JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Influence of the Brewing Process on Furfuryl Ethyl Ether Formation during Beer Aging

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In beer, the development of a solvent-like stale flavor is associated with the formation of furfuryl ethyl ether. The synthesis rate of this important flavor compound is proportional to the concentration of furfuryl alcohol in beer. This study shows that furfuryl alcohol in beer is mainly formed by Maillard reactions initiated during wort boiling and malt production. A mechanism for its formation from α-(1,4)-oligoglucans and amino acids in wort and beer is proposed. During wort boiling, a quadratic relationship was found between the wort extract concentration, on the one hand, and the increase of furfuryl alcohol and furfural, on the other. The reduction of furfural by yeast during fermentation further increases the furfuryl alcohol content. In pale beers, the furfuryl alcohol concentration is essentially determined by the thermal load on wort during brewing operations. In dark beers, a considerable fraction of furfuryl alcohol may, however, come from the dark malts used. These results lead to important practical conclusions concerning the control over furfuryl ethyl ether in beer.

KEYWORDS: Furfuryl ethyl ether; furfuryl alcohol; beer; aging; flavor stability; staling; brewing

INTRODUCTION

In previous studies (*1*, *2*), furfuryl ethyl ether (FEE) was identified as an important aging flavor in beer. Particularly in specialty beers, its concentration often exceeds the threshold of 6 μg/L, resulting in a solvent-like aging flavor. The formation rate of FEE in beer is proportional with the concentration of its two precursors: ethanol and furfuryl alcohol (FALC). FEE is formed in an acid-catalyzed S_N2 substitution reaction of these precursors. Because the FALC concentration is rate limiting for the formation of FEE, the formation of FALC during brewing is an important factor for practical control of FEE development during staling (*1*).

Saccharomyces cerevisiae cells can reduce furfural to FALC (*3*). In fact, this type of conversion was one of the first biotransformations known to occur in yeast (*4*). It was suggested that furfural competes with acetaldehyde in a reduction process catalyzed by alcohol dehydrogenase (*5*). However, furfural was also shown to have inhibitory effects on yeast metabolism (*6*, *7*). The synthesis of furfural in a Maillard reaction of pentoses is well-known (*8*). The reaction mechanism is analogous to the formation of 5-hydroxymethyl-2-furfural from hexoses. During the kilning of barley malt and the boiling process of wort, high levels of sugars and amino acids are subjected to high temperatures. These conditions are optimal for extensive Maillard and caramelization reactions (*9*, *10*).

FALC has also been identified as a wort component (*11*). Information on its origin in the brewing process is scarce, although it has been identified as a side product in various Maillard model systems (*12*−*15*). A mechanism for the formation of FALC from glucose in aqueous systems was given by Yaylayan and colleagues (*16*, *17*). The reaction involves the oxidation of glucose to gluconic acid, which is decarboxylated to a pentitol and followed by dehydration and cyclization to FALC.

However, because these reaction mechanisms were derived from Maillard model systems, they are not necessarily applicable to conditions of wort production. Therefore, FALC formation during the brewing process and beer storage had to be investigated in more depth. In this paper, we study several parameters affecting FALC formation and we propose practical measures for the control of its concentration in beer.

MATERIALS AND METHODS

Chemicals. The following substances with corresponding purity were supplied by Sigma Aldrich Chemie GmbH (Munich, Germany): 3-methylbutanal (98%), 2-furfural (99%), 5-methyl-2-furfural (99%), 2-acetylfuran (99+%), 2-furfuryl alcohol (99%), 2-heptanol (99%), aminoguanidine hydrochloride (98+%), sodium sulfite (99%), glycine (99.5%), xylose (99%), fructose (99%), glucose (99%), sucrose (99%), 2-deoxyribose (97%), gluconic acid (99%), maltose (95%), maltotriose (95%), maltotetraose (97%), maltopentaose (98%), maltohexaose (95%) maltoheptaose (92%), and rhamnose (95%). The 2-furfuryl ethyl ether with a purity of 95% was purchased from Narchem Corp. (Chicago, IL). Sodium hydroxide (50%) and sulfuric acid (98%) were supplied by Merck (Darmstadt, Germany).

Pilot-Scale Brewing. A pilsner malt (Boortmalt, Boortmeerbeek, Belgium) was ground with a disk mill (Engl Maschinen, Schwebheim, Germany). Mashing was performed using 85 kg of milled malt and

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10.1021/jf0490854 CCC: $27.50
Published on Web 10/12/2004

210 L of water in a mash tun of a 5 hL pilot brewery. The pH of the mash was adjusted with lactic acid to 5.5, and a specific temperature−time profile (step infusion method) was followed (10 min at 55 °C, 50 min at 63 °C, 20 min at 73 °C, and raised to 78 °C). A traditional lauter tun at 80 °C was used for separation of the wort and the spent grains. The extract content of the sweet wort was adjusted to 12 °P.

Sweet wort was adjusted to pH 5.2 with lactic acid and boiled for 75 min in a pilot-scale brew kettle. Evaporation during boiling was ∼5%. The boiled wort was transferred to a whirlpool for clarification (30 min) and subsequently aerated and cooled to 18 °C.

Wort Prepared from Different Barley Malt Varieties. Pilsner (Scarlett Pils; Weyermann, Bamberg, Germany), colored (Melanoidinen Malt; Weyermann, Bamberg, Germany), caramel (Cara-aroma; Weyermann), and roasted (Carafa II; Weyermann) barley malts were ground in a disk mill (Buhler-Miag, Minneapolis, MN) set for fine mill. In the case of pilsner malt (Scarlett Pils), 70 g was used in standard laboratory Congress wort preparations according to Analytica-EBC (*18*). In the case of specialty malts (Melanoidin, Cara-aroma, Carafa), 35 g of specialty malt and 35 g of pale malt were used in Congress wort brewing. Sweet wort samples of ∼12 °P of different malt varieties were obtained and submitted to a laboratory-scale boiling process.

Wort Prepared from Dried Extracts. Some worts were prepared by dissolving spray-dried malt extract powder (Spraymalt Light; Muntons, Suffolk, U.K.) in distilled water. Worts with various extract concentrations (6, 8, 10, 12, 14, 16, 18, and 20 g/100 mL) were prepared to study the effect of the wort density on FALC and furfural formation. To investigate the origin and formation mechanism of FALC and furfural during boiling, solutions with 160 g/L of wort extract were supplemented with sodium sulfite, aminoguanidine hydrochloride, sodium hydroxide (20%), or sulfuric acid (20%), and solutions with 120 g/L of wort extract were supplemented with xylose, glucose, fructose, maltose, sucrose, xylitol, gluconic acid, glycine, or 2-deoxyribose in various concentrations. Except for the samples with sodium hydroxide or sulfuric acid addition, the pH was corrected to 5.5. Samples were submitted to a laboratory-scale boiling process.

Laboratory-Scale Wort Boiling. Exactly 200 mL of wort was boiled under reflux in a glycerol bath (120 min, 106 °C) and then cooled to 20 °C in a water bath.

