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Release of Deuterated (*E*)-2-Nonenal during Beer Aging from Labeled Precursors Synthesized before Boiling

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Although lipid autoxidation in the boiling kettle is a key determinant of the cardboard flavor of aged beers, recent results show that mashing is another significant source of wort nonenal potential, the well-known indicator of how a beer will release (*E*)-2-nonenal during storage. Although unstable, deuterated (*E*)-2-nonenal nitrogen adducts created during mashing can in some cases partially persist in the pitching wort, to release deuterated (*E*)-2-nonenal during beer aging. In the experiment described here, the relative contributions of mashing and boiling were estimated at 30 and 70%, respectively. The presence of oxygen during mashing and, to a lesser extent, high lipoxygenase activity can intensify the stale cardboard flavor.

KEYWORDS: Flavor; beer; aging; oxidation; lipoxygenase

INTRODUCTION

Flavor is a key attribute of beer, and its deterioration during storage after packaging has long been a source of concern for brewers. Among the key flavor molecules contributing to staling, alkenals having seven to nine carbon atoms (*1*−*3*) and, in particular, (*E*)-2-nonenal (*3*−*7*), dimethyl trisulfide (*8*), and β-damascenone (*9*, *10*) can rise during storage to levels above their threshold concentrations.

Mechanisms leading to the release of (*E*)-2-nonenal have received special attention, because this compound is known to impart a cardboard character to aged beers when present at levels as low as 0.035 μg/L (*11*). Whereas 0.2−0.5 μg/L is usually detected after 3−5 months at 20 °C or after 3−5 days at 40 °C (*6*, *7*), as little as 0.1−0.2 μg/L can be measured after 6 months (*5*). According to Schieberle and Komarek (*12*), the concentration is below the aroma threshold even after 12 months of natural aging at 20 °C or after 1 month at 37 °C. This is probably due to the fact that free (*E*)-2-nonenal can convert to 3-hydroxynonanal through hydration or to nonenoic acid through oxidation, as evidenced in model solutions by Noël and Collin (*13*). Meilgaard (*1*) has described a cardboard flavor appearing after 4 weeks of agitated storage at 33 °C, peaking after 8−12 weeks and then dropping off as other flavors become more intense. Wang and Siebert (*4*) observed a similar profile for the (*E*)-2-nonenal concentration over storage at 38 °C.

Recently, studies on a model solution allowed us to propose a nonoxidative mechanism for the appearance of (*E*)-2-nonenal in aged beer (*6*). Wort amino acids and proteins can bind (*E*)-2-nonenal. Due to their Shiff base structure, the resulting adducts are fortunately partially lost in the brewhouse. Residual adducts can protect (*E*)-2-nonenal from the reducing activity of yeast and further release by acidic hydrolysis free (*E*)-2-nonenal during storage. The nonenal potential, defined by Drost et al. (*5*) as the potential of wort to release (*E*)-2-nonenal after treatment for 2 h at 100 °C at pH 4 under argon, is a means of quantifying reversibly bound (*E*)-2-nonenal in the wort (*6*, *14*, *15*).

As previously demonstrated with labeled compounds, the nonenal potential arising from linoleic acid autoxidation during boiling can partially persist in the final beer, leading to the release of free (*E*)-2-nonenal through aging (*7*). The amount of (*E*)-2-nonenal released through natural or forced aging correlates with the nonenal potential of the pitching wort (*6*, *7*). The antioxidant activity of the boiling wort, as measured by means of the 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced linoleic acid oxidation assay (*16*), can be used to predict the intrinsic resistance of a wort to (*E*)-2-nonenal synthesis, according to the hop variety used (*17*). Other methods also appear to be very useful for predicting beer flavor stability, such as the measurement of radicals by the application of electron spin resonance (ESR) spectroscopy (*18*−*21*), the decoloration of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical as a measure of reducing power in beer (*22*, *23*), or the measurement of chemiluminescence (*24*−*26*).

Many authors have suggested that malt lipoxygenase (LOX) activity is also related to beer aging (*5*, *27*). The experience of brewers shows, however, that this is not always a crucial parameter. During mashing, the LOX-1 isoenzyme converts linoleic acid to 9-hydroperoxide (*5*, *28*−*32*), which is further degraded to *cis*-3- and (*E*)-2-nonenal (*5*, *33*, *34*). In an experiment where 100 μg/L deuterated (*E*)-2-nonenal was added to a laboratory-scale wort (produced with a malt having low antioxidant activity in the absence of any special protection against oxidation), Noël et al. (*7*) found that only 0.2 μg/L of the labeled nonenal potential produced persisted up to the

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boiling-kettle stage. They detected no trace of labeled (*E*)-2-nonenal in the final beer after aging, even for a spiking of 1.5 mg/L. Although these data clearly demonstrate the instability of alkenals in an oxidized medium, they are insufficient to rule out the presence of enzymatically produced (*E*)-2-nonenal in the final beers produced from other worts.

