Review article

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Transition metals in brewing and their role in wort and beer oxidative stability: a review

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Beer inevitably changes over time: the colour will darken, haze may form, and stale flavours develop, while others fade. The challenge of maintaining the fresh flavour quality of beer (over a typical 9-12 month storage period) is generally the determining factor of a beer's shelf-life for brewers, as opposed to colloidal or microbiological stability. Fortunately, as early as the brewhouse, oxidative degradation can - to a certain extent - be controlled, enabling the shelf-life to be increased. This review considers the general issues of oxidative stability, mechanisms of ageing, ways of quantifying staleness and staling potential, and current practical approaches to prevent oxidative beer ageing. Emphasis is placed on the catalytic role of iron, copper and manganese on oxidation during brewing and storage; and how the removal and/or inhibition of these prooxidative transition metal ions leads to prolonged beer (flavour) stability.

Keywords: Beer ageing; flavour stability; staling; oxidation; transition metal catalysts; chelation

Introduction

Beer is the most widely consumed alcoholic drink and - together with coffee and tea - one of the most popular beverages. The beer market continues to expand *(1)*, as does consumer knowledge and demand for high(er) quality. Beer is expected to be fresh and free of any contamination or inappropriate haze. Fortunately, the latter are no longer serious problems for the brewer to control, as microbial and colloidal stability are, respectively, taken care of by removal of bacteria and wild yeast (e.g. by hygienic working practices, pasteurisation, 0.45 μm membrane filtration) and application of stabilisation agents (e.g. PVPP, silica gel, tannic acid, etc.) *(2)*. Accordingly, flavour stability - the ability of a product to retain freshness and resist physicochemical changes – is the key factor in determining beer shelf-life.

Beer flavour stability has been the subject of more than 500 publications (SciFinder). This is because, for any business to attract and keep customers, the production and delivery of a consistent quality product is key. Creating a well known identity is vital to maintain customer selection preference *(3)*. Delaying in-pack flavour changes as long as possible is of great commercial importance *(4)*.

The issue of beer flavour (in)stability is more relevant than ever, given the international market where packaged beer can be shipped around the globe, often under harsh conditions (e.g. hot containers, vibrating trucks) and for extended periods of time *(5)*. Even though it has been intensively studied since the 1960s, the science behind beer staling is still not fully understood and controversies remain.

The chemical non-equilibrium of beer, emanating from its intricate matrix composition, gives rise to a complex set of ageing phenomena; suggesting that fresh beer, as a natural product, will never be a fully stable commodity *(6)*. Nonetheless, considerable improvements in beer flavour stability have been made over the years and further advances can be expected, because of ongoing research and the continuous improvement of technology.

Noticeable flavour changes may become apparent three months from packaging (at room temperature) *(7)*, but this is a rough and general estimate. Actual dates will vary depending on multiple factors, including beer style, storage conditions, total package oxygen (TPO), packaging, and agitation *(8)*. Oxidative processes are widely recognised to be the main force behind product degradation *(9–12)* and the resulting flavours are described as 'oxidised', 'aged' or 'stale'. These are umbrella terms for describing a combination of oxidation notes found in beer, such as cardboard/papery, sherry/Madeira, honey, ribes/blackcurrant/catty, leathery, etc.

With high oxygen ingress (e.g. during the brewing process, filtration *(13)*, poor filling practices, or usage of PET bottles *(14)* or inadequate bottle caps *(15)*), these flavour transitions take place more rapidly. Off-flavour formation occurs, but also degradation of initial, fresh flavours, such as hop aromas and pleasant bitterness. The organoleptic outcome of the shifts will depend on the concentration of the formed and degraded substances, and their respective flavour activities.

Besides flavour deterioration, aged beers typically appear darker than their fresh counterparts, due to the oxidised polyphenols being colourants *(16)*. These oxidised polyphenols can also cause colloidal and foam stability issues, due to the polymerisation of protein-polyphenol-metal complexes (forming haze), which concurrently diminishes the foaming ability through precipitation of foam positive polypeptides *(17,18)*.

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Beer ageing mechanisms

Oxidative beer ageing

Oxygen exposure is detrimental to beer stability and causes accelerated aldehyde formation *(19)*. Fresh beer typically only contains low levels of aldehydes which are below the flavour threshold. In the past, the aldehyde *trans*-2-nonenal was subject to a lot of attention since increasing concentrations of this potent, volatile carbonyl were often seen in conjunction with beer staling, contributing to the 'aged beer' flavour characterised by stale, oxidised, and cardboard/papery notes. These days, an array of marker aldehydes is typically used to determine flavour instability *(20,21)*: including Strecker degradation aldehydes (2- and 3-methylbutanal, 2-methylpropanal, methional, benzaldehyde, and phenylacetaldehyde), lipid oxidation aldehydes (hexanal and *trans*-2-nonenal), and aldehydes formed during Maillard reactions (furfural). With the exception of furfural, these aldehydes can be formed through oxidative reactions *(6)*.

Oxidative degradation is the main cause of rapid beer staling *(22)* and, in most cases, the principal oxidising agent will be oxygen. However, as is later discussed in 'oxygen-free beer ageing', other reagents can act as an oxidant, such as mineral ions (e.g. Fe^{3+} or Cu^{2+}), oxidised organic compounds (e.g. melanoidins, phenols), and halogens (e.g. from cleaners/sanitisers). Regardless, the presence of oxygen will invariably promote oxidation.

Remarkably, molecular oxygen ($^3O_2$) - partially dissolved in beer, present in the headspace and diffusing through the crown cap liner during storage - is relatively unreactive, as the reaction of oxygen with organic compounds is hindered kinetically *(23)*. However, ground state oxygen can be converted to highly reactive forms, either by chemical reduction or collision with other organic radicals, which can be formed during high energy stages, including kilning of malt, wort boiling, beer pasteurisation, exposure to light *(24)*, and even milling *(25)*. These highly reactive oxygen forms are collectively termed 'reactive oxygen species' (ROS) and are potent oxidisers. Each is more reactive than oxygen itself and, in increasing order of reduction state and reactivity, involve superoxide anion ($O_2^{\bullet-}$) < perhydroxyl radical ($HO_2^{\bullet}$) < hydrogen peroxide (H_2O_2) < hydroxyl radical ($OH^{\bullet}$) *(6)*.

Bamforth and Parsons *(26)* first noted the importance of ROS in beer regarding flavour stability. At the time (1985), little was known about the mechanisms at play; but, involvement of the Fenton and Haber-Weiss mechanism was likely, as transition metal ions in beer are known to catalyse these radical-creating reactions. In 1988, Kaneda and colleagues *(27)* first monitored free radicals in beer by electron spin resonance (ESR) spectroscopy. In further studies *(28–33)*, they uncovered the effects these radicals have on beer flavour deterioration, and the pathways involved in their formation, by trapping the short-lived radicals with spin-trapping reagents. The technique to trap short-lived radicals, creating detectable long-lived spin adducts is still used in beer ageing research today.

Supporting Bamforth's claim, Kaneda and colleagues suggested the free radicals formed in beer, to be hydroxyl radicals which, as the most reactive oxygen species, attack almost every substance with poor selectivity. While this is indeed taking place, Andersen and Skibsted *(34)* later showed the 1-hydroxyethyl radical to be the most abundant radical in beer, due to hydroxyl radicals reacting with ethanol a good radical scavenger and copious in beer. The generated alkyl radicals subsequently form acetaldehyde and hydroperoxyl radicals, after electron loss by metal ions or oxygen (see Figure 1) *(34,35)*. The dominant radical species in alcohol-free beer is unknown, but presumably, would also be carbon centred, since, in the absence of ethanol, the nonselective hydroxyl and alkoxyl radicals will react in a non-specific manner with any nearby carbohydrate, protein, polyphenol, or other organic molecules present in the matrix *(36)*.

Although the mechanisms in Figure 1 are well understood, and their significance is not in question, it is still uncertain exactly how big a part they play in the overall staling picture. Oxidation, for example, also occurs enzymically in malting and mashing through the action of lipoxygenase (LOX) *(37)*. There are however some caveats, as iron besides promoting free radical and ROS formation through Fenton and Haber-Weiss reactions, also plays a role in lipoxygenase-induced oxidation *(38,39)*. To further illustrate the complexity, other enzymes such as catalase conversely protect against oxidative damage *(36,40)*.

