Stability of beer through control of minerals in sweet wort

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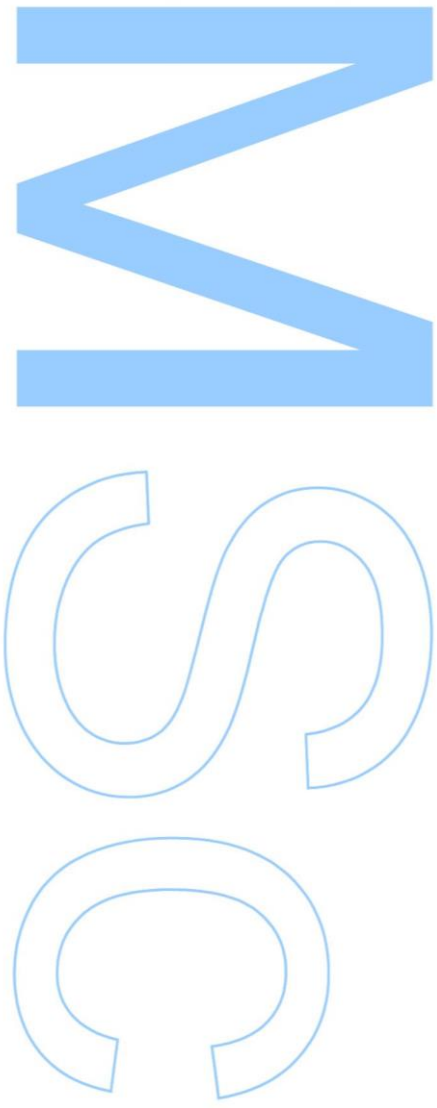

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Resumo

A produção de cerveja é um processo complexo que combina métodos empíricos e ciência. Consiste num antigo processo biotecnológico no qual os passos unitários se mantêm inalterados há centenas de anos. Desta forma, o estudo científico na indústria cervejeira não só garantiu que a produção da cerveja moderna seja altamente controlada, com elevado grau de consistência e qualidade como muitas outras industrias de produção de bebidas fermentadas.

Sabe-se que a concentração iónica em metais pode ter um impacto negativo na produção de cerveja. Apesar de alguns minerais serem essenciais durante os passos de maceração e fermentação, é atribuído ao cobre e ferro, de forma geral, um impacto negativo na estabilidade oxidativa da cerveja. Metaais de transição como o ferro e o cobre têm a capacidade de catalizar a formação de espécies reativas de oxigénio na presença e ausência de compostos orgânicos, contribuindo deste modo para a oxidação durante a produção de cerveja. A reação Haber-Weiss, correlacionada diretamente com a reação Fenton, é considerada o principal mecanismo através do qual se dá a formação do radical hidroxil, na maioria dos sistemas biológicos.

O malte é a principal fonte de compostos iónicos metálicos, sendo que a transferência dos mesmos se dá maioritariamente durante a maceração. Estudos indicam que a composição iónica do mosto doce depende principalmente da taxa de remoção dos minerais durante o processo de maceração e filtração. Para além disto, sabe-se que a torrefação do malte aumenta o teor de ferro e diminui o de cobre no mosto doce. O principal objetivo deste trabalho foi a identificação e análise da influência da torrefação do malte e do teor de ferro e cobre na estabilidade oxidativa do mosto doce, de forma a prever a estabilidade da cerveja final. A importância da quantificação de ferro e cobre no mosto baseia-se na possibilidade do estabelecimento de uma ligação entre a estabilidade oxidativa da cerveja e o tempo de prateleira.

O conteúdo em metais nas amostras de mosto foi determinado por espectrometria de emissão atómica por plasma acoplado indutivamente. A estabilidade oxidativa foi avaliada por espectroscopia de ressonância de spin eletrónico e através da taxa de consumo de oxigénio.

Malte to tipo *pilsner* de um único lote foi torrado a 125ºC e 190ºC. Amostras de mosto doce foram produzidas a partir de malte não torrado e de malte torrado de acordo com as temperaturas mencionadas. Sendo que 50µM de ferro e cobre foram adicionados no início

da empastagem e comparados com as amostras em branco. Posteriormente, ao malte *pilsner* não torrado foi adicionado 10% de cada tipo de malte torrado e 10% de *pilsner* foi adicionado a malte torrado a 190ºC. Tendo sido também analisado o conteúdo em metais e estabilidade oxidativa dos mostos doces resultantes.

Os resultados deste trabalho demonstram uma forte e significativa remoção de ambos os metais durante a empastagem e filtração, sendo removidos essencialmente durante a filtração. A remoção do ferro diminuiu significativamente com o aumento da temperatura de torrefação e o efeito oposto foi verificado para o Cu ($p<0.05$).

Um decréscimo na estabilidade oxidativa foi relacionado a torrefação do malte ($p<0.05$) e aumento do conteúdo em ferro ($p<0.05$), mas não se tendo verificado os mesmos resultados para o cobre.

O plano de mistura selecionado não afetou significativamente a remoção dos metais comparando com as amostras puras. No entanto, verificou-se um aumento significativo da estabilidade oxidativa ($p<0.05$) com a adição de 10% de malte pilsner a malte torrado a 190ºC.

Abstract

Brewing beer is a complicated process, combining craft and science. This is an ancient biotechnological process, in which the unit processes have not changed in hundreds of years. Equally, scientific study within the brewing industry not only has ensured that modern beer making is highly controlled, leading to highly consistent, high-quality, healthful beverages, but also has informed many other fermentation-based industries.

Metal ion concentrations affect the success of beer brewing. Although the presence of some minerals is crucial during mashing and fermentation, copper and iron generally have a negative impact on the oxidative stability of the final beer. Malt is the biggest source of metal ions and therefore, the main uptake of iron and copper ions happens during mashing. Past research has indicated that the ionic composition of sweet wort primarily depends on the ion removal rate during mash filtration and that malt modification by roasting increases the sweet wort levels of Fe and decreases Cu. Transition-metals like Fe and Cu are catalysts in radical generation and oxidation during beer and wort aging. The Haber–Weiss reaction, which makes use of Fenton chemistry, is considered to be the major mechanism by which the highly reactive hydroxyl radical is generated in biological systems.

The main purpose of this research project is the identification and measurement of the influence of malt roasting and metal content on the oxidative stability of sweet wort in order to predict oxidative stability of the final beer. The importance of the quantification of iron and copper in wort comes from the possibility to establish a correlation between the oxidative stability of beer and its shelf life.
The metal content was determined by inductively coupled plasma optical emission spectroscopy and the oxidative stability by electron spin resonance (spin trapping) as well as by measuring the oxygen consumption of the sweet wort samples.

Pilsner malt from a single batch was roasted at 125ºC and 190ºC. Sweet wort samples were produced from the three types of malts and 50µM of iron and copper were added right at the beginning of the mash and compared to the blanks. The three types of malts were also mixed in a realistic approach and the same parameters were evaluated. 10% of roasted malts were added to untreated pilsner malt, respectively. Finally, 10% of pilsner malt was added to 190ºC roasted malt.

The results of this project show a strong levelling effect for both metals during mash and filtration. The removal of Fe during mash decreased significantly with the increase of roasting temperature and the opposite effect was verified for Cu.

The decrease of oxidative stability was related to the increase of roasting temperature and increase in iron content.

Mixing the malts did not significantly influence the levelling effect for both metals, comparing to the pure sweet wort samples.

Finally, the addition of roasted malts did not affect the oxidative stability comparing to pure pilsner wort. However, the addition of 10% of pilsner significantly ($p<0.05$) increased the oxidative stability of the wort when comparing to 190ºC sweet wort.

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List of abbreviations and symbols

BSG – Brewer spent grains

CAS - Chemical Abstracts Service

ESR - electron spin resonance

M – metal

MR – Maillard Reaction

MRP – Maillard Reaction Products

OC – Oxygen consumption

POBN - α-(4-Pyridyl 1-oxide)-N-tert-butylnitrone

ppb – parts per billion

ppm – part per million

1. Introduction

I. State of the art

i. Beer

ii. Historical Framework

iii. Beer production

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v. Types of beer

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State of the art

Beer

Nowadays, beer consumption ranks first in Europe, among alcoholic beverages, slightly above wine consumption, according to the World Health Organization[1]. The definition of beer is ambiguous, though it is mostly defined as a fermented aqueous drink made from starch (usually malted grains) and hops. The production of beer involves three consecutive biochemical processes: the formation of enzymes in the germinating grain, the breakdown of starch to sugar by these enzymes and the fermentation of the sugar to alcohol and CO_2[2]. According to the *Article 1 of Decree-Law no. 93/94 of 7 April (Portugal),* beer is a drink *"obtained by alcoholic fermentation, by means of yeasts selected from the genus Saccharomyces"* and the wort made *"from malt of cereals, mainly barley, and other starchy or sugary raw materials" ("Ordinance No. 1/96", 1996)*[3]*.* Actually, *beer* is a generic term of a wide range of drinks with distinct organoleptic characteristics. Besides the unavoidable overlap of some raw materials, various drinks called beer can distinctly grow apart from the original concept.

Historical Framework

Archaeological findings on ancient fragments of broken pottery show that Chinese villagers brewed fermented alcoholic drinks as far back as 7000 BC, breaking the previous record for the oldest evidence of brewing, found in Iran and dated at about 5400 BC[4].

The history of beer is intimately connected with the evolution of Humanity. From the moment nomad populations had started to settle, domesticate animals and store cereals and plants, the discovery of fermentation was unavoidable. When realizing the benefits of such apparently ‘spontaneous’ phenomena, men developed techniques to reproduce it[5].

After their discovery, fermented drinks took a privileged place in civilization’s economy, culture and social events. The Babylonian civilization developed the art of fermenting cereals, specially barley and Farro wheat *(Triticum turgidum var. Dicoccum),* to the point of producing around twenty different types of beer, following strict regulations on techniques and raw materials described in the *Hammurabi Code* (manuscript that compiled rules and laws of the Babylonian civilization during the reign of the king Hammurabi). Egyptian civilization is another example of an ancestral society where beer played a crucial role as an essential part of people’s diet, in religious celebrations and medicine.

The culture and technology of cereal fermentation had expanded from Mesopotamia to the Mediterranean Europe during the 3th century[5] . In the Romanian civilization it was barely consumed in comparison to wine, essentially for cultural and ideological reasons, as it was seen as the Barbarian's drink, who occupied at the time the North and East regions of Europe[6]. After the fall of the Romanic Empire, beer became the most consumed drink in Europe due to the occupation of the Germanic tribes. During this period and until the 12th century beer production was exclusively produced in monasteries, being also used as currency trading. From the 16th century, monks started to add hops to beer a practice that prevails until nowadays[6].

On the 16th century the high demand of beer in the big urban centers burst the specialization of procedures and techniques of brewing to an industrial scale. The commercial position of the brewer became established, but it was subjected to municipal laws from purchase of raw materials to the manufacture of the final product and its sale. These laws also included regulations about the price and quality of the yeast, which primarily looked after the interests of the bakers, who obtained their yeast from the brewers[2]. Meanwhile shortages of raw materials as a result of poor harvests and other circumstances, led to the use of raw materials other than those previously customarily employed. Thus hops were often replaced by other flavouring plants. Cereals for bread making or cheaper oats were also used in the grist for brewing. There was even a health risk from some of the hop replacements. In 1516 the *Reinheitsgebot or Bavarian Purity Law* was signed by the jointly ruling Dukes Wilhelm IV and Ludwig X. It was established that beer could be produced only from barley, hops and water. This law was enacted in the interest of consumer protection, regulating the manufacturing of the product and establishing the price in relation to the product quality. The Purity Law can therefore be described as the first consumer protection law in the world[2, 7].

In the 19th century, the Industrial Revolution reinforced the technology and specialization of brewing procedures, introducing more efficient production methods. Also during this period is created the first *Lager and Pilsner* by the brewer Josef Groll in *Plzen* (Czech Republic), 1842[8]. During the mid-nineteenth century, a new science – Microbiology - was discovered which dramatically contributed to the understanding of the alcoholic fermentation, demonstrated by *Louis Pasteur* between 1855 and 1875, when the role of yeast in the brewing process was understood. A few years later *Emil Hansen* isolated yeast strains for the first time, establishing one of main processes in the food and particularly the brewing industry, which is also an important scientific field[2, 8].

Beer production

Beer has four basic ingredients: water, malt, hops and yeast. These elements are common to all types of beer[2]. Different mixtures and combinations of these components are common in the brewing industry, being in fact a crucial determinant in the differences and definition of types of beer.

Figure 1 depicts simplified scheme of the brewing process emphasizing the inputs of the raw materials and the outputs in each stage.

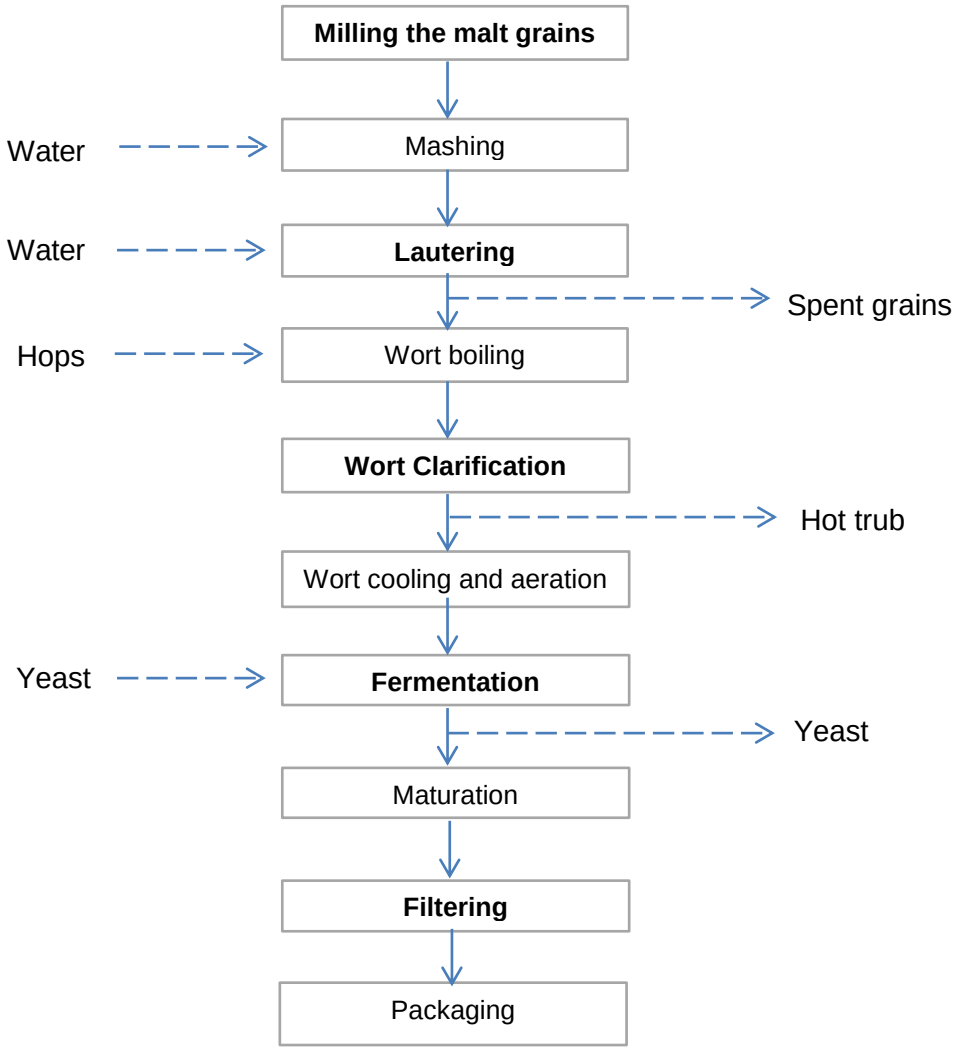

Figure 1. Simplified scheme of the brewing process. Own elaboration based on Bamforth, C. W., Brewing Materials and Processes A Practical Approach to Beer Excellence. Academic Press. Elsevier, 2016.

Raw materials

Water

Beer contains about 91-92% of water and it's by far the most important component of a beer[2]. Diverse chemical and mineralogical characteristics lead to different types of beer; consequently, these parameters have to be adjusted according to the type of beer desired. The content of dissolved salts and the water's pH are the most important parameters to evaluate due to the direct influence on the chemical balance through all the brewing processes[9].

Malt

Beer production permits the use of diverse types of cereals (malted or not), although barley *(Hordeum vulgare, vulgare L.)* is unique in brewing for sensorial, technological and even historical reasons. Compared to other cereals, the germination process is easier to control. The malting process allows the grain to partially germinate, releasing amylolytic and proteolytic enzymes from the aleurone layer. These enzymes are responsible to convert starch into fermentable sugars that will be used by the yeast[7]. Enzymes such as ß-amylase, exo-peptidase and carboxy-peptidase are present in the starchy endosperm of the barley, and other enzymes, such as β-glucanase, endo-proteases, α-amylase and pentosanases are formed in the aleurone layer of the barley during malting. The formation and activation of these enzymes is promoted by the increasing moisture and oxygen during the steep. Malting begins with the hydration of barley grains. During *steeping*, (immersing the grains in water) the grain moisture content increases from around 12% to 40-45%[6]. It is followed by *germination* during which malt enzymes develop and changes occur on the grain's structure. The final stage of malting is roasting or *kilning* reducing the grain moisture content and interrupting the germination. This process can be conducted in different ways, leading to diverse types of malt and subsequently different beers[5].

Malt influences beer color, aroma and flavour. These characteristics are mainly developed during kilning/roasting – the last phase of the malting process. During roasting are featured, among other reactions: Maillard reactions, caramelization, and degradation of proteins, lipids and phenolic compounds[2, 10]. Depending on the time/temperature applied during kilning, malt can be classified in two groups: pale (lager) or dark (speciality)[10]. The purpose of the pale malt (>95%) in the recipe is to provide diastatic enzymes and fermentable sugars. By itself, however, it does not provide the brewer with enough variability to produce all beer styles[11]. Therefore, in order to provide different flavor profiles, the brewer uses specialty malts (<5%). Specialty malts are made in many different ways and the selection of the grains used and its

quantity is made in accordance with the desired type of beer. They can be considered into two groups; those that are prepared by a simple heating process, such as *amber, diamber, brown, chocolate and black malts*, and *crystal* and *caramel* malts in which the wet malts are *stewed* so that the endosperm contents are liquefied before they are dried and cooked[5].

Adjuncts

Adjuncts are materials, other than malt, that are sources of extract. These are less expensive and can also be used to impart desirable characteristics to the beer. Although a wide range of brewing raw materials fall within this definition, the most common are: solid unmalted raw materials (e.g. corn or rice), liquid adjuncts (syrups) and other than barley malted cereals, , such as wheat and sorghum.[12]

Hops

The hop plant (*Humulus lupulus*) is a climbing, herbaceous perennial, from the *Cannabaceae* family[7]. Hop resins, from the lupulin glands, contain alpha and beta acids plus a variety of chemical compounds. Their effect on the final taste of the beer depends on the relative proportion of compounds and the length of time these compounds are allowed to boil in the wort[13]. Hops are used primarily as a flavouring, stability and antimicrobial agent in beer, to which they impart bitter, zesty, or citric flavours[2]. Hops antimicrobial properties have been associated to its extracts as humulone, lupulone and xanthohumol. These compounds have shown antibacterial properties, antibiofilm activity in vitro and anti-infective agent of virus[14].

