2016**, *5*, 35
2. ISO 13301:2002
3. Hough, Methven & Faulkner, *J Sens Stud*, **2013**, *28*, 414-421.

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- Icons retrieved from www.flaticon.com, authored by Freepik.

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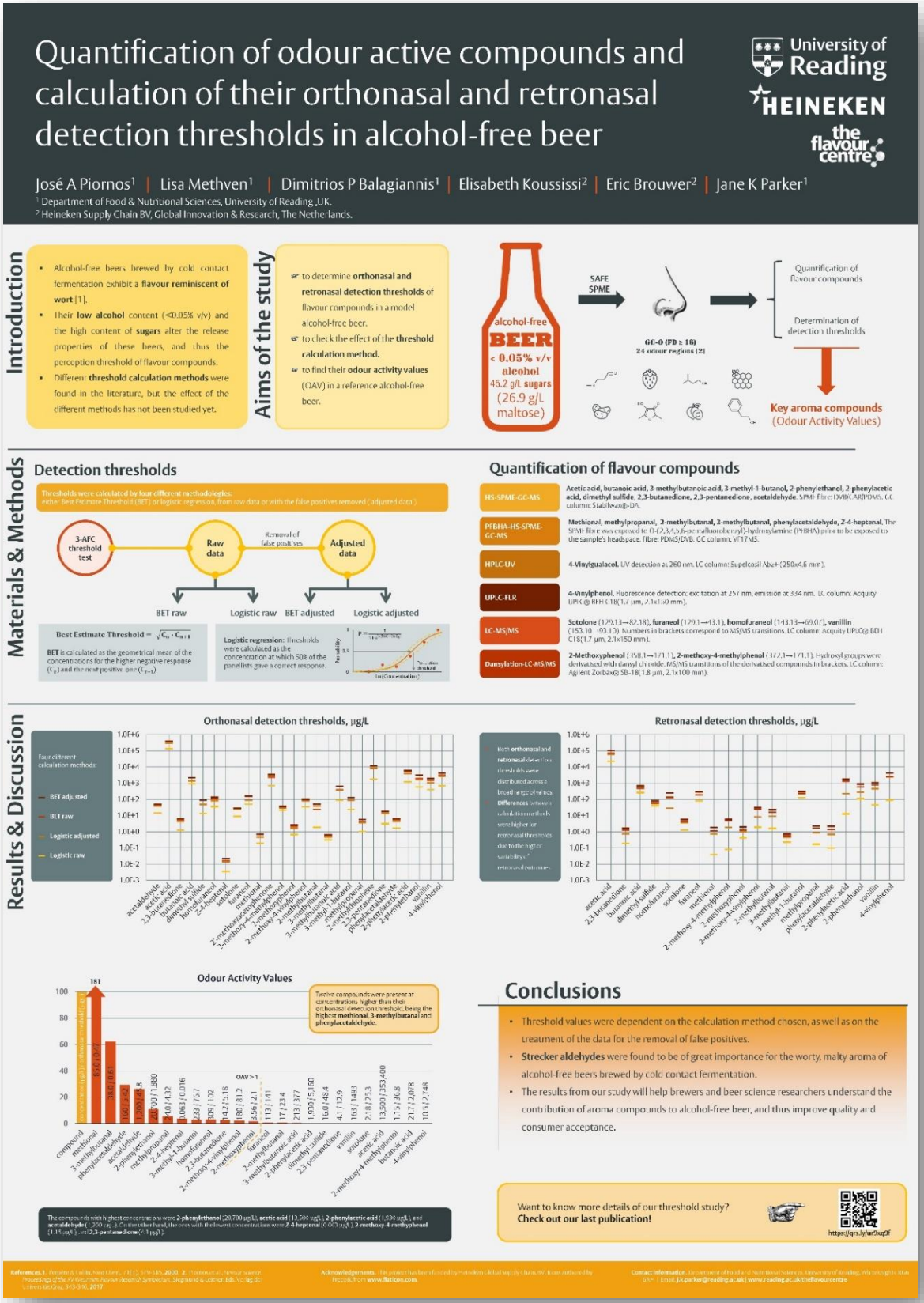
Compusense.