Fermentation Conditions. Small-scale fermentation experiments were carried out in cylindrical Schott flasks (5 L). A *Saccharomyces cerevisiae cerevisiae* strain (KUL CMBS 213) was first propagated by inoculating 25 mL of 12 °P wort with 1 mL of stock culture and incubation at 20 °C on an orbital shaker (Edmund Bühler, Hechingen, Germany) at 150 rpm for 2 days. The culture was then transferred to 250 mL of fresh culture medium and incubated under the same conditions for 2 days.

Yeast cells at the late exponential growth phase were inoculated (1×10^7 cells/mL) in aerated pilot brewery wort (4 L; dissolved oxygen, 8 mg/L). Two types of fermentation experiments were carried out at 20 °C: without or with 115 mg/L of furfural added to the original wort. An air-lock system was used to prevent the entrance of air during fermentation.

Analysis of Volatile Compounds in Wort and Beer. Prior to analysis, 200 μL of internal standard solution (250 mg/L 2-heptanol) and 200 μL of a 10% antifoam solution (Sigma Aldrich Chemie GmbH, Munich, Germany) were added to 50 mL of degassed beer or wort. Beer was degassed by kieselguhr filtration. Five milliliters of medium was transferred by a Tekmar-Dohrman Aquatek 70 autosampler (Emerson, Mason, USA) into the Tekmar-Dohrman 3000 purge and trap concentrator (Emerson) unit with a Vocarb 3000 trap (Supelco, Bellefonte, PA). For beer analysis, the following conditions were used: helium was the carrier gas, a 10 min purge at 140 °C, an 8 min dry purge at 140 °C, 6 min of desorption at 250 °C, and 10 min of baking at 260 °C. For wort analysis, the trap temperature during purge and dry purge was set at 100 °C, whereas other parameters were the same as for beer. The temperatures are those of the adsorbing trap, whereas the beer sample temperature was kept at 20 °C during purging. Before entering the GC, volatiles were concentrated using a cold trap with an MFA 815 control unit (ThermoFinnigan, San Jose, CA) in the following conditions: initial temperature, −70 °C; final temperature, 200 °C. GC was performed using a Fisons GC 8000 gas chromatograph equipped with a Chrompack CP-WAX-52-CB column (length = 50 m, internal diameter = 0.32 mm, film thickness = 1.2 μm; Varian, Palo Alto, CA). The temperature program was as follows: 3 min at 50 °C, rasied at 6 °C min^{-1} and held for 3 min at 240 °C. Total ion mass chromatograms were obtained in the Fisons MD 800 quadrupole mass spectrometer (ionization energy, 70 eV; source temperature, 250 °C) and analyzed using the Masslab software program for identification and quantification of volatiles. Quantification was performed using standard reference compounds. Peak areas were normalized using 2-heptanol as an internal standard. Calibration factors were determined using the standard addition method and creating linear regression models. Target ions were used in the identification and quantification of the component.

Addition of Substances to Beer. Additions of compounds to fresh top-fermented beer were made by opening the bottle, adding the substance to the beer, evacuating the headspace air by foaming, and again capping the bottle. To study the formation of FALC from its precursors, aminoguanidine hydrochloride, sodium sulfite, xylose, glucose, maltose, glycine, and 2-deoxyribose were added. The beers were then submitted to aging at 60 °C for 1 week.

Beer Aging Conditions. To study the Maillard reaction during beer storage, a fresh pasteurized top-fermented beer was subjected to storage at 60 °C, and after 0, 3, 7, 14, and 21 days, samples were analyzed in duplicate.

Amino Acid Analysis in Beer. Amino acids in beer were analyzed as described by Krause et al. (*19*). Samples were first deproteinized by ultrafiltration insert (UFC; M_r cutoff, 5000; Millipore, Bedford, MA) and then derivatized by the dabsyl chloride reagent (12 mM in acetone, Sigma) using an automated precolumn dabsylation device (AS3500; ThermoFinnigan). With an acetonitrile/acetate buffer as a mobile phase, the derivatized amino acids were separated on an Alltima C_{18} column (particle size, 5 μm; length, 150 mm; and i.d., 4.6 mm; Alltech, Deerfield, IL), including a guard cartridge (length, 30 mm; and i.d., 4.6 mm) and detected at 365 nm with a UV detector (UV3000HR; ThermoFinnigan). Results were analyzed using PC1000 system software (version 3.5; ThermoFinnigan).

Carbohydrate Analysis in Beer. Prior to the analysis, 200 μL of beer was run through cation (Dowex 50WX8-200; Acros Organics, Fisher Chemicals, Fair Lawn, NJ) and anion (Dowex 1X8-200, Acros Organics Fisher Chemicals) exchangers. Afterward, samples were diluted: seven times with Milli-Q-water and four times with azide water (0.02% w/v) followed by centrifugation (5 min, 16000*g*). Then, 25 μL of diluted sample was used for analysis, and carbohydrates were separated by high-pressure anion exchange chromatography and quantified by pulsed amperometric detection (Dionex, Sunnyvale, CA). A Carbopac PA-100 guard column and a Carbopac PA-100 (4 × 250) column (Dionex) in series were equilibrated with 90 mM NaOH for 9 min. The elution (1.0 mL/min at 2400 psi and 32 °C) was performed with NaOH (90 mM) and sodium acetate gradient as follows: 0−6 min, 10 mM; 6−16 min, 10−100 mM; 16−26 min, 100−175 mM. Finally, the column was regenerated with 90 mM NaOH and 500 mM sodium acetate during 5 min. Rhamnose was used as an internal standard. Calibration factors for glucose, fructose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were obtained by injecting 25 μL of a standard solution containing 50 mg/L of the pure compounds.

RESULTS AND DISCUSSION

The mechanism and parameters that may affect the FALC concentration in beer were investigated by monitoring its formation during different brewing operations. This was followed by a study of the kinetics and formation mechanism of FALC and furfural during wort boiling. Finally, experiments were set up to study the Maillard reactions during beer aging, with particular focus on FALC formation.

Origin of FEE Precursors in the Brewing Process. *Malt Production.* In the production of dark specialty beers, several types of dark malts are used. For their production, barley is subjected to different kilning or roasting conditions (e.g.,

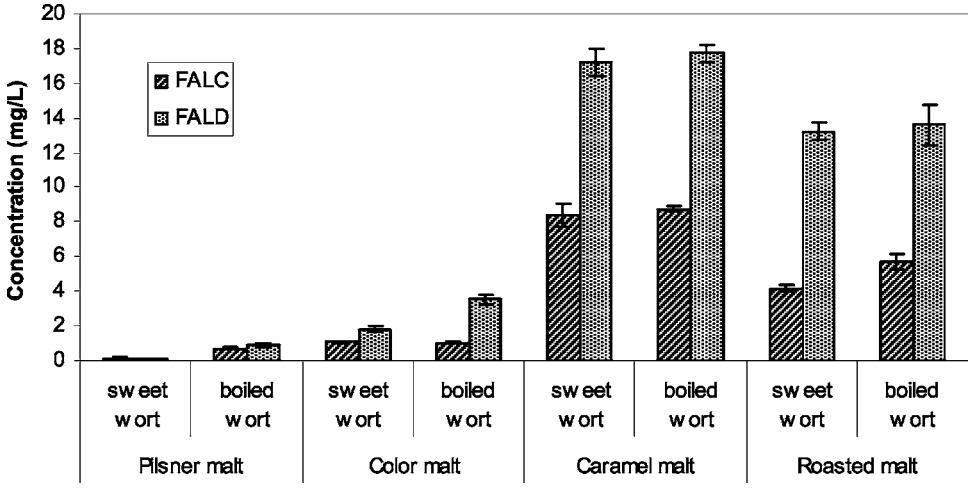

Figure 1. Furfuryl alcohol (FALC) and furfural (FALD) concentrations in sweet and boiled wort produced from pilsner, color, caramel, and roasted malts. For each data point the standard deviation (SD) is given ($n = 2$).