Yeast

Technically a *fungus*, yeast is a single-celled eukaryotic organism with a significant impact on the final flavour. It is responsible for the anaerobic conversion of fermentable sugars in wort to ethanol and carbon dioxide. Furthermore, yeast also yields an extensive array of other compounds that will influence the beer's flavour and decrease its pH[2]. Brewing yeasts can be generally divided into "top-fermenting" and "bottom-fermenting." The most common strain of top-fermenting yeast is *Saccharomyces cerevisiae*. It is most active at a temperature between 15 and 21 °C (commonly used for *ales*). *Saccharomyces pastorianus* is a bottom-fermenting yeast – also known as *lager yeast* – and , unlike *S. cerevisiae*, remains active at lower temperatures, as low as 4 °C[7].

Types of Beer

Beer styles are defined based on the nature of raw materials, type of yeast used and also the country or region where it is produced. Moreover, the beer style cannot be dissociated from the historic factor, techniques and base ingredients. Table 1 contains eight of the most popular.

Table 1. Classic types of beer. Adapted from Strong, 2015

Beer style	Origin	Type of fermentation	ABV [alcohol by volume]	Description
Pilsner	Bohemia (Czech Republic)	Low	5%	Lager. Pale and hoppy. Produced with mild water.
Bock	Einbeck (Germany)	Low	5,5-7,5%	Dark brown. Mild hoppy flavour.
Stout	Ireland	High	3-7%	Strong hoppy flavour, black and dark brown degradé colour.
Lager	Czech Republic	Low	3-5%	Characterized for being fermented and maturated at low temperatures (0-6ºC). It may be pale, golden, amber, or dark.
Saison	Wallonia (Belgium)	High	5,5-6%	Highly carbonated, fruity, spicy, Cloudy golden flavour.
Weiss	Bavaria (Germany)	High	4,0-5,6%	Light hop flavour. Phenolic flavour.
Indian Pale Ale	UK	High	5,5-7,5%	Strong hop bitterness, and moderate grapefruit and pine flavours.
Alt	Germany	High and Low	4-5%	Red/Copper colour. Hop, flower and spicy flavour.

Brewing Process

Due to its high complexity in chemical and technical terms, only the fundamental processes of brewing are summarized in this sub-chapter. The brewing process can be divided into four main stages: mashing, boiling, fermentation and bottling[6], Figure 2.

1. **Mashing**
    a. Milling
    b. Mashing
    c. Lautering

2. **Boiling**
    a. Clarification
    b. Cooling down

3. **Fermentation**
    a. Fermentation
    b. Maturation (and colloidal stabilization)

4. **Bottling**
    a. Filtering
    b. Packaging
    c. Pasteurization
    d. Labelling
    e. Conditioning

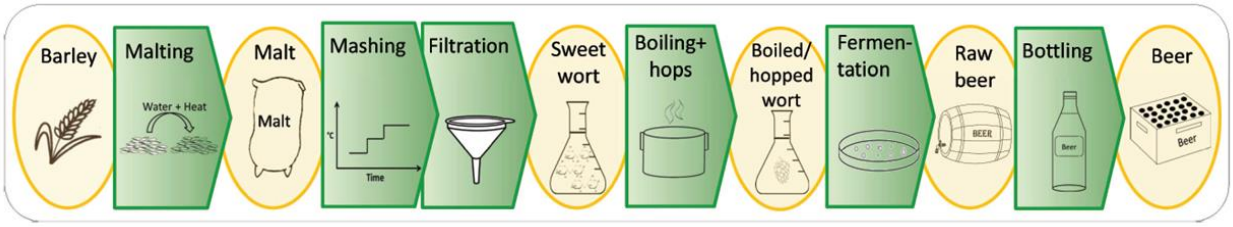

Figure 2. Simplified scheme of the brewing process.

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Before milling, grain is screened and aspirated to remove impurities and `thin' corns. The main purpose of milling is to expose the endosperm, enabling an easier solubilisation and hydrolysis of the starch. Afterwards milled malt and solid adjuncts are mixed with hot water to form the mash[5].

Mashing process

Mashing is the first biochemical process step of brewing and completes the enzymatic degradation procedures started during malting. The mashing process is of highest technological relevance for all following processes of wort and beer manufacturing. The process of mashing relies on the different biochemical characteristics of the enzymes involved. Mashing is performed over different time/temperature frames, according to the various types of enzymes in action and their optimal activation temperature. During this stage occurs the conversion of starch molecules into fermentable sugars (mainly glucose, maltose and maltotriose) and dextrines. The principal enzymes responsible for this conversion are *α-* and *β-amylases, β-glucanases* and *proteases*, creating a *liquor* of mixed sugars, peptides and amino acids[5].

The enzyme *α-amylase* is largely responsible for the breakdown of starch into lower molecular weight sugars and dextrins. *β-amylase* has a limited effect on starch, though it rapidly breaks down dextrins to form the fermentable sugar maltose and maltotriose[8]. Another important difference between these enzymes is their thermal and biochemical stability, Table 2.

Table 2. pH and temperature optima of some barley enzymes in mash. Adapted from Helinger, 2009

Enzyme		**Temperature *optima* (ºC)**	**pH *optima***	**Substrate**	**Product**
Cytolysis	β-glucan solubilase	62-65	6.8	matrix bound β-glucan	Soluble high molecular β-glucan
	Endo-1-3-β-glucanase	<60	4.6	Soluble high molecular β-glucan	Low molecular β-glucan, cellobiose
	Exo-β-glucanase	<40	4.5	cellobiose	glucose
Proteolysis	endopeptidase	45-50	3.9-5.5	proteins	Peptides, free amino acids
	carboxypeptidase	50	4.6-4.8	Proteins, peptides	Free amino acids
	aminopeptidase	45	8.8	dipeptides	Free amino acids
Amylosys	α-amylase	65-75	5.6-5.8	High molecular and low molecular α-glucans	Melagosaccharides, oligosaccharides
Other	β-amylase	60-65	5.4-5.6	α-glucans	Maltose

enzymes	maltase	35-40	6.0	maltose	glucose
	Limit dextrinase	55-65	5.1	Limit dextrin	Dextrins
	lipase	55-65	6.8-7.0	lipids	Glycerine, free long-chain fatty acids
	peroxidase	45-55	6.5-7.0	Organic and inorganic susbtrates	Free radicals

The wort composition is essential for beer quality, and a number of factors influence the mashing process and, thus, wort composition and properties. In addition to temperature and pH there are other factors, such as ionic strength, water composition, grist composition, pH, mash thickness, dissolved oxygen, among other[15]. Another key parameter when discussing the properties of the sweet wort (and eventually the finished beer) is the mash profile (i.e., temperatures and time).[15] Each of these physical and chemical parameters must be considered and optimized in order for an enzymatic reaction to be accurate and reproducible.

The purpose of mashing is economically to prepare wort of the correct composition, flavor and color in the highest practical yield and in the shortest time. There are two basic schemes for mashing[5]: Single Temperature - a compromise temperature for all the mash enzymes, and Multi-Rest- where two or more temperatures are used to favor different enzyme groups. The mash can be heated in two ways: by the addition of hot water (Infusion) or by heating the mash tun directly. There is also a combination method, called Decoction Mashing, where part of the mash is heated on the stove and added back to the main mash to raise the temperature[2]. All of these mashing schemes are designed to achieve *saccharification*. Though, the route taken to that goal can have a considerable influence on the overall wort character[9, 15].

Today, most malts are well modified and respond well to a single infusion mash when an all-malt beer is being brewed. The modification of the malt refers to the degree to which cell walls and the protein/carbohydrate matrix in the endosperm is broken down during malting[16]. The lesser modified the malt is, the more needs to be done in the mash. This is where decoction mashes and step mashes came from. Nevertheless, nowadays it is hard to come by malt that is less than well modified[7]. The mash temperature of a single infusion mash is almost always in the 64–72 °C range. Step infusion mashes, in which the mash is rested at two or more temperatures. A common single step mash is one with a rest in the beta-glucanase range 45–50 °C followed by a rest in the saccharification range 64–72 °C[2].

After mashing it is necessary to clear the sweet wort from the insoluble matter (rich in protein, fatty material and polyphenols) that will not be used by the yeast in the fermentation step.

The mash filtration process in a brewery's brewhouse is a central process step, whereby wort and spent grains are separated in two phases: the first one involves the run-off of the wort in a separation or filtration process. During the second phase, the remaining wort in the spent grains and the embedded sugar molecules are then washed out using hot brewing water: a process known as *sparging*. The objective of lautering is to achieve a high yield with low sparging water in the shortest possible time. For this task, breweries have been predominantly using *lauter tuns* or *mash filters*. The decisive reason is the type of grinding, as mash filters offer the option to filter mashes from the finest ground raw materials. This is not possible with the lauter tun, because in this case, the husks have to form the necessary depth filter layer[12].

During *lautering*, the insoluble material is allowed to settle in the mash tun to be used as filter bed. The sweet wort is filtered through it and the residual fraction obtained is called brewer's residual spent grains (BSG) (usually used for animal feed), Figure 3.

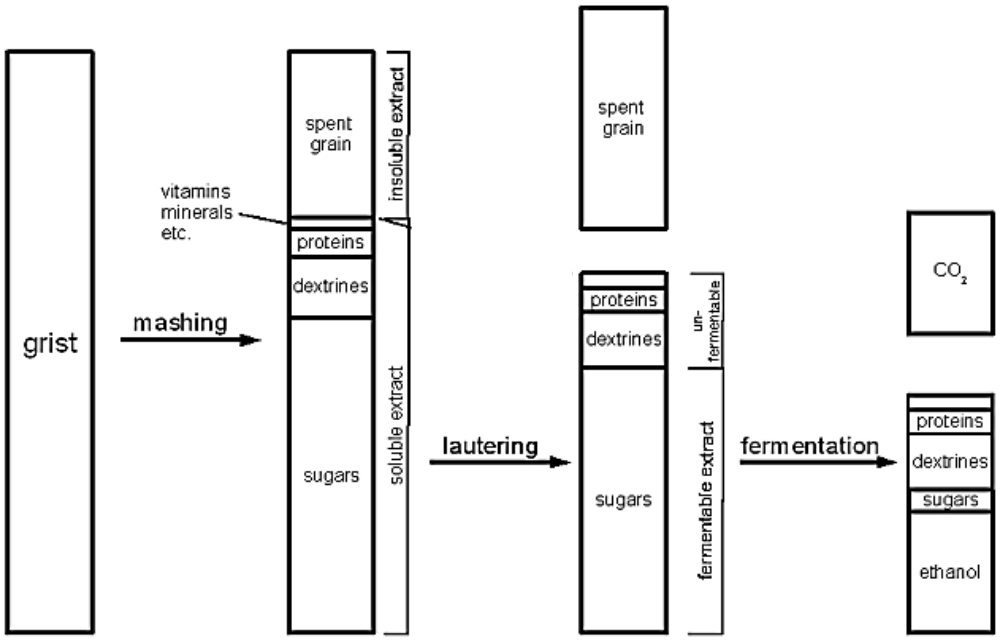

Figure 3. Simplified scheme of the brewing process, focusing on the main products of each step. (Mosher, 2017)

BSG is basically a lignocellulosic material, the major constituents of which are fibre (20-25% hemicellulose and 12-25% of cellulose), protein (19-30%) and lignin (12-28%)[17]. It contains vitamins[17] and high amounts of minerals, such calcium, phosphorus, copper, iron and manganese[18]. BSG contains high levels of phytic acid, a strong chelating agent and binds

copper, iron and zinc ions as well as calcium ions[17]. Other mechanism that might be responsible for the adsorption capacity of BSG to metals is the presence of ester and carboxyl groups suitable for metal ion adsorption. Ionic exchange affinity increase with ionic valence and at the same charge value the cation with larger ionic radius is preferentially adsorbed. For example, the *Pauling ionic radius* of Cu^{2+} and Fe^{2+} is respectively 1.86 and 2.08 Å[19]. This general trend may be explained by the fact that the metal ions with smaller ionic radius diffuse faster in aqueous systems. This implies that ion with smaller ionic size would easily interact with the biomass resulting in a higher surface coverage than for ions of larger sizes. Naturally Cu^{2+} has a better affinity to the BSG surface, though deviations may occur due to the different ability of the complex formation of these metal ions[20].

After lautering, sweet wort is characterized by being viscous, sweet, dense and coloured[21]. Substances present include simple sugars, dextrins, glucans, pentosans, phosphates, dissolved inorganic ions, proteins, peptides and amino acids, nucleic acid breakdown products, lipids, vitamins, organic acids, bases and phenolic substances.[2] A typical sweet wort may contain solids consisting of about 90-92% carbohydrates, 4-5% nitrogen-containing substances and 1.5-2% ash[21].

Then, sweet wort is boiled in the brewing kettle from 60 to 90 minutes with the addition of hops. Boiling aims to sterilize the extract and solubilize aromatic molecules, precipitate proteins and tannins, evaporate unwanted flavor substances like DMS (dimethyl sulphides) and adjust the extract content in wort[6].

At the end of the boiling wort contains trubaceous matter (hot break) and suspended fragments of hops. For separation of these residues, it is transferred to a kettle called *Whirlpool*. Afterwards, cooled and aerated wort has to be mixed rapidly with yeast (pitching) in order to prevent proliferation of bacteria[12].

The principal role of brewing yeast during fermentation is to produce ethanol, carbon dioxide and other flavor-active compounds.

Traditionally, ales are fermented with *top yeasts* which rise to the top of the beer in the head of foam. These are pitched at about 16 ºC and fermentation is carried out at 15-20 ºC for 2±3 days. Traditional lagers are fermented with *bottom yeasts*, which settle to the base of the fermenter. These are pitched at lower temperatures (e.g., 7-10 ºC) and fermentations are also carried out at lower temperatures (e.g., 10-15 ºC). Consequently, they take longer than ale fermentations. Yeasts are selected with reference to: their rate and extent of growth; the rate and extent of fermentation; the *flavor* and aroma of the beer produced.

Fermentation conditions of each brewery have to be carefully monitored in order to avoid contaminations and obtain the desired metabolites and by-products. Also, fermentation is

dependent upon the composition of the wort in carbohydrates, amino acids, free fatty acids, and trace minerals such as zinc, manganese and calcium[2].

The *green* or immature beer yield from the *primary* fermentation is stored for a period of maturation or secondary fermentation. During this process the flavor of the mature beer is refined. At this stage there's an adjustment of undesirable compounds such as ketones, hydrogen sulfide, acetaldehyde and diacetyl. Maturation is usually carried out at low temperatures in order to favor precipitation of flocculated yeast, proteins and phenolic complexes for colloidal stabilization. The main goal of this step is to achieve microbiologic, physical and sensorial stabilization of beer. The length of the period required for maturation is also directly related to the type of beer.[2, 7].

After conditioning in bulk, most beers are filtered or centrifuged to remove residual yeast, proteins and polyphenols. It should be performed at low temperatures to avoid colloids redissolution[2].

At the final stage of brewing beers are carbonated, and transferred into bottles, cans, kegs, or bulk tanks. Nitrogen gas is sometimes added to the package, because the entrance of air would make the product completely inappropriate for consumption in a short period of time. To guarantee microbiologic stability pasteurization is often performed before or after the packaging. Flash pasteurization is used for continuous treatment of bulk beer prior to filling. It is typically carried out in a plate heat-exchanger before shifting the beer to the bright-beer tank. Tunnel pasteurization is used principally for in-pack treatment following the crowning of the bottles. After labelling the final product is ready to be distributed and consumed[5, 8].

Beer Staling

The loss of quality during beer storage is one of the main concerns of the Brewing Industry. The staling process is characterized by oxidation reactions of natural beer components (higher alcohols, melanoidins, amino acids, fatty acids, hop resins)[22].

During aging, bitterness is reduced, whereas sweetish and caramel notes are amplified. A cardboard flavor as well as ribes-like flavor can develop, but also disappears after time, and even bread, wood, or sherry flavors can be presented after longer storage times. Additionally, fresh aromas and masking effects are diminished. However, the development of a certain *flavour* cannot be correlated to a single compound[23]. Several components are formed, of which the carbonyl compounds, particularly aldehydes with 6 to 12 carbon atoms,

are the most important *stale flavor* compounds (e.g., 5-hydroxymetilfurfural, 3-methylbutanal, 2-furfural, 5-methylfurfural, n-hexanal)[5, 22]. Among these *flavour* compounds, acetaldehyde was considered as one of the most important factors[23].

Generally, high concentrations usually cause an unpleasant pungent aroma, while appropriate acetaldehyde content can contribute a pleasant green apple aroma[23].

References regarding reasons for the staling process indicate that it is impossible to refer to only one mechanism or to a limited series of mechanisms identifying the processes inducing the degradation of the beer *aroma*[24]. There are suggestions for different schemes of the degradation processes. A simple scheme that highlights the role of transition metals in this process is shown in Figure 4.

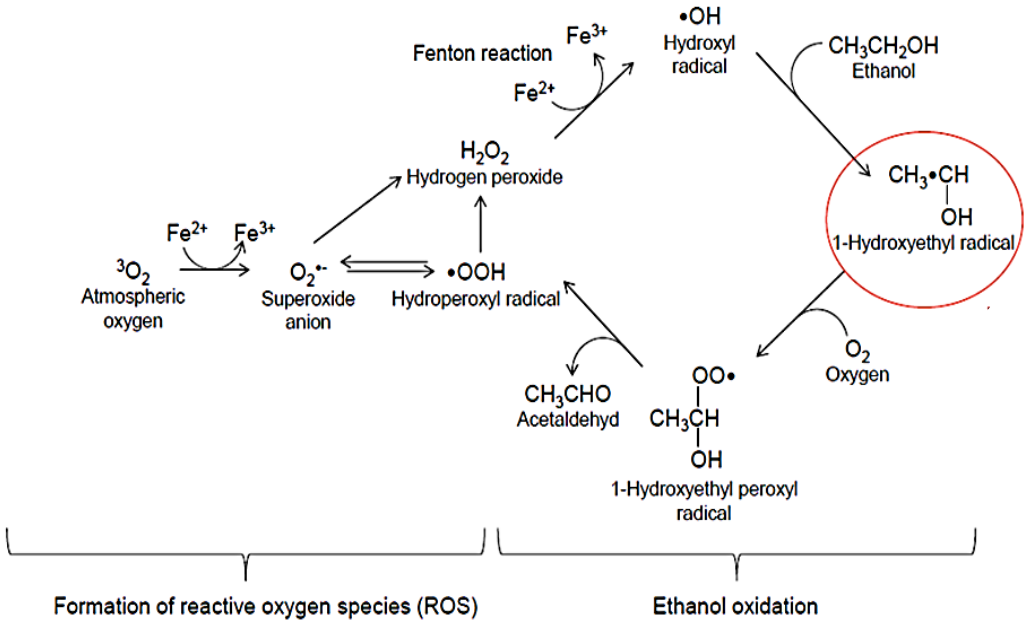

Figure 4. Simplified scheme of the degradation process of organic compounds present in beer, highlighting the role of transition metals. (Lund,2015)

The reaction between ethanol and hydroxyl radicals is proposed to give rise to the formation 1-hydroxyethyl radical in the presence of hydrogen peroxide and transition metal ions, such as iron(II) and copper(II)[25]. Hydroxyl radicals can virtually react with all wort and beer components, including bitter acids from hops, phenolic compounds, and thiol-containing peptides and proteins. They are ultimately responsible for the generation of staling compounds, resulting in changes in *flavor* and colloidal stability[26].

Oxygen plays an important role; as direct oxidation agent of compounds; and indirectly for the reduction of reducing compounds contained in wort and beer. Nevertheless, oxygen is a

critical component of fermentation, and best practices include the injection of concentrated oxygen (8-20 ppm) into wort[5]. Besides, the performance and technological function of a lauter tun in the brew house is one of the most important steps in the brewing process with regard to efficiency, product quality, and capacity. Already at this early stage, oxygen uptake has a direct influence on turbidity and color as well as on the quality of bitterness. Oxygenation of wort is dictated by the specific gravity of the wort and the type of yeast used for fermentation. Post-fermentation is when the quest for low oxygen begins, as measuring focuses on eliminating as many unwanted sources of oxygen as possible[7].

