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Formation of Aldehydes by Direct Oxidative Degradation of Amino Acids via Hydroxyl and Ethoxy Radical Attack in Buffered Model Solutions

The formation of aldehydes in bottled beer is promoted by the presence of oxygen and transition metal ions. In this paper, a so far unrevealed pathway to explain this phenomenon is presented. Leucine, isoleucine, and phenylalanine were oxidized by H_2O_2-Fe^{2+} in 'beer-like' buffered model solutions (pH 4.5; 5 % (v/v) ethanol) at room temperature thereby yielding 3-methylbutanal, 2-methylbutanal, phenylacetaldehyde, and benzaldehyde, respectively, as measured and identified by solvent extraction and HRGC-MS. Further trials revealed that the aldehydes formed were significantly correlated with radical concentration as determined by electron spin resonance (ESR) spectroscopy indicating that hydroxyl radicals (•OH) and ethoxy radicals (EtO•) are involved in the pathway. A reaction route for 'beer-like' model systems is featured and confirmed by a storage trial in which a steady increase of aldehydes over a time span of 18 days could be demonstrated.

Descriptors: aldehydes, amino acids, electron spin resonance spectroscopy, hydroxyl radicals

1 Introduction

Beer instability has many faces and can be divided into a number of categories such as physical-, flavor-, microbiological-, foam-, and light (in)stability. Non-oxidative and microbiological deterioration processes are most widely under control and have been conquered by technical improvements and cautious industrial hygiene. Next to a myriad of flavor-active compounds deriving from the yeast metabolism contributing to the perception of bottled beer, unwanted off-flavors arise during storage which is a result of complex reactions occurring in the final product. Estery and floral aromas that are initially recognized as pleasant will decrease. The bitterness quality and sulfury notes decline while other off-flavor notes like bready-, sweet-, caramel-, and sherry-like notes are recognized [17, 20, 45, 67, 68, 71]. Research from the last years and decades gave evidence that carbonyl compounds are primarily responsible for the appearance of such off-flavors as they exceed their individual flavor thresholds during storage or are thought to be involved in a synergistic interplay [35]. Although an uncountable number of compounds are involved in the perception of stale flavor, researchers agreed that specific aldehydes provoke such in bottled beer and are consequently used as markers for analytically identifying and monitoring stale flavor during storage. The individual aldehydes greatly vary in their contribution to the aged odor and flavor of beer. In comparison to other carbonyls, (E)-2-nonenal has a very low determined flavor threshold of 0.03–0.05 ppb [57, 66] and is accompanied with the so-called "cardboard" or "bready" flavors [6, 7, 34, 38, 46]. The odor thresholds of 2-furfural and 5-hydroxymethylfurfural were determined with odor thresholds of 15.16 and 35.78 ppm, respectively, and are perceived as caramel-like and bready [27, 46, 57]. Additionally, *De Clippeleer* et al. [19] demonstrated that 2-furfural has a remarkable effect on beer astringency and mouthfeel even when present in a sub-threshold flavor concentration of 400 ppb. Further compounds comprise the group of the so-called Strecker aldehydes which involves 2-methylpropanal (86 ppb*; grainy, fruity), 2-methylbutanal (45 ppb*; almond-like, malty), 3-methylbutanal (56 ppb*; malty, chocolate-, cherry-, almond-like), methional (4.2 ppb; like cooked potatoes, worty), phenylacetaldehyde (105 ppb; flowery, hyacinth-, roses-like), and benzaldehyde (515 ppb; almond-, cherry-, stone-like) [46, 57]. Their respective odor thresholds (marked with an asterisk), flavor thresholds and descriptors are mentioned in parentheses.

In beer and during beer production, all these compounds are formed in interplay of many constituents from the raw materials (malt, hops, water, yeast) and through complex reactions in which factors like temperature, oxygen, light irradiation, enzymatic, and pro-oxidative and antioxidative interactions are involved. Enzymatic oxidation by oxygen oxidoreductases, and photo- and autoxidation of unsaturated fatty acids, such as linoleic and linolenic acid, contribute to the formation of fatty acid derivatives such as (E)-2-nonenal [21, 34, 61] and hexanal [24]. The non-enzymatic browning or Maillard-reactions occur primarily during wort boiling in beer production and give rise to many different products. One of the most-established and surveyed reaction in this context is the reaction of an amino acid with an α-dicarbonyl compound yielding Strecker aldehydes which contain one carbon atom less than

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the respective amino acid ('Strecker degradation'). Although all amino acids can theoretically react in such way, only the Strecker aldehydes from valine (2-methylpropanal), leucine (3-methylbutanal), isoleucine (2-methylbutanal), methionine (methional) and phenylalanine (phenylacetaldehyde and benzaldehyde) are relevant in beer because of these amino acids concentrations in beer and their Strecker degradation products' flavor thresholds [46, 57]. According to *Coghe, Derdelinckx* and *Delvaux* [15], the Maillard reaction mainly commences at elevated temperatures (above 50 °C) and in a slightly acidic pH range (pH 4–7). There is evidence that Maillard reactions also occur at lower temperatures, e.g. during beer storage, thereby consequently contributing to the appearance of off-flavors [10, 11]. However, outcomes from this study are partly ambiguous because evidence was based on blocking α-dicarbonyls with aminoguanidine [10]. In addition to rendering α-dicarbonyls 'harmless', aminoguanidine is also capable of reacting with monocarbonyls directly [1], accordingly reducing stale aroma in beer.