Quantification of odour active compounds and calculation of their orthonasal and retronasal detection thresholds in alcohol-free beer
University of Reading
HEINEKEN
the flavour centre
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Introduction
Alcohol-free beers brewed by cold contact fermentation exhibit a flavour reminiscent of wort [1].
Their low alcohol content (<0.05% v/v) and the high content of sugars alter the release properties of these beers, and thus the perception threshold of flavour compounds.
Different threshold calculation methods were found in the literature, but the effect of the different methods has not been studied yet.
Aims of the study
to determine orthonasal and retronasal detection thresholds of flavour compounds in a model alcohol-free beer.
to check the effect of the threshold calculation method.
to find their odour activity values (OAV) in a reference alcohol-free beer.
alcohol-free BEER
< 0.05% v/v alcohol
45.2 g/L sugars (26.9 g/L maltose)
SAFE SPME
GC-O (FD ≥ 16)
24 odour regions [2]
Quantification of flavour compounds
Determination of detection thresholds
Key aroma compounds (Odour Activity Values)
Materials & Methods
Detection thresholds
Thresholds were calculated by four different methodologies: either Best Estimate Threshold (BET) or logistic regression, from raw data or with the false positives removed ('adjusted data')
3-AFC threshold test
Raw data
Removal of false positives
Adjusted data
BET raw
Logistic raw
BET adjusted
Logistic adjusted
Best Estimate Threshold = $\sqrt{C_n \cdot C_{n-1}}$
BET is calculated as the geometrical mean of the concentrations for the higher negative response (C_n) and the next positive one (C_{n-1})
Logistic regression: Thresholds were calculated as the concentration at which 50% of the panellists gave a correct response.
Probability
Ln(Concentration)
Quantification of flavour compounds
HS-SPME-GC-MS
Acetic acid, butanoic acid, 3-methylbutanoic acid, 3-methyl-1-butanol, 2-phenylethanol, 2-phenylacetic acid, dimethyl sulfide, 2,3-butanedione, 2,3-pentanedione, acetaldehyde. SPME fibre: DVB/CAR/PDMS. GC column: Stabilwax®-DA.
PFBHA-HS-SPME-GC-MS
Methional, methylpropanal, 2-methylbutanal, 3-methylbutanal, phenylacetaldehyde, 2-4-heptenal. The SPME fibre was exposed to O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine (PFBHA) prior to be exposed to the sample's headspace. fibre: PDMS/DVB. GC column: VF17MS.
HPLC-UV
4-Vinylguaiacol. UV detection at 260 nm. LC column: Supelcosil Abz+ (250x4.6 mm).
UPLC-FLR
4-Vinylphenol. Fluorescence detection: excitation at 257 nm, emission at 334 nm. LC column: Acquity UPLC @ BEH C18(1.7 µm, 2.1x150 mm).
LC-MS/MS
Sotolone (129.13→82.18), furaneol (129.1→43.1), homofuraneol (143.13→69.07), vanillin (152.10 →93.10). Numbers in brackets correspond to MS/MS transitions. LC column: Acquity UPLC@ BEH C18(1.7 µm, 2.1x150 mm).
Dansylation-LC-MS/MS
2-Methoxyphenol ([M+H]+ 318.1→171.1), 2-methoxy-4-methylphenol ([M+H]+ 332.1→171.1). Hydroxyl groups were derivatised with dansyl chloride. MS/MS transitions of the derivatised compounds in brackets. LC column: Agilent Zorbax® SB-18(1.8 µm, 2.1x100 mm).
Results & Discussion
Orthonasal detection thresholds, µg/L
Four different calculation methods:
BET adjusted
BET raw
Logistic adjusted
Logistic raw
1.0E+6
1.0E+5
1.0E+4
1.0E+3
1.0E+2
1.0E+1
1.0E+0
1.0E-1
1.0E-2
1.0E-3
acetaldehyde
acetic acid
2,3-butanedione
butanoic acid
dimethyl sulfide
homofuraneol
2-4-heptenal
sotolone
furaneol
methional
2-methoxy-4-ethylphenol
2-methoxy-4-methylphenol
2-methoxy-4-vinylphenol
2-methylbutanal
3-methylbutanal
3-methyl-1-butanol
3-methylbutanoic acid
2-methylpropanal
2-methylbutanone
2,3-pentanedione
phenylacetaldehyde
2-phenylacetic acid
2-phenylethanol
vanillin
4-vinylphenol
Retronasal detection thresholds, µg/L
Both orthonasal and retronasal detection thresholds were distributed across a broad range of values.
Differences between calculation methods were higher for retronasal thresholds due to the higher variability of retronasal responses.
Odour Activity Values
Twelve compounds were present at concentrations higher than their orthonasal detection threshold, being the highest methional, 3-methylbutanal and phenylacetaldehyde.
OAV > 1
compound
methional
3-methylbutanal
phenylacetaldehyde
acetaldehyde
2-phenylethanol
methylpropanal
2-4-heptenal
3-methyl-1-butanol
homofuraneol
2,3-butanedione
2-methoxy-4-vinylphenol
2-methoxyphenol
furaneol
2-methylbutanal
3-methylbutanoic acid
2-phenylacetic acid
dimethyl sulfide
2,3-pentanedione
vanillin
sotolone
acetic acid
2-methoxy-4-methylphenol
butanoic acid
4-vinylphenol
The compounds with highest concentrations were 2-phenylethanol (20,700 µg/L), acetic acid (13,300 µg/L), 2-phenylacetic acid (1,030 µg/L), and acetaldehyde (200 µg/L). On the other hand, the ones with the lowest concentrations were 2-4 heptenal (0.063 µg/L), 2-methoxy-4-methylphenol (1.15 µg/L), and 2,3 pentanedione (4.1 µg/L).
Conclusions
Threshold values were dependent on the calculation method chosen, as well as on the treatment of the data for the removal of false positives.
Strecker aldehydes were found to be of great importance for the worty, malty aroma of alcohol-free beers brewed by cold contact fermentation.
The results from our study will help brewers and beer science researchers understand the contribution of aroma compounds to alcohol-free beer, and thus improve quality and consumer acceptance.
Want to know more details of our threshold study?
Check out our last publication!
https://qrs.ly/ur5wq9f
References.1.
Acknowledgements.
Contact information.

José Antonio Piornos Martínez

- PhD thesis -

July 2020