Fenton reaction

The Fenton reaction generally occurs in chemical and biological systems as well as in the natural environment. The importance of Fenton chemistry has been long recognized among others in food chemistry[27].

Transition-metals like Fe and Cu are catalysts in radical generation and oxidation during beer and wort aging[28]. The Haber–Weiss reaction, which makes use of Fenton chemistry, is considered to be the major mechanism by which the highly reactive hydroxyl radical is generated in biological systems[27].

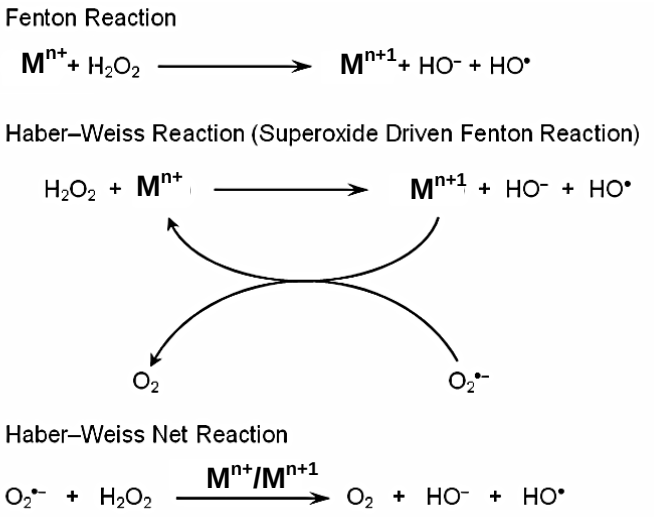

Figure 5. Basic free radical mechanisms for the Fenton and Haber-Weiss reaction. Adapted from BARBUSIŃSKI,2009.

The reduced form of transition metals (M^{n+}) is oxidized trough the Fenton reaction by hydrogen peroxide (H_2O_2), leading to the generation of $OH^\bullet$ and OH^-. M^{n+1} can react with a further molecule of H_2O_2 generating a superoxide radical ($O_2^{\bullet-}$). Superoxide radical reacts

with oxidized form of M^{n+1} in the Haber-Weiss reaction leading to the production of M^{n+} and O_2. Which then again affects redox cycling. These radicals formed from both Fenton and Haber-Weiss schemes are very reactive and give rise to chain reactions[28].

Factors that affect metal content during the brewing process

Metals are very important and influential in the brewing processes, especially in terms of proper growth and metabolism of yeasts. Some of them are essential co-factors for numerous fermentative enzymes, components of transport systems fulfilling charge-balancing or structural roles[29]. The most important metals that influence yeast fermentation are potassium, magnesium, calcium, manganese, iron, copper, and zinc[5].

Brewing water

Water, in terms of quantity, is the most important raw material of beer. The chemical and biological composition of water therefore has a significant relevance in beer production, and there is no step in the brewing process that is not influenced by the constituents of water[29].

Natural waters originate from many different geological formations and therefore every rarely conform to the quality required for brewing water. For this reason treatment is required before water from such sources can be used for brewing. The actual extent of water conditioning is governed by the concentrations of anions, the dissolved organic substances, and the presence of aggressive gases in the untreated water.
Water treatment needs to be addressed in two aspects[7]:

- Treatment of crude water to fulfil legal criteria.
- Treatment of drinking water due to technological brewing requirements.

Progress in water treatment technology has allowed the use of water sources previously not regarded as suitable for brewing purposes. The aim of water treatment can be defined as providing water of the required quality and in sufficient quantity for the different purposes in a Brewery.

Some general guideline requirements for brewing water[30] are summarized in Table 3.

Table 3. General guideline requirements for brew water. Adapted from Eumann, 2012

Parameter	Limits
Fe (ppm)	<0.1
Mn (ppm)	<0.05
Ca^{2+} (ppm)	70-90
Mg^{2+} (ppm)	0-10
Na^{+} (ppm)	0-20
Cl^{-} (ppm)	0-50
SO_4^{2-} (ppm)	100-150
NO_3^{-} (ppm)	0-25
pH	<5
Total H_2S (ppb)	<5
$CaCO_3$ (ppm)	10-50

Generally, the metal ion composition of brewing liquors remarkably changes during all brewing process. All natural components used such as malt, hops, water, yeast, brewing equipment's are sources of metals in beer. Metal ion entry is affected by chemical properties of the specific elements and physical-chemical factors, such as temperature, extract concentration, pH among others. The concentration of metals in the intermediate stages depends not only on their content in the raw materials, but also on their ability to transfer into solution during the brewing process[18]

Determinations of Cu content in barley malt ranged from 2.0 to 6.038 ppm and from 13.40 to 34.0 ppm[28, 31-32] for Fe. These variations in the metal content may be due to the different origins (Germany, Ethiopia, Iran, Poland and Check-Republic), differences in soil types on which barley is grown, agrochemical treatments and environmental pollution.

Čejka et al. found evidence that metals which pass into beer, primarily from raw materials, distribute themselves into the waste (spent grain, trub, yeast) during the brewing process. Therefore, only a negligible fraction of them passes into beer (Cd, Pb, Ni, Cr, Hg, Se)[29]. Cu was reported to have a great affinity to bind to proteins, thus precipitating during boiling and being removed to some part with the hot trub. Fe is lost to a high extent during fermentation[28, 31]. Mash filtration and hot trub separation are stated to be the processes that mainly affect the ionic composition of the wort (Ca, Mg, Fe, Cu and Zn)[32].

Another study compared sweet wort samples, spiked with Fe (50μM) at the beginning of the mashing process with blank samples (without any addition of metals)[25]. No significant differences were found on the Fe content between them, which suggests that Fe becomes trapped during mashing. It correlates with the fact that BSG efficiently binds the iron.

It has been demonstrated that the ionic composition of the wort depends mainly on the removal rate of ions from the wort during mash filtration and hot trub separation, rather than on the actual amount of ions in the raw materials[32].

Effects of malt roasting on the oxidative stability of wort

Maillard reaction (MR), also known as nonenzymatic browning, is of particular importance for food browning and the formation of aroma and flavor substances[33]. The compounds involved are carbonyl and amino compounds, which include reducing carbohydrates and the free amino groups of amino acids, peptides or proteins. It can be described as series of subsequent and parallel reactions, which can be divided into three stages: the early, advanced and final MR steps. Maillard reaction products (MRPs) are not a single type and include diverse glycoforms[34]. Differences in MRP concentrations are caused not only by differences in the chemical composition of the ingredients but also by variations in the production process. The results of early-stage MR are called *Amadori products* whereas when higher temperatures are applied for longer times, advanced brown pigment termed melanoidins (MLD) are formed[24]. The reaction parameters that affect the type and quantity of MRPs are, among others: weight ratio of amino group to reducing sugar, reaction time, water activity (relative humidity), amino acid and reducing sugar characteristics, pH and temperature[34].

In the brewing process, the reaction parameters that affect the type and quantity of MRPs are, among others: weight ratio of amino group to reducing sugar, reaction time, water activity (relative humidity), amino acid and reducing sugar characteristics, pH and temperature. In the brewing process MR occurs when malt is kilned and further occurs during wort boiling[22].

MLD have been widely studied and have shown many interesting biological reactions, as fiber-like action, bacteriostatic and bactericide activity, antioxidative[35] but also pro-oxidative effects[36]. It has been reported that MLD have the ability to scavenge hydroxyl radicals, chelate metals and reducing power[36]. MLD behave as anionic hydrophilic polymers with the

ability to form stable complexes with metal cations. At pH values close to those found in most foods, melanoidins have a negative net charge and are able to bind metallic ions[37]. The reductant ability of MLD is related to an overall increase of radical formation in a Fenton system, involving iron reduction[36].

The influence of malt roasting on beer stability is preferably studied in wort as sulfite produced during fermentation has an impact on beer stability and may mask the influence of the roasting[38]. The most commonly used methods to assay the antioxidant capacity of MLD are based on molecular absorption spectrophotometry UV–Vis due to their simplicity to handle and low cost. These are indirect methods, based on the pre-formation of a free radical from an aromatic organic compound, like DPPH (2,2-diphenyl-1-picrylhydrazyl)[39]. They measure the scavenging activity using a semi stable radical that has high reactivity toward many types of compounds. These methods often result in antioxidative activity, whereas pro-oxidative effects are neglected. On the other hand, Electron Spin Resonance (ESR)-based experiments are established on a complete system providing information about the competition between pro- and antioxidants. In this method the spin adduct formation is expected to be a result of a competition between the pro-oxidative effects, leading to the formation of radicals, and the antioxidative effects that quench radicals[25, 36]

ESR technology offers a very practical means of observing oxidation points in a brewing process, especially in beer filtration and finishing. By using this method to survey the brewing process, these oxidation points can be located and improvements can be made.

The quantification of radicals was achieved by the addition of the spin trap α-(4-pyridyl-1-oxide)-N-tert-butylnitrone (POBN). The highly reactive hydroxyl radicals – originated by the Fenton/Haber-Weiss reaction - react immediately with ethanol forming α-hydroxyethyl radicals. It was found that the rate of spin adduct formation in wort could be enhanced by the addition of ethanol.[40] The α-hydroxyethyl radicals are trapped by POBN or may react with antioxidants (AOH) present in the sample, generating ethanol and a semi-inert antioxidant radical. The spin adduct (PBN/CH_3·CHOH) was detected by ESR and was used as an indicator of antioxidant or pro-oxidant activity of the added samples. A decrease of the spin adduct signal is representative of an antioxidant activity due to a scavenging of short-lived radicals by antioxidants present in the sample. An increase of the intensity of the signal, on the other hand, is representative of a pro-oxidant effect, as a result of an induction of radical formation.[36]

In a Fenton-based system assay (spin trapping and ESR detection)[36] wort samples produced from *black malt* have shown a 50% lower antioxidant effect compared to *pilsner* and *melano malts*. Also, in this study, the pro-oxidative properties induced by *melano* and

black malts were tested in a Fenton assay in the absence of H_2O_2 and/or reactants (Fe^{2+}). In the absence of Fe^{2+} no significant differences were found in the formation of spin adducts between the different types of malt. However, in the presence of Fe^{2+} and atmospheric O_2 there was a significant increase of the pro-oxidative effect of *black malt*, intensifying the radical formation by the Fenton reaction[36].

It is known that metal ions can form complexes of various properties with MRP, oxidize Amadori compounds and their derivatives[41]. Though, the chemical mechanism of incorporation of metal ions into MRP in the different reaction stages remains unclear because of the complexity and high variability of the reaction and its products[42].

The effect of malt roasting on the oxidative stability of sweet wort using a standardized roasting process (125ºC, 135ºC, 145ºC, 160ºC, 190ºC) was evaluated based on Fe and Cu content and radical intensity[38]. The ability of radical formation was examined by quantification of the amount of radicals measured by ESR spectroscopy. The results showed that dark sweet worts are more unstable than lighter sweet worts. Also, malt roasting was found to influence the Fe and Cu content in sweet wort. Cu concentration decreased from the lighter to the darker sweet worts. Fe concentrations were generally higher and increased in temperature. It indicated that roasting reduces the ability of the malt solids to bind Fe but improves the ability to bind Cu[38]. The results propose that malt roasting, radical intensity and Fe content are closely linked, indicating that MRP act as pro-oxidants. An explanation could be that reductones are formed during MR, reducing Fe^{3+} to Fe^{2+} leading to the formation of reactive radicals compounds by the Fenton reaction[38].

2. Objectives

This project aims to evaluate beer stability through control of minerals in wort. For this purpose the influence of malt roasting on metal content and levelling effect of the mash were analysed.

More specific objectives aimed to measure how roasting temperature and addition of metals right at the beginning of the mash may influence:

- Metal content in sweet wort
- Levelling effect of the mash
- Oxidative stability of the sweet wort

The ultimate goal of this project is to understand how mixing different types of malt in a realistic approach influence the previously mentioned parameters.

Identification of the metals

The importance of the quantification of iron and copper in wort comes from the possibility to establish a correlation between the oxidative stability of beer and its shelf life.

The identification of their influence on the oxidative stability of sweet wort could be useful for the brewing industry. Therefore, the content of these metals in the raw materials can be adjusted and by this way turning the process simpler and cheaper, according to the mashing-levelling capacity.

3. Experimental procedures

i. Material and equipment

ii. Methods

iii. Overview of the process

iv. Experimental design

v. Statistical analysis

Materials

- Finnpipette
  5.00 ± 0.01 mL (Class A, Thermo Scientific)
  10.00 ± 0.50 mL (Class A, Thermo Scientific)
- Volumetric flasks:
  10.00 ± 0.1 ml (Class A, Scherf)
  100.0 ± 0.1 ml (Class A, Scherf)
- 25 mL conic flask (Class A, Scherf)
- Funnel
- Pasteur Pipette
- 50 µL micropipettes (Brand GMBH, Wertheim, Germany)
- Plastic tubes, 50mL (Ref: 02-572-8001, Lot: 50CC108B; nerbeplus)
- Open-folded filter (#614 1/4, Macherey-Nagel, Germany)

Equipment

- Manual mill, Ref: 851-923-0031, Jack Schmidling Productions
- Coffee-mill, type 6720. OBH Nordica
- Analytical Balance (Sartorius, BP 110S, máx: 110g ± 0.0001g)
- Precision Balance (Mettler Toledo PM 4600, máx. 4100g±0.1g)
- Milli-Q® Integral 3 Water Purification System, Ref: ZRXQ003WW, Merck KGaA, Darmstadt, Germany
- pH Meter 691, 3D1/496 Type 1.691.0020 (Metrohm, Göttingen Germany)
- Anton-Paar© Density meter 58-V1.9 (United States)
- Cintra 40 Spectrofotometer (GBC, Melbourne, Australia)
- Water Bath (Heto Laboratory Equipment, Allerød, Denmark)
- MA-001 Mashing Apparatus (Lg-automatic ApS, Frederiksvaerk, Denmark)
- Inductively coupled plasma optical emission spectrometry (ICP-OES) (Agilent 5100 ICP-OES, United States). Hollow cathode-lamp Fe-Cu-Zn-Mn – Brand PerkinElmer Lumina
- HQ 30d Luminescent Dissolved Oxygen (LDO) sensor (Hach Lange, Düsseldorf, Germany)
- Electron spin resonance spectrometer - Miniscope MS 200 (Magnettech, Germany)

Chemicals

The reagents used in the preparation of the solutions required throughout the project and their general characteristics are resumed on Table 4.

Table 4. Reagents used in the elaboration of required solutions

Reagent	Molecular Formula	Nº CAS	Brand	Assay
Iron(II) sulfate heptahydrate	$FeSO_4.7H_2O$	7782-63-0	ACROS	99.5%
Copper(II) sulfate pentahydrate	$CuSO_4.5H_2O$	7758-99-8	ACROS	100%
Nitric acid (65% w/w)	HNO_3	7697-37-2	Roth, Karlsruhe, Germany	65%
Hydrogen peroxide (30% w/w)	H_2O_2	7722-84-1	Merck KGaA	30%
α-(4-Pyridyl N-oxide)-N-tert-butylnitrone (POBN)	$C_{10}H_{14}N_2O_2$	56893-81-0	Merck KGaA	99%
Ethanol	C_2H_6O	64-17-5	Danish Distillers	96%
2,2,6,6-Tetramethyl-1-piperidinyloxy (TEMPO)	$C_9H_{18}NO$	2564-83-2	Merck KGaA	98%
ICP multi-element standard solution IV **(23 elements in diluted nitric acid) 1000 mg/l: Ag, Al, B, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Sr, Tl, Zn Certipur®**	$Fe(HNO_3)_2$ $Cu(HNO_3)_2$	-	Merck KGaA	-

Preparation of Iron and Copper Stock solutions:

To prepare a stock solution of Iron (II) sulfate heptahydrate (4000µM), 0.111g of $FeSO_4.7H_2O$ were rigorously weighed. It was transferred to a volumetric flask of 100mL and the volume was completed with Ultra-pure water.
For the stock solution of Copper (II) sulfate pentahydrate (4000µM), 0.099g of $CuSO_4.5H_2O$ were dissolved in 100 mL of ultrapure water in a volumetric flask.

The 50 µM concentration was calculated for the 400mL final volume of the mashing process according to *European Brewing Congress*-Mashing 4.4[43], by adding 5mL of the stock solution at the beginning of the mash. The data used is summarized in Table 5.

Table 5. Data used for the calculation of the concentration of the stock solutions

Compound	V_{final} (mL)	Ci µM	C_{final} µM	$V_{initial}$ (mL)	n_{final}
$FeSO_4.7H_2O$	400	4000	50	5	20
$CuSO_4.5H_2O$	400	4000	50	5	20

For the calculation of the mass needed of iron (II) sulphate heptatahydrate and copper (II) sulfate pentahydrate the purity and the molar mass were taken into account, Table 6.

Table 6. Data used for the elaboration of the stock solutions

Compound	Molar Mass	V_{final} (dm^3)	C_{final} (M)	Purity (%)	$Mass_{weighted}$ (g)
$FeSO_4.7H_2O$	278.01	0.100	0.004	99.5	0.111
$CuSO_4.5H_2O$	249.69	0.100	0.004	100	0.099

Methods

Sample characterization

Organic Pilsner malt (two-row spring barley (Hordeum vulgare), harvest 2016) was purchased from Weyermann, Bamberg, Germany through Maltbazaren, Copenhagen, Denmark.

Brewing analytical methods

A number of scientific organizations carry out collaborative tests of brewing analytical methods and confer official status upon those that are deemed to perform satisfactorily. These organizations include the American Society of Brewing Chemists (ASBC), the Brewery Convention of Japan (BCOJ), the European Brewery Convention (EBC), the Institute of Brewing and Distilling (IBD), and the Mitteleuropaeische Brautechnische Analysenkommission [the Central European Brewing Technology Analysis Commission] (MEBAK). Each of these organizations publishes its official methods and the use of the specification is to minimize variation within analysis and measurements.

EBC - Congress Mashing

A congress mashing was carried out with modifications. Malt samples were milled in a hand-driven mill (0.2mm in line joining, according to Analytica EBC 1.1: “Care and Adjustment of Apparatus”[43]) and mashed in at 46 °C in 200 mL of Ultra-pure water for 30 min followed by an increase in temperature to 70 °C for 25 min (increase of 1 °C per min). A 100 mL amount of Ultra-pure water was added to the mash when the temperature reached 70 °C. The temperature was held at 70 °C for 60 min (saccharification rest) after which the mash was cooled to room temperature (Figure 6). To ensure the same wet content of all samples, each beaker was adjusted to the same weight of 450.0 g by the addition of Ultra-pure water. The mash was subsequently filtered through an open-folded filter, and the sweet wort samples were frozen immediately after filtration. Wort samples were collected at the end of the mashing program and adjusted to a total weight of 450 g before filtration by the addition of ultra-pure water to the beaker.

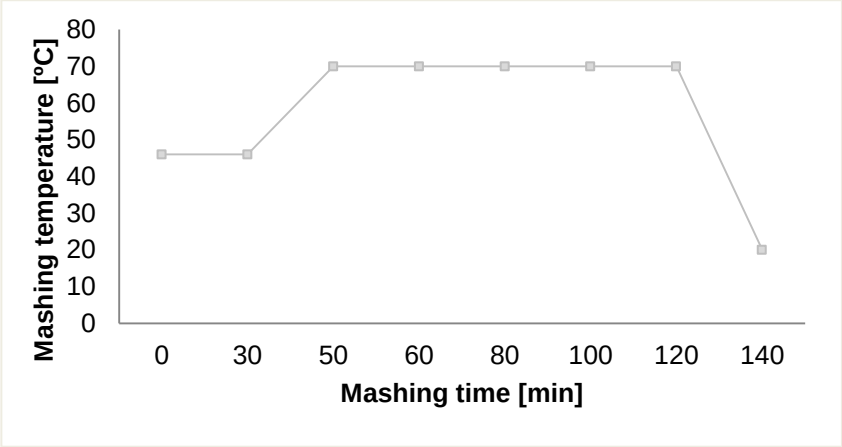

Figure 6. Mashing temperature profile applied in this work.