In addition, staling compounds (such as aldehydes) that are formed during malting and mashing, may bind to other compounds, resulting in adduct formation *(41,42)*. While the influence of oxygen (and other parameters) on the formation of bound state aldehydes has yet to be fully investigated *(43)*, their gradual release may be considered an indirect form of oxidative staling, as they progressively become 'unmasked' during storage *(44–47)*. Protecting the malt and wort from oxidation could prove beneficial to shelf-life, since it would limit the endogenous 'ageing potential' carried over to the fresh beer. However, it is still unknown to which degree oxidised substances in the free or bound state are formed during malting and brewing, or how much of the intrinsic antioxidative power of malt and wort is lost during these stages, and how substantial this is in the ageing of packaged beer. In summary, beer staling is an immensely complex interplay of reactions, with its kinetics still mostly unclear (e.g. enzymatic versus non-enzymatic) *(48)*.

As briefly mentioned, and displayed in Figure 1, transition metals, such as ferrous (Fe^{2+}) and cuprous (Cu^{+}) ions, play a vital role in ROS formation *(49)*. The participation of manganese ions (Mn^{2+}) in generating radicals has been investigated but to a lesser extent. Although, like iron and copper, manganese is a d-block element (found from the third group to the twelfth group of the modern periodic table), making it a viable metal catalyst, as it shares the tendency to exhibit two or more oxidation states. Since the initial report by Zufall and Tyrell in 2008 *(50)*, few recent studies have confirmed the augmenting effects of manganese ions on beer staling *(51,52)*.

Fe^{2+}, Cu^{+} and Mn^{2+} serve as electron donors to reduce oxygen species and are oxidised to Fe^{3+}, Cu^{2+} and Mn^{3+}. Oxygen ($^3O_2$) capturing an electron forms superoxide ($O_2^{\bullet-}$), which can become protonated to generate the perhydroxyl ion ($HO_2^{\bullet}$), or is further reduced to peroxide (O_2^{2-}) which is then protonated twice, forming hydrogen peroxide (H_2O_2). Both perhydroxyl and hydrogen peroxide are reduced to hydroxyl ($OH^{\bullet}$) via the Fenton and Haber-Weiss reactions. Because beer is relatively acidic (pH ~ 4.3), most of the superoxide will be in the more reactive perhydroxyl radical form (pKa 4.8).

Prooxidant molecules present in beer can reduce oxidised metal ions back to their reduced state, so that they can contribute to the activation of ground state oxygen (or a ROS) again. This is aggravated by a process of 'free radical atom abstraction', where the prooxidant itself can turn radical, furthermore degrading or reacting with other compounds, producing off-flavours. Examples of wort/beer species, which have easily abstractable hydrogen and can become secondary organo-radicals, are alcohols (primarily ethanol), sugars (e.g. glucose), free-thiol group (e.g. cysteine) containing

Fenton reaction:

$$M^n + H_2O_2 \longrightarrow M^{n+1} + OH^\bullet + OH^-$$
$$M^{n+1} + H_2O_2 \longrightarrow M^n + O_2^{\bullet-} + 2H^+$$

Net: $2H_2O_2 \xrightarrow{M} OH^\bullet + OH^- + O_2^{\bullet-} + 2H^+$

Haber-Weiss reaction:

$$M^{n+1} + O_2^{\bullet-} \longrightarrow M^n + O_2$$
$$M^n + H_2O_2 \longrightarrow M^{n+1} + OH^\bullet + OH^-$$

Net: $O_2^{\bullet-} + H_2O_2 \xrightarrow{M} O_2 + OH^\bullet + OH^-$

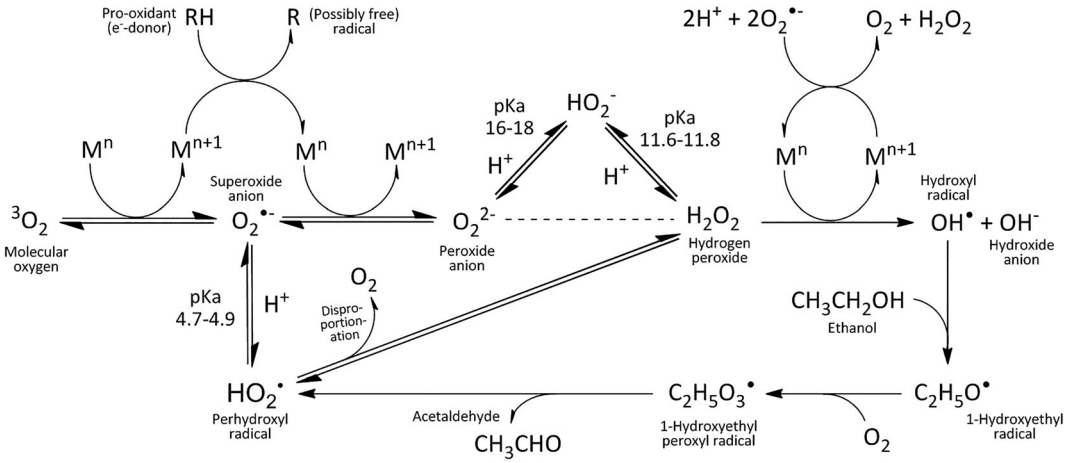

Figure 1. Mechanism of reactive oxygen species formation in beer, adapted from *(33,34)*

proteins, organic acids (e.g. isohumulones), and phenolic substances (e.g. hydroquinone, catechols) *(53–56)*.

Some prooxidants, such as cysteine or other sulfhydryl-group containing proteins, can act as a direct source of H_2O_2, when heated or illuminated *(57)*. Examples of compounds that can act as prooxidative electron donors are reductones (e.g. early and intermediate stage Maillard reaction products) *(58)*, polyphenols, sugars, iso-humulones, oxidised melanoidins, and alcohols *(33)*.

Antioxidants, on the other hand, inhibit the effects of oxygen, by either chelating metal ions and/or capturing ROS and other free radicals ('quenching') *(59)*. Some native beer and wort components - when present in appropriate (typically high) amounts - already exhibit these protective properties, including yeast produced sulphite and certain malt and hop products (such as tocopherols, flavonoids, tannoids, phenolic acids, reduced/unoxidised melanoidins, reductones, and specific amino acids and peptides *(60)*).

Sulphite tends to get the most attention, as it is arguably the best naturally occurring antioxidant found in beer that can, in addition, mask aged flavours by forming flavour inactive adducts with carbonyl staling compounds *(61–63)*. Nevertheless, some authors claim that there may be an upper limit regarding the ideal SO_2 content in a beer *(22,64)*. Yeast, for example, cannot readily reduce carbonyl-SO_2 adducts. Reduced formation of sulphite during fermentation will equate to higher levels of free aldehydes, which the yeast can then reduce to alcohols. This may mitigate the carbonyl 'blooms' often seen during (warm) beer storage *(65)*. Additionally, when it comes to anti/prooxidants, concentration is decisive. Even with sulphite, a recent study by Foster and Ranguelova *(66)* found that when the total beer SO_2 is in excess of what is needed for the quenching of free radicals, reduction of H_2O_2 and adduct formation, excessive HSO_3^- is converted to $SO_3^{\bullet-}$ (a free radical detrimental to beer flavour stability).

The oxidative mechanisms in wort and beer are an intertwined chain of electron transfer (oxidation-reduction/redox) reactions. Each reaction has a reaction rate (the speed at which a chemical reaction proceeds) and this rate is affected by the type of reaction, the substrate concentration, and the temperature. Thus, that a reaction is thermodynamically probable, does not guarantee that it happens under storage conditions.

As noted - apart from obvious parameters (storage temperature and in-pack oxygen) - transition metals play a key role in the loss of beer freshness *(22)*. They may even be responsible for the majority of oxidative degradation reactions in foods, as oxidation in the absence of catalysts is often negligible *(67)*. Transition metal ions influence a myriad of chemical reactions and aid in the creation of free radicals. This causes oxidative degradation of various organic molecules in wort and beer, including iso-humulones *(68)*, unsaturated fatty acids *(69)*, amino acids *(70)*, polyphenols *(68)*, sugars *(71)*, alcohols *(19)*, and melanoidins *(72)* - although melanoidins require high temperatures to oxidise *(72)*.