Table 1. Malt and Resultant Sweet Wort Properties

	malt	sweet wort		
	color (EBC)	color (EBC)	density (g/100 g)	pH
pilsner malt	6.2	9.0	12.50	5.79
color malt	67.0	61.8	12.50	5.32
caramel malt	481	388	11.90	4.93
roasted malt	1218	886	12.00	5.10

temperature and time) to produce dark pigments (melanoidins) in Maillard and caramelization reactions. Because FALC and furfural are intermediates in these reactions, sweet and boiled worts produced from different malt varieties according to standard laboratory Congress wort preparations (*18*) were analyzed. The malt and corresponding wort properties are given in **Table 1**. The pH value of the wort samples decreased with the malt color. The occurrence of acid reductones and acid melanoidins may have contributed to the lower pH values in dark malts (*20*). Furthermore, the extract content decreased with increasing malt color. These findings can be explained by the occurrence of more intensive Maillard reactions and by the inactivation of enzymes during curing or roasting (*20*).

FALC and furfural concentrations detected in wort (**Figure 1**) are linked to the malt production conditions. Pilsner malts are kilned at relatively low temperatures (maximally 85 °C). This apparently results in limited levels of FALC and furfural in the sweet (unboiled) wort. Boiling of the pale wort, on the other hand, is sufficient to increase their concentration. The typical higher kilning temperatures (up to 115 °C) of colored malts resulted in increased FALC and furfural concentrations in unboiled wort. The highest FALC and furfural concentrations were observed for caramel malts. Initially, the production of these malts proceeds at 60 °C which, in combination with a high malt moisture content (±43%), leads to saccharification. A subsequent temperature increase to 180 °C in a roasting drum causes the preformed carbohydrates and amino acids to react and produce high amounts of Maillard intermediates (*20*, *21*). Although the roasted malt has a darker color than the caramel malt, the levels of FALC and furfural were lower in the sweet wort. For the production of roasted malts, pilsner malt is rapidly heated to temperatures of 200−220 °C. This leads to malt dehydration and extensive Maillard, caramelization, and pyrolysis reactions, producing many dark melanoidins (*20*, *21*). These conditions might favor further reactions between Maillard intermediates such as FALC and furfural, so that the concentrations of the components in the resulting wort is lower than when more moderate conditions are applied.

In these experiments, wort was prepared with 50% specialty malts and 50% pilsner malt according to the Congress wort preparation procedures. However, the use of caramel and roasted malts is generally limited to 5% in the production of dark beers. Consequently, in practice, the FALC levels in dark beers will be lower than indicated in **Figure 1**.

Wort Boiling. To study the effects of wort boiling and further brewing operations on pilot scale (boiling, clarification, cooling, and aeration), sweet pilsner wort produced in a pilot brewery was used. The effects of boiling were also studied in a small-scale boiling process under reflux. The concentration changes of FALC and furfural during both experiments are respectively given in panels **A** and **B** of **Figure 2**. The initial concentration differences in sweet wort between the two experiments are due to the use of wort from different production batches. Both panels show a significant increase during boiling of FALC and furfural. During the 120 min laboratory boiling process, FALC and furfural increased almost linearly from 0.049 to 1.0 mg/L and from 17 to 150 μg/L, respectively (**Figure 2A**). Concentrations of FALC were higher than for furfural in the boiled wort. Similar results were obtained for pilot-scale wort production (**Figure 2B**), which consisted of wort heating to boiling temperature (30 min), wort boiling (75 min), wort clarification in the whirlpool (30 min), and wort cooling and aeration (5 min). When the temperature regimen of the different steps is compared to the FALC increase rate, it becomes clear that the FALC increase is strongly linked with the combined total "thermal load" the wort receives during brewing operations. Whereas furfural showed a linear increase with boiling time in the small-scale process, this was less evident in the more practical brewing conditions. This may be related to the higher volatility of furfural than that of FALC, causing more evaporation losses during the pilot-scale boiling process compared to small-scale boiling under reflux. This hypothesis is also supported by Narziss (*22*, *23*), who showed reduced furfural levels in wort to be related with higher total evaporation percentages during boiling.

Fermentation. Wort from a pilot brewery, after boiling, was supplemented with or without 115 mg/L furfural and fermented with a top-fermenting yeast strain at 20 °C. The changes in the FALC and furfural concentrations are shown in panels **A** and **B** of **Figure 3**, respectively. In the unsupplemented wort (**Figure 3A**), the furfural concentration decreased from 0.253 mg/L to a constant level of 0.010 mg/L within 8 h, whereas the FALC

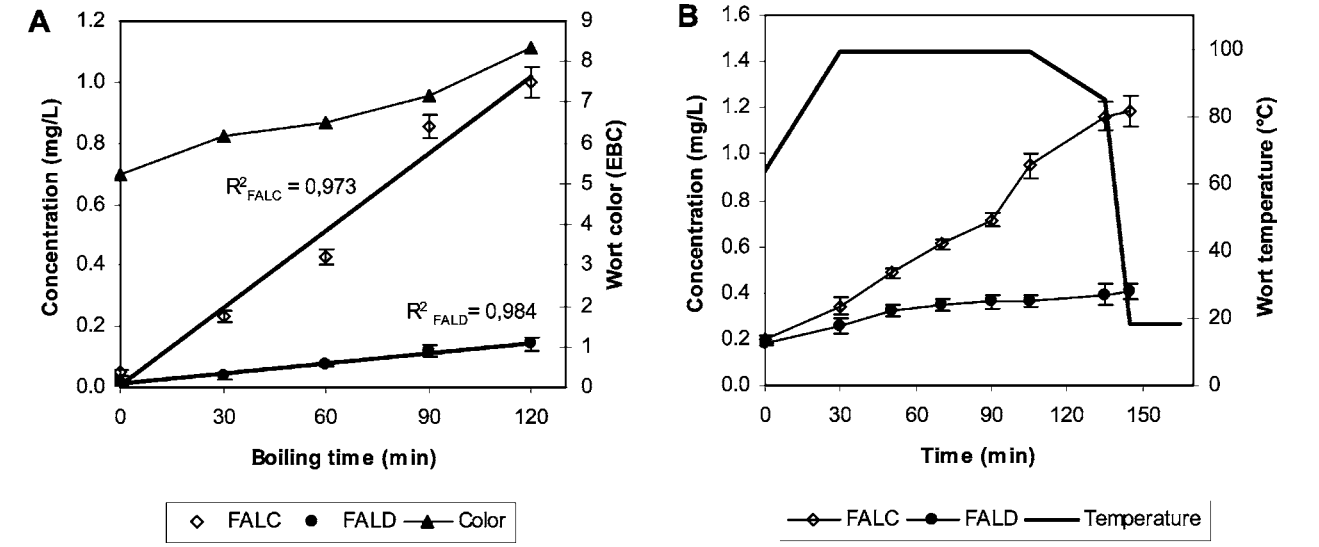

Figure 2. Concentration increases of FALC and FALD in pilsner wort during small-scale boiling under reflux (**A**) and a pilot-scale wort under heating, boiling, clarification, cooling, and aeration processes (**B**). For each data point of FALC and FALD the SD is given ($n = 2$).