Color

For the estimation of beer color according to the respective EBC method 8.5 *Colour of Wort: Spectrophotometric Method*[43], aliquots of sweet wort samples were transferred to a cuvette (d=10 mm). The absorption at 430 nm was measured photometrically against water as the blank. If necessary, sweet wort samples were diluted to keep the absorption below a value of 1.0. The EBC value was calculated as follows:

$C = f \times 25 \times A_{430}$

where C is the color in EBC units, f is the dilution factor, A_{430} is the absorbance at 430 nm, and 25 is a multiplication factor.

pH

pH values were measured using a pH Meter, [*pH Meter 691, 3D1/496 Type 1.691.0020 (Metrohm, Göttingen Germany)].*

Plato

Plato values were then calculated according to Analytica EBC 8.3 *Extract of wort*[43]($^{o}Plato$ = -460,234 + 662.649 * SG – 202.414 * SG2 (Equation 1). From the density values (g/cm^3) obtained the specific gravity (SG, Equation 2) was calculated by dividing the density of the samples by the water density at 20ºC **Error! Reference source not found.**

$^{o}Plato = -460{,}234 + 662.649 * SG - 202.414 * SG^2$ (*Equation 1*)

$SG = \frac{\rho Sample}{\rho Water}$ (*Equation 2*)

Microwave digestion and Determination of Metal Ion Concentrations in Test Samples by Inductively Coupled Plasma - Atomic Emission Spectrometry (ICP-OES)

Metal analysis of all wort samples was performed by microwave, digesting the samples with HNO_3 (65% w/w) and H_2O_2 (30% w/w) followed by determining the metal ion concentration using an Agilent 5100 ICP-OES system fitted with a CID 86 detector and argon as the carrier gas.

Preparation of multi-element stock solutions

From the ICP multi-element standard solution IV ([Fe] and [Cu]= 10^6ppb) stock solutions 1 and 2 were elaborated according to the next table.

Table 7. Data used for the preparation of Multi-element stock solutions. Final volume was fulfilled with ultra-pure water

Stock solution	**$V_{ICP\ multi\text{-}element\ standard\ solution\ IV}$ µL**	**$V_{stock\ solution\ 1}$ µL**	**V_{final} mL**	**$[Fe]_{final}$ and $[Cu]_{final}$ ppb**
1	1000	-	50	20 000
2	-	2500	50	1000

The elaboration of the standards for the calibration curve were prepared in 10mL volumetric flasks, according to Table 8 and the volume completed with ultra-pure water.

Table 8. Data of standard solutions and pippeted volumes used for the elaboration of the calibration curve

Standard	**[Cu]i and [Fe]i ppb**	**Vi_{stock1} µL**	**Vi_{stock2} µL**	**V_{final} mL**	**[Fe]f and [Cu]f ppb**
1	1000	-	1000	10	100
2	20000	750	-	10	1500
3	20000	1500	-	10	3000

The sample preparation procedure was as follows: 5 mL of HNO_3 (65% w/w) and 2 mL of H_2O_2 (30% w/w) were added to 10mL of sample and the sample mixtures were allowed to react for 24 hr at room temperature. Then, the samples were exposed to microwave heat using the following temperature program: heating to 160°C in 5 min, holding the temperature for 20 min, and cooling down for 30 min.

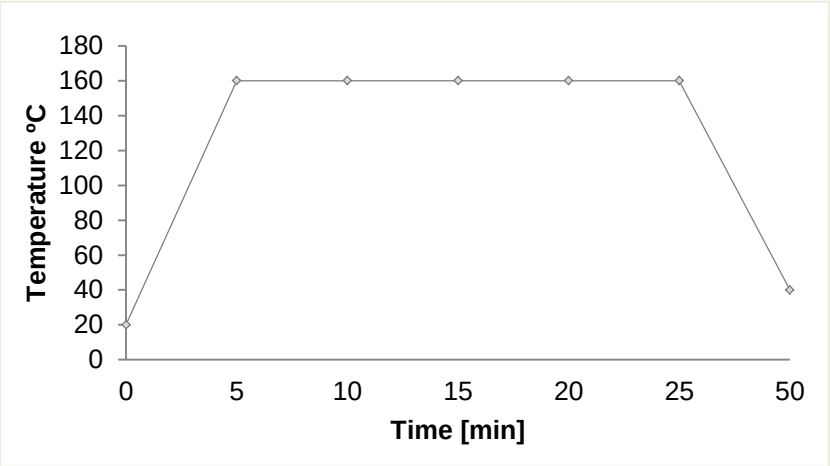

Figure 7. Microwave temperature program.

After heat exposure, the samples were transferred to 25-mL volumetric flasks, and made up to volume with ultra-pure water.

All the glass wear used for the sample preparation was previously acid washed in a 2% HNO_3 plus 2% HCl acid bath.

For the final analysis at the ICP instrument, the following parameters were used: RF power, 1,150 W; argon gas flow rates, auxiliary at 0.5 L/min and nebulizer at 0.5 L/min; sample flow rate, 4.0 mL/min. The analytical wavelengths used for the determination are stated in Table 9

Table 9. Analytical wavelengths used for the determination of Iron and Copper

Element	Wavelenghts (nm)
Fe	259.940
Cu	324.754

Oxygen consumption

25mL conic flasks were filled with 20mL of wort sample which had been freshly saturated with atmospheric air by bubbling for 2 min. The oxygen measurements were started immediately after the flask was filled. The oxygen concentration was recorded every 5 min, and the sensor had initially been calibrated against air saturated (for 45 minutes) demineralized water at 25 °C. The oxygen consumptions were measured during 8 h.

Electron spin resonance (ESR) and spin trapping

The quantification of radicals was achieved by the addition of the spin trap α-(4-pyridyl-1-oxide)-N-tert-butylnitrone (POBN). A 500µL solution of 600 mM POBN in ethanol was prepared by weighing 58,3 mg into a micro tube. 1,9 ml of wort sample was added to a 20 ml glass tube with a stopper plus 100µl of POBN solution, the final concentration of ethanol was 5%. Samples were stirred for 10 minutes at room temperature. The bottles were transferred to a preheated water bath (T 60 °C). Samples (50 µL) were taken at given time intervals. ESR spectra of the wort samples were recorded using 50 µL micropipettes as sample cells. The settings used were as follows: microwave power, 10 mW; sweep width, 49.82 G; modulation frequency, 2000 mG; receiver gain, 800; and sweep time, 30 s. All spectra, consisting of four scans, were recorded at room temperature. The amplitudes of the spectra were measured and are reported as the height of the central doublet relative to the height of the central line in the ESR signal of an aqueous TEMPO solution (2 µM). The TEMPO standard was measured as the first and last sample of the day. The results were presented as spin adduct concentration (uM) and it was obtained diving the signal of the sample per the average of the EMPO signal of the same day. All samples were measured in duplicate.

Overview of the process

Malt was roasted by distributing it in a single layer on a baking paper on a baking tray and heating it in an oven at 125 °C and 190 °C for 50 min. The chosen temperatures[38] as well as the metal concentrations[25] were taken from previous work. The following scheme resumes the sampling plan. All the samples were produced in duplicate.

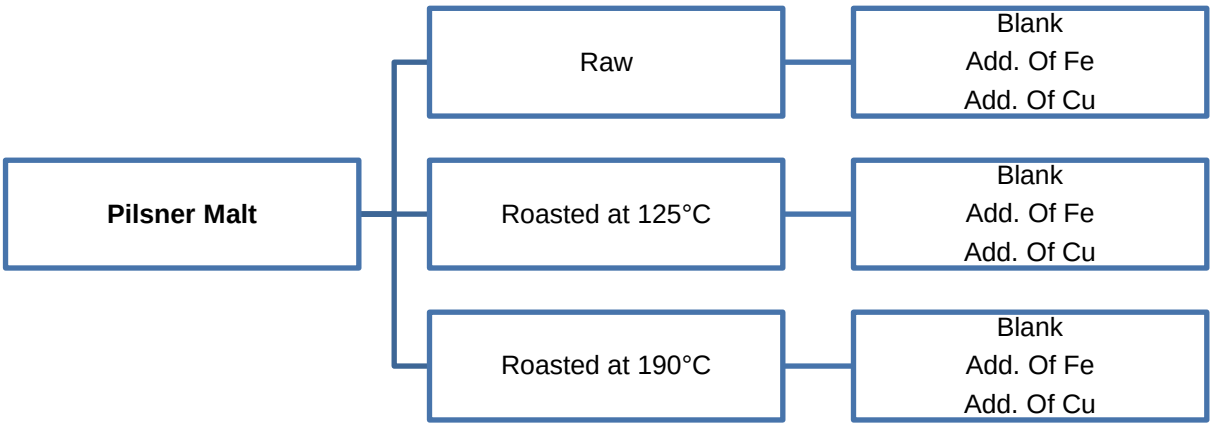

Figure 8. Overview of the process – Selected roasting temperatures used for the pure sweet wort samples production . 50μM of iron and copper were added at the beginning of the mashing process.

50 μM of $FeSO_4.7H_2O$ and $CuSO_4.5H_2O$ were added to the wort samples at the beginning of the mashing and compared to a control wort (blank) to which the metals were not added.

Usually in brewing, beer is produced from a mixture of different types of malt and not from a single one. In order to continue the study in a more realistic approach, sweet wort samples were produced from a mixture of the previous malt samples. According to the literature, average fractions of roasted malt (according to EBC units) in % used in Brewing are for: Dark beers: >90% of roasted malt, <10% pilsner malt and for Lighter beers:<10% roasted malt; >90% pilsner malt[2, 36, 44]. All the samples were produced in duplicate.

Table 10. Fraction in % of Pilsner (unroasted), 125ºC and 190ºC of roasted Pilsner malts for sweet wort production

Sample	Fraction in % of Pilsner malts					
	Pilsner	**g**	**125ºC**	**g**	**190ºC**	**g**
A	90	45.00	0	0.00	10.00	5.00
B	90	45.00	10	5.00	0.00	0.00
C	10	5.00	0	0.00	90.00	45.00

The following scheme represents the sampling plan for the realistic approach of wort production. Metal additions were performed as previous samples.

Milling effect

In order to analyse if different grades of milling affect the metal levelling effect of the mashing process, wort samples were produced from unmodified pilsner and 190ºC roasted malt, finely ground by using a coffee-mill. To the wort samples 50 µM of $FeSO_4.7H_2O$ and $CuSO_4.5H_2O$ were added at the beginning of the mash and were compared to a control wort (blank) to which the metals were not added (n=1)

Filtrate effect

After mashing, samples were filtered for 1 h 20 min. Then, 25 wt.% of the spent grains were mixed with the spiked solutions in a beaker for 1 min before filtering. The samples were then transferred to a new filter paper and 100 mL of the following samples were filtered through it:

1. Wort sample containing 50 µM of Fe added at the end of filtration (sample FE_Wo)
2. Ultra-pure water containing 50 µM of Fe (sample FE_Wa)

All the samples were produced in duplicate.

Recovery experiment - Spiking after mashing and filtration

In a plastic tube of 20mL 0.25mL of stock solution of Iron(II) sulfate heptahydrate (50 µM) were added. The total volume was fulfilled with an aliquot of the blank sample.

Experimental Design

The scheme of the experimental design is represented in Figure 1.

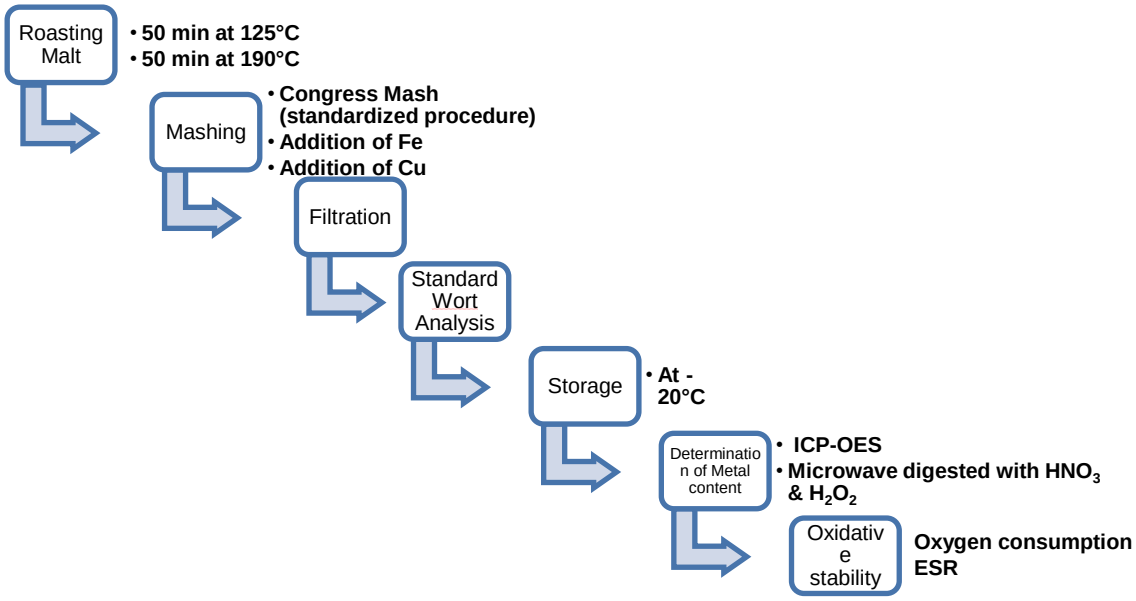

Figure 9. Schematic representation of the experimental design used for test sample production and analysis.

Statistical analysis

Results are expressed as means ± standard deviations (SD) of at least two independent experiments. Statistical analysis was performed using Excel software, (Microsoft, Redmond, WA, U.S.A).

Data were treated by the analysis of one-way ANOVA to determine the significance of the main effects. Significant ($p<0.05$) differences between means were identified by the least significant (LSD) test. Homoscedasticy, a formal requirement for some statistical analysis[45], has been considered in the statistical tests performed. A *p* value less than 0.05 ($p<0.05$) was considered to indicate a statistical significant difference.

3. Results and Discussion

i. Characteristics of sweet worts applied in this study

ii. Recovery experiment

iii. Milling effect

iv. Filtrate effect

v. Effect of malt roasting on the metal content of sweet wort

vi. Effect of mixing malts on the metal content of sweet wort

vii. Influence of Fe/Cu on the concentration of Mn and Zn

viii. Oxidative stability of sweet wort samples

ix. Electron spin resonance (ESR) and spin trapping

x. Oxygen consumption (OC)

Characteristics of sweet worts applied in this study

Color, pH and Plato measurements were taken to characterize the test samples. The characteristics of the blank samples are resumed in Table 11.

Table 11. Characteristics of the three sweet worts applied in this study

Sample	Roasting TºC	Wort pH	Wort ºPlato	Wort color (EBC units)
1	untreated	5.89 ± 0.21	8.3826 ± 0.2500	6.61 ± 1.94
2	125ºC	5.62 ± 0.24	8.3929 ± 0.4454	20.97 ± 0.87
3	190ºC	4.86 ± 0.25	2.9639 ± 0.7358	107.08 ± 24.42
A	-	5.69 ± 0.02	8.7903 ± 0.0917	19.81 ± 1.70
B	-	5.85 ± 0.06	8.7714 ± 0.0477	6.80 ± 0.82
C	-	5.05 ± 0.43	7.7690 ± 0.6451	112.36 ± 6.47

The addition of metals to the different samples doesn't seem to affect significantly the color, plato or pH (Annex II).

Results indicate a decrease in pH with roasting temperature. Mixing to the proportion of $1_{190ºC}$:9 $_{pilsner}$ (sample A), $1_{pilsner}$:$9_{125ºC}$ (sample B), didn't significantly decreased the pH of A and B samples comparing to pure pilsner. The proportion of $1_{pilsner}$:$9_{190ºC}$ (sample C) didn't significantly increase the pH compared to pure 190ºC wort. A significant decrease in Plato in 190ºC sample was verified when comparing to lighter worts. The addition of roasted malts in samples A and B slightly decreased its values, although this difference is not considered significant. However, the proportion of $1_{pilsner}$:$9_{190ºC}$ (sample C) had a strong increase in Plato value comparing to pure 190ºC.

An increase in color with roasting temperature was observed. Color EBC in sample A significantly increased comparing to pure pilsner. Similar values were found for sample B and pure pilsner, as well as for 190ºC and sample C.

Recovery experiment

Our results show a high level of accuracy for Fe concentration through the methodology used for the sample preparation and metal analysis.

The obtained results are presented in Table 12.

Table 12. Concentration of Iron in sweet wort samples spiked after mash and filtration

Metal	Add. to Pilsner blank	Measured	SD
Fe	50 uM	49.32 uM	0.24 uM

Milling effect

No significant variation between normal mill and coffee-milled samples was observed for iron and copper content (Table 13). Our results indicate that the possible variation between mills in a hand driven mill don't seem to influence the metal content in wort samples.

Table 13. Iron and Copper content (uM) in pure wort samples, hand-milled and coffee-milled

	Sample	*n*	*[Fe]uM*	*SD*	*[Cu] uM*	*SD*
Normal Mill (hand driven mill)	**Pilsner_blank**	2	1.08	0.05	3.46	0.04
	Pilsner_Fe	2	1.63	0.30	3.22	0.44
	Pilsner_Cu	2	0.83	0.27	28.83	6.88
	190_blank	2	2.61	0.47	0.34	0.02
	190_Fe	2	11.40	0.81	0.59	0.35
	190_Cu	2	3.57	0.80	3.99	5.16
Coffee-Mill	**Pilsner_blank**	1	0.97	-	3.58	-
	Pilsner_Fe	1	3.41	-	5.62	-
	Pilsner_Cu	1	0.85	-	31.27	-
	190_blank	1	2.23	-	0.19	-
	190_Fe	1	9.99	-	0.22	-
	190_Cu	1	1.06	-	5.43	-

Filtrate effect

In order to analyze the spent grain's impact on the iron levelling effect, wort samples produced with unroasted pilsner malt were analyzed. As expected, our results indicate an effective binding of Fe through filtration (BSG and filter paper). From the initial 50uM of iron in wort solution, final sample contained 25.63±1.46 uM (FE_Wo). The water sample with the same initial 50uM of iron, after filtration contained 12.71±2.11 uM (FE_Wa), as shown in Table 14. It suggests that wort contains compounds that effectively bind to iron, as its content is more or less double than what found in water sample.

As previously mentioned, wort is a complex mixture of substances, some naturally present in malt as phenolic compounds, phosphates and phytic acid, MRP as well as compounds from enzymatic activity, such as peptides and free amino acids. All of these compounds have the capacity to form complexes with iron[46-49]. Besides the presence of porous microstructures allied to the presence of reactive functional groups in BSG enhance its ability to adsorb metallic ions[48]. However, comparing to pilsner blank and pilsner spiked with iron, there is a significantly lower content on the later samples. These results might be due to the smaller amount of BSG and wort sample filtrated, which considerably reduced the filtration time – less contact time of metals with BSG and filter paper (10 minutes vs 2h00). Another factor could be the stirring time, as a prolonged time could increase the binding of the metals to sweet wort compounds.

Table 14. Iron content (uM) in wort and water samples. Comparison to pilsner blank and spiked with Fe

Sample	n	[Fe]uM	SD
FE_Wo	2	25.63	1.46
FE_Wa	2	12.71	2.11
Pilsner_blank	2	1.08	0.05
Pilsner_Fe	2	1.63	0.30

Besides, the experimental design is intrinsically different, as in this experiment iron is added after the mash what reduces, as well, the contact time with wort compounds and BSG before filtration.