Iron, copper and manganese are directly or indirectly involved in the formation of aldehydes (stale flavours) and (di)ketones *(27,70)*. These contribute to a rapid alteration of the original/intended flavour profile of a beer. Due to their abundance in beer, the degradation of hop bittering acids (mainly iso-α) is especially noticeable; making the continuous loss in bitterness over time a defining parameter of beer ageing, which can be used as a quantitative measure for staling (*trans/cis*-ratio) *(73)*. To produce beers that are

consistently bitter, even after prolonged storage, brewers may use tetrahydro-iso-α-acids, which are resistant to oxidative deterioration, more bitter and enhance foam stability *(17)*.

The concentration of metals is important. Even more significant, however, is the chemical state (speciation) in which these metallic ions are present *(74,75)*, which depends heavily on the physicochemical conditions of the system: pH, composition, temperature, and oxidation-reduction potential. These same variables also influence precipitation, dissolution, redox and complexation reactions. The ligand speciation can also drastically affect the nature and characteristics of the formed metallic complexes *(76)*. The chelator size and the strength of the complex, for example, determine whether the electron is transferred by an inner- or outer-sphere mechanism, which influences the reactivity of the complexed metal *(77)*. An inner-sphere mechanism involves electron transfer through a bridging ligand, with bonds being broken and new ones formed. An outer-sphere mechanism involves electron transfer between complexes that do not undergo substitution, with no bonds broken or formed.

While determining the speciation of metals in beer is important, only limited experimental research has been reported with the pioneering work of Svendsen and Lund *(78)*, and subsequent studies of Pohl and colleagues *(79–82)* on metal species in beer. Both iron and copper seem to be mostly complexed *(78,80,83)*. The state of the bound iron is reported to be negatively charged (although later findings also imply positive and neutral species), while copper was found as neutrally, negatively ($\sim$ 70%) and positively ($<$ 30%) charged species *(78,80,84)*. Manganese (which has a much weaker ability to form complexes with beer components), remains mostly unbound ($>$ 90% at pH 4.0), as simple Mn^{2+} cations *(78,80)*. The small bound fraction presumably exists as polyphenolic complexes.

Even from the limited data available, it is obvious that a wide range of ligands are at play. More research into the different forms of Cu, Fe and Mn in beer is needed to learn more about which reactions drive flavour instability.

Oxygen-free beer ageing. Although oxygen is the main staling agent, it would be incorrect to regard (O_2-driven) oxidation as the sole force behind beer ageing. Glycoside and ester hydrolysis, ester and etherification and Maillard reactions are all flavour deteriorating reactions that can operate non-oxidatively *(6)*. Even *trans*-2-nonenal which is a major indicator of beer staling and an important oxidative off-flavour is released during beer storage, independently of oxygen content *(46)*.

Reactive oxygen species are not the only (re)active molecules in beer. Melanoidins, for example, can oxidise higher alcohols to aldehydes in the absence of oxygen although O_2 does accelerate the reaction *(19)* and metals can catalyse some (e.g. fatty acid) radical formation independent of oxygen *(85)*. β-Damascenone - an aroma compound that behaves similarly to *trans*-2-nonenal during extended storage of beer - has been reported to develop independently of the total oxygen and SO_2 content in bottled beer *(86,87)*. It has also been reported that, in the presence of suitable electron acceptors, flavour active beer constituents (e.g. five-membered-ring hop derivatives, including *trans*-isohumulones, dihydroisohumulones, tetrahydroisohumulones, and humulinones) can be oxidatively degraded, even without the involvement of any oxygen containing entities *(88)*.

Aldol condensation such as the formation of *trans*-2-nonenal, by condensation of acetaldehyde and heptanal *(89)* is another oxygen independent process linked to staling. It is unclear, however, whether this non-oxidative reaction causes off-flavours in a significant way *(90)*. Similarly, Strecker degradation of amino acids in beer can happen in the absence of oxygen, but is of questionable relevance at low total package oxygen (TPO) *(70,91)*.

A further example of oxygen free off-flavour formation is beer becoming 'sun-struck', 'light-struck' or 'skunked'. This involves the occurrence of a sulphury, skunky note when beer is exposed to visible and ultra-violet (UV) light. Light-struck flavour is strongly associated with isohumulones, which decompose - under the influence of light and the photosensitiser riboflavin - to 3-methyl-2-butene-1-thiol (MBT), the main chemical related to the odour and flavour of light struck beer *(92)*. Interestingly, Lusk et al *(93)* found that MBT can also form in beer in the absence of light, through thermal ageing, although slowly.

Measuring beer staling and stability

Over the years, multiple techniques have been developed to monitor beer staling. Sensory analysis is a well-established approach *(94)*, but suffers from poor reproducibility and requires a lot of resources (time, people). Chemical analyses are usually more sensitive but unfortunately, there is no absolute test or all-encompassing assay for quantifying beer staling or flavour stability, as flavour changes are not due to one single reaction *(95)*. The most used methods are listed in Table 1.

Of these methods, two address the direct and indirect detection of free radicals and ROS in real-time. Electron spin resonance spectroscopy (ESR) measures electrical signals whilst chemiluminescence uses photochemical detection. In both methods, samples are forcibly oxidised (typically by applying heat) and the resulting free radicals measured. The time before detection and formation rate of the radicals offers information about the oxidative stability and staling potential. Both methods, however, require expensive equipment with high operating costs and the need for skilled operators.

The ESR assay is a widely used holistic and established technique for predicting beer flavour stability and the technology is increasingly available commercially. The main advantage of ESR is its capability to unambiguously detect unpaired electrons in complex biological samples (quantification), and its capacity to shed light on the molecular structure of the free radicals (identification), while requiring only small amounts of sample *(96–98)*.

Uchida and Ono *(99,100)* first applied ESR to predict beer flavour stability in 1996. They observed, when force ageing beer, that long lived spin adducts were not detected immediately after the start of heating but were produced after a period of incubation. This inhibited oxidation phase ('lag time') is the result of naturally present antioxidants in the beer quenching the generated radicals or ROS. Once the antioxidants are sufficiently depleted, the radical products react covalently with the spin-trapping agent to form (more) stable adducts, which can then be determined. Figure 2 displays two typical ESR spectra of wort and beer, with successive measurements taken during forced ageing.

The endogenous antioxidant activity of beer - its natural ability to quench radicals - can be estimated through its lag time determined (using N-tert-butyl-α-phenylnitron/PBN as spin-trap) *(27)* or the endogenous antioxidant potential (EAP) value (using α(4-pyridyl-1-oxide)-N-tert-butylnitrone/POBN as spin-trap) *(101)*. Both metrics correlate strongly with the sulphite content of the beer and give an indication of its inherent antioxidant capacity and potential flavour stability. Only the EAP value shows a linear correlation with the SO_2 content, while the lag time portrays an

Table 1. Methods for determining staleness, staling potential and flavour stability

Type	Method
I	**Measuring fluctuations in marker compounds**
	Increase of staling components, such as
	• 5-Hydroxymethyl furfural (5-HMF) *(256)*
	• Acetaldehyde *(257)*
	• Ethylene *(258)*
	• Furfural *(259)* and other marker aldehydes (2- and 3-methylbutanal, hexanal, 2-methyl-propanal, etc.) *(20)*
	• β-Damascenone *(86)*
	Decrease of 'fresh' components, such as
	• Iso-α-acids (bitterness); *trans/cis*-ratio *(73)*
	• Pro-anthocyanidins *(20)*
	• Sulphur dioxide (SO_2) *(260)*
	• Total flavanoids *(261)*
II	**Antioxidant capacity assays**
	• 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging or Trolox-equivalent antioxidant capacity (TEAC) *(262)*
	• 2,2-Diphenyl-1-picrylhydrazyl (DPPH) reducing activity *(263,264)*
	• Cupric reducing antioxidant capacity (CUPRAC) *(265)*
	• Ferric reducing antioxidant power (FRAP) *(266)*
	• Hydrogen peroxide scavenging *(267)*
	• Linoleic acid (LA) assay *(268)*
	• Metal-chelating activity (of Fe^{2+} with ferrozine) *(269,270)*
	• Oxygen radical absorbance capacity (ORAC) *(271)*
	• Peroxyl radical scavenging (β-carotene bleaching) *(272)*
	• Superoxide and hydroxyl radical scavenging *(273)*
	• Thiobarbituric acid (TBA) index *(274,275)*
	• Total reactive antioxidant potential (TRAP) *(276)*
III	**Others**
	• Chemiluminescence (CL) *(277–279)*
	• Electron spin resonance (ESR) spectroscopy *(99,100)*
	• Nonenal potential concept *(69)*
	• Peroxide challenge test (PCT) *(96)*