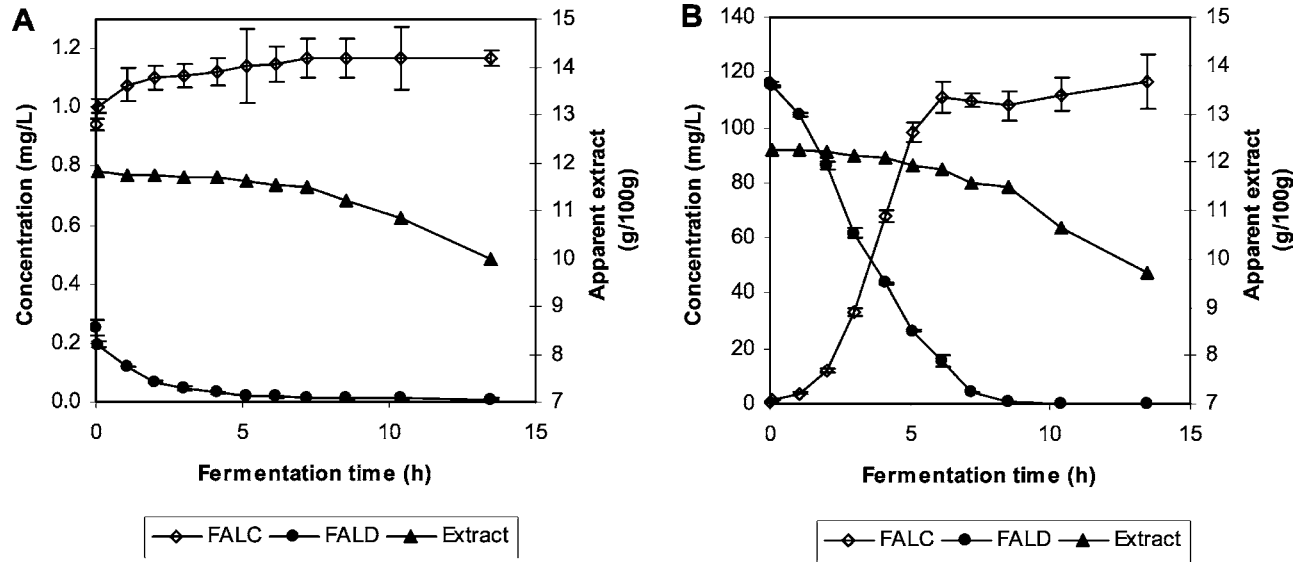

Figure 3. FALC and FALD concentration changes during fermentation of unsupplemented wort (**A**) and wort supplemented with 115 mg/L furfural (**B**). For each data point of FALC and FALD the SD is given ($n = 2$).

concentration increased from 0.943 to 1.167 mg/L. Although in the supplemented wort the furfural concentration was almost 500 times higher, it was mainly reduced to FALC within the first 8 h of fermentation (**Figure 3B**). These results confirm the yeast's ability to reduce furfural during fermentation. Very likely, the yeast reductases use the aldehyde to help restore the cells' redox balance, when excess levels of reduced coenzymes are formed during fermentation (*7*). Although furfural has a toxic effect on yeast cells (*5*, *7*), this was not observed, as the fermentation rates (decline of apparent extract) were almost identical in supplemented and nonsupplemented medium. Furthermore, during fermentation, not only is furfural converted to furfuryl alcohol, but small amounts of side products may also be formed (*5*).

Beer Storage. The precursors of FEE in beer are FALC and ethanol. From the results above, it is clear that the FEE concentration in pale beers is essentially determined by FALC and furfural formation during the thermal processes inherent to wort production. In contrast to dark beers, only a limited level of FALC in pale beers originates from the malts used. During fermentation, furfural in wort is almost completely reduced to FALC.

As shown in **Figure 4**, the FALC concentration in beer may further increase during storage. It increased at 60 °C from 1.810 to 3.634 mg/L in the first 7 days and remained relatively constant afterward. FEE, on the other hand, constantly increased from 0.007 mg/L in fresh beer to 0.294 mg/L after 3 weeks. In a previous study (*2*), FALC increases were also observed when beer was stored for 6 months at 20 and 40 °C. This means that, in practice, FALC levels will significantly increase after relatively long storage periods or during (short) storage at high temperatures.

Formation of FEE Precursors during Wort Boiling. *Kinetic Aspects.* The kinetic behavior of FALC and furfural formation was examined by measuring the FALC or furfural concentration increase ($\Delta C_{boiling}$) at different wort extract concentrations (C_{extr}), after 120 min of boiling under reflux. Wort samples were prepared by dissolving different amounts of a spray-dried malt extract powder in distilled water. When

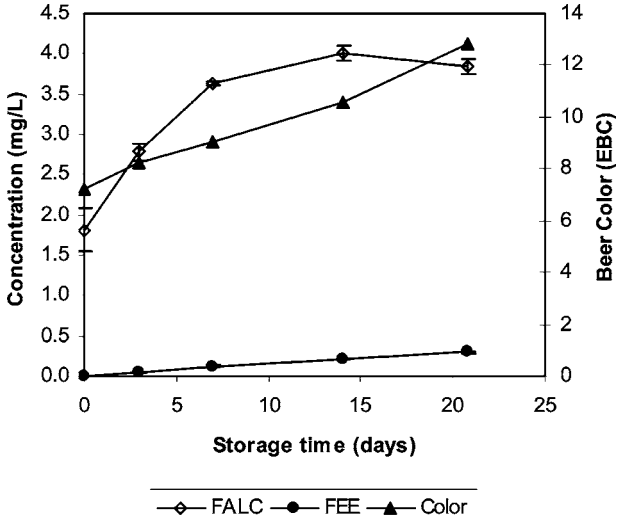

Figure 4. FALC and furfuryl ethyl ether (FEE) evolution during beer storage at 60 °C. For each data point of FALC and FEE the SD is given ($n = 2$).

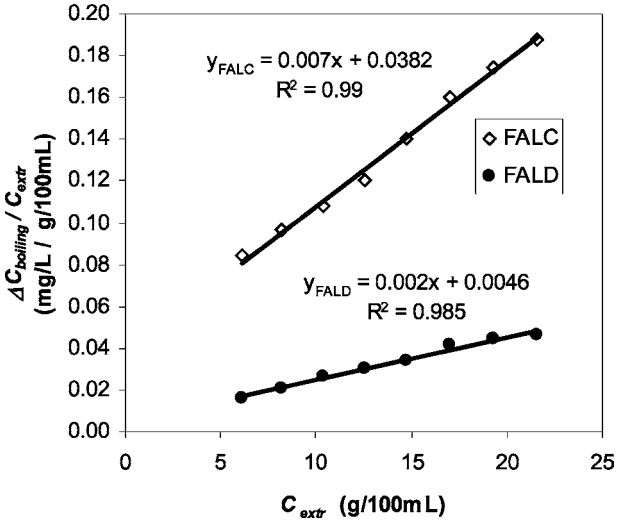

Figure 5. FALC and FALD formation during wort boiling as a function of extract concentration.