Finally, in this experiment, BSG and filter paper were used for the filtration (mimicking a *mash filter*). In further studies only the BSG without the filter paper would also be relevant to test (mimicking a *lauter tun*).

Effect of malt roasting on the metal content of sweet wort

The capacity to remove Fe and Cu during mashing has been evaluated in this study. Malt modification by roasting increases the sweet wort levels of Fe and decreases Cu, being our data in accordance with previous results[38] (Figure 10).

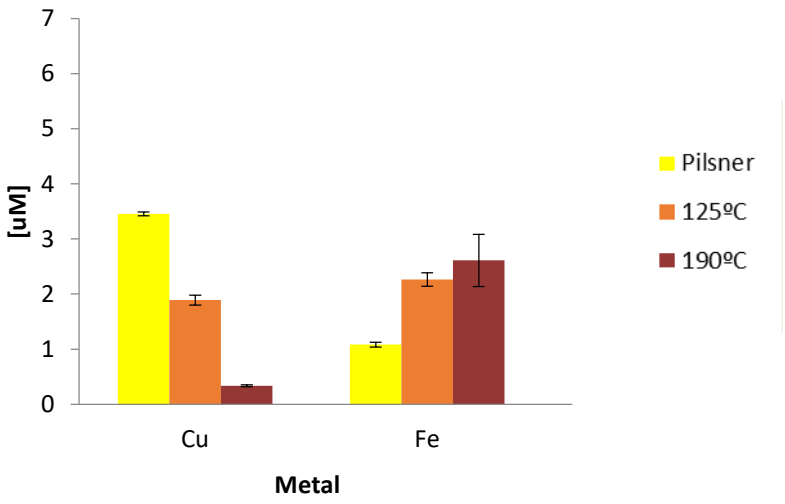

Figure 10. Iron and Copper concentration (uM) in blank wort samples according roasting temperature (ºC). Bars represent mean ± SD.

Almost no difference was found for Fe concentration between the spiked samples and the blanks (Table 15) when using pure pilsner malt, suggesting that there is an efficient binding of this metal. Roasting significantly decreased the removal of Fe while increasing the removal of Cu.

According to our previous results, it should be expected that sample A ($1_{190ºC}$:$9_{pilsner}$) and sample B ($1_{125ºC}$:$9_{pilsner}$) would have higher content in Fe and lower in Cu than pure pilsner wort. In addition, Sample C ($1_{pilsner}$:$9_{190ºC}$) would have higher content in Cu and lower in Fe than pure 190ºC wort.

Comparing pure pilsner wort to samples A, no significant differences in Fe and Cu content were found between the blanks neither the spiked samples, respectively. Besides, no differences were found between the blanks and spiked samples of pure pilsner to sample B, respectively.

When mixing $1_{pilsner}$:$9_{190ºC}$ and comparing to pure 190ºC, no significant differences were found between the blanks and spiked samples of pure 190ºC, respectively.

This suggests that the realistic mixture of pure malts designed in this study doesn't significantly affect the levelling effect of pure pilsner and 190ºC roasted malts, as shown in Table 6.

Table 15. Fe and Cu content (uM) in the analysed samples

		[Fe] uM		***[Cu] uM***	
Sample	*n*	*Average*	*SD*	*Average*	*SD*
Pilsner_blank	2	1.08	0.05	3.46	0.04
Pilsner_Fe	2	1.63	0.30	3.22	0.44
Pilsner_Cu	2	0.83	0.27	28.83	6.88
125_blank	2	2.27	0.12	1.89	0.09
125_Fe	2	4.82	0.28	2.45	0.46
125_Cu	2	2.40	0.22	24.38	4.28
190_blank	2	2.61	0.47	0.34	0.02
190_Fe	2	11.40	0.81	0.59	0.35
190_Cu	2	3.57	0.80	6.83	1.16
A_blank	2	1.44	0.42	3.15	0.04
A_add. Fe	2	2.53	0.70	3.33	0.81
A_add. Cu	2	0.98	0.28	21.89	2.36
B_blank	2	1.09	0.00	3.46	0.14
B_add. Fe	2	1.63	0.08	3.41	0.26
B_add. Cu	2	0.56	0.15	24.53	3.09
C_blank	2	3.63	0.18	0.85	0.10
C_add. Fe	2	12.23	0.40	0.72	0.05
C_add. Cu	2	3.23	0.02	9.11	0.51

It is noteworthy that the addition of iron at the beginning of the mashing did not increase the iron content in pilsner wort samples, whereas the direct addiction of iron to the wort after the mashing substantially increase the iron concentration (Recovery experiment). Apparently, high concentrations of iron in the wort are prevented from being detected by their efficient binding to the solids that are removed by filtration or lautering in the brew house. Our findings for the pure samples are in agreement with previous results[25, 29, 38]

Though, the investigation of the effect of mixing different types of malt on metal content of sweet wort hasn't been made focusing on the roasting temperatures of malt. Nevertheless, replacing barley malt with wheat malt on the concentration of magnesium, manganese, iron

and zinc ions in brewer's wort was investigated. Despite wheat malt natural higher content in metals, it was concluded that the loss of ions via the by-products has a bigger impact on their content in wort than the actual concentration in the raw materials[32]. Even though the high impact of mash and filtration on metal removal[29, 32,18] has been demonstrated the mechanism behind this effect remains unclear.

Since the type of malt and roasting temperature influence the metal content on sweet wort, further investigations will focus on whether the roasting temperature and higher amounts of spiked metals affect its concentration in wort and in the final beer.

Influence of Fe/Cu on the concentration of Mn and Zn

The mineral nutrition of yeasts is relevant to brewers as they seek to increase fermentative capacity, improve ethanol yields and maintain product consistency[2]. A general agreement exists, based on wide range of biochemical and microbiological experiments that yeast requires number of metals in trace quantities in order to grow and ferment. Moreover, metal ions can impact significantly on the progress and efficiency of industrial fermentations[6].

The bioavailability of certain key minerals is limited and it can adversely affect yeast fermentation processes[7]. As instance, zinc and manganese levels may decrease during mashing and wort boiling as the metal ion becomes complexed in precipitated trub[29]. Lower concentrations of zinc and manganese can result in slower fermentations[50]. Due to the essential nature of zinc and manganese in fermentation these metals were analyzed in all samples.

Although manganese is crucial for yeast activity, its presence in beer has been associated with decrease in oxidative of beer, along with iron and copper[51].

In wort samples made from a single type of malt, the results show that roasting pilsner malt significantly increased the content in Mn and Zn (Figure 11).

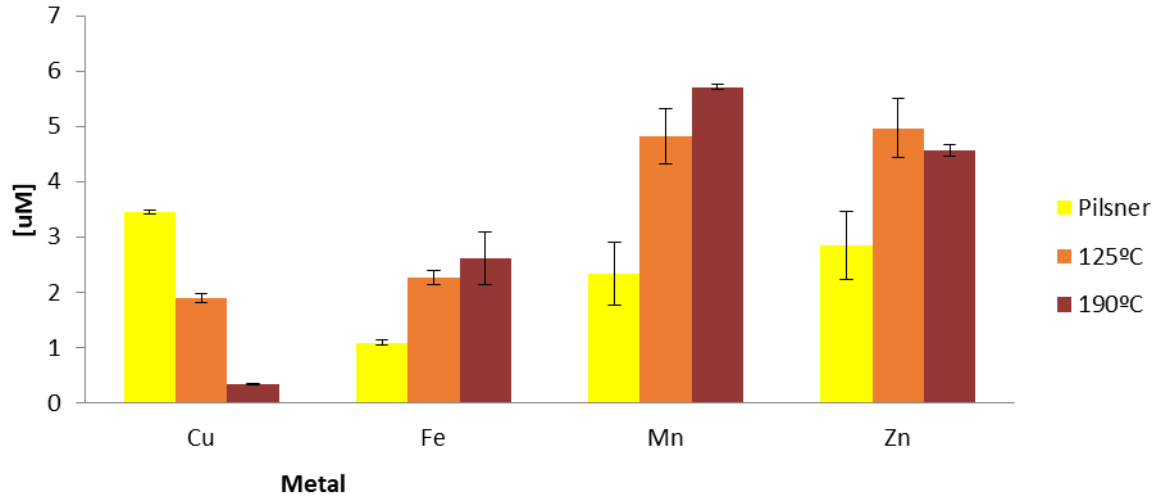

Figure 11. Metal concentrations (uM) in blank wort samples according to roasting temperature (ºC). Bars represent mean ± SD.

Our results also indicated that the addition of Fe and Cu didn't influence the content of Zn and Mn. (Table 16).

Table 16. Manganese and Zinc concentrations in uM in pure and mixed samples

Sample	***n***	***[Mn] uM***		***[Zn] uM***	
		Average	*SD*	*Average*	*SD*
Pilsner_blank	*2*	2.33	0.57	2.84	0.61
Pilsner_Fe	2	2.60	0.45	3.06	0.34
Pilsner_Cu	2	2.78	0.80	2.52	0.57
125_blank	2	4.81	0.50	4.96	0.53
125_Fe	2	5.20	0.71	5.31	0.69
125_Cu	2	5.08	0.58	4.99	0.36
190_blank	2	5.71	0.05	4.56	0.10
190_Fe	2	5.97	0.56	4.84	0.37
190_Cu	2	5.88	0.51	4.79	0.34
A_blank	2	3.10	1.17	3.93	0.66
A_add. Fe	2	2.37	1.41	2.70	1.63
A_add. Cu	2	2.64	1.08	2.35	0.80
B_blank	2	2.98	0.54	3.75	0.74
B_add. Fe	2	2.61	0.56	3.08	0.75
B_add. Cu	2	2.71	0.83	2.61	0.89
C_blank	2	5.27	0.87	5.41	1.02
C_add. Fe	2	4.98	0.63	5.20	0.43
C_add. Cu	2	4.48	0.19	4.92	0.32

As observed for Fe and Cu, the addition of roasted malts didn't affect the levelling capacity of pure pilsner wort, as shown in Figure 12.

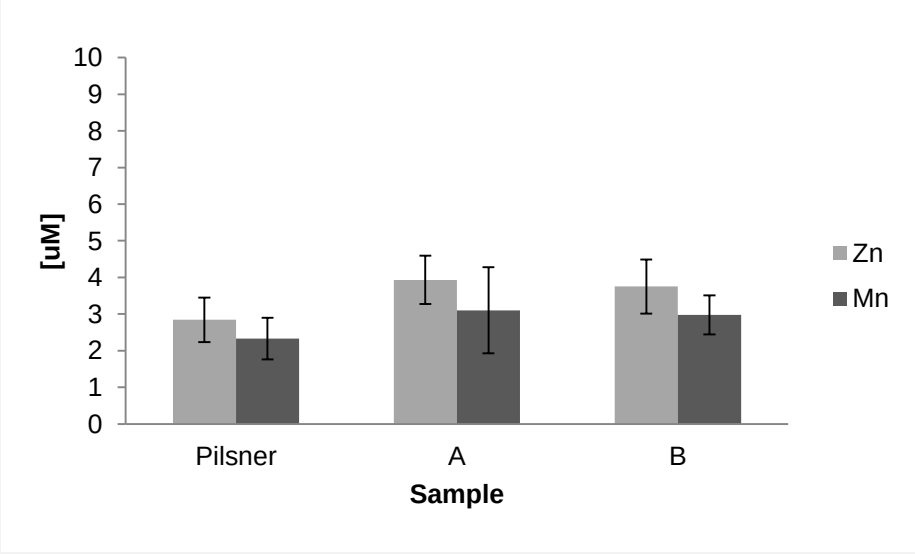

Figure 12. Zinc and Manganese content (uM) in samples A, B and pure pilsner wort. Bars represent mean ± SD.

The addition of 10% of pilsner didn't seem to influence as well the levelling capacity of pure 190ºC wort (Figure 13).

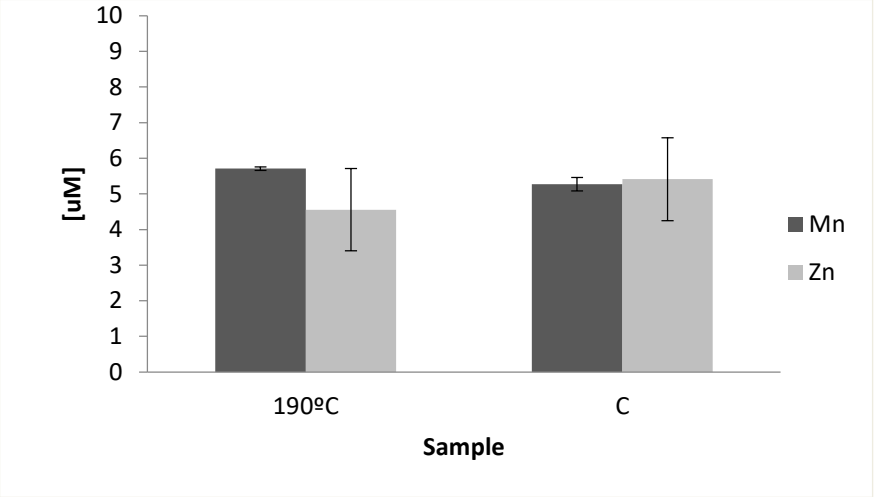

Figure 13. Zinc and Manganese content (uM) in sample C and pure 190ºC roasted malt. Bars represent mean ± SD.

Reactive functional groups such as hydroxyl, amine and carboxyl in BSG are responsible for the binding of metal ions[20]. Has been reported on the potential use of BSG in removal of heavy metals from aqueous systems[52]. Though, the mechanisms underlying the effective binding of Fe and Cu remains unclear[52]. Also, our results suggest that these minerals don't compete during the mashing/filtration process, as the addition of Fe and Cu doesn't affect the content of Mn and Zn, in spiked and blank samples, as shown in Table 16.

Oxidative stability of sweet wort samples

Electron spin resonance (ESR) and spin trapping

The chemical composition of wort/beer is a dynamic system and changes throughout storage. This phenomenon, referred as *beer staling* is a highly complex process owing to the many different oxidative and non-oxidative reactions that take place[23]. Several chemical species are involved in these reactions (such as metal ions, proteins, polyphenols). Thus, no single character-impact compound is responsible for the staling of beer, but rather a variety of products from different reactions. Initially, the radical generation is delayed by the *endogenous antioxidative activity* of sweet wort. After the consumption of antioxidants, the ESR signal increases when spin-trap adducts, mainly hydroxyethyl radicals, are generated[53].

The increase of roasting temperature significantly increase the amount of spin adduct concentration in pure wort samples, though not between pilsner and 125ºC roasted malt, as presented in Figure 14.

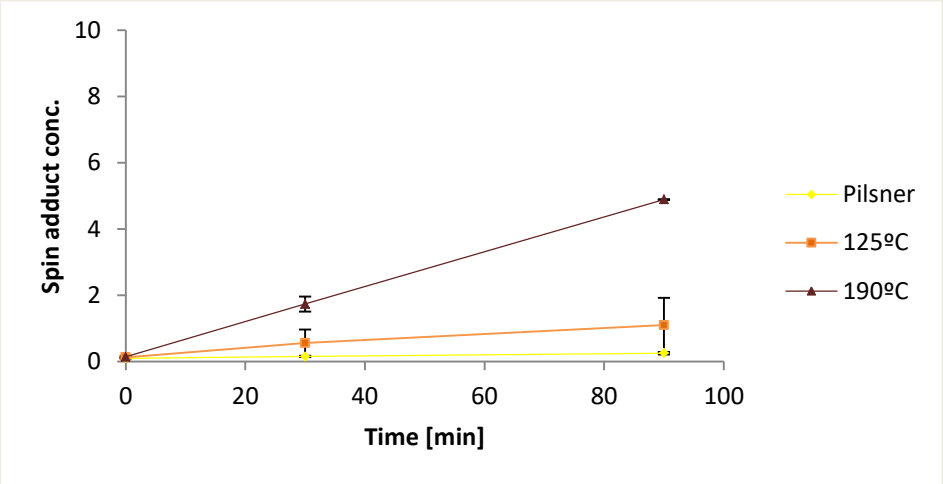

Figure 14. Spin adduct concentration for pure wort samples, made from pilsner, 125ºC and 190ºC roasted malt. Bars represent mean ± SD.

Our results show that in pure pilsner wort the addition of Fe and Cu didn't significantly alter the oxidative stability of the sample when comparing to the blank (Figure 15).

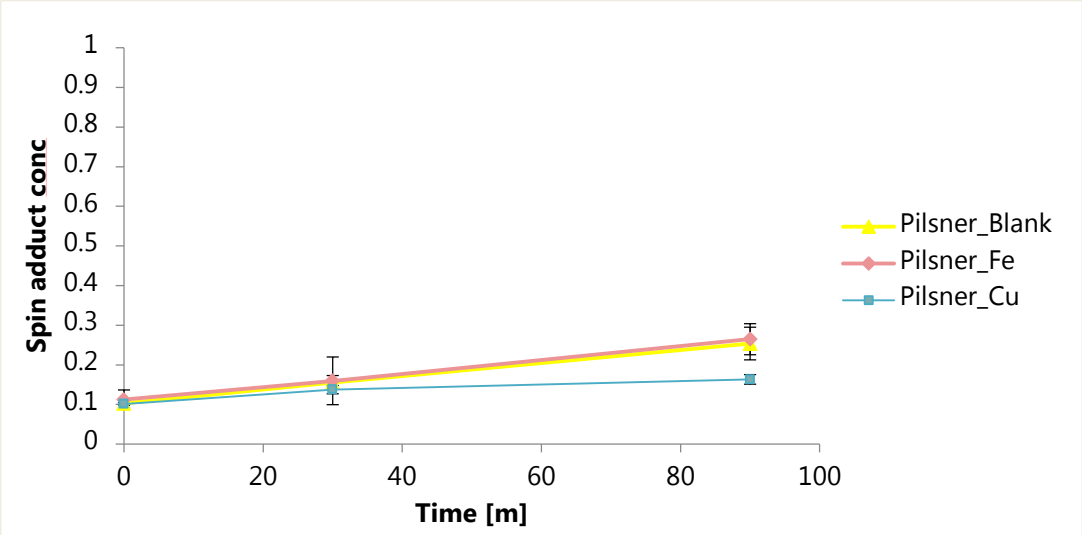

Figure 15. Spin adduct concentration for pure pilsner wort samples, blank and spiked with iron and copper. Bars represent mean ± SD.

When comparing the ESR results to the metal content, it should be expected the same spin adduct concentration for *pilsner* blank and sample spiked with Fe, as no difference between them was found for Fe content. However, higher ESR values for sample spiked with Cu were surprisingly lower (0.163±0.012 spin adduct conc.), comparing to the blank (0.253±0.041 spin adduct conc.).

Roasting malt to 125ºC didn't significantly change the spin adduct concentration (0.653±0.071 spin adduct conc.) when comparing pilsner wort (0.254±0.041 spin adduct conc.), $p<0.05$

However, a significant increase in the amount of spin adducts was observed when adding Fe to 125ºC wort, compared to the blank and sample spiked with Cu. This is in accordance with the higher concentration of Fe found in the spiked sample. Although Cu concentration was much higher in 125ºC spiked than in blank sample the amount of spin adducts was observed to be similar after 90 minutes of forced aging (0.708±0.012 and 0.653±0.071 spin adduct conc., respectively) (Figure 16).