The rate of beer staling and the appearance of off-flavors are often linked to the presence of oxygen and transition metal ions in bottled beer. In particular Strecker aldehydes were found in higher concentrations when oxygen was present at elevated levels [54]. *Clapperton* [14] observed that the ribes flavor in beer was closely connected to the amount of air in the headspace. *Blockmans, Devreux*, and *Masschelein* [9] added the amino acids valine and leucine to beer and noticed increased concentrations of the corresponding aldehydes, 2-methylpropanal and 3-methylbutanal, when oxygen was present. This effect was strengthened by addition of copper and iron ions. Studies from *Miedaner, Narziss* and *Eichhorn* [51] verified these findings and observed also higher concentration of Strecker aldehydes when oxygen was abundant in bottled beer while other carbonyls, e.g. 2-furfural, were unaffected. It is not fully understood which reactions are accountable for these observations. The Strecker degradation of amino acids cannot be held solely accountable for this phenomenon. *Hashimoto* and *Eshima* [30, 31] proposed the melanoidin-catalyzed oxidation of relevant higher alcohols and the aldol condensation of unsaturated aldehydes as further potential pathways. *Barker* et al. [7] doubted their relevance for beer storage and anticipated that aldehydes are mainly formed during wort boiling, subsequently binding to bisulfite during fermentation and forming reversible complexes which are then released during storage. The existence of sulfite-adducts was also studied and confirmed by *Kaneda* et al. [39]. *Baert* et al. [4, 5] studied these possibilities in greater detail and furthermore suspected that imine adducts can be also be a source of carbonyls. *Suda* et al. [62] added C^{13} labeled amino acids to filtered wort before boiling for 90 minutes and claimed that 85 % of the Strecker aldehydes present after 2 weeks at 37 °C aging were derived from wort boiling and 15 % were derived from Strecker degradation in the bottled beer itself. A potential reaction pathway involving oxygen was proposed by De Clippeleer [18] in which the oxidative degradation products of isohumulones, the hydroxy-allo-isohumulones, act in a Strecker-like reaction thereby yielding the corresponding aldehydes. However, this reaction pathway which has been postulated from studies in model systems may be negligible in beer because of the low oxygen concentrations in beer. Further studies in model systems revealed that transition metal ion-catalyzed oxidation of Amadori-compounds also contributes to the formation of Strecker aldehydes [32, 56]. It remains open, though, if these reactions are also applicable for beer systems.

The role of oxygen and transition metal ions is closely connected to the discovery of reactive oxygen species (ROS) [37, 64, 65]. Studies using electron spin resonance (ESR) spectroscopy helped to verify their existence and further elucidate their influencing factors during beer production and in the final product [2, 3, 25, 41–44, 47–50, 55, 63, 72]. ROS, and in particular the highly reactive hydroxyl radical ($^{\bullet}OH$), are formed in the Fe- and Cu-catalyzed Fenton- and Haber-Weiss-reaction systems. Hydroxyl radicals, in turn, react nonspecifically with organic beer constituents but in principal with ethanol thereby generating ethoxy radicals ($EtO^{\bullet}$) because ethanol is, after water, the second most occurring organic compound in beer [3].

Stadtman and *Berlett* [60] were the first to describe a pathway involving transition metal-catalyzed oxidation of amino acids, and found ammonium ions, α-keto acids, CO_2, oximes and aldehydes or carboxylic acids with one carbon atom less as the corresponding amino acids as the major reaction products. The oxidation products of leucine were studied in greater detail and 3-methyl-2-oxobutyric acid, 3-methylbutanoic acid ethyl ester and 3-methylbutanal were identified as its derived oxidation products [59]. At acidic pH ranges, this pathway leading to the formation of derived aldehydes, ammonium ions and CO_2 appears more likely to happen [16, 59]. All these trials, however, were carried out within the scope of cell ageing at a physiological pH and without the presence of ethanol.

There is evidence that 'beer-radicals' are closely connected to beer deterioration reactions; however, the direct impact of those radicals for beer flavor deterioration reactions such as the formation of staling-related aldehydes has not been elucidated thus far. Both the existence of oxygen-derived 'beer-radicals' and the published claims that more Strecker aldehydes are formed when elevated levels of oxygen are present in bottled beer support the conclusion of the existence of a direct oxidation of amino acids by radical attack as proposed by Stadtman and Berlett [60]. The objective of the study presented was to investigate if the mechanism as described by Stadtman and Berlett [60] is also applicable for a 'beer-like' model system with a particular focus on the concomitant formation of staling-related aldehydes.

2 Materials and Methods

Chemicals. Acetic acid (glacial), α-(4-pyridyl-1-oxide)-N-tert-butylnitrone (POBN), iron(II)sulfate heptahydrate, iron(III)chloride hexahydrate, leucine, and phenylalanine were obtained from Sigma Aldrich Inc., Steinheim, Germany. Hydrogen peroxide and isoleucine were purchased from AppliChem GmbH, Darmstadt, Germany. Ethylenediaminetetraacetic acid (EDTA), sodium acetate trihydrate, sodium carbonate, and sodium sulfate were obtained from Merck KGaA, Darmstadt, Germany. Diethylether and anhydrous ethanol were purchased from VWR international GmbH, Darmstadt, Germany. All chemicals were of analytical grade or higher. All solutions were made with double-distilled water and prepared freshly every day.

2.1 Determination of volatile carbonyls in buffered model solutions

Aldehyde concentrations were determined following a modified literature procedure described by *Engel, Bahr* and *Schieberle* [23]. This procedure uses the solvent assisted flavor evaporation (SAFE) technique and high resolution gas chromatography (HRGC) together with mass spectrometry (MS) analysis. In the present study, the distillation step using the SAFE apparatus was skipped because no non-volatile interfering material was expected in the buffered model solutions. An aliquot (100 mL) of sample was spiked with 1 µg of pentanal as an internal standard (IS). The sample was subsequently extracted twice with 150 mL diethyl ether, washed twice with a 0.5 M Na_2CO_3 solution and water, respectively, dried over Na_2SO_4 for 1 h, and concentrated to 5 mL using a Vigreux column at a temperature of 46–48 °C. A sample (1 µL) of the concentrated distillate was applied via a cold injection system (Gerstel, Mülheim, Germany) in 10:1 split mode to a gas chromatograph (6890, Agilent Technologies, Waldbronn, Germany) fitted with a capillary column (VF-5 MS, 60 m x 0.25 mm, 0.25 µm film, Varian, Darmstadt, Germany). The following temperature program was used for the GC oven: after 12 min at 35 °C, the oven temperature was raised to 150 °C at a rate of 12 °C/min and then to 250 °C at 30 °C/min where it was held for 5 min. The flow rate of the helium carrier gas was 0.6 mL/min. The MS analysis was performed by an MSD 5973 mass spectrometer (Agilent Technologies, Waldbronn, Germany). Mass spectra in the electron impact mode (MS/EI) were generated at 70 eV using selected ion monitoring (SIM). The retention times and monitored ions are depicted in table 1. For the quantification of the aldehydes, separate calibration curves for 3-methylbutanal, 2-methylbutanal, benzaldehyde, and phenylacetaldehyde were determined at final concentrations ranging from 0 to 1000 ppb. The compounds were solved individually in the buffer/ethanol mixture and, after following the extraction procedure as described above, measured by using HRGC-MS. All calibrations produced a linear response with an r^2 value > 0.97 over the whole concentration range analyzed.