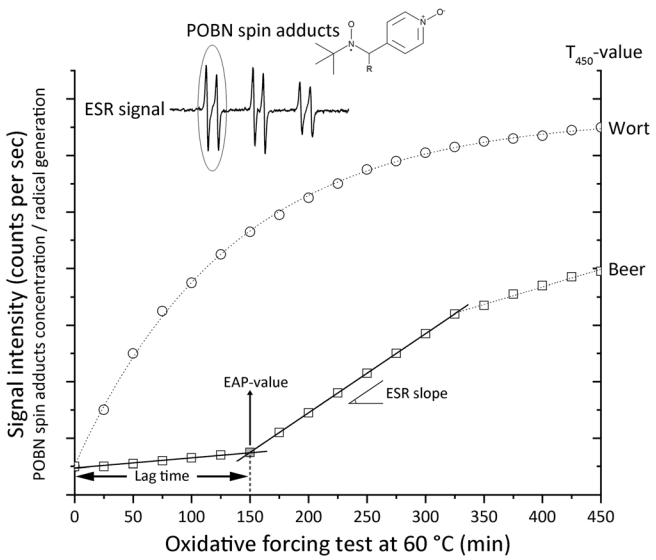

Figure 2. ESR analysis of wort and beer during an oxidative forcing test at 60°C. An increase in free radical formation is observed in beer after the lag phase. Wort typically does not have a lag phase. Note: while the lag time and EAP-value might seem interchangeable, consider that lag time is determined by using PBN as a spin-trap reagent, while EAP-value (and T-value) is determined by using POBN.

exponential relationship *(102)*. Because of the linearity between sulphite and EAP value, the time to consume one mg of SO_2 per litre of beer can be determined. Expressed in min*L/mg, this ratio is termed the Beverage Antioxidant IndeX or BAX value and provides information about the interplay of anti- and prooxidative beer components independent of the sulphite content and the rate of consumption of the existing antioxidative potential during storage *(102)*.

Beers with high endogenous antioxidant activity show retarded formation of stale flavours *(103)*. A supplementary ESR metric is the T-value: an indicator for the quantitative radical generation after a certain time, typically around minute 450 (although T_{150} and T_{600} are also used). It is mainly influenced by pH and substances that suppress or promote radical formation, such as complexing agents, transition metals, and intermediates of the Maillard reaction *(99,102,104)*. A high T-value has been shown to correlate with the rapid development of Strecker aldehydes *(65)*. Thus, an ESR graph provides information about the anti/prooxidative balance in a wort or beer, where a longer lag phase = higher EAP value = greater antioxidant potential = higher flavour stability; and a steeper slope = higher radical generation = higher T-value = greater prooxidant potential = faster staling.

However, the ESR technique is not free from criticism. Studies *(77,105–107)* have demonstrated certain chelators to form strong oxidative metal complexes, capable of oxidising biomolecules, that

are not ESR-detectable (by being spin-trap inaccessible). Accordingly, ESR does not detect every oxidative species; contrary to chemiluminescence, which does not rely on spin-trapping.

It is important that a spin-trap reagent does not affect the pH, as a small change in acidity will dramatically affect the autoxidation rate of a metal *(77)*. However, several studies make use of PBN as the ESR spin-trap, which can increase the sample pH. This causes lag time measurements to be significantly distorted (up to 500% in high EAP beers) through accelerated radical generation *(102)*. For this reason, using a spin-trap agent without any effect on pH (such as POBN) is recommended *(101,102,108)*.

A further concern involves the typical ESR measurement being conducted in vials that are open to the atmosphere, leading to oxygen ingress and volatilisation of ethanol. However, a recent development rectifies this by encapsulating the beer sample in a sealed capillary tube *(66)*. This substantially reduces sample oxidation during analysis and better represents packaged beer in trade.

Lastly, the calculated lag time is not always an ideal metric in predicting sensory flavour stability, as it can be imprecise and inaccurate due to high variability of the fitted sigmoidal curve *(22)*. This is especially the case with beer styles that have low or no lag time (stale lagers, red beers, dark beers) *(22)*. The ESR area under the curve has been suggested as a better metric, since it correlates more strongly with sensory data and consumer acceptance *(22)*. Others, because of the complexity of beer ageing, propose an even broader approach to measuring flavour stability *(109)*, such as the 'stability index' (SI) method, which combines the results of four different antiradical analyses *(110,111)*.

Preventing beer ageing

Keep it dark, cool, and still. A principle for slowing down any chemical reaction, including those involved in beer staling, is to keep the system energy low. For beer, this typically involves no light irradiation (keep dark) and low temperatures (keep cold). Less significant, but not inconsequential, is to limit vibrational energy (keep still), particularly during transport *(112)*. Agitation enhances the diffusion of any headspace oxygen into the beer, such that oxygen involved reactions proceed at a faster rate.

In terms of minimising the penetration of light, cans and kegs inevitably outperform glass bottles. With glass, brown/amber bottles are best at protecting the product against the damaging effects of ultraviolet light, with green bottles being a poor second, followed by blue/cobalt and uncoloured/flint glass *(113)*. Besides preventing beer from being light-struck, it is important to shield beer from irradiation to prevent it undergoing other photo-oxidative reactions. Examples include the photochemical oxidation of unsaturated fatty acids through the formation of singlet oxygen via photosensitisers (e.g. riboflavin) *(45)*, and the photo-oxidation of sulphur containing amino acids/polypeptides/proteins by triplet-excited riboflavin (and other flavins), delivering S-centred radicals *(114)*.

Formation of 3-methyl-2-butene-1-thiol (MBT) can also be avoided by using modified/advanced hop products, such as rho, tetra and hexa hop extracts (containing rho-, tetra-, and hexahydro-iso-α-acids). Because these hop products do not photodecompose into MBT, there is the misconception that these compounds are light stable. However, they do break down, but into light-struck flavours with higher aroma thresholds *(114)*.

It is a general rule of thumb for most food systems that a 10°C decrease in temperature roughly halves the rate of all chemically based deterioration reactions (i.e. a Q_{10} temperature coefficient of ~2; Figure 3) *(115,116)*. Accordingly, a beer held at 0-4°C will keep four times longer than one held at room temperature (Figure 3) *(95)*. Refrigerated transport and storage is used by some brewing companies but is a logistically complex and expensive affair. So, despite being one of the most powerful tools to prevent staling, it is not always an economically feasible option *(117)*.

It is not only during storage that (high) temperatures drive staling. Throughout the brewing process, and especially during the high heat stages, a variety of staling relevant compounds are formed. These include the Maillard reaction products (reductones and melanoidins), formed via the reaction of reducing sugars with proteins or amino acids (present in malt, wort, and beer). They add colour and flavour to malt and beer and are responsible for a myriad of (pro- and anti) oxidative effects throughout the production chain and in the final product *(102,118)*.

The TBA method (Table 1) is used to gauge heat load in the brewhouse. Thiobarbituric acid forms complexes with many Maillard intermediates but is particularly sensitive towards furfurals. One complex (5-hydroxymethylfurfural-TBA) acts as a yellow indicator, with a maximum absorption at 448 nm that can be used as a quantitative measure for thermal load *(119)*. A downside of the TBA method is its limited specificity, since thiobarbituric acid can also react with other substances, including proteins and sugars, to form coloured species that can interfere with the assay.

The heat/thermal load received by wort, beer, and malt, gives an indication of the expected flavour stability of beer. A high (er) heat load equates to high (er) rates of unwanted staling reactions (free radical generation, autoxidation of unsaturated fatty acids, Maillard reactions, Strecker degradation), which equates to high (er) levels of aldehydes, ageing precursors and prooxidative compounds.