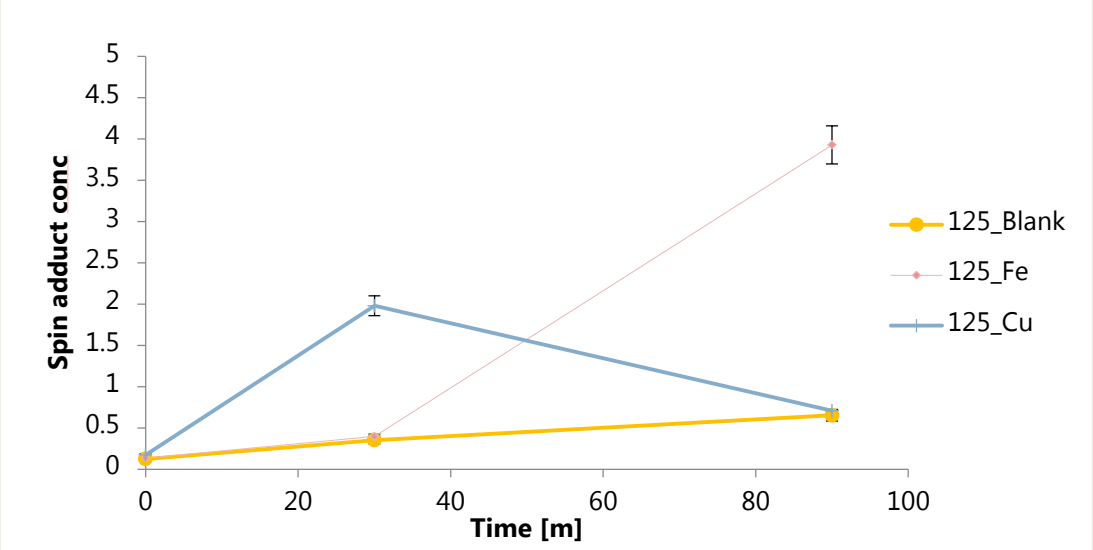

Figure 16. Spin adduct concentration for pure 125ºC wort samples, blank and spiked with iron and copper. Bars represent mean ± SD.

Roasting to 190ºC significantly decreased the oxidative stability of the sweet wort when comparing to pilsner and 125ºC, as can be seen in Figure 14. As previously observed the addition of Cu to the 190ºC roasted malt surprisingly decreased the spin adduct concentration comparing to the blank (2.162±0.831 and 4.888±0.001 spin adduct conc., respectively). However, the addition of Fe (10.698±0.177 spin adduct conc.) significantly decreased the oxidative stability comparing to the blank (4.888±0.006 spin adduct conc.) (Figure 17).

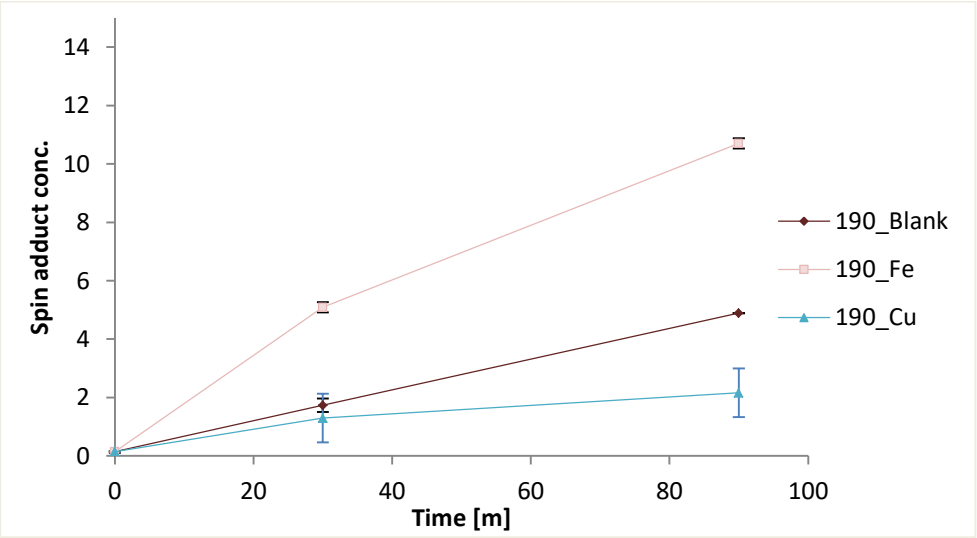

Figure 17. . Spin adduct concentration for pure 190ºC wort samples, blank and spiked with iron and copper. Bars represent mean ± SD.

Mixing effect

Oxidative stability of sweet wort only produced from pilsner didn't significantly differ from the blank and spiked samples A and B, (Figure 18 and Figure 19).

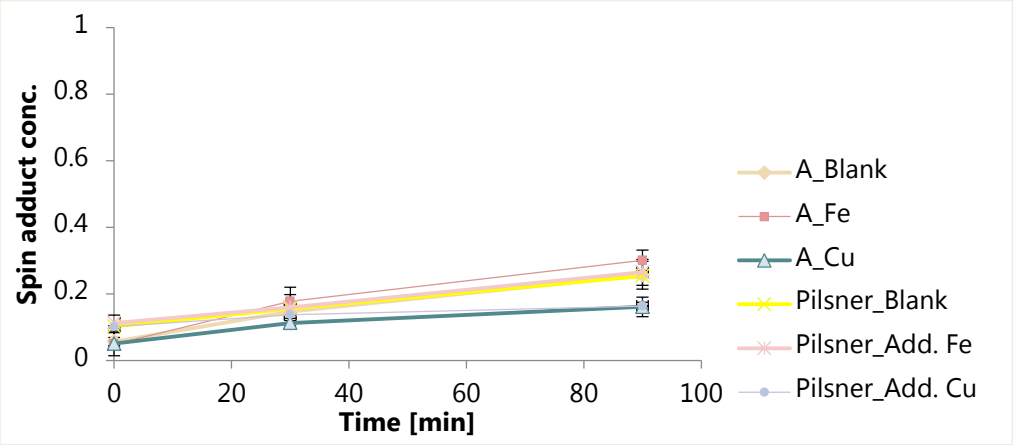

Figure 18. Spin adduct concentration for A and pure pilsner wort samples. Bars represent mean ± SD.

The metal content in blank and spiked samples from mixture A and B, as mentioned before, didn't differ from pure pilsner wort. In agreement the ESR values for mixture A were also very similar.

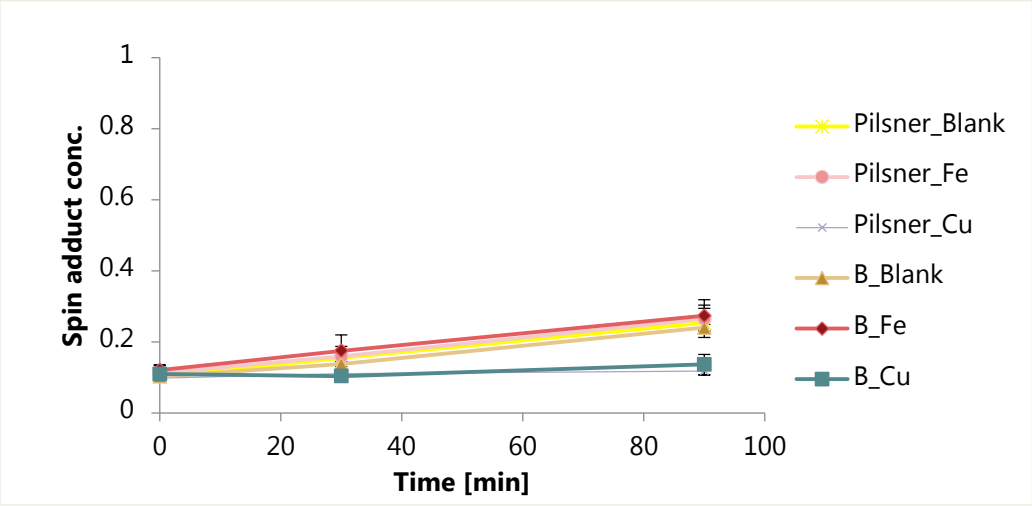

Figure 19. Spin adduct concentration for B and pure pilsner wort samples. Bars represent mean ± SD.

When analyzing the mixture $1_{pilsner}$:$9_{190ºC}$ (sample C) blank samples had shown significant increase in oxidative stability comparing to pure 190ºC (Figure 20).

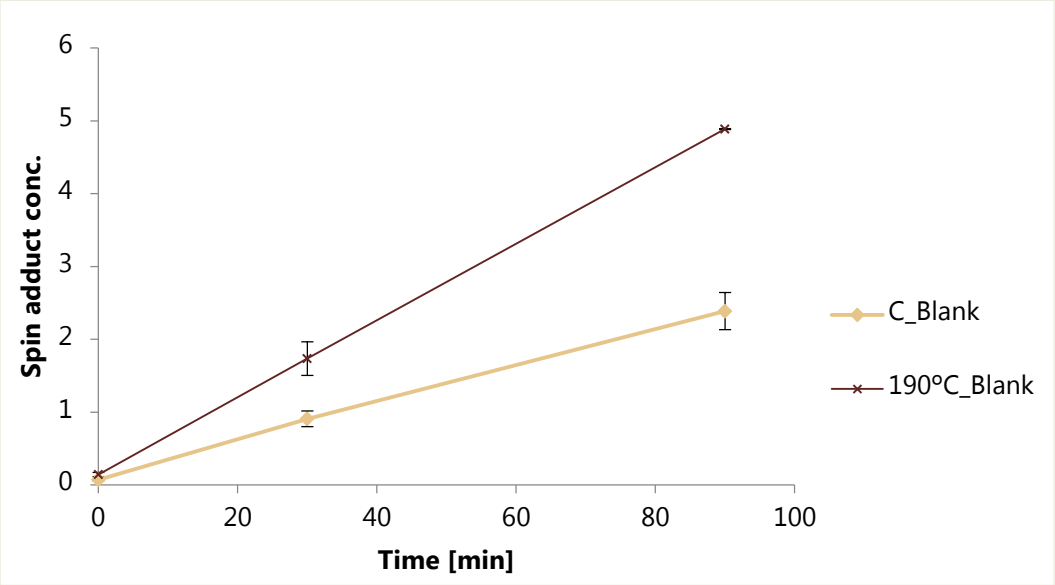

Figure 20. Spin adduct concentration for C and pure 190ºC wort blank samples. Bars represent mean ± SD.

As the metal content between both blank samples was very similar, it suggests that the increase in the oxidative stability of sample C is due to the antioxidants present in pilsner malt.

Roasting contributes to the formation of MRP which have been associated with a pro-oxidative effect, decreasing the oxidative stability of malt and beer[10]. Though, MLD have been widely studied and have shown many interesting biological reactions, as fiber-like action[54], bacteriostatic and bactericide activity, antioxidative[35] and also pro-oxidative effects[36]. As previously mentioned, the difference in results regarding the antioxidant capacity of MRP is probably due to the difference in methodologies. Indirect methods, based on the pre-formation of a free radical from an aromatic organic compound, like DPPH (2,2-diphenyl-1-picrylhydrazyl)[39], measure the scavenging activity using a semi stable radical that has high reactivity toward many types of compounds. These methods often result in antioxidant activity, whereas pro-oxidative effects are neglected. On the other hand, Electron Spin Resonance (ESR)-based experiments are established on a complete system providing information about the competition between pro- and antioxidants[38].

Our results corroborate the hypothesis that MRP act as pro-oxidant, as roasting at 190ºC significantly decreased the oxidative stability comparing to pilsner malt. Furthermore, the

pilsner antioxidant effect verified in the mixture of 10% pilsner to 90% 190ºC roasted malt may be due to the natural presence of polyphenols (principally catechin and ferulic acid)[10] and vitamins (particularly E and B) presents in the raw malt. Besides, thermal processes contribute to the loss of these compounds[55] what can also affect the oxidative stability of malt roasted at high temperatures.

The same *pilsner antioxidant effect* was verified in spiked samples with Fe, as the metal content didn't significantly differ, as shown in Figure 21.

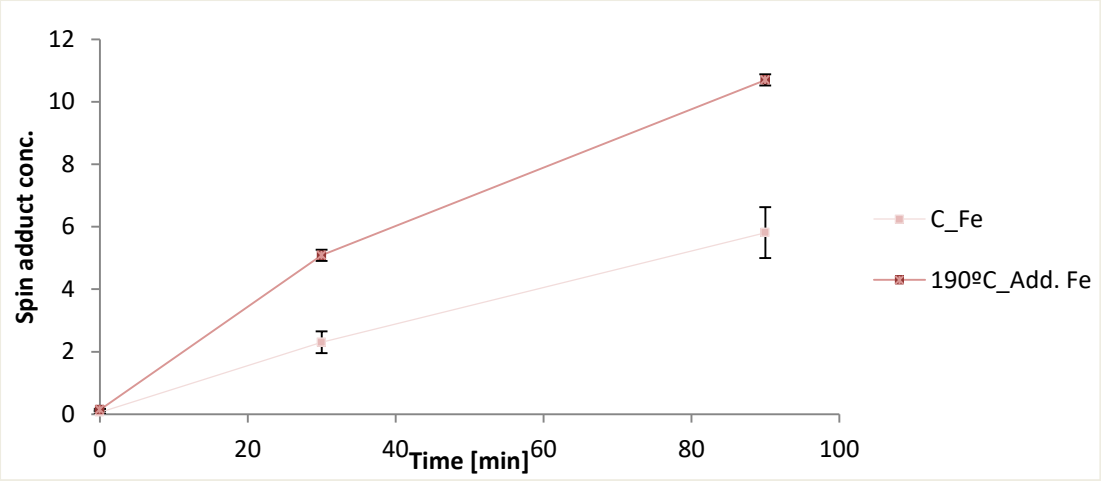

Figure 21. Spin adduct concentration for C and pure 190ºC wort samples spiked with Fe. Bars represent mean ± SD.

It is noteworthy that ESR results for spiked samples with Cu were very similar to the blanks, though its amount significantly increased in the spiked samples (Figure 22).

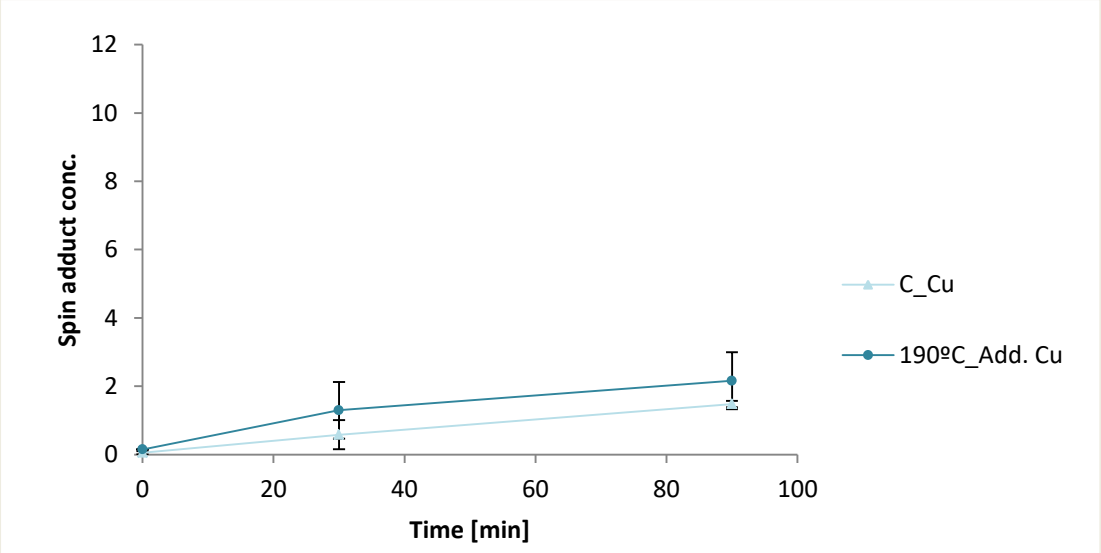

Figure 22. Spin adduct concentration for C and pure 190ºC wort blank samples spiked with Cu. Bars represent mean ± SD.

In a recent study[51], different concentrations of transition metal ions (Fe(II), Fe(III), Mn(II) and Cu(II)) were added to four different beers immediately prior to assessment for oxidative stability using the ESR assay.

Manganese produced the highest peak intensity when 160 ppb (highest amount dosed) was added to beer, followed by iron, the control, and copper, respectively. The addition of iron directly to beer resulted in an earlier generation of spin adducts and higher overall production with greater iron concentrations. However, the formation of spin adducts during the oxidative stability assay when copper concentration was increased clearly differed. They were consistently reduced in beers as copper was increased, in strong contrast to the results of iron. Copper produced more spin adducts early in the assay compared with the control but a smaller maximum peak was achieved with the increase in concentration

The smaller peaks detected for Cu, (Figure 17,18, 20 and Figure 22) are currently unexplained, though some potential reasons were given. Lower peaks might suggest that Cu generates less stable spin adducts with POBN. Also, that Cu might be interacting with pro-oxidant compounds in the beer system. Finally, that Cu ions can form stable complexes with proteins[29] being unable to react and this way potentially reduce the overall spin adduct formation[51].

Our results show that malt roasting has a larger influence on the oxidative stability of sweet wort and that light and dark worts behave very differently. Light sweet worts were less reactive toward oxidation with low radical intensity, low oxygen consumption rate and low Fe content. The dark sweet worts were found to be less stable with high radical intensities and high Fe content. However, the presence of Cu seems not to significantly affect the oxidative stability of the light wort samples. An increase in Cu content in light worts didn’t increase the amount of spin adducts and rate of oxygen consumption. Surprisingly and contrary to what expected they were slightly reduced comparing to the blank samples, though not significantly.

Oxygen consumption (OC)

Oxygen is a central reactant in various oxidative reactions and the rate of oxygen consumption is therefore expected to provide quantitative information about the overall rate of oxidation reactions in the tested sweet wort[56].

Results indicate an increase in oxygen consumption rate with the increase of roasting temperature. The addition of Fe and Cu to pilsner and 125ºC sweet wort didn't change the oxygen consumption rate compared to the blank samples. Also, the addition of Cu to 190ºC didn't alter the oxygen consumption rate comparing to the blank sample. However, the addition of Fe significantly increased the oxygen consumption.

The rate of oxygen consumption didn't differ between pilsner and samples A, blanks and spiked with metals respectively. Besides, no difference was found between pilsner and samples B.

The oxygen consumption rate from pure 190ºC wort and sample C didn't differ between blank and spiked samples, except for sample 190ºC added with iron, which had a significant higher rate.

All the results are in agreement with the relative oxidative stabilities that were observed with the ESR-based spin trapping method, except for the sample $1_{pilsner}:9_{190ºC}$, in which ESR values were significantly lower than pure 190ºC blank although the oxygen consumption rate is similar.

In ESR, the determination of the *endogenous antioxidative potential* is based on the detection of the radical generation during accelerated wort aging (60°C). In OC it is measured through the rate of oxygen consumption at room temperature.

Forced-aging is a pervasive but discriminative way to accelerate the processes that occur during the natural aging of beer and thus predict flavor stability. Since brewers are not able to wait several weeks or months until the first perceivable changes occur, they depend on sensory and analytical tools for the rapid estimation of flavor stability in beer[53].

The difference observed between ESR and OC, for sample $1_{pilsner}:9_{190ºC}$ may be due to the difference in methodologies.

Results showed that the beer ageing rates at 50 and 60°C were 30 and 56 times as fast as those at room temperature, respectively[23]. Accordingly, the same results would be expected,

for both ESR and OC, though more time would be required for the OC experiment to achieve the same outcome.

Oxygen consumption results and represented in Figure 40.

The final oxygen concentration in sweet wort is significantly lower in 190ºC sample than pilsner sample ($p<0.05$).