The aim of the present study was therefore to investigate the fate of labeled (*E*)-2-nonenal added to a wort having high antioxidant activity and, thereby, to clarify the contribution of mashing to the cardboard off-flavor of aged beer. The effects of oxygen and lipoxygenase activity on the nonenal potential of the pitching wort were also investigated.

MATERIALS AND METHODS

Chemicals. Ammonium sulfate p.a. was purchased from Vel (Leuven, Belgium), argon from Air Liquide (Liège, Belgium), dichloromethane from Romil (Cambridge, U.K.), and (*E*)-2-nonenal 97% from Aldrich Chemical Co. (Bornem, Belgium). Absolute ethanol p.a., phosphoric acid (85%), and Tween 20 were obtained from Merck (Overijse, Belgium) and Bis-Tris 98%, bovine serum albumin 99% (BSA), iminodiacetic acid, and linoleic acid 99% from Sigma Chemical Co. (Bornem, Belgium).

Labeled and Unlabeled (*E*)-2-Nonenal Analysis. Carbonyl compounds were extracted by vacuum distillation, transferred to dichloromethane, and concentrated before analysis by gas chromatography−mass spectrometry, as previously described by Lermusieau et al. (*6*). Selected ion monitoring analyses were used with *m*/*z* 57, 70, 83, and 85 for unlabeled (*E*)-2-nonenal, as mentioned by Lermusieau et al. (*6*), and with *m*/*z* 46, 58, and 71 for deuterated (*E*)-2-nonenal, as mentioned by Noël et al. (*7*). All analyses were performed in duplicate, and their global variation coefficient was under 10%.

Nonenal Potential Experiment [Based on the Method of Drost et al. (5)]. The pH of 1.5 L of wort or beer was adjusted to 4 with 85% phosphoric acid. After being purged for 15 min with argon to reduce the oxygen level, the sample was heated at 100 °C for 2 h in a 2 L closed vessel and then cooled to 4 °C and kept at that temperature overnight prior to (*E*)-2-nonenal analysis. All analyses were performed in duplicate, and their global variation coefficient was under 15%.

Antioxidant Assay. The antioxidant activity was measured according to the method of Liégeois et al. (*16*) based on the AAPH-induced oxidation of an aqueous dispersion of linoleic acid. UV spectroscopy was used to monitor the appearance of oxidation products at 234 nm. Antioxidant activity was assessed by the inhibition time (T_{inh}), as the point of intersection between the tangents to the inhibition and propagation phase curves, under precise oxidation conditions. This activity varied according to the antioxidant and its concentration. All analyses were done in duplicate. All variation coefficients were under 3%.

Total Lipoxygenase Extraction. Malt was ground in a coffee mill for 30 s with 10 volumes of cold 0.1 M potassium phosphate buffer, pH 7, and then stirred gently for 40 min at 4 °C. After centrifugation (14000*g* for 30 min at 4 °C), the total lipoxygenase activity of the supernatant was immediately assayed.

Lipoxygenase Isoenzyme Separation and Characterization. Lipoxygenase isoenzymes were separated directly on a hydroxyapatite column after precipitation with ammonium sulfate (to 50% saturation) as described by Yang et al. (*30*). All steps were carried out at 4 °C. Column chromatography was performed on an FPLC system (Pharmacia, LKB Biotechnology, Uppsala, Sweden).

Solid ammonium sulfate was added to the crude extract and the mixture homogenized for 40 min. The fraction precipitating between 20 and 50% saturation was collected by centrifugation (14000*g* for 20 min). The pellet was resuspended in 5 mL of 10 mM potassium phosphate buffer, pH 6.5, and applied to a hydroxyapatite column (Econo-Pac HTP, 12.4 × 38 mm, 5 mL; Bio-Rad, Richmond, CA) equilibrated with the same buffer. Bound proteins were eluted from the column with a linear gradient (10−300 mM) of potassium phosphate buffer, pH 6.5. The flow rate was kept at 0.5 mL/min, and 0.5-mL fractions were collected. Two completely resolved active peaks were identified as the LOX-1 and LOX-2 isoenzymes by isoelectric point determination (checked on a MonoP HR 5/20 column from Pharmacia in the 7−4 pH range, according to the manufacturer's instructions).