Reduction of heat load typically involves reducing the time and/or temperature of the high heat process steps (kilning, mashing, wort boiling, pasteurisation). Mashing-in above 63°C can, for instance, limit unnecessary thermal stress. Additionally, it will inhibit enzymatic (lipid) oxidation of unsaturated fatty acids to *trans*-2-nonenal by deactivating the lipoxygenase (LOX) enzyme *(120)*.

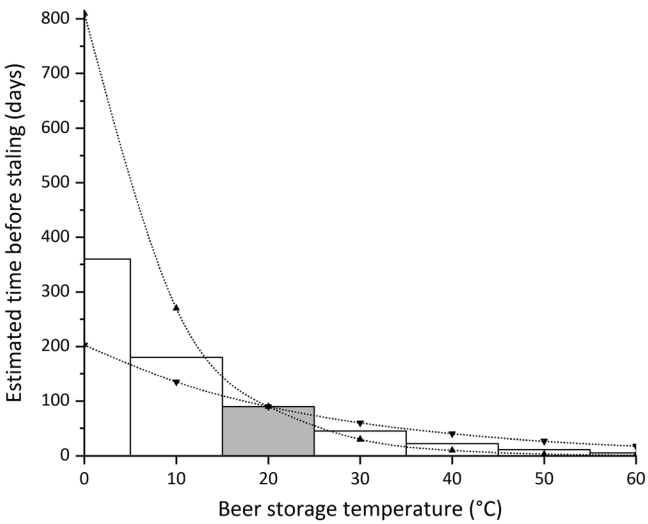

Figure 3. Calculated time before noticeable beer staling in relation to storage temperature, based on a 90 day shelf-life assumption at room temperature (20°C), with the following Q_{10} values: 1.5 (▼-line), 2 (bars) and 3 (▲-line). Note: The Q_{10} describes the ratio by which reaction rates change when the temperature is increased by 10°C and is used to predict the expected shelf-life of a food product. Typically, a Q_{10} value of 2 is used as an initial shelf-life estimate, but it can range anywhere from 1.1-3 for beer (depending on the temperature and the system/product).

Another possibility to lower heat stress is to brew with raw (unmalted, unkilned) barley *(121)* or with green (germinated, unkilned) malt *(122,123)*; to cool the wort before the whirlpool rest with the added benefit of limiting SMM (DMS precursor) cleavage *(124)*, or to centrifuge the wort instead of using a whirlpool. With boiling, many alternative systems that reduce heat load and save energy are available, including internal boiling/heating system *(9)*, thin-film evaporation *(125,126)*, dynamic low-pressure boiling *(127)*, vacuum boiling *(128)* and innovative wort production (with mashing-off at 95°C) *(129)*.

Avoid oxygen. To battle oxidative ageing, exposure to oxygen must be avoided as best as possible. This means oxygen ingress throughout the brewing process is as low as is reasonably achievable, and headspace oxygen in packaging is as low as is practically attainable, with avoidance of any oxygen ingress during storage. Bamforth *(64)* estimated that 0.1 μg/L of oxygen would incite oxidative mechanisms to a damaging degree. With modern filling machines achieving in-pack dissolved oxygen levels of 20-50 μg/L in beer *(130,131)*, it is debatable whether we should continue to strive for even lower oxygen concentrations.

Good manufacturing practices will improve the shelf-life of beer by minimising the formation of ROS. Kuchel et al *(132)* suggest that lowering the in-pack oxygen of beer to 1 μg/L should, at a minimum, extend its shelf-life beyond the typical 120 days. It can also be reasonably assumed that 'downstream' oxygen ingress is a bigger concern than 'upstream' oxidation *(95)*; notwithstanding that both should be prevented. Upstream entails the materials needed for production and any part of the production involving the extraction (making of wort); downstream includes processing after fermentation, the finished product (packaged beer), distribution and retail.

For this reason, any unnecessary transfer of beer should be prevented, whether in the brewery or in retail. When required, it should be done gently, to avoid splashing and turbulence, which leads to aeration *(133)*. Where possible, wort and beer should be 'pushed' with CO_2 or another inert gas. It is best to purge any container (bottles, kegs, cans, carboys, tanks) with CO_2 or N_2 before filling and to fill from the bottom up. Hoses and pumps can also be purged, or better still, prefilled with deaerated water to expel any air. When bottling or canning, always 'cap on foam' by agitating/fobbing the beer slightly so that headspace oxygen is minimised *(134)*.

The caps on glass bottles are another unavoidable contributor to flavour instability, as air can permeate into the headspace. There are, however, differences among cap/crown types when it comes to oxygen ingress *(135)*. Pry-off bottle caps are better at keeping oxygen out than twist-offs *(14,136,137)*. The crown liner material is also instrumental, as polymers vary in the extent of oxygen permeation *(135,138,139)*. To minimise the problem, innovative oxygen-scavenging caps can be used. The scavengers within the liner react with gaseous oxygen, reducing the overall oxygen content in the bottle *(140–142)*.

With keg beer, gases can permeate through some grades of dispense tubing with CO_2 leaving and air entering the system *(143)*. This is of greater concern with keg beers that are 'slow moving'. The use of CO_2 as top pressure gas for beer dispense may contribute oxygen, as commercially available food-grade CO_2 contains trace amounts of O_2 *(131)*. Conversely, canned beer has zero oxygen ingress after sealing, but bears the risk of having a higher TPO than bottled beer (Table 2), as cans cannot be vacuum evacuated without collapsing and have wide mouths (which impedes the pre-seal fobbing of beer). Regardless of the container type, beer should be stored upright and vibration/transportation minimised. This way, the beer has less surface area to interact with trace amounts of oxygen in the headspace, slowing down oxidation.

Table 2. Industry standard oxygen levels across the brewery *(131)*

Stage	Oxygen content (μg/L)
Aerated/oxygenated wort	8000 – 17000+
Fermentation	< 10 – 30
Filtration	5 – 50
Bright beer after filtration	10 – 50
Beer at the filler	10 – 30
Package dissolved O_2 (bottle)	20 – 50
Package dissolved O_2 (can)	30 – 60
Total package O_2 (dissolved + headspace)	40 – 150

A practical but niche option to lower in-pack oxygen is to add yeast to beer in bottle or can, which scavenges some of the remaining free amino acids and removes oxygen *(144,145)*. In addition, the yeast also removes aged flavour notes (such as aldehydes) from the beer, prolonging its overall freshness *(146,147)*. However, refermentation or secondary conditioning is a complex biochemical process that involves more than oxygen removal. Suspended yeast can cause haze and the additional carbon dioxide may lead to excess carbonation and gushing. Furthermore, there is the risk of yeast autolysis and the release of intracellular enzymes, lipids, amino acids, and metal ions, which can increase beer pH and cause off-flavours *(148–151)*.

Add antioxidants. Antioxidants are substances that prevent, delay or remove oxidative damage, either by eliminating superoxide, hydroxyl radicals or other reactive species (like peroxides, which sulphite will quench), or by inactivating trace amounts of Fe, Cu and Mn (through complexation/chelation) *(61)*. They are already present in beer and play a vital role as endogenous staling inhibitors, even at low concentrations. However, there are ways to naturally enrich the antioxidant content, such as ageing the beer in wooden barrels *(152)*, by using ingredients or brewing processes that favour a high polyphenol content in wort and/or beer or, as noted above, the use of re-fermentation, which will produce in-pack SO_2 *(146–148)*. Other options to enhance the sulphite content are to use a high-sulphite producing yeast strain *(153,154)*, zinc addition *(155)*, reduced fermentation temperature *(155,156)*, lower dissolved oxygen in pitching wort *(154,157)*, clear/bright wort *(154,158,159)*, and a higher wort pH *(160)*.

Depending on the regional legislation, antioxidants may be added to the brewing process or the final product to potentially prolong shelf-life. However, better packaging technologies with lower oxygen pick-up have made the practice of adding exogenous antioxidants less common *(161)*. Additionally, regulations have become stricter in recent years and consumers increasingly prefer 'clean label' products with no artificial ingredients or synthetic chemicals. In Europe and the United States, beers with sulphite contents > 10 mg/L must be labelled as such, as high sulphite residues can trigger allergenic effects in susceptible individuals *(63)*.