$\Delta C_{boiling}$ is devided by C_{extr}, the amount of furfural or FALC synthesized per unit of wort extract is obtained. In **Figure 5** $\Delta C_{boiling}/C_{extr}$ is plotted as a function of the extract concentration (C_{extr}). In both FALC and furfural, a quasi directly proportional relation was found between both variables. This means that the FALC and furfural concentration increases ($\Delta C_{boiling}$) do not vary proportionally with the wort extract concentration.

The linear relationships observed in **Figure 5** can be approximated by eq 1 in which k' is a constant.

$$\frac{\Delta C_{boiling}}{C_{extr}} = k' C_{extr} \quad (1)$$

Figure 2A indicates that FALC and furfural levels increased linearly during a 120 min (Δt) boiling process under reflux. This means that in these conditions, the reaction rate (v) of both formation processes can be approximated by a constant. The FALC or furfural concentration increases during boiling can thus be expressed by eq 2.

$$\Delta C_{boiling} = v\Delta t \quad (2)$$

Using eq 2, eq 1 can be transformed to eq 3.

$$v = \frac{k'}{\Delta t} C_{extr}^{\ 2} \quad (3)$$

In eq 3, $k'/\Delta t$ can be replaced by a constant k, which has the dimensions of a rate constant. A rate law (eq 4) is then obtained, which gives the relationship between the synthesis rate of FALC or furfural and the wort extract concentration.

$$v = kC_{extr}^{\ 2} \quad (4)$$

This indicates that FALC and furfural formation during wort boiling follows a second-order kinetics. The apparent constant rate of FALC and furfural formation during boiling is due to the persistent limited substrate conversion after 120 min.

Due to the complexity of the Maillard reactions, it is unrealistic to draw mechanistic conclusions from the reaction kinetic properties. However, some important practical implications become apparent concerning the synthesis of FEE during beer storage. Previous results for aged commercial beers (*1*) indicated that the formation of FEE is strongly correlated with the beer ethanol content, but it now becomes evident that in fact a combined effect of two processes is involved. The FEE formation rate is proportional with the concentration of each precursor (ethanol and FALC). However, both concentrations are related. A higher alcohol content of a beer implies that wort with a higher extract concentration was used. This in turn causes the quadratical increase of FALC and furfural synthesis during wort boiling. Higher original wort extracts lead to more FALC and furfural and to more alcohol as well.

The technique of *high-gravity brewing*, frequently applied in most modern breweries, also stimulates the formation of FEE during beer aging. For example, when beer is produced from 18 °P wort and the final beer is diluted to an original extract of 12 °P, it may theoretically contain 1.5 times more FALC than beer with the same alcohol percentage produced from 12 °P wort. This value was obtained by multiplying the ratio of FALC synthesis rates at 18 and 12 °P ($= 18^2/12^2$; see eq 4) by the dilution necessary to obtain beers with the same alcohol percentage ($= 12/18$).

Formation Reactions. The mechanism of FALC and furfural formation during wort boiling was studied by analyzing both components in a wort prepared with 120 g/L of malt extract, supplemented with various Maillard reaction substrates (**Table 2**). Without any additions, the boiled wort contained 2.100 mg/L FALC and 0.419 mg/L furfural. The addition of 40 g/L more malt extract clearly increased the FALC and furfural concentrations after boiling, as could be expected from the results presented in the previous section.

When wort carbohydrates such as xylose, glucose, fructose, maltose, and sucrose were added, the furfural concentration increased only by the addition of xylose. A 6 times higher concentration was observed with the addition of 40 g/L of xylose to wort than in the control sample. The Maillard reaction mechanism from pentoses to furfural is well-known (*8*), and the obtained results confirm that this reaction takes place during wort boiling. On the other hand, the different sugars showed only a limited effect on FALC formation.

The amino acid content of wort was increased by adding glycine. This addition clearly increased the FALC concentration in boiled wort, whereas it decreased the furfural concentration. Stimulation of FALC formation by adding an amino acid confirms that FALC can indeed be formed in a Maillard reaction during wort boiling. The negative effect of glycine on furfural

Table 2. Furfuryl Alcohol (FALC) and Furfural Concentrations[a] in Boiled Wort (120 min) Prepared with 120 g/L Malt Extract and Various Additions

addition to wort[b]	FALC (mg/L)	furfural (mg/L)	color increase (EBC)
no addition	2.100	0.419	6.63
40 g/L malt extract	3.105	0.508	8.15
40 g/L xylose	1.828	2.521	6.89
40 g/L glucose	2.168	0.434	5.89
40 g/L fructose	1.961	0.435	9.01
40 g/L maltose	2.126	0.429	5.33
40 g/L sucrose	1.934	0.371	5.32
2 g/L glycine	3.431	0.372	11.70
10 g/L glycine	4.052	0.088	15.02
40 g/L glycine	4.247	0.030	20.94
1 g/L xylitol	2.468	0.473	7.80
4 g/L xylitol	2.267	0.417	7.58
10 g/L gluconic acid	2.406	0.421	7.72
40 g/L gluconic acid	2.718	0.419	10.07
40 g/L maltose + 40 g/L glycine	5.770	0.114	25.64
1 g/L 2-deoxyribose	37.088	0.638	8.20

[a] Coefficients of variance: furfuryl alcohol = 7.4%, furfural = 3.8%. [b] The sweet wort had a color of 8.53 EBC and contained 0.593 mg/L furfuryl alcohol and 0.042 mg/L furfural.

concentration is probably related to the increased involvement of this Maillard intermediate in further Maillard reactions (*24*).

Although addition of glucose to wort did not lead to a higher FALC synthesis, the pathway of glucose to FALC according to Yaylayan and Keyhani (*16*) was further investigated. The addition to wort of the pathway intermediates gluconic acid and xylitol (a pentitol) produced no evidence for the involvement of these compounds or the proposed reaction pathway during boiling. The reaction conditions are probably not suited for ring closure of xylitol, which requires a nucleophilic attack of an alcohol function on a primary carbon atom followed by the expulsion of a water molecule.

Maltose represents ~50% of all wort sugars, and its concentration greatly exceeds that of amino acids. Hence, the addition of more maltose to wort might most likely have little effect on the rate of its Maillard reaction. Therefore, maltose and glycine were added together each in a concentration of 40 g/L. The effect on furfural formation was comparable to the effect obtained with only glycine. On the other hand, the addition of maltose and glycine obviously accelerated the FALC formation during boiling more than the addition of glycine alone. These results suggest that FALC can be formed in a Maillard reaction of maltose during wort boiling.

Other characteristics of the reactions leading to FALC and furfural were investigated by analysis of both components in wort at different pH values and wort with added aminoguanidine and sulfite (**Figure 6**).