Table 1 Retention times (t_R) and parameters for selected ion monitoring of the measured aldehydes

		Selected ion monitoring, m/z	
Name	t_R [min]	ion 1	ion 2
3-Methylbutanal	10.6	58	86
2-Methylbutanal	11.1	58	86
Pentanal (IS)	13.1	58	86
Benzaldehyde	23.3	106	77
Phenylacetaldehyde	25.1	91	120

2.2 Determination of •OH and EtO• concentration in sample solutions

The concentration of •OH and EtO• in the samples were determined by using ESR spectroscopy. The principle of the measurement relies on spin-trapping with α-(4-pyridyl-1-oxide)-N-tert-butylnitrone (4-POBN) and measuring ESR signal intensities using an ESR spectrometer (e-scan, Bruker, BioSpin, Rheinstetten, Germany). The ESR spectrometer was used with the following settings: centre field: 3.475 G; attenuation: 0 dB; sweep width: 100 G; receiver gain: 2.0×10^3; resolution: 512; modulation amplitude: 1.49 G; modulation frequency: 86 kHz; conversion time: 10 ms; time constant: 40 ms; scans: 50. Typical reactions were started by adding 1 mL of a 50 mM H_2O_2 solution to a 9 mL aliquot of the reaction mixtures to be assayed. The ESR measurement was started immediately after the H_2O_2 solution was added. The ESR signal intensity was evaluated automatically by using the software WinEPR, version 4.3 (Bruker Biospin GmbH, Rheinstetten, Germany). The samples' radical concentration is determined as ESR signal intensity. All measurements were carried out at ambient temperature (20–22 °C).

2.3 Influence of ethylenediaminetetraacetic acid (EDTA)-Fe^{2+} ratio on radical formation in buffered model solutions

An ethylenediaminetetraacetic acid (EDTA)-Fe^{2+} complex was used in this study as Fenton reagent for the generation of radicals. The stoichiometry in this complex has a great influence on the effectiveness of radical generation [8, 28]. To study this effect in further detail, a trial was conducted in which solutions of EDTA and $FeSO_4 \times 7\ H_2O$ were mixed and pre-incubated for 5 minutes at different ratios. The tested ratios were 0:100 µM, 100:100 µM, 200:100 µM, and 100:200 µM (EDTA:Fe^{2+}). The trials were conducted in buffer/ethanol mixtures (acetate buffer, pH 4.5, 0.2 mM; 5 % (v/v) ethanol) containing 7.5 mM POBN, and reactions were started by addition of 5 mM H_2O_2. The ESR settings and measurement procedure were the same as described above.

2.4 Studies of aldehyde formation from amino acids (AA) in buffered model solutions

Aldehyde formation in model solutions from AA by direct oxidation via a Fenton reagent was elucidated by preparing reaction mixtures containing 5 % (v/v) of anhydrous ethanol, 100 µM $FeSO_4 \times 7\ H_2O$, and 5 mM of the assayed AA. Typical reactions were started by adding 10 mL of a 300 mM H_2O_2 solution to 90 mL aliquots of the reaction mixtures. The reaction mixtures were then incubated for 1 hour at 20 °C and aldehydes were isolated and determined using the procedure described above.

To elucidate the dependency of aldehyde formation from •OH/EtO• concentration, two experiments were carried out.

In a first set of experiments, the dependency of aldehyde formation from radical concentration was elucidated with the same buffered model solutions as described above. However, in this experiment, instead of adding $FeSO_4 \times 7\ H_2O$ at a fixed concentration, a solution containing 0–200 µM of an EDTA-Fe^{2+} complex (EDTA:Fe^{2+}, 1:1) was added with the goal to vary the radical concentration. Mixing Fe^{2+} with EDTA in a ratio of 1:1 and using this complex should diminish Fe^{2+} autoxidation to Fe^{3+} while hydroxyl radicals are still formed from this complex. As a reference, one sample was prepared by pre-incubated Fe^{3+} with EDTA instead of Fe^{2+} because Fe^{3+} is not capable of acting catalytically in the Fenton reaction. The final H_2O_2 concentration in the model systems used in these trials was 5 mM. Reactions were carried out for 5 days

at 20 °C in the dark and the samples' aldehyde concentrations were determined as described above using diethylether extraction followed by HRGC-MS.

In a separate trial, the test solutions' $^{\bullet}OH/EtO^{\bullet}$ concentrations were determined using ESR measurement as described above. Prior to the measurement, sample solutions were prepared by adding 5 % (v/v) of ethanol, 0200 µM of EDTA-Fe^{2+} (EDTA:Fe^{2+}, 1:1), and 7.5 mM POBN to an acetate buffer (pH 4.5, 20 mM). The reactions were started again by adding 5 mM H_2O_2.

To study the effect of increasing $EtO^{\bullet}$ concentrations on the formation of aldehydes, a second set of experiments was carried out. The previously described assay was modified and the ethanol concentration was adjusted to 0–10 % (v/v) in steps of 2.5 % (v/v) while all the other parameters were kept constant (100 µM EDTA-Fe^{2+} (EDTA:Fe^{2+}, 1:1), 5 mM of the assayed AA). The test samples' aldehyde concentration was determined after 5 days storage at 20 °C in the dark. In a separate trial, the reaction mixtures' $^{\bullet}OH$-/$EtO^{\bullet}$-POBN concentration was determined again by using the same ESR settings and experiment design as described above; however, with the modification that the EDTA-Fe^{2+} concentration was kept constant at 100 µM, and the samples' ethanol concentrations were adjusted from 0–10 % (v/v) in steps of 2.5 % (v/v). All trials were done in triplicate.