Common antioxidants used in brewing are ascorbic acid (vitamin C; E300) and sulphur dioxide *(90)*. The latter can be administered by dissolving (potassium or sodium) metabisulphite at various stages in the brewing process. Apart from the quenching of ROS *(61)*, sulphites will form adducts with unwanted aldehydes (acetaldehyde and other carbonyl compounds *(162,163)*), making them less flavour active, although this masking effect may only be temporary *(43,65)*. Ascorbic acid, like most antioxidants, also has prooxidative capabilities *(164,165)*. This is due to ascorbate, being a typical reductone, having a strong affinity to reduce oxidised metal ions back to their catalytic state (e.g. Fe^{3+} to Fe^{2+}) so that they are again available to activate oxygen by electron transfer *(58)*.

Whether a chemical entity behaves as an anti- or prooxidant depends greatly on the type and the concentration of the compound, oxidation state, pH, the type and concentration of transition metals present and the matrix. It can be difficult to make clear cut predictions, as what works in one medium might not work in another. This may explain why, for some substances, conflicting results have been reported in the literature. Indeed, in this review, melanoidins have had both pro- and antioxidative effects attributed to them (catalysing oxidation of higher alcohols into their equivalent aldehydes, but also chelating transition metals) *(58,166–169)*. The same is true of certain phenolic compounds (chelating transition metals, but also reducing them back to their prooxidative form) *(170–172)*.

A novel antioxidative compound that can be employed during brewing is punicalagin *(173)* a water-soluble ellagitannin, found in pomegranates, which can be hydrolysed to smaller phenolic compounds (such as ellagic acid). Both punicalagin and ellagic acid are capable of forming chelates with iron and copper ions at beer and wort pH *(174,175)*, inhibiting the production of reactive oxygen species and scavenging them *(176)*.

Remove transition metals. Metals in beer are mainly derived from the raw materials (malt, hops, water, yeast), but also from the brewing equipment and additives (filter media *(102)*, pipes, tanks, vessels, packaging *(177,178)*, adjuncts, stabilisers, pesticides *(179)*). Table 3 summarises the range of concentrations reported for iron, copper and manganese from different raw materials and stages of the brewing process. Whether good or bad, their presence plays a substantial and overlooked part in the palatability, conservation and overall stability of beer. Positive effects include supporting yeast and fermentation and contributing to the nutritional value. Negative effects involve spoilage - due to haze formation, oxidative processes, gushing - and other sensory defects *(31,180)*.

The metal content of a beer varies depending on the quality of the materials and processing aids used, though it is unavoidable that a portion of the metal ions are present in the final beer. A study by Wietstock et al demonstrated transfer rates, from raw materials to the finished beer, of 0.1% Fe, 0.4% Zn, 3.1% Cu, 6.3% Ca and 15.1% Mg *(181)*, with the biggest metal source being (pale lager) malt (ca. 96%). With darker malt types, such as crystal or roasted malts, different transfer ratios would be anticipated; namely, higher for Fe and Mn and lower for Cu, due to changes in the binding capacity of malt solids *(166,173,182)*.

Beer has a much lower transition metal content than wort *(183)*, which make the ransfer rates appear low. The explanation for this is that a large fraction of the metals in wort are bound to nitrogenous and polyphenolic compounds and are removed with the hot break and trub during mashing, wort boiling, and in the whirlpool *(181,184–186)*. The spent grains, left after lautering or mash filtration, are also a great sink for metals, particularly transition metal ions *(187,188)*. Moreover, yeast in addition to removing oxygen *(145,189)*, scavenges metals during fermentation (especially Cu, Fe and Zn), lowering the final metal content in beer *(190–193)*. In contrast, manganese is not significantly lost during the brewing process, making it a potent beer prooxidant *(50,52)*.

However, it is important to note that the metal concentration in finished beer does not need to be high to cause noticeable defects. The damaging properties of copper occur at < 50 μg/L *(50)* and a transition metal addition of 10 μg/L results in a measurable decrease in oxidative stability *(52,102,194)*. Some metal ions may be introduced after the brewhouse and fermentation stages, bypassing the protective effects of spent grains, hot/cold break/trub formation, and yeast. The increasingly popular practice of dry hopping is one such example. Considering that hops are rich in metal ions *(181)*, dry-hopping is presumably detrimental to beer flavour stability, although this has yet to be adequately investigated *(51,179,195,196)*. Furthermore, such additions to finished beer, can inadvertently result in oxygen pick up.

It is likely that a high amount of transition metals, present during brewing, will negatively impact the final quality and stability of the finished beer; even though a beer made from an iron-rich wort might finish with a similar iron content as a beer from an iron-poor wort. This notion is often overlooked, despite the known capacity of (metal ion) oxidation catalysts to facilitate oxidative degradation of wort (and its antioxidative compounds) especially during high heat stages and regardless of the catalysts being in a bound state or not.

To reduce any negative flavour effects, the content of transition metals (Fe, Cu and Mn) needs to be lowered in some way, ideally as early as possible. One approach is by complexing metals during

Table 3. Transition metals throughout the brewing process

	Fe	Cu	Mn
Malt (mg/kg dm)	25-32[a]	2-3[a]	8-12[a]
Hops (mg/kg dm)	300-800[b]	5-10[b]	40-60[b]
Filtered wort (μg/L)	200-500[a]	50-100[a]	70-150[a]
Pitching wort (μg/L)	≥ 200[a]	≥ 70[a]	≥ 70[a]
Beer (μg/L)	20-80[a]	20-60[a]	70-130[a]

[a] Range according to Zufall and Tyrell *(50)*.
[b] Range according to Lie et al. *(280)*.

the brewing process so that they are no longer chemically involved in activating oxygen (Reaction 1). Certain chelators can do this by one or more of the following: interruption of the metal redox cycle; occupation of all coordination sites; formation of insoluble metal complexes; steric hindrance between metals and oxidation intermediates (e.g. peroxides) *(197)*. However, perfect 'catch-all' chelators may not exist *(49)*.

$$\text{(Fe, Cu, Mn)} + \text{chelator} \longrightarrow \text{(Fe, Cu, Mn)-chelate} \quad \text{(Reaction 1)}$$

Some native wort/beer components seem to portray such characteristics naturally, through donor N-, O- and S-atoms, and include polyphenols *(198,199)*, amino acids *(192)*, phytic acid *(200,201)*, melanoidins *(202–204)*, and hop acids *(205,206)*. These mostly derive from malt, but also from hops. So, even without the addition of complexing agents, a natural and complex equilibrium already exists between free and bound metal ions in wort and beer with an inclination to the bound, organometallic state *(78)*.

The benefit of scavenging metal ions is clear, but the reality proves challenging. To prevent Fenton chemistry, a chelator must stabilise the metal ion in a state inert to either oxidation by H_2O_2 or reduction by reducing agents *(207)*. But the bound-state metal ions can even - depending on the concentration and type of chelator - end up promoting oxygen radical formation (Reaction 2 and 3, forming a cycle), instead of quenching it *(90,99,133)*.

$$\text{(Fe, Cu, Mn)}^{n}\text{-chelate} + O_2 \longrightarrow O_2^{\bullet -} + \text{(Fe, Cu, Mn)}^{n+1}\text{-chelate} \quad \text{(Reaction 2)}$$

$$\text{(Fe, Cu, Mn)}^{n+1}\text{-chelate} + RH \longrightarrow R^{\bullet} + H^{+} + \text{(Fe, Cu, Mn)}^{n}\text{-chelate} \quad \text{(Reaction 3)}$$

The degradation of ascorbic acid serves as an illustration of this. The oxidation of ascorbate is catalysed by both copper and iron, with free Cu^{+2} being roughly 80 times more potent than unbound Fe^{3+}. The presence of EDTA diminishes the ability of copper to catalyse ascorbate oxidation. However, this is not the case for Fe^{3+}, as EDTA bound iron is four times more potent in degrading ascorbate than its free form *(208)*.

The extent of metal ion binding does not necessarily correlate with the extent of protection against oxidation from that metal. This may also explain why there are still disagreements on whether some compounds are anti- or prooxidative (e.g. melanoidins and polyphenols), especially in complex systems such as wort and beer. Model studies with lipid peroxidation show that when the ratio of chelator (EDTA or DTPA) to transition metal (Fe) is high (> 1), oxidation is inhibited. When the ratio is low (< 1), oxidation is stimulated *(209,210)*. This suggests that chelator/metal-ratio also plays an important role and that chelators must be present at sufficiently high concentrations.