The pH is an important parameter in Maillard reactions because various steps are acid−base catalyzed. The open chain form of the sugar and the nonprotonated form of the amino group are considered to be the reactive forms and are favored at higher pH values (*25*). However, when specific Maillard intermediates such as FALC and furfural are studied, the effect of pH is more complex. The overall effect on initial and intermediate reaction stages as well as the effect on further degradation steps and competitive reactions is determining. **Figure 6A** shows that the pH had no significant effect on FALC formation during wort boiling. On the other hand, furfural formation was enhanced at a lower pH (**Figure 6B**). At pH 6.5, the furfural concentration increased only by 0.011 mg/L during boiling, but this was raised significantly to 2.384 mg/L at pH 4.5.

Aminoguanidine is an inhibitor of certain Maillard reactions due to its ability to convert reactive intermediate α-dicarbonyl compounds to stable 3-amino-1,2,4-triazines (*26*). The fact that FALC synthesis is not changed (**Figure 6C**) by the presence of aminoguanidine indicates that α-dicarbonyl compounds are not involved in its synthesis. In contrast, α-dicarbonyl compounds are well-known intermediates in furfural formation, which is confirmed by the reduced furfural concentrations on addition of aminoguanidine (**Figure 6D**). Moreover, reaction of furfural with aminoguanidine may further reduce the concentration of furfural.

Although sulfite is also an inhibitor of Maillard reactions and is known to reduce furfural formation, its addition to wort did not influence furfural and FALC production during boiling (**Figure 6E**,**F**). Probably, during boiling, sulfite is rapidly converted to gaseous SO_2 before it can interfere with FALC or furfural formation pathways.

The results suggest that Maillard reactions of maltose particularly produce FALC during wort boiling. A recent study of Hollnagel and Kroh (*27*) revealed that 3-deoxypentosulose is the predominating α-dicarbonyl compound in aqueous model solutions at 100 °C of maltose or maltotriose and glycine. Furthermore, the 3-deoxypentosulose is a specific intermediate of Maillard reactions of α-(1,4)-oligo- and polyglucans and is not formed with glucose. A mechanism was proposed in which a 1-amino-1,4-dideoxyhexosulose would be formed by vinylogous β-elimination from the 2,3-enediol structure after Amadori rearrangement, favored by planar alignment of the bonds between the C1 and C4 of the glucose units. A retro-Claisen reaction of an enolization product of 1-amino-1,4-dideoxyhexosulose produces a pentose intermediate and imidic acid. Oxidation of the pentose intermediate would produce 3-deoxypentosulose (**Figure 7**).

During wort boiling, maltose and other α-(1,4)-oligoglucans may thus be involved in this Maillard reaction producing the pentose intermediate. Besides reacting to 3-deoxypentosulose, the pentose intermediate may also react at the weak acidic pH of wort to a 2-deoxypentose. To confirm whether the 2-deoxypentose reacts to FALC under wort boiling conditions, 1 g/L of 2-deoxyribose was added to wort. This deoxy sugar should react in the same way as the 2-deoxypentose intermediate in the pathway from maltose. **Table 2** indicates that 2-deoxyribose is a very reactive precursor in the formation of FALC. On the basis of these findings, there is strong evidence that FALC in wort is formed by the Maillard reaction of maltose and other α-(1,4)-oligoglucans as shown in **Figure 7**.

Maillard Reactions during Beer Storage. *Behavior of Beer Carbohydrates and Amino Acids during Storage.* The main substrates in beer of Maillard reactions are amino acids and carbohydrates. Their changes in concentration were monitored during storage of beer. Concerning the amino acids (**Table 3**), 12 acids increased (aspartate, methionine, lysine, leucine, histidine, serine, phenylalanine, isoleucine, threonine, glycine, valine, and arginine), 5 acids remained constant (asparagine, alanine, tyrosine, proline, and glutamate), and 3 acids decreased (glutamine, tryptophan, and cysteine) in concentration. The largest relative increase was observed for aspartate, and the largest relative decrease was observed for cysteine. As a result, the total level of amino acids in beer increased with aging. Probably, this is related to degradation of proteins and faster

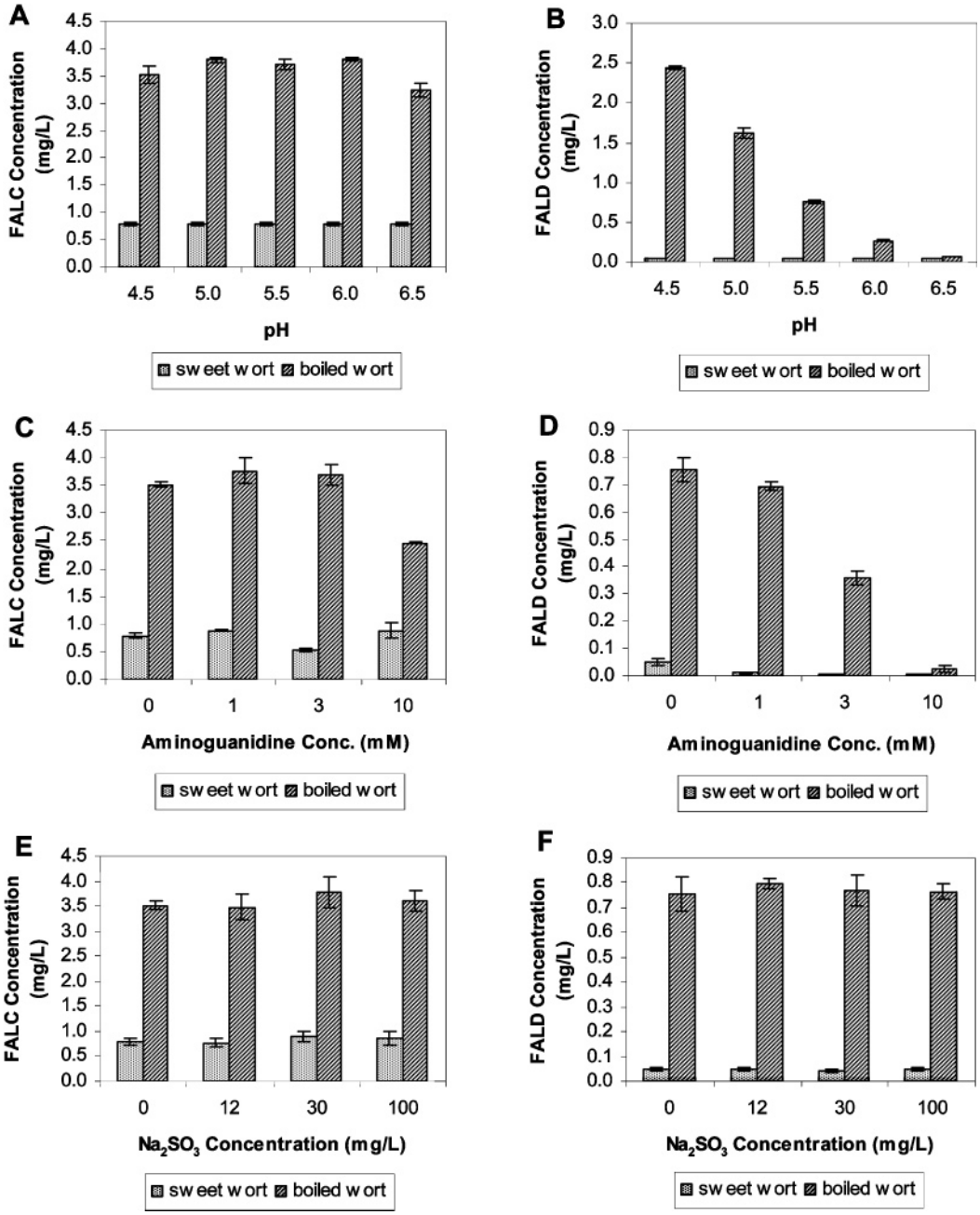

Figure 6. Effect of pH (**A**, **B**), aminoguanidine (**C**, **D**), and sulfite (**E**, **F**) on the formation of FALC and FALD during boiling of wort prepared with 160 g/L malt extract. For each data point the SD is given ($n = 2$).