2.5 Monitoring the rate of the formation of aldehydes by oxidative degradation of AAs in buffered model solutions

The rate of the formation of aldehydes in buffered model solutions was examined by dissolving 5 mM AAs (leucine, isoleucine, phenylalanine), 100 µM EDTA-$FeSO_4 \times 7\,H_2O$ (EDTA:Fe^{2+}, 1:1), and 5 mM H_2O_2 in 100 mL of a buffer/ethanol mixture (acetate buffer, pH 4.5, 0.2 mM; 5 % (v/v) ethanol) and storing these mixtures for 18 days at room temperature. In total, 6 swing top bottles (V = 180 mL) were prepared. The level of aldehydes was determined from the single bottles after 0, 1, 2, 3, 12, and 18 days with the method described above. For the bottle representing day 0, no H_2O_2 was added and the bottle was measured directly after preparing the samples assuming that no reactions occurred, yet.

3 Results and Discussion

Beer is a very complex matrix and it is thus practically impossible to examine and to give proof of a single reaction pathway. In the present study, the complex beer matrix was simplified and reduced to the presence of AAs and Fe^{2+}. Furthermore, a 0.2 mM acetate buffer at a pH of 4.5 with 5 % (v/v) ethanol was used as the base. Typical reactions were then started by the addition of 5 mM H_2O_2 to the reaction mixtures containing 100 µM Fe^{2+} and 5 mM of AAs. In a first trial, leucine was added to the reaction mixture and incubated for 1 hour at 20 °C. The volatiles from this solution were isolated by solvent extraction, and the isolate was evaluated using HRGC-MS in SIM mode. From this isolate, 3-methylbutanal (t_R = 10.6; m/z = 58/86) was identified as the prevalent peak (Fig. 1, II) and an amount of 0.56 ± 0.11 µM was determined (n = 2). A sample where no leucine was added served as a reference (Fig. 1, I). Further trials were carried out where Fe^{2+} or H_2O_2 were omitted from the test solution in individual trials and double-distilled water was added instead. The omission of Fe^{2+} or H_2O_2 led to a drastic decrease of the amounts of 3-methylbutanal being detected by 83.8 % and 96.7 %, respectively, as compared to the trial where both substances were included. This finding implies that both play a major role in the reaction leading to the formation of 3-methylbutanal. The observations further signify that H_2O_2 may be more reactive in the formation of 3-methylbutanal indicating a direct oxidative degradation of leucine by H_2O_2. A further trial where solely leucine and neither Fe^{2+} nor H_2O_2 was added still revealed a diminutive peak at a retention time of 10.6 minutes indicating that the product which was used for the study was already contaminated with minimal amounts of 3-methylbutanal. The concentration of this peak was determined as 0.015–0.023 µM indicating a contamination of the product by 0.005 %.

Phosphate buffer (0.2 mM; pH 4.5) with 5 % (v/v) ethanol was also tested in the assay and yielded 18.2 % lower amounts of 3-methylbutanal as compared to the use of an acetate buffer (data not shown). The reason may lie in the phosphate buffer's ability

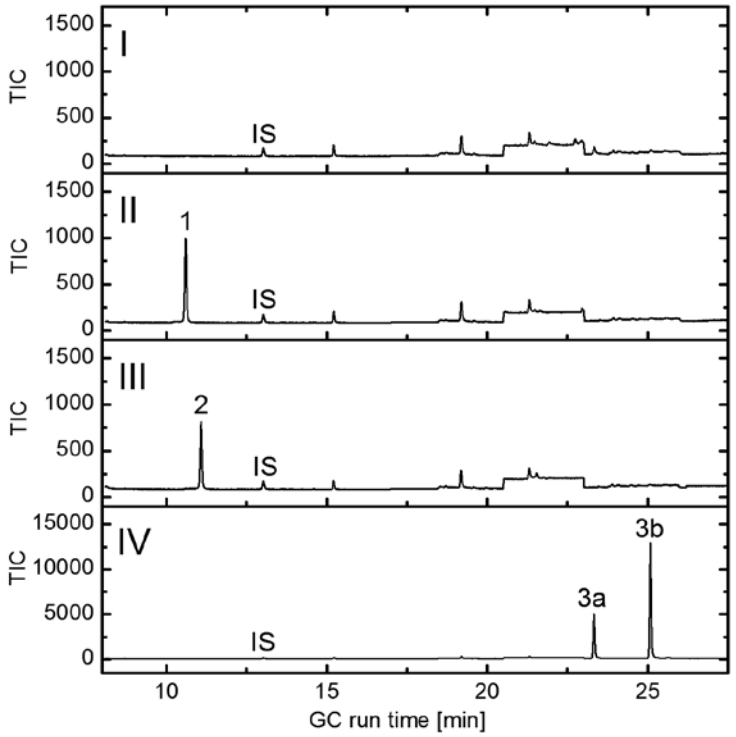

Fig. 1 HRGC-MS chromatograms of the reaction products of Fe^{2+}-H_2O_2 without addition of amino acids (I), and with addition of leucine (II), isoleucine (III), and phenylalanine (IV), respectively. The samples were incubated for 1 hour at 20 °C in a buffer/ethanol mixture (acetate buffer pH 4.5 0.2 mM; 5 % (v/v) ethanol) and isolated by extraction with diethylether. The MS was operated in EI mode (70 eV) using selected ion monitoring.

1: 3-methylbutanal, t_R = 10.6 min, m/z = 58/86;

2: 2-methylbutanal, t_R = 11.1 min, m/z = 58/86;

3a: benzaldehyde, t_R = 23.3 min, m/z = 106/77;

3b: phenylacetaldehyde, t_R = 25.9 min, m/z = 91/120;

IS: pentanal, t_R = 13.1 min, m/z = 58/86

to enhance Fe^{2+} autoxidation to Fe^{3+} [70] thereby decreasing the Fe^{2+} concentration available for the Fenton reaction.