Overall, chelation properties are influenced greatly by the pH of the solution, which in turn will also influence the reactivity of the metal species present *(211)*. The complexity of metal and chelation/coordination chemistry is immense. In addition to the factors already noted, many others will affect the outcome, including chelator size, nature of the ligand (and whether it is capable of forming a multiligand complex), buffer system, competing ions, and the matrix *(77,212)*.

'Simple' chelation might not suffice in protecting wort and beer from oxidative damage. A chelator that forms an insoluble metal complex has a better chance of diminishing oxidation, by decreasing the mobility, and the reactivity of the metal. Ideally, a chelator should also possess a high enough binding affinity for transition metals, so that it can strip them away from other molecules, protecting possibly flavour relevant molecules from site-specific degradation by the metal-catalysed radicals *(213)*. Good examples of site-specific degradation reactions in beer are when isohumulones or polyphenols are directly attacked by $^{\bullet}OH$ radicals, generated by iron ions that they captured, destroying them; respectively resulting in cardboard off-flavour and haze formation *(31)*. In addition, chelators must be food safe and flavour neutral; not remove metals required for brewing (yeast requires trace amounts of Zn^{2+}, Ca^{2+} and Mg^{2+}) *(84)*; they must perform well at high temperature and low pH; destroy $O_2^{\bullet -}$ and H_2O_2 without reducing agents; and, preferably, be low in cost and practical in use.

In 1999, Bamforth et al *(133)* called for a deeper exploration of the aspects of chelation, but little has been subsequently been published. A few studies have examined the metal ion scavenging capabilities in beer of added, exogenous compounds. Effective ones include diethylenetriaminepentaacetic acid (DTPA) *(26)*, ethylenediaminetetraacetic acid (EDTA) *(31,99,165)*, egtazic acid (EGTA) *(214)*, bipyridine *(99)*, phenanthroline *(99)*, Divergan® HM *(102,215)*, gallotannins/tannic acid (Brewtan®) *(13,216,217)*.

All these chemicals are foreign to beer, but a few chelation studies in brewing have shown promising results with native beer and wort compounds. As noted, phytic acid is a strong antioxidant because of its metal scavenging ability (Cu and Fe, but also Zn and Ca) *(184,218,219)*. Proteins of all fractions especially bind Cu and Fe ions, with their amino acids acting as ligands *(214,220)*, which explains why trub contains a high amount of metals. Despite this, the nature and extent of the binding do not prevent copper from participating in oxidative reactions *(221)*. Hop α-acids (isohumulones) and hop β-acids can firmly bind Fe ions by complex formation *(195,205,222)*. The organic compound citric acid forms complexes with Fe and Cu *(80,214)*. Melanoidins are known to strongly capture Cu, Fe and Zn strongly. Beer flavonols (e.g. from hops) can bind Cu at beer pH; myricetin and quercetin can chelate Fe *(183,223)*. Oxalic acid (oxalate) is reported to modulate the activity of iron *(224)*.

It is important to note that most of these studies were conducted by examining chelating agents in finished beer. However, chelators are often unstable at low pH *(211,225)* and typically perform better in less acidic environments *(35,67,212)*. Accordingly, it might prove more effective to add chelators during mashing, as wort has a higher pH. Additionally, the high level of amino acids in wort may help complex formation (amino acid + organic acid + metal ion). Such mixed complexes are often stronger than those composed solely of organic and amino acids *(226)*.

A recent study explored the binding capacities of 19 chelators added during mashing *(173)*. The findings highlight the advantageous effect of some chelating agents, of which the most effective were green tea extract, tannic acid and pomegranate extract. The latter two were especially successful in reducing the iron content of wort after lautering. The addition of pomegranate extract (60 mg/L, 90% ellagic acid) resulted in an 80% decrease in radical generation. The study also showed that excessive release of iron and manganese during mashing can be avoided by not acidifying the mash, but instead mashing at a 'natural' pH of 5.6. This is in accordance with the findings of Narziß et al *(227)*, who observed

an increase of ageing related compounds with the reduction of the mash pH (from 5.8 to 5.5 and 5.2). Not acidifying the mash might also result in a less acidic beer, which according to Grigsby et al and Kaneda et al *(32,228)*, will lead to a more flavour stable beer that tastes less oxidised when aged. At lower pH, more of the superoxide radical will be in its protonated state (the more damaging perhydroxyl radical).

The free radical and ROS scavenging properties that some chelators also possess should not be overlooked. Caffeic acid shows low iron binding ability but has outstanding antioxidant properties, as it can scavenge several reactive species (DPPH, peroxyl and hydroxyl radicals) *(229,230)*. There is still much room for research in the field of 'chelation therapy' with respect to improvement of beer oxidative stability. It is well known that phenolic acids, containing the catechol or galloyl moiety (an ortho-dihydroxy functional group), are effective in chelating transition metals *(231,232)*; and that beer contains a number of these acids, including caffeic acid, chlorogenic acid, protocatechuic acid, and gallic acid. Accordingly, enhancing these phenolic acids during the brewing process could potentially improve the shelf-life of beer *(233)*. However, at certain concentrations - depending on the polyphenol type and beer composition - they may lead to the formation of protein-polyphenol hazes over time *(234)*.

Control of flavour stability in practice

Table 4 provides a summary of the strategies to prevent beer ageing.

Raw materials, equipment and additives. The excellence of the raw materials (malt, water, hops, yeast), processing aids, and equipment is paramount to ensure good beer quality. They will influence the overall metal load throughout the entire process. Brewing water can be used 'as is', if the local raw water quality is adequate, but will commonly have to be deionised to remove minerals and unwanted metal ions. Water treatment, such as reverse osmosis, may come with high costs and process complexity, as salts must be added back to the brewing liquor *(235,236)*. The metal content of the other inputs is not always as transparent or easy to control. The use of whole hops, rather than pellets or extracts, raises the antioxidative polyphenol content of the wort but also tends to be higher in heavy metals *(237)*. With malt, the wort metal content can vary by variety, cultivation, and roast intensity *(182,185)*. Similar considerations also apply to spices, herbs, and other additives *(238)*. Brewing equipment should not leach metal ions into the wort or beer and accordingly, passivated stainless steel 'coppers' should be used for wort boiling, rather than copper. Similarly, membrane filtration of beer is recommended rather than filtration through kieselguhr/diatomaceous earth, which is rich in iron *(216,239)*. The design of process equipment (pumps, stirrers, pipes, vessels) should be to reduce high mechanical stress and shear, to minimise oxygen pick-up, to impede β-glucan extraction during mashing, and to avoid disruption of any aggregates, such as coagulated protein/polyphenols. The primary purpose is to prevent reduced filterability by averting the formation of large hydrogel complexes and, thus, poorer flavour stability.

With dark, roasted malts, more metal ions are transferred to the wort during mashing *(173)*. This explains why dark beers, such as stouts, typically have a higher Fe content *(52,240)*. Roasted malts also contribute higher levels of organic radicals, deterioration-relevant carbonyl compounds (Strecker aldehydes), and reductone compounds (Maillard reaction products), due to the elevated heat stress that these malts undergo during kilning, resulting in decreased beer flavour stability *(166,168,241)*.

Brewing with green malt or unmalted barley (or a combination of both, making use of the high diastatic power of green malt to compensate for the enzyme deficiency in raw barley) would reduce the staling potential considerably, since these starch sources do not have the above defects - although they might contribute other shortcomings. Green malt, for example, must be produced and used on-site, as it is microbiologically unstable raw material with a limited shelf-life *(123)*. Contrary to expectation, the heightened LOX content of green malt does not result in beer with significant taints or obvious defects, even when using it at a 100% *(122)*.

Milling. Aggressive milling of malt results in excessive fracture of the husks and embryo, which increases wort polyphenol levels, but also leads to increased LOX, lipase, and lipid release, leading to elevated nonenal potential *(60,242)*. Finely milled grist (from hammer milling) would, typically be utilised by modern mash filters, which provide reduced filtration time, less heat load, lower oxygen pick-up, and shorter mashing times (due to the larger surface-to-volume ratio of the fine grist, leading to higher enzyme and extract yields).

Alternatively, or in addition, the malt acrospire can be kept intact by employing steep conditioned or wet milling. Wet milling systems can use deaerated water to minimise oxidation during milling and mashing. To achieve this with steep conditioned milling, the milling and mashing chambers should be flooded with nitrogen or carbon dioxide.