amino acid release than utilization in processes such as Maillard reaction and Strecker degradation. Similar observations were made for amino acids during prolonged aging of wine (*28*).

Concerning the major beer carbohydrates (**Table 4**), no storage dependent significant changes were observed for glucose and α-(1,4)-oligoglucans. Fructose was absent in fresh beer but was detected after storage. Further research is required to reveal the fructose formation mechanism during beer storage. The relatively high glucose content is due to the addition of this sugar just before bottling to the commercial top-fermented beer examined.

Promoting and Inhibition Effects on Maillard Reactions in Beer. Some of the volatile compounds that increase in stored beer are well-known intermediates of Maillard reactions. However, it is unclear which process is responsible for their increase: an initial Maillard reaction between carbohydrates and amino acids or a conversion of reactive Maillard intermediates (e.g., α-dicarbonyl compounds), which are present in fresh beer. For example, α-dicarbonyl compounds may appear during the thermal stages of the brewing process (malt production, wort boiling, or whirlpool process) and "survive" in the final beer. During beer storage, these compounds may be further converted. From **Table 4**, it appears that no noticeable change in the concentration of carbohydrates during beer storage was found. However, the analytical procedure used might not be sensitive enough to detect concentration changes in the milligrams per liter range of beer carbohydrates.

Therefore, the Maillard reaction was further investigated by adding carbohydrates and amino acids to fresh beer and by monitoring the volatile compounds formed during aging (**Table 5**). The addition of xylose resulted in much larger levels of furfural in the aged beer, and the Strecker aldehyde 3-methylbutanal also increased. On the other hand, the addition of glucose or maltose seemed to have no effect. Glycine stimulated

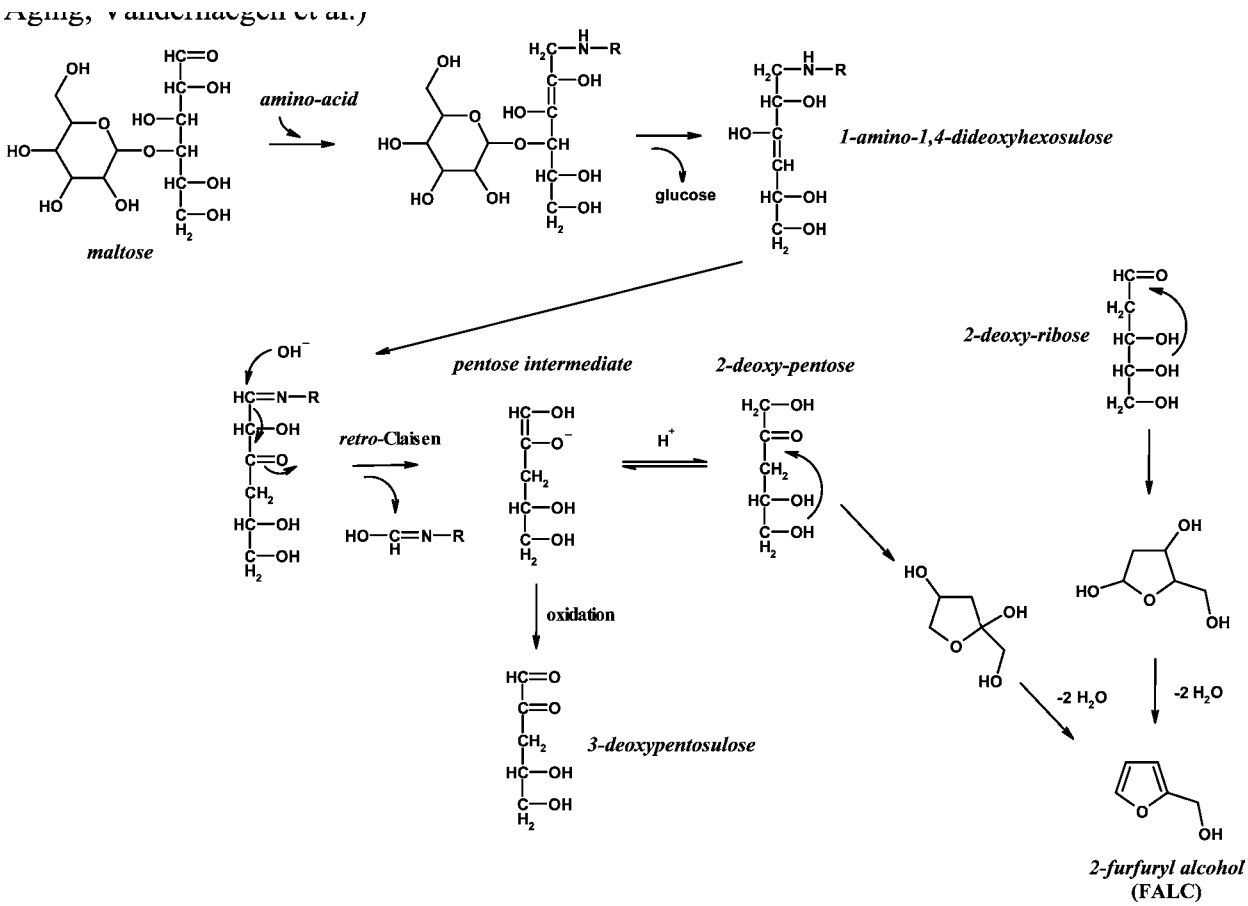

Figure 7. Proposed pathway for the formation of furfuryl alcohol in Maillard reactions during wort boiling. The scheme is partly based on the pathway from maltose to 3-deoxypentosulose according to that presented by Hollnagel and Kroh (*27*).