As a complement to the previous trial where leucine was tested, isoleucine and phenylalanine were also examined in individual experiments and incubated for 1 hour at 20 °C. From the isolates, 2-methylbutanal at a concentration of 0.81 µM was obtained when isoleucine was added (Fig. 1, III), and, interestingly phenylacetaldehyde (1.66 µM) and benzaldehyde (6.78 µM) were found when phenylalanine was added to the test solutions (Fig. 1, IV). The appearance of benzaldehyde as derived by oxidative degradation from phenylacetaldehyde was reported recently by *Chu* and *Yaylayan* [13]. The pathway introduced by them also involves an oxygen-induced free-radical mechanism and therefore confirms the observations from this study.

Taken together, these results imply that hydroxyl radicals ($^{\bullet}OH$) which are generated in the Fenton reaction with Fe^{2+} as a catalyst are involved in these reactions. By adding H_2O_2 to the model solutions, the Fenton reaction cascade is initiated and $^{\bullet}OH$ radicals are formed. Ethanol is a major quencher of these radicals and because it is the most abundant compound in the model solutions used, it will be the primary reactant of $^{\bullet}OH$, yielding ethoxy radicals ($EtO^{\bullet}$) [3, 43].

To study the role of $^{\bullet}OH$ and $EtO^{\bullet}$ in these reactions, two additional trials were carried out using the same experiment design as described in the materials and methods section. In a first experiment, varying concentrations of $^{\bullet}OH/EtO^{\bullet}$ radicals were produced by adding different quantities of the Fenton reagent EDTA-Fe^{2+} to the reaction mixtures. This complex was used because pre-incubating Fe^{2+} with EDTA diminishes its autoxidation to Fe^{3+}. Furthermore, iron can provoke site localized reactions with the compound of interest when bound to it [12, 58]. By binding Fe^{2+} to EDTA prior to adding it, these site localized reactions are prevented while Fe^{2+} is still capable of acting as a catalyst in the Fenton reaction thus allowing the 'production' of radicals. For reviews on Fenton chemistry and the efficiency of chelators please consult the following references [29, 70, 74].

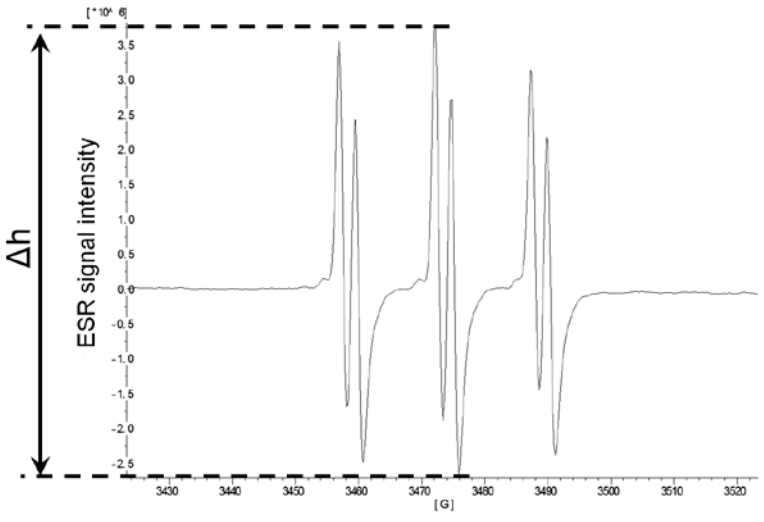

Fig. 2 ESR spectrum of •OH/EtO•-POBN spin trap adducts at room temperature following addition of POBN (7.5 mM), EDTA-$FeSO_4$ × 7 H_2O (EDTA:Fe^{2+}; 100:100 µM), and H_2O_2 (5 mM) to a buffer/ethanol mixture (acetate buffer, pH 4.5, 0.2 mM; 5 % (v/v) ethanol)

The stoichiometry of the EDTA-Fe^{2+} complex has a great influence on the efficiency of radical formation [8, 28]. Therefore, preliminary trials were carried out to study the effect of the EDTA-Fe^{2+} stoichiometry on radical formation efficiency in greater detail. Figure 2 shows an ESR signal as received when measuring $^{\bullet}OH/EtO^{\bullet}$-POBN adducts [3, 43]. The height of the signal (Δ h) is proportional to the concentration of formed spin adducts in the system and was consequently used to evaluate the sample's radical concentration.

Table 2 Influence of EDTA-Fe^{2+} ratio on ESR signal intensity [a]

ESR signal intensity			
no EDTA	EDTA:Fe^{2+} ratio		
	100:100 µM (1:1)	100:200 µM (1:2)	200:100 µM (2:1)
736990 ± 12098	674164 ± 10145	311066 ± 8527	701026 ± 6888

[a] Data represents the means of a triplicate experiment ± 1 standard deviation.

The data as shown in table 2 demonstrate that equimolar EDTA-Fe^{2+} ratios of 100:100 µM resulted only in a slightly diminished formation of radicals (8.5 %) as compared to adding 100 µM Fe^{2+} only. Supermolar EDTA-Fe^{2+} ratios (EDTA:Fe^{2+}; 200:100 µM) yielded a distinct reduction of the ESR signal intensity by 57.8 % while submolar ratios (EDTA:Fe^{2+}; 100:200 µM) resulted again in only a slight decrease of 4.9 % as compared to adding Fe^{2+} only. Others found similar results [28].

The ratio of 1:1 (EDTA:Fe^{2+}) was finally chosen to study the dependency of aldehyde formation from $^{\bullet}OH/EtO^{\bullet}$ attack because all the ferrous iron is then complexed while an adequate quantity of radicals is produced. EDTA-Fe^{2+} was added in concentrations of 0–200 µM to the reaction mixtures containing AAs and 5 % (v/v) ethanol and the reactions were started again by adding 5 mM H_2O_2. After incubation for 5 days at 20 °C in the dark, the sample solutions were extracted and the isolates were measured using HRGC-MS. In a separate trial, the radical generation from these samples was determined using ESR spectroscopy as described in the materials and methods section. The results are depicted in figure 3. The concentrations of phenylacetaldehyde and benzaldehyde were divided by a factor 10 for a better visibility in the graph.