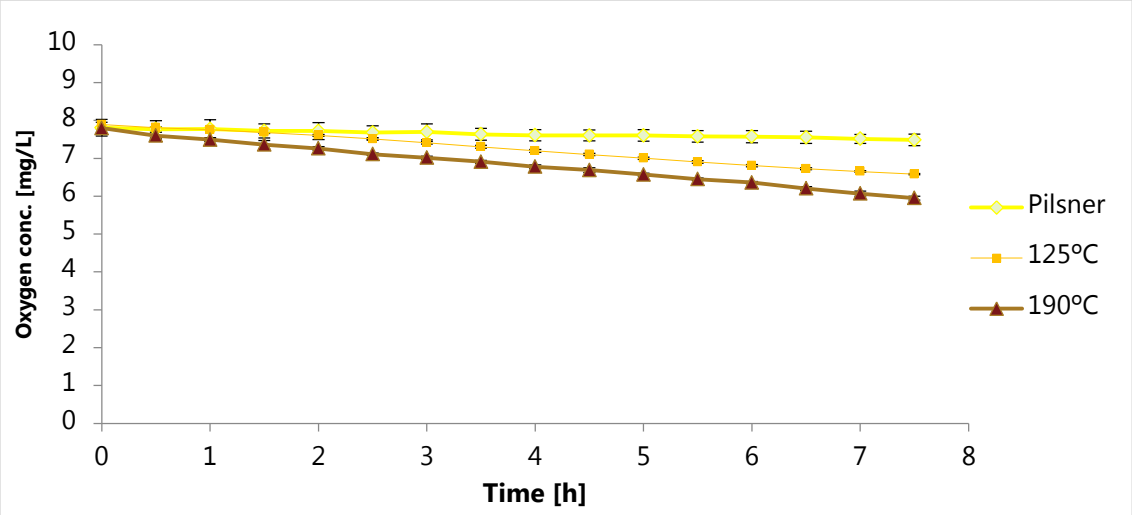

Figure 23. Oxygen concentration [mg/L] for pure wort samples, made from pilsner, 125ºC and 190ºC roasted malt. Bars represent mean ± SD.

The addition of metals didn't affect the rate of oxygen consumption in all pilsner samples, Figure 33.

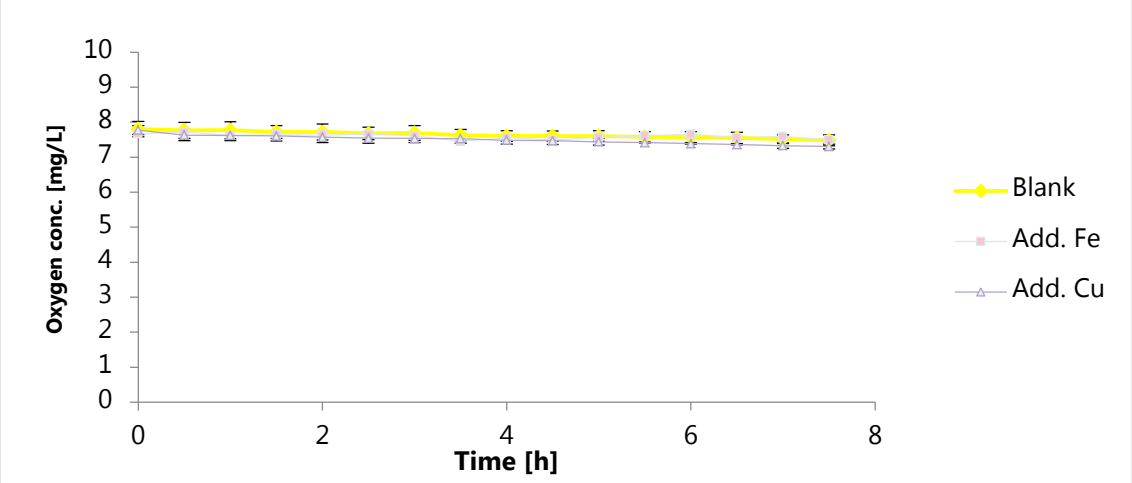

Figure 24. Oxygen concentration [mg/L] for pilsner pure wort samples, blank and spiked with Fe and Cu samples. Bars represent mean ± SD.

The addition of Fe and Cu didn't significantly affect the rate of oxygen consumption in 125ºC wort sample, Figue 34.

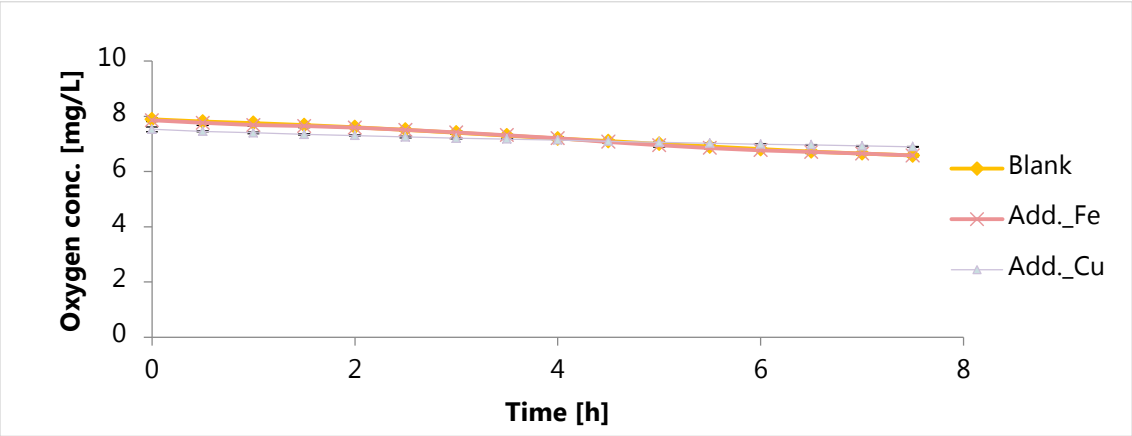

Figure 25. Oxygen concentration [mg/L] for pilsner pure 125ºC roasted wort, blank and spiked samples. Bars represent mean ± SD.

For 190ºC wort sample the addition of Cu didn't influence the final concentration of oxygen compared to the blank sample. Though, the addition of Fe significantly ($p<0.05$) increased the rate of oxygen consumption, Figure 35.

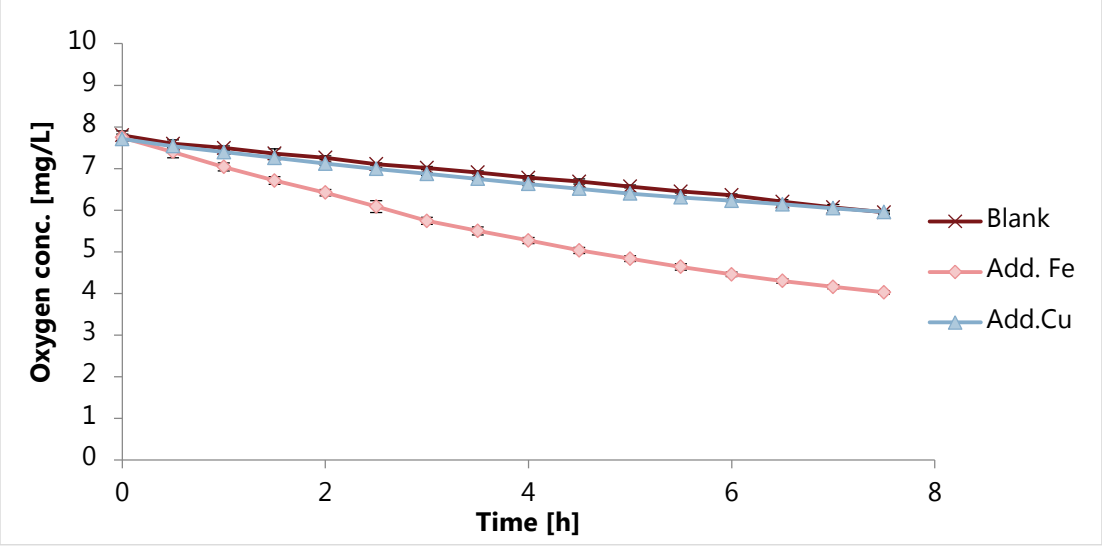

Figure 26. Oxygen concentration [mg/L] for pilsner pure 190ºC roasted wort, blank and spiked samples. Bars represent mean ± SD.

When analyzing the mixing effect between pure pilsner sweet wort and the proportion $1_{190ºC}$:$9_{pilsner,}$ no significant differences were found between the blank and spiked, respectively, Figure 36.

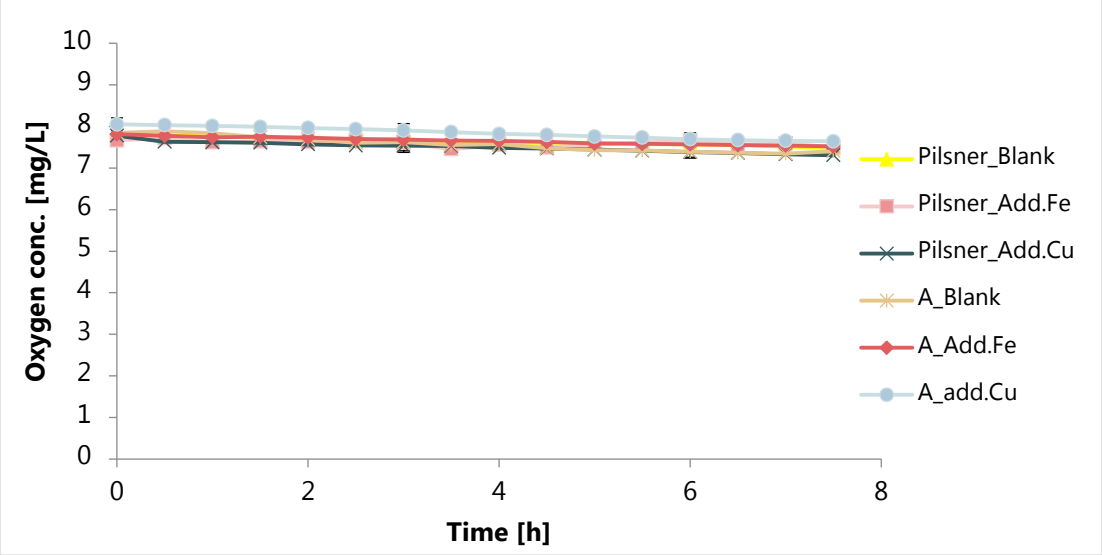

Figure 27. Oxygen concentration [mg/L] for pilsner pure pilsner and A wort, blank and spiked samples. Bars represent mean ± SD.

Also, the results of oxygen consumption of the proportion of $1_{125ºC}$:$9_{pilsner}$ (sample B) comparing to pure pilsner sweet wort didn't differ from blank and spiked samples, respectively, Figure 37.

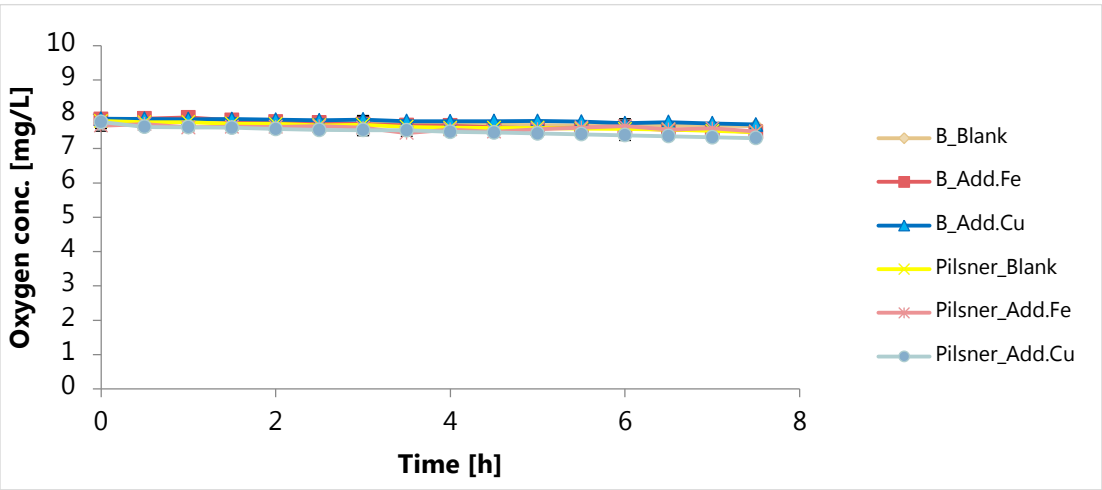

Figure 28. Oxygen concentration [mg/L] for pilsner pure pilsner and B wort , blank and spiked samples. Bars represent mean ± SD.

The results for the mixture $1_{pilsner}$:$9_{190ºC}$ (sample C) show that only the addition of Fe significantly increased the rate of oxygen consumption, comparing to the blank sample and spiked with Cu. Besides, final concentration of oxygen in the pure and spiked samples of 190ºC wort did not differ between them and samples C (blank and spiked with Cu).

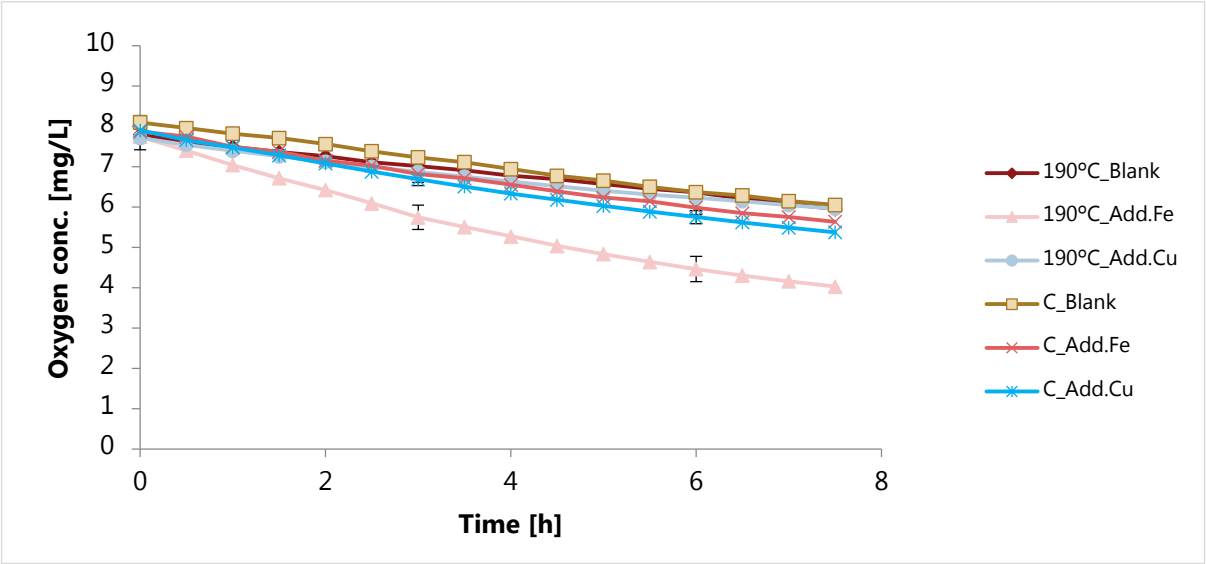

Figure 29. Oxygen concentration [mg/L] for pilsner pure 190ºC roasted and C wort, blank and spiked samples. Bars represent mean ± SD.

4. Conclusions and future perspectives

The main aim of this study was to evaluate the impact of Fe and Cu content on the oxidative stability of sweet wort and how malt modification by roasting temperature would influence the metal content after mash and filtration as well as the oxidative stability of the sweet wort.

Pilsner malt from a single batch was roasted at 125ºC and 190ºC and sweet wort samples were produced from the three types, respectively. The results obtained allowed to conclude that roasting malt has a significant effect on the metal levelling during the mash and filtration process. Untreated pilsner malt demonstrated a strong levelling effect for Fe but not for Cu. The reason for this effect is unknown.

It was also verified that the increase of roasting temperature decreases the removal of Fe and increases significantly the removal for Cu (higher levelling effect for 190ºC, though not as strong as pilsner levelling for Fe).
The oxidative stability of sweet worts decreased significantly with the increase of roasting temperatures and addition of Fe. Our results didn't demonstrate an impact of Cu content on the oxidative stability of wort.

In order to proceed the study in a more realistic approach the three previous types of malts were mixed to produce new sweet wort samples. It aimed to verify if the addition of 10% of roasted malts to untreated pilsner malt would affect significantly the levelling effect and oxidative stability demonstrated for pure pilsner worts. It was further tested if the addition of 10% of untreated malt would affect the same parameters for sweet wort produced only from 190ºC roasted malt.

Our results indicate that the realistic mixture of malts didn't affect significantly the levelling effect for both metals, though we could see the expected tendency for Fe and Cu – Fe content slightly increased with the addition of roasted malts and Cu decreased, respectively.

This project results indicate that antioxidant compounds present in unroasted pilsner malt significantly contribute for the oxidative stability of sweet wort. The addition of 10% of pilsner significantly reduced the amount of spin adducts comparing to pure 190ºC sweet worts.

Nevertheless, the increase in Fe content significantly decreased the oxidative stability, though again no impact of Cu in this parameter was verified.

Although copper is often postulated to have a similar pro-oxidant role to iron in contributing to *flavor* instability, its influence has not been demonstrated in this study. Further research is

needed to better understand its impact on wort and beer oxidative stability through ESR and OC methodologies.

The *upper levelling capacity* of the mashing process, for Fe and Cu is still unknown and would be relevant to test it through increasing the amount of these metals at the beginning of the mash. In addition, further studies are needed to evaluate the impact of higher Fe concentrations on the oxidative stability of pure pilsner sweet wort.

From a practical point of view, it is important to consider that all raw materials used for brewing (malt, hops, water, yeast, adjuncts) influence *flavor* stability and the staling potential of the resultant beer. Even though the *Congress Mashing* used in this study is *outdated* from the mashing process typically used in the brew houses, our results demonstrate a tendency for the levelling effect and oxidative stability. In this way, a further step would be to test the same variables in a pilot scale brew house and additionally analyze the impact of the content of the metals at the beginning of the mash, further in *boiled wort*, *immature* and the *mature* final beer.

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Annex

Annex I

Intensity values of the standard solutions and calibration curve

Intensity values for the standard solutions of Copper and Iron are resumed inTable 17Table 18, respectively.

Table 17. Register of intensity values of Cu

Standard	Intensity	Average	SD	%RSD	Concentration (mg/L)
1	2415.56 2431.96 2430.42	2425.98	9.05	0.37	100
2	27224.97 26767.99 26731.38	26908.11	275.01	1.02	1500
3	55577.12 55063.32 55096.66	55245.7	287.5	0.52	3000

Table 18. Register of intensity values of Fe

Standard	Intensity	Average	SD	%RSD	Concentration (mg/L)
1	1556.18 1534.97 1536.82	1610.41	11.75	0.76	100
2	2592.76 2561.61 2548.28	17018.65	22.83	0.89	1500
3	119352.56 116815.1 119582.15	34974.59	1535.55	1.29	3000

In Figure 30 and Figure 31are represented, respectively, the calibration curves for Cu and Fe.

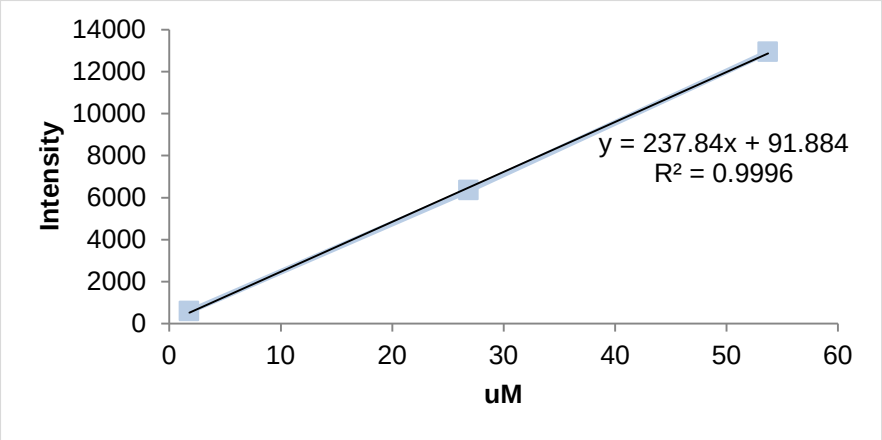

Figure 30. Calibration curve for Cu.