Table 3. Changes in the Amino Acid Concentrations of a Top-Fermented Beer during Storage at 60 °C

		concn (mg/L)				
amino acid	CV[a] (%)	fresh	3 days	7 days	14 days	21 days
aspartate	6.4	3.4	6.5	10.0	16.9	26.4
methionine	20.2	0.6	0.5	1.1	1.9	2.2
lysine	11.6	3.9	4.8	6.5	8.3	10.5
leucine	2.3	9.7	11.2	12.2	13.9	15.4
histidine	3.6	12.8	14.8	16.9	18.0	19.0
serine	12.8	10.5	12.6	11.9	14.6	14.9
phenylalanine	1.9	23.3	24.1	24.8	26.4	29.0
isoleucine	1.5	16.0	17.3	17.8	18.5	19.8
threonine	0.7	23.9	25.9	26.0	28.1	27.9
glycine	1.4	33.8	35.7	35.8	37.2	39.2
valine	0.4	32.3	33.4	34.3	35.1	37.2
arginine	1.0	33.0	35.9	37.5	38.8	37.4
asparagine	5.1	22.2	23.4	20.6	25.1	24.4
alanine	3.1	68.6	71.2	70.3	69.7	72.4
tyrosine	0.8	38.7	39.6	38.5	39.4	40.8
proline	2.0	473.9	480.8	465.9	462.9	468.8
glutamate	1.3	11.9	12.7	11.5	11.2	11.5
glutamine	34.6	2.4	1.4	1.5	1.6	2.1
tryptophan	1.2	30.6	30.2	27.8	26.6	25.2
cysteine	6.0	11.1	11.5	9.0	8.4	8.8
total AA		862.6	893.6	880.1	902.6	932.7
total AA without proline		388.7	412.8	414.2	439.7	463.9

[a] Coefficient of variance.

Table 4. Changes in the Carbohydrate Concentrations of a Top-Fermented Beer during Storage at 60 °C

		concn (g/L)				
sugar	CV[a] (%)	fresh	3 days	7 days	14 days	21 days
glucose	6.8	7.7	7.3	7.1	7.3	7.1
fructose	2.1	0.0	0.2	0.5	0.7	0.7
maltose	1.6	0.8	0.8	0.8	0.8	0.9
maltotriose	4.4	2.9	2.8	2.8	2.7	2.8
maltotetraose	7.5	7.4	7.3	7.3	7.2	7.4
maltopentaose	1.9	2.7	2.5	2.6	2.7	2.7
maltohexaose	11.1	1.7	1.6	1.7	1.6	1.8
maltoheptaose	2.6	0.6	0.6	0.6	0.5	0.6

[a] Coefficient of variance.

the synthesis of all volatiles except for the concentration of furfural, which was increased at 1 g/L, but reduced at 5 g/L glycine. These results indicate that some Maillard reactions between amino acids and carbohydrates may occur during beer storage, particularly with pentoses. Furthermore, α-dicarbonyl intermediates may stimulate a Strecker degradation during storage.

The possibility that the pathway to FALC (**Figure 7**) occurs in stored beer was investigated. The addition to beer of maltose plus glycine clearly promoted FALC formation, which in turn produced more furfuryl ethyl ether. The 2-deoxyribose was a very reactive precursor in the syntheses of FALC and FEE, the concentrations of which increased up to 3 mg/L (**Table 5**). The deoxy sugar can be considered to react in the same way as the 2-deoxypentose intermediate in the pathway from maltose. The addition of glycine also stimulated FALC and FEE formation, although the maltose concentration in beer was relatively low (**Table 4**). Therefore, it seems likely that the FALC increase during beer storage (**Figure 4**) is presumably due to Maillard reactions involving unfermentable α-(1,4)-oligoglucans in beer.

Table 5. Concentration of Maillard Components[a] in Fresh Beer and in Beer Aged at 60 °C for 7 Days with Added Maillard Reaction Substrates and Inhibitors

addition to beer	furfuryl alcohol (mg/L)	furfuryl ethyl ether (mg/L)	furfural (mg/L)	3-methylbutanal (mg/L)	2-acetylfuran (mg/L)	5-methyl-2-furfural (mg/L)
fresh beer	1.741	0.006	0.045	0.007	0.020	0.008
no additions	3.363	0.127	0.946	0.056	0.065	0.026
10 g/L maltose	3.235	0.127	1.167	0.057	0.062	0.027
10 g/L glucose	3.487	0.130	1.013	0.053	0.069	0.028
10 g/L xylose	3.447	0.128	12.95	0.096	0.058	0.026
1 g/L glycine	3.851	0.132	1.152	0.056	0.086	0.035
5 g/L glycine	4.215	0.137	0.892	0.069	0.083	0.034
10 g/L maltose + 5 g/L glycine	8.023	0.145	0.807	0.075	0.069	0.029
2 g/L deoxyribose	146.6	2.986	1.526	0.061	0.066	0.032
1 mM aminoguanidine	3.243	0.126	0.512	0.041	0.058	0.021
3 mM aminoguanidine	3.385	0.128	0.074	0.019	0.041	0.012
100 mg/L Na_2SO_3	2.322	0.115	0.627	0.051	0.051	0.024
500 mg/L Na_2SO_3	1.904	0.094	0.196	0.020	0.027	0.016

[a] Coefficients of variance (%): furfuryl alcohol = 7.4%, furfuryl ethyl ether = 3.7%, furfural = 3.8%, 3-methylbutanal = 4.5%, 2-acetylfuran = 4.1%, 5-methyl-2-furfural = 6.2%.

The oligoglucans as well as maltose react with amino acids and then release fragments with one glucose unit less than the original oligoglucan.

Finally, the study of the Maillard reaction inhibitor, aminoguanidine, indicated that it could reduce the formation of furfural, 3-methylbutanal, 2-acetylfuran, and 5-methyl-2-furfural. This is explained by the formation of unreactive 3-amino-1,2,4-triazines from α-dicarbonyls, which are intermediates in the formations of these compounds. However, FALC and FEE syntheses were unaffected by the addition of aminoguanidine. This agrees with the proposed formation mechanism of FALC formation. In contrast, sulfite reduced FALC production and the synthesis of FEE. Sulfite is known (*29*) to inhibit Maillard browning by nucleophilic reaction with the double bond of Maillard intermediates, such as 3,4-dideoxyhexosulos-3-ene (3-DDH). The reaction of sulfite with unsaturated structures in the reaction pathway of FALC may then explain the inhibitory effects of sulfite. The concentrations of other Maillard components shown in **Table 5** were also reduced by sulfite. Besides inhibition of their Maillard formation pathway, the reactions of sulfite with the carbonyl functions of these components are also involved.

In conclusion, the results of this work combined with the conclusions obtained from previous studies (*1*, *2*) provide insight into the controlling factors of FEE, which has stale characteristics during beer aging. Our study indicates that FEE and possibly other furanic ethers are typical staling components of specialty beers. Indeed, the beer parameters that stimulate FEE synthesis during storage are high ethanol content, dark color, or low pH. Most specialty beers differ from pilsner beers by at least one of these beer parameters. The identification of these flavor compounds may explain the distinct sensory differences between aged pilsner and aged specialty beers. Although FEE formation may induce a major flavor change during storage, the rate of the overall flavor deterioration during beer storage depends also on the rate of other staling processes and on characteristics of the fresh beer flavor profile. The beer parameters promoting FEE formation may affect the rate of other staling processes in different ways. Moreover, some flavors of fresh beers may mask the appearance of stale flavors.

Furthermore, FEE development in beer increased with the total thermal load on wort during the production stages on pilot scale. The process conditions or technological innovations, which reduce this parameter, will produce beers that are less susceptible to FEE stale flavor formation during storage.

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Received for review June 7, 2004. Revised manuscript received August 30, 2004. Accepted September 3, 2004.

JF0490854