As expected, the four aldehydes 3-methylbutanal, 2-methylbutanal, phenylacetaledehyde, and benzaldehyde were detected in the isolates. Their concentrations followed a linear response ($r^2 > 0.97$) and were increased with higher EDTA-Fe^{2+} concentrations, except for benzaldehyde which showed no linear behavior ($r^2 = 0.57$) and no remarkable increase. The same behavior was observed for the ESR signal intensity and therefore the free radical concentration ($r^2 > 0.99$). Replacement of Fe^{2+} by Fe^{3+} revealed only little amounts of 0.02 µM of the aldehydes being detected. Most likely, the detected aldehyde concentrations can be traced back to impurities of the AA products used because Fe^{3+} is thought to be incapable of acting catalytically in the Fenton reaction. This was strengthened by the observations from an ESR experiment

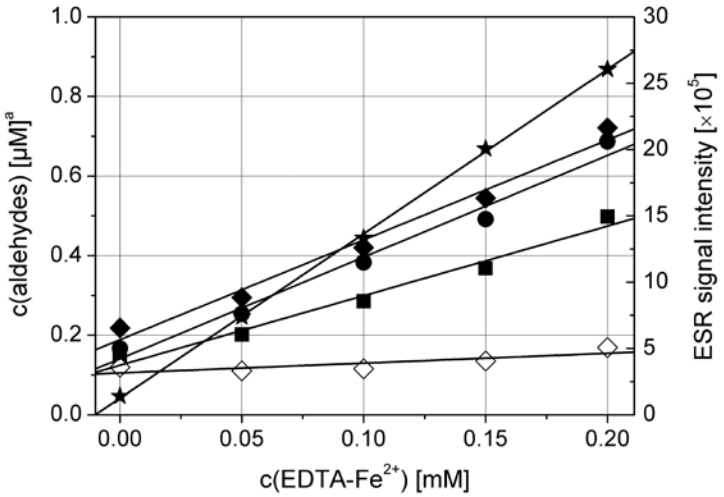

Fig. 3 **Dependency of ESR signal intensity (★), and the formation of 3-methylbutanal (■), 2-methylbutanal (●), benzaldehyde (◇), and phenylacetaldehyde (◆), from the EDTA-Fe^{2+} concentration (0–200 µM) in buffered model solutions (acetate buffer, pH 4.5, 0.2 mM; 5 % (v/v) ethanol). The model solutions contained 5 mM of leucine, isoleucine, and phenylalanine, respectively, and reactions were started by the addition of 5 mM H_2O_2. The reaction mixtures were incubated for 5 days at 20 °C in the dark. The ESR signal intensity was measured by adding 0–200 µM of EDTA-Fe^{2+} (EDTA:Fe^{2+}, 1:1) and 7.5 mM POBN to an acetate buffer (pH 4.5, 20 mM) containing 5 % (v/v) of ethanol. The reactions were started by adding 5 mM H_2O_2 and the ESR measurement was started immediately. The ESR measurements were carried out at ambient temperature (20–22 °C). The lines represent linear fittings of the data.**

[a] The concentrations of benzaldehyde and phenylacetaldehyde were divided by a factor 10 to fit them into the graph

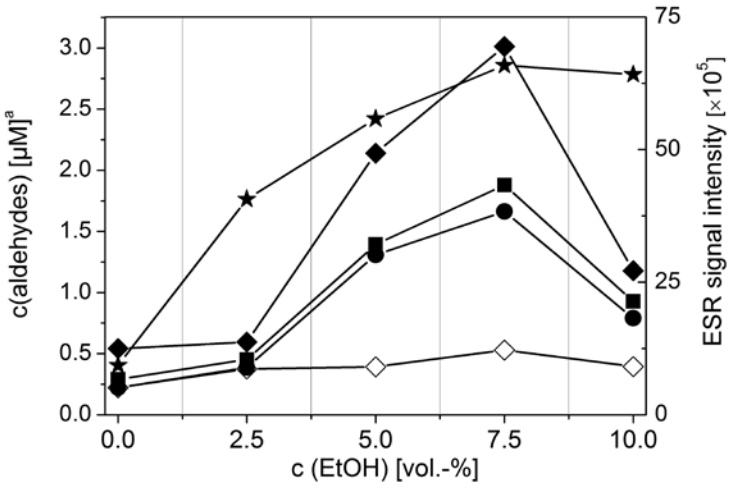

Fig. 4 **Dependency of ESR signal intensity (★), and formation of 3-methylbutanal (■), 2-methylbutanal (●), benzaldehyde (◇), and phenylacetaldehyde (◆), respectively, from ethanol concentration (0–10 % (v/v)) in buffered model solutions (acetate buffer, pH 4.5, 0.2 mM). The model solutions contained 100 µM EDTA-Fe^{2+}, 5 mM of leucine, isoleucine, and phenylalanine, respectively, and reactions were started by the addition of 5 mM H_2O_2. The reaction mixtures were incubated for 5 days at 20 °C in the dark. The ESR signal intensity was measured by adding 0–10 % (v/v) of ethanol, 100 µM of EDTA-Fe^{2+} (EDTA:Fe^{2+}, 1:1), and 7.5 mM POBN to an acetate buffer (pH 4.5, 20 mM). The reactions were started by adding 5 mM H_2O_2 and the ESR measurement was started immediately. The ESR measurements were carried out at ambient temperature (20–22 °C). The lines represent linear fittings of the data.**

[a] The concentrations of benzaldehyde and phenylacetaldehyde were divided by a factor 10 to fit them into the graph

where no ESR signal could be detected when using Fe^{3+} instead of Fe^{2+} (data not shown).