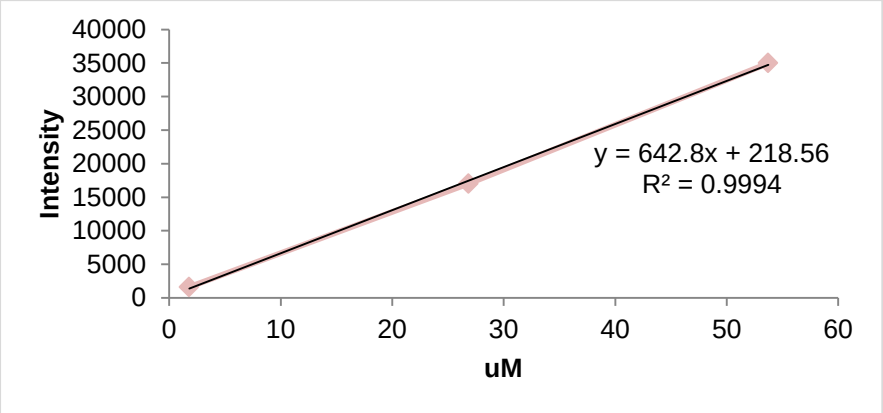

Figure 31. Calibration curve for Fe.

Annex II

The addition of metals to the pure wort samples doesn't seem to affect significantly the pH, Color and Plato, Figure 32, 27 and 28, respectively.

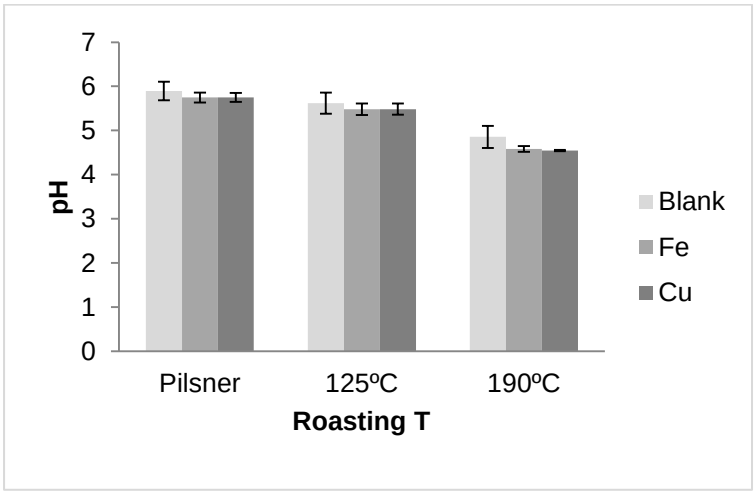

Figure 32. pH values for pure wort samples according to roasting temperature (ºC). Bars represent mean ± SD.

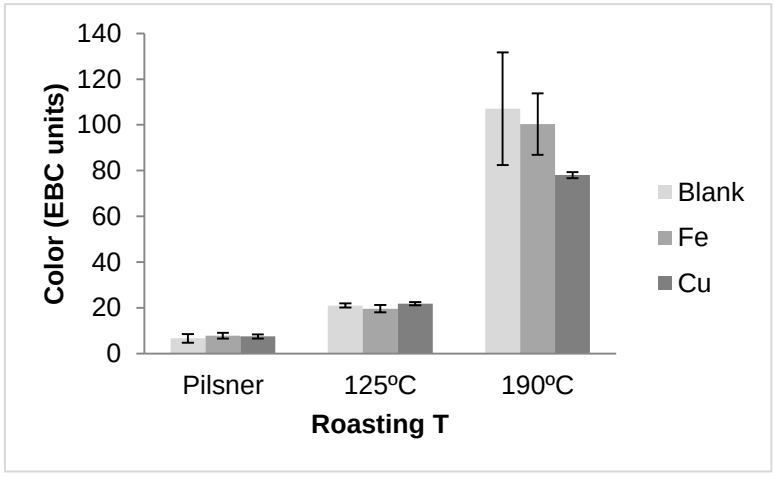

Figure 33. Color in EBC units for pure wort samples according to roasting temperature. Bars represent mean ± SD.

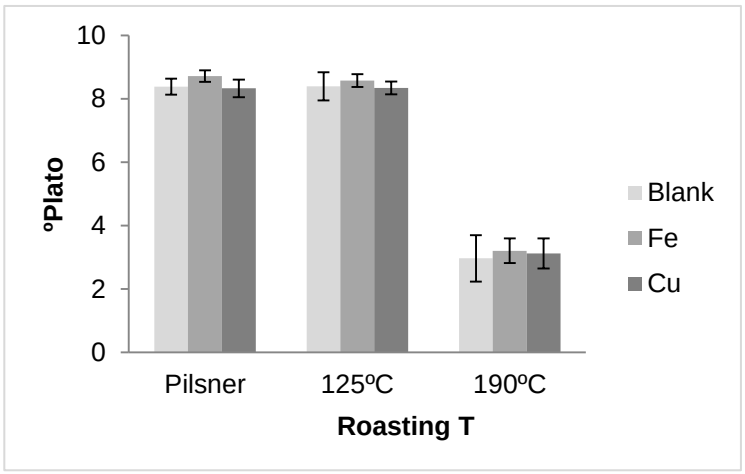

Figure 34. ºPlato (%) values for pure wort samples according to roasting temperature (ºC). Bars represent mean ± SD.

The addition of metals to the mixed wort samples doesn't seem to affect significantly the pH, Color and Plato,Figure 35 to Figure 36.

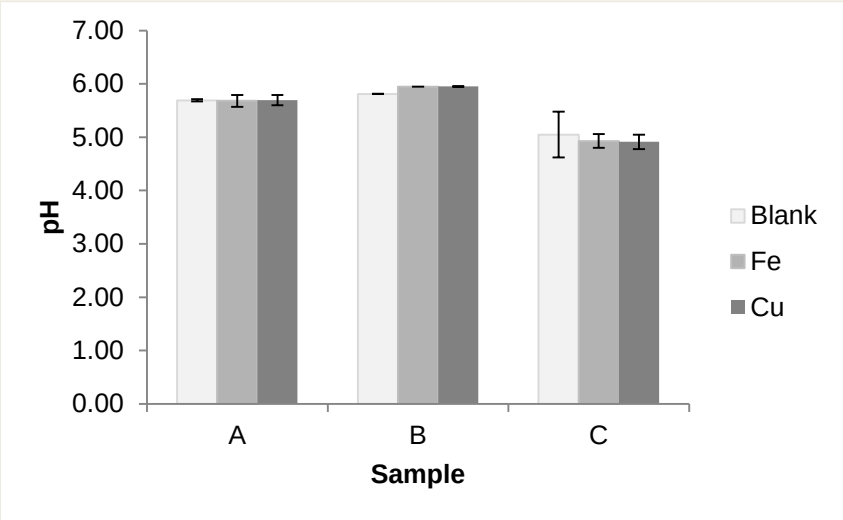

Figure 35. pH values for mixed wort samples according to roasting temperature (ºC). Bars represent mean ± SD.

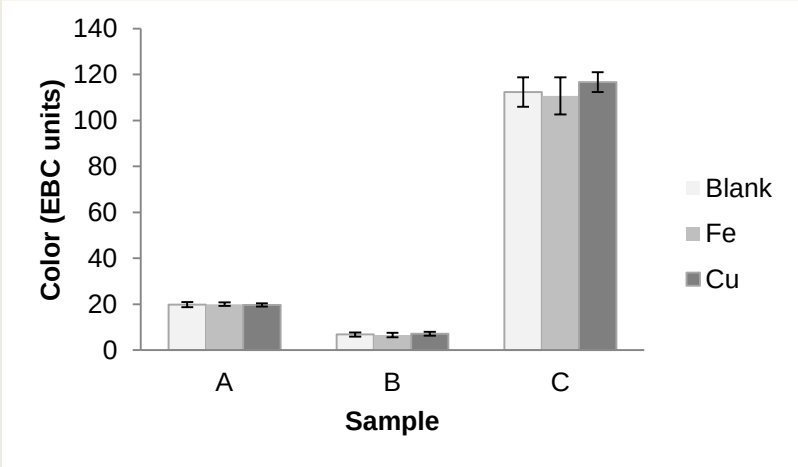

Figure 36. Color (EBC units) values for pure wort samples according to roasting temperature (ºC). Bars represent mean ± SD.

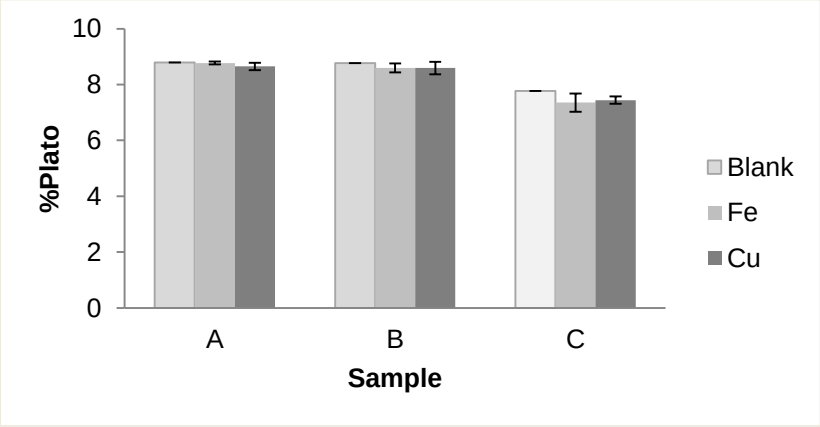

Figure 37. ºPlato (%) values for mixed wort samples according to roasting temperature (ºC). Bars represent mean ± SD.

Annex III

Centrifuged 190ºC sweet wort samples

First ESR measurements of pure 190ºC samples showed a variation between the duplicates that made impossible the assumption of any effects, Figure 42

In order to analyse if the haze from the 190ºC wort samples would influence the results, all 190ºC samples were centrifuged and measured again. Our results show a significant reduction in the standard deviation; though around the same average values for the spin adduct concentration, Figure 39.

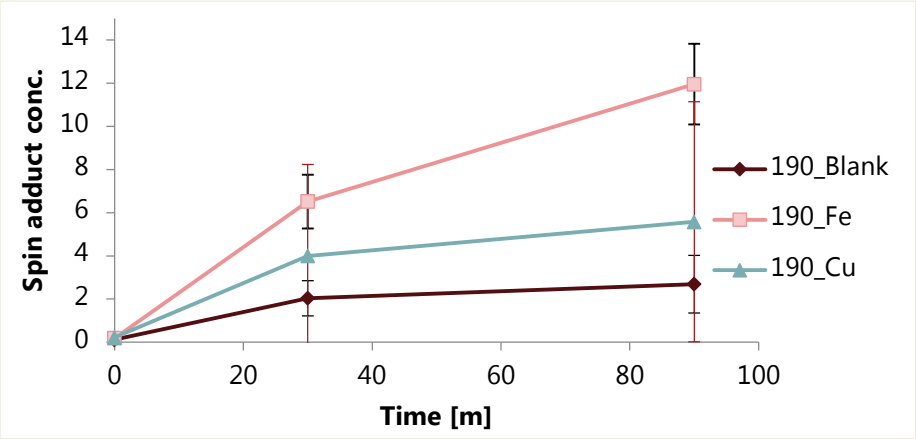

Figure 42. Spin adduct concentration for pure 190ºC wort samples, blank and spiked with iron and copper. Bars represent mean ± SD.

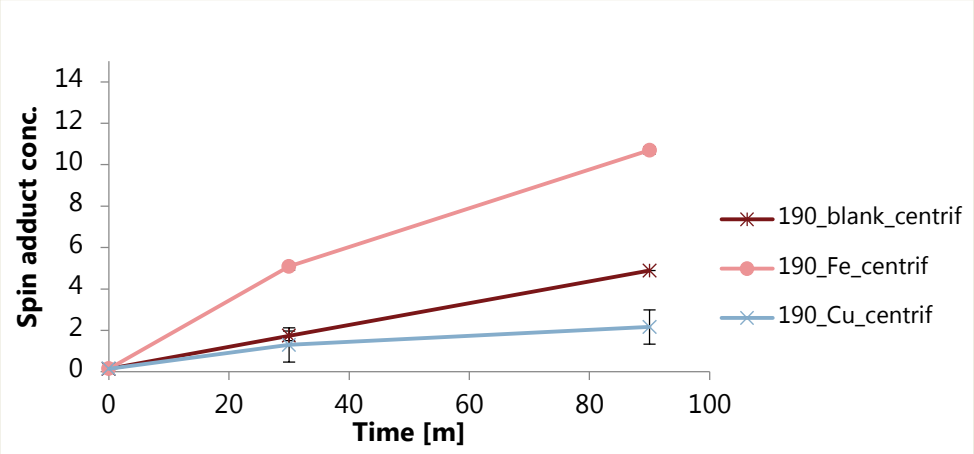

Figure 41. Spin adduct concentration for pure 190ºC centrifuged wort samples, blank and spiked with iron and copper. Bars represent mean ± SD.

A demonstrative sampling of hazed samples is virtually impossible due to the small volume measured in ESR. Centrifuging the samples allowed a more homogeneous sampling. These results indicate that haze compounds have an impact on the oxidative stability of sweet wort.

Annex IV

Oxygen consumption - Preliminary Tests

In order to test the protocol for the Oxygen Consumption measurements were analysed two samples of aged beer (Royal Classic® Pale Ale, alc. vol. 4.6%)

The measurement mode was set as:

25mL conic flasks were filled with 20mL of wort sample which had been freshly saturated with atmospheric air by bubbling for 2 min. The oxygen measurements were started immediately after the flask was filled. The oxygen concentration was recorded every 5 min, and the sensor had initially been calibrated against air saturated (for 45 minutes) demineralized water at 25 °C. The oxygen consumptions were measured during 8 h.

The oxygen concentration was recorded twice per beer sample, every:

- 5 min for 8h
- 10 sec for 8h

The results for Oxygen concentration and Temperature were slightly different between the probes and the measurement settings, for the same sample, though not considered significative. For this project was chosen the 5min measurement interval for 8h.

Results for beer 1 are presented in Figure 43 and Figure 44.

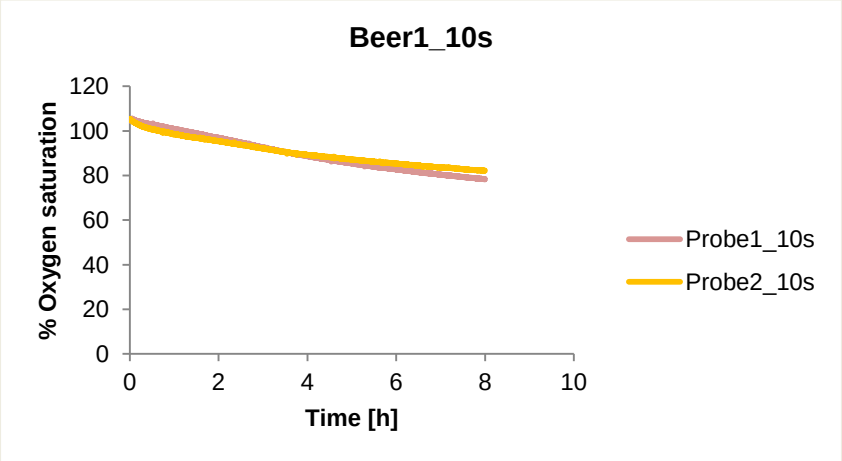

Figure 43. % Oxygen saturantion in beer nº1. 10secs measurement interval for total time frame of 8h.

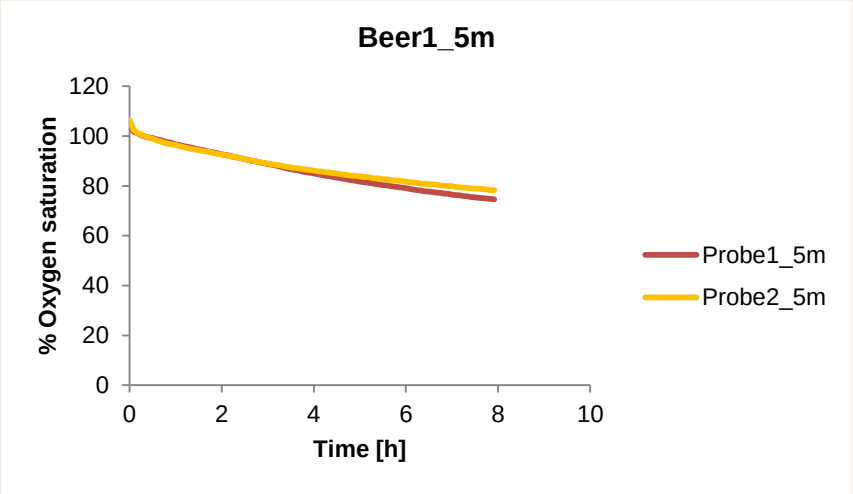

Figure 44. % % Oxygen saturantion in beer nº1. 5min measurement interval for total time frame of 8h.

Results for beer 2 are presented in Figure 45 and Figure 46.

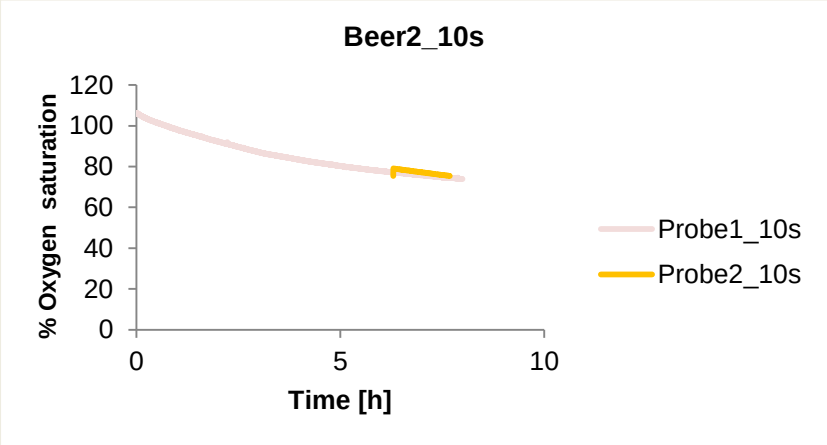

Figure 45. % Oxygen saturantion in beer nº2. 10 secs measurement interval for total time frame of 8h.

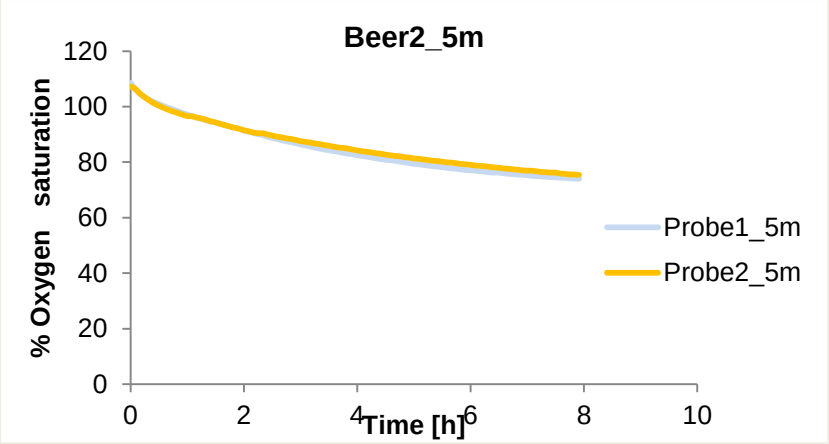

Figure 46. % Oxygen saturantion in beer nº2. 5 min measurement interval for total time frame of 8h.