Mashing, wort separation, boiling, and clarification. Although chelating agents, such as tannic acid and ellagic acid, can be used successfully across the brewing process, addition during the mashing stage is recommended. Firstly, the early elimination of transition metals will more promptly negate their catalytic effects on oxidation. Secondly, wort has a higher, more suitable pH for chelation, compared to beer *(206,212)*. Hop acids also possess chelating capacity, of which the non-isomerised α-acids have the highest binding efficiency *(206)*. Application of optimised hopping regimes, where hops are added incrementally - rather than a single dosage at the beginning of wort boiling - can achieve lower levels of iron and copper in the pitching wort *(195)*.

At mashing-in temperatures of ≥ 63°C, LOX is inactivated and fewer staling components are retained in the beer *(241,243)*. A further advantage of mashing-in above the gelatinisation temperature of barley malt starch is shortened vessel occupation time, which in turn limits the total heat damage *(244)*. Wort kettles are usually fitted with vents that aid the removal of unwanted volatiles (such as staling aldehydes and DMS), and condensate traps that prevent their re-entrance *(129)*. The addition of oxygen to warm wort (upstream oxidation or 'hot side aeration') should be avoided as oxidation of wort compounds (proteins, fatty acids, melanoidins, polyphenols) will happen rapidly at high temperatures *(72)*. Wort should be aerated/oxygenated on the cold side of the heat exchanger. The hot wort should be treated gently, by filling the mash tun and kettle from the bottom up and avoiding turbulence during transfers. Where possible, mashing and sparging should use deaerated liquor. Better still perform mashing, wort filtration, and boiling anaerobically (under an inert atmosphere). Another 'innovative' concept to reduce hot side aeration, heat load, and energy costs is to brew without wort boiling, which can either be done through the use of near-boiling temperatures and stripping *(129)* or by omitting wort boiling entirely. Traditional no-boil beers (or raw ales) have been brewed for centuries including Finnish sahti,

Table 4. Strategies for preventing wort and beer staling (adapted from *(281)*)

Process stage	Raw materials, equipment, and additives	Milling	Mashing and wort separation	Boiling and clarification	Fermentation and conditioning	Downstream processing and packaging	Distribution and storage
High relevance, low cost, effort or risk	Add antioxidants: sulphur dioxide, whole hops Add effective chelators: tannic acid, punicalagin/ ellagic acid		Avoid excess iron and manganese: mash pH ~ 5.6	Limit heat load: avoid prolonged heating, cool efficiently and timely Avoid excess of iron and copper: optimised hopping regime	Ensure vigorous fermentation: use healthy yeast, adequate pitching rates, recommended temperature	Minimise in-pack oxygen: cap on foam, purge containers, limit transfers and filtration Avoid pick-up of iron, copper and manganese	Keep it dark
High relevance,high (er) cost, effort or risk	Avoid pick-up of iron, copper and manganese Limit heat load: pale malt, green malt and/or unmalted barley		Limit heat load: alternative boiling systems			Minimise in-pack oxygen: oxygen scavenging bottle caps, cans	Keep it cold Limit storage time: stock rotation and logistics
Low (er) relevance, low cost, effort or risk		Non-aggressive milling	Limit hot side aeration Inhibit LOX: mash-in ≥ 63 °C	Remove trub and break	Bright/clear worts		Store it upright Keep it still
Low (er) relevance, high (er) cost, effort or risk	Use lipoxygenase-null barley	Flush grist with inert gas Mill anaerobically				Bottle conditioning with yeast	

Danish gammeltøl, Norwegian kornøl, and German Berliner Weisse *(245)*.

There has been much debate about the impact of trub rich (cloudy or turbid) worts on beer quality and flavour stability, with most research focusing on the potential negative effects of the higher lipid content *(246,247)*. However, little consideration is given to trub being high in iron and copper *(181,248)*. In terms of transition metal content, trub removal for a bright/clear wort is recommended.

Fermentation and conditioning. Healthy yeast and vigorous fermentation are important for flavour stability *(95,249)*. Yeast reduces aldehydes to their corresponding alcohols and produces low levels of sulphur dioxide. The use of yeast of high viability and good physiological state enhances flavour stability and the organoleptic properties of the final beer *(250)*. Appropriate levels of zinc (and magnesium) are required in the pitching wort to facilitate yeast performance *(251,252)*.

Anaerobic, repitched yeast requires trace amounts of dissolved oxygen (5-20 mg/L) in the wort to synthesise sterols and unsaturated fatty acids, which are needed for membrane formation and cell multiplication. Accordingly, pitching wort is aerated or oxygenated, post heat exchanger, on route to the fermenter. Although a necessary process, the addition of oxygen to wort is counter intuitive in managing flavour stability. To limit the oxidative damage, oxygen is added on the cold side and yeast is pitched without delay.

Alternatives to wort aeration have been explored, including the direct addition of oxygen to yeast slurries. In one study *(253)*, pitching yeast was exposed to olive oil prior to fermentation, so as to supply unsaturated fatty acids (such as oleic acid). This approach does not satisfy the nutritional need for sterols (anabolic or exogenous) but was said to produce beers that were less oxidised.

Downstream processing and packaging. In-pack oxygen as low as is reasonably achievable is critical for the prolonged shelf-life of beer. Due to improved beer processing and packaging techniques, the TPO can be as low as 40-150 μg/L. But even at these levels, oxidative staling of beer is still taking place. While it is true that the oxygen initially present is already enough for oxidation to occur (as it will be recycled through the Fenton and Haber-Weiss reaction), a large factor is 'new' oxygen finding its way into the beer package by penetration through the closure and/or packaging material. As such, a continuous dynamic situation exists, where in-pack oxygen lost through reaction with beer constituents, can be supplemented by atmospheric oxygen. This is why aged bottled beer can still have dissolved oxygen levels of 30 μg/L *(254)*, rather than near-zero as with aged canned beer *(255)*. In the absence of oxygen scavenging caps, ingress rates of 1-5 μg/L O_2 per day can be anticipated *(131,135)*.

Distribution and storage. Storage temperature may be the single most important quality factor in beer stability. However, there is generally limited control over the distribution and retail conditions, such as temperature, light, motion, time in warehouses, distribution, wholesalers, and retailers. Ideally, the distribution and retail chain are temperature controlled, with short transport and storage duration, rapid turnover, and stock rotation. Further, thermal insulation and vibration damping can be employed. Consumers should also be encouraged to store beer refrigerated.

Best practice should be applied to stock rotation with FIFO (first in, first out), where older stock is preferentially sold. Where economically viable, brewers and retailers can agree on positive release systems, where the beers released for consumption are (still) true to their brand specification. Additionally, brand owners can enforce 'pull dates' - deadlines where unsold beer should return to the brewery, usually ranging from 60 to 180 days.

Concluding remarks

Fresh beer is not in chemical equilibrium and flavour shifts inevitably occur over time. This inherent flavour instability of beer remains a major challenge facing brewers. Each of the reactions involved is subject to numerous determinants, including temperature, oxygen, time, transition metal content and speciation, pH, and beer composition. The multitude of variables make beer ageing an immensely complex chemical process that is not fully understood. Although multiple methods for measuring beer staling and stability are available, none are absolute. ESR spectroscopy has been among the most adopted analytical techniques in recent years and gives valuable information about the endogenous antioxidant potential and the interplay between the pro- and antioxidants in wort or beer.

Although oxygen and oxidation are not the sole reasons for staling, they play a central role in beer ageing, together with transition metals. Iron, copper and manganese are major drivers of oxidation, as they catalyse the production of reactive oxygen species. As brewing and packaging technology may be approaching the practical limit for in-pack oxygen, it is wise to explore other pathways in restricting oxidation, such as the depletion and inhibition of transition metal catalysts. Their chemical or physical removal from the brewing process is desirable and can be achieved by chelation, an uncharted area in brewing science. Because of the complexity, contradictory results are found in the literature about the anti- or prooxidative effects of chelating compounds, such as polyphenols, melanoidins, and ascorbic acid. Nevertheless, chelation and flavour stability warrant further investigation.

Author contributions

Tuur Mertens: writing (original draft).
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Conflicts of interest

The authors declare no conflicts of interest.

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