Correlating the concentrations of 3-methylbutanal, 2-methylbutanal, and phenylacetaldehyde with the ESR signal intensity and therefore with the free radical concentration yielded Pearson correlation coefficients > 0.98 indicating a clear relationship between radical concentration and aldehyde formation. Interestingly, leucine and isoleucine seem to have a similar and 10-fold lower trapping capacity for $^{\bullet}OH/EtO^{\bullet}$ attack than that of phenylalanine. Benzaldehyde was formed in higher concentrations than 3-methylbutanal and 2-methylbutanal but in lower concentration as compared to phenylacetaldehyde. The explanation may lie in its formation indirectly from phenylacetaldehyde. The correlation of benzaldehyde concentration with ESR signal intensity still yielded a Pearson correlation coefficient = 0.83, suggesting that radicals are involved in the abstraction of the carboxyl group from phenylacetaldehyde thereby forming benzaldehyde. The demonstrated results are consistent with published claims that amino acids, such as phenylalanine are better `sinks' for radical attack [26, 69] but also imply that leucine and isoleucine are susceptible too for $^{\bullet}OH/EtO^{\bullet}$ attack thereby forming the corresponding aldehydes.

The influence of the ethanol concentration and therefore the reactivity of AAs towards $EtO^{\bullet}$ attack was tested in a second set of experiments. The same experiment design was used again with the modification that in this trial, the ethanol concentration was increased stepwise from 0 to 10 % (v/v) and the EDTA-Fe^{2+} concentrations and ratio was kept constant at 100 µM. The concentration of radicals was determined again in a separate experiment by using ESR spectroscopy.

The aldehyde concentrations in the isolates after 5 days of storage at 20 °C show a clear trend and increase with higher ethanol concentration up to a concentration of 7.5 % (v/v) (Fig. 4). At 10 % (v/v) ethanol concentration, the concentration dropped by 49.4 %, 47.5 %, 39.1 %, and 74.5 % for 3-methylbutanal, 2-methylbutanal, phenylacetaldehyde, and benzaldehyde, respectively. When taking this data point not into account, the aldehydes' ethanol dependent behavior can be described by a linear behavior for the individual aldehydes (3-methylbutanal, $r^2 > 0.94$; 2-methylbutanal, $r^2 > 0.93$; phenylacetaldehyde, $r^2 > 0.93$; benzaldehyde, $r^2 > 0.91$). The ESR signal intensity showed the same trend as the aldehydes and also followed a linear response ($r^2 > 0.91$) up to an ethanol concentration of 7.5 % (v/v). An explanation for the increase in ESR signal intensity lies in the stability of the POBN spin trap adducts. The spin trap adducts of $EtO^{\bullet}$ with POBN have a greater half-life period (ca. 16 minutes) than those of $^{\bullet}OH$ with POBN (< 1 minute) [43, 47]. Up to an ethanol concentration of 7.5 % (v/v), in addition to the POBN-$HO^{\bullet}$ adducts, more POBN-$EtO^{\bullet}$ adducts are formed which 'survive' the way to the ESR's measuring chamber and the measurement span consequently producing an increased ESR signal intensity. As the ethanol concentration of 7.5 % (v/v) is exceeded, it is assumed that ethanol provokes a dilution effect

thereby causing a diminished radical formation (unpublished work; details are available from Thomas Kunz on request). A further increase of ethanol concentration to 10 % (v/v), led to a leveling off of the ESR signal intensity This dilution effect may also clarify the decrease in the formation of aldehydes at this data point.

Excluding the data point at 10 % (v/v) ethanol revealed significant correlations between the ethanol concentration and the aldehyde concentrations (Pearson correlation coefficients > 0.95) and the ESR signal intensity and the aldehyde concentrations (Pearson correlation coefficients > 0.86) indicating that the AAs used in this study are also susceptible by $EtO^{\bullet}$ attack.

To exclude the possibility that the ethanol additions caused an alkaline pH shift which may affect the occurring reactions, the test solutions' pH was also measured. A slight pH increase by 0.08 units from 0 to 10 % (v/v) could be observed. It is unlikely that this minimal pH shift is accountable for the observations.

Taking together, these data give evidence of a reaction route for the formation of the aldehydes 3-methylbutanal, 2-methylbutanal, phenylacetaldehyde, and benzaldehyde in a beer-like model system via direct oxidation of leucine, isoleucine, and phenylalanine, respectively, by attack from $^{\bullet}OH/EtO^{\bullet}$ without α-dicarbonyls present. A proposal of the reaction route was adapted from Stadtman [47, 59] and is depicted in figure 5 with leucine as an example: Catalyzed by Fe^{2+}, H_2O_2 decomposes to ^{-}OH and $^{\bullet}OH$ (Fenton reaction), from which $^{\bullet}OH$ further reacts with organic radicals ($R^{\bullet}$) such as ethanol. The primary attack on leucine (I) by $^{\bullet}OH/R^{\bullet}$ involves abstraction of a hydrogen atom from the α-carbon to form a carbon-centered radical (II) (reaction a). O_2 addition yields a peroxy radical derivative (III) (reaction b), which, upon reaction with a superoxide anion radical (or its conjugate acid, $HOO^{\bullet}$) leads to the production of O_2 and an alkylperoxide derivative (IV) (reaction c). An imino derivative (V) can then be formed by spontaneous decomposition of the alkylperoxide (reaction d) thereby dissociating H_2O_2. The imino derivative, in turn, can undergo hydrolysis to form an ammonium ion, carbon dioxide, and the aldehyde 3-methylbutanal (V) (reaction e).

The rate at which a chemical reaction proceeds is typically influenced by the amount of each reactant present, the ambient reaction conditions such as pH, and the temperature of the reaction. Based on the results and depicted reaction route, it may be assumed that the rate of attack of $^{\bullet}OH/EtO^{\bullet}$ on leucine, isoleucine, and phenylalanine is constant with time, as long as AA, Fe^{2+}, H_2O_2 and ethanol are present, and that the evolving aldehydes are a measure of the $^{\bullet}OH/EtO^{\bullet}$ attack. The rate at which the Fenton reaction occurs is strongly pH-dependent [40, 52, 73] but generally is completed within the order of seconds or minutes [22, 36, 73]. Therefore, the H_2O_2 present should be consumed rapidly. Yet, with proposal of the reaction route (Fig. 5) it can be anticipated that the H_2O_2 formed in reaction d becomes available for the Fenton reaction again and, as long as reactants are present, the reaction starts over again.

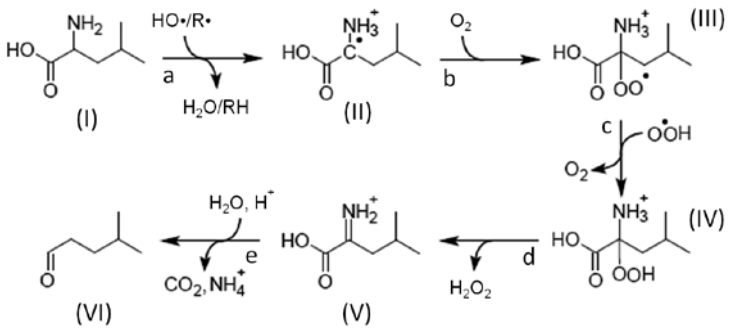

Fig. 5 Formation of 3-methylbutanal via oxidative degradation of leucine by $^{\bullet}OH/EtO^{\bullet}$ attack (modified reaction route adapted from Stadtmann [59]).

To test this hypothesis, the time-dependent reaction course over a span of 18 days was investigated again working with similar reaction conditions as in the previous trials and measuring aldehyde concentrations after 0, 1, 2, 3, 12, and 18 days.

Over the time-span measured, a steady increase of aldehydes could be observed (Fig. 6) which strengthens the previously proposed theory. Certainly, higher concentrations of H_2O_2 and Fe^{2+} or higher temperatures will affect the rate at which these reactions occur. Stadtman and Berlett [60] observed in their investigations that the metal-catalyzed oxidation of leucine is almost completely dependent of the presence of bicarbonate ions such as $NaHCO_3$. This may be an explanation for the overall low yields of the reaction products (aldehydes) found in the present study. As bicarbonate ions are present in beer, further trials should be carried out under addition of bicarbonate ions to the reaction mixture and examining the effect on the reaction rate.

In the series of experiments presented in this study, all reactions were started by the addition of H_2O_2 and with higher concentrations

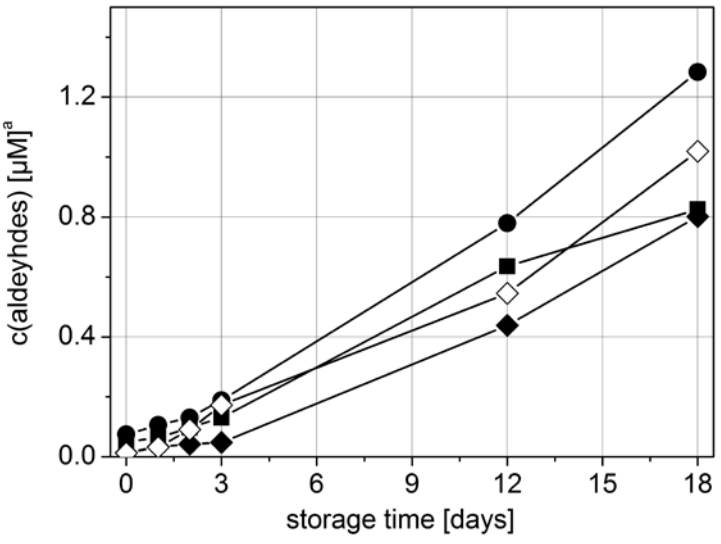

Fig. 6 Time-course of the formation of 3-methylbutanal (■), 2-methylbutanal (●), benzaldehyde (◇), and phenylacetaldehyde (◆), respectively, in buffered model systems (acetate buffer, pH 4.5, 0.2 mM, 5 % (v/v) ethanol). The solutions contained 5 mM leucine, isoleucine, and phenylalanine, respectively, 100 µM EDTA-$FeSO_4$ × 7 H_2O (EDTA:Fe^{2+}, 1:1), and were started by the addition of 5 mM H_2O_2. For the bottle representing day 0, no H_2O_2 was added and the bottle was measured directly after preparing.

[a] The concentrations of benzaldehyde and phenylacetaldehyde were divided by a factor 10 to fit them into the graph.

of AAs and Fe^{2+} than supposed to be in beer. In beer, however, the appearance and formation of H_2O_2 is believed to be a product of various oxidation systems in which oxygen, transition metal ions, ethanol, polyphenols, hop bitter acids, and melanoidins play a role [33, 53]. Furthermore, oxidative reactions are the results of a very complex interplay in which antioxidants and pro-oxidants such as sulfite, ascorbic acid, reductones, etc. are involved. Accordingly, outcomes from this study certainly not reflect 'real' conditions which disputes to some extent the adaptability and relevance of this reaction route for beer. Yet, the knowledge of the existence of radicals in beer and the observation that more aldehydes are formed during beer aging in the presence of oxygen and promoted by elevated iron levels support the relevance of this pathway.

4 Conclusions

The series of experiments presented, demonstrated that an additional mechanism leading from α-amino acids to aldehydes in 'beer-like' model solutions exists in addition to the already published mechanisms such as the Strecker degradation of amino acids. Deductions from this study relate the role of 'beer-radicals' with aldehyde formation and consequently provide a possible explanation for increased Strecker aldehyde concentrations in aged beer when elevated oxygen levels were present. This information can therefore contribute to some extent to the clarification of the processes which promote the formation of aroma-active aldehydes in bottled beer and other beverages. The results obtained from this study demand continued research aiming to further clarify the role of this pathway and gain more insight into the relevance of this radical-dependent mechanism as related to aldehyde formation in beer and other foods. The final goal of this research, certainly, is to facilitate the preparation of beers with an enhanced shelf-life and consistency to allow a longer lasting pleasant flavor.

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Received 02 July 2013, accepted 9 August 2013