Lipoxygenase Assay and Protein Determination. Lipoxygenase activity was determined polarographically using a thermostated Clark-type oxygen electrode (Rank Brothers, Bottisham, U.K.) with air-saturated reagents at 30 °C. Potassium phosphate buffer (2.45 mL, 0.2 M, pH 7) was added to the cell and stirred until equilibrated at 30 °C. Then 0.5 mL of a linoleic acid stock solution was added [16 mM linoleic acid dispersed in 0.05% Tween 20 as described by Surrey (*35*)] and the reaction started by addition of the enzyme (50 μL). The decrease in oxygen concentration was monitored for 5−10 min, and the enzyme activity was calculated from the initial rate of oxygen uptake, assuming an initial oxygen concentration of 203 μM. Activity was expressed in nanomoles of oxygen consumed per second (nkat). Protein concentrations were determined according to the Bradford method (*36*), using BSA as a standard.

Beer Production with Addition of Deuterated (*E*)-2-Nonenal through Mashing. Mashing was performed under nitrogen bubbling. Cork malt flour (deoxygenated in a vacuum freeze-drying apparatus after milling) and deoxygenated mashing water (Millipore water containing 35 mg/L $CaSO_4 \cdot 2H_2O$, 10 mg/L $MgCl_2 \cdot 6\ H_2O$, and 30 mg/L NaCl) were mixed at the ratio of 1 kg for 2.5 L. The temperature program applied was 36 °C for 15 min, to 50 °C at 2.8 °C/min, 50 °C for 30 min, to 63 °C at 1.3 °C/min, 63 °C for 30 min, to 72 °C at 0.6 °C/min, and 72 °C for 30 min. When the temperature reached 63 °C, deuterated (*E*)-2-nonenal was added (1.84 mg/L as calculated for an equivalent 12 °P wort). Before addition, the labeled (*E*)-2-nonenal, synthesized as described by Noël et al. (*7*), was transferred from dichloromethane to absolute ethanol and dissolved in deionized water/ethanol (9:1 v/v). After mashing, the temperature was raised to 80 °C at 0.6 °C/min for filtration on a 2001 mash filter (Meura, Belgium). The wort was diluted to 12 °P with mashing water and boiled with liquid CO_2 hop extract (3.14 g of α-acids/hL) for 90 min. After clarification for 20 min, the hot break was removed and the wort quickly cooled to 12 °C. After another adjustment to 12 °P with mashing water, 0.3 mg/L $ZnCl_2$ was added to the clarified wort. Fermentation was carried out in 3-L EBC tubes with an ale yeast (*Saccharomyces cerevisiae*, pitching rate = 7.5×10^6 cells/mL) at 20 °C for 7 days and at 7 °C for 7 days. Yeast cells were removed from the beer by continuous centrifugation (15000 rpm; Contrifuge 17RS, Heraus Sepatech). Accelerated aging was carried out at 40 °C for 5 days.

Wort Production in the Presence or Absence of Oxygen during Mashing. The protocol described above was used [except that no labeled (*E*)-2-nonenal was added]. Either air or nitrogen was bubbled through the mixture during mashing. In one experiment performed with air bubbling, non-deoxygenated malt flour was used.

Wort Production from Three Pale Malts with Different Lipoxygenase Activities. Mashing was performed without specific bubbling. Non-deoxygenated malt flour and deionized water were mixed at the ratio of 1 kg for 5 L. The following temperature program was applied: 36 °C for 15 min, to 49 °C at 1.3 °C/min, 49 °C for 30 min, to 63 °C at 1.3 °C/min, 63 °C for 15 min, to 76 °C at 2.2 °C/min, and 76 °C for 10 min. Filtration, boiling, and clarification were performed as described above.

RESULTS AND DISCUSSION

Addition of Deuterated (*E*)-2-Nonenal To Mimic the Effect of Lipoxygenase Activity. To mimic the effect of lipoxygenase activity, 1.84 mg/L of deuterated (*E*)-2-nonenal was added to the wort after the 50 °C mashing stage. Addition of such a high level of deuterated (*E*)-2-nonenal was necessary to balance the high sensitivity to degradation of such a spiking compared to real enzymatic production. To avoid the excessive oxidation linked to laboratory-scale experiments, mashing was also carried out under nitrogen bubbling. In this way, similar concentrations of natural (*E*)-2-nonenal (1.6 μg/L) and added deuterated (*E*)-2-nonenal (1.1 μg/L) were measured in the wort after filtration. In both cases, huge amounts were definitely lost in the spent grains (*7*).

Table 1. Labeled (-D) and Unlabeled (-H) (*E*)-2-Nonenal and Nonenal Potential (Micrograms per Liter) in Wort and Beer Samples after Addition of 1.84 mg/L Deuterated (*E*)-2-Nonenal during Mashing[a]

	(*E*)-2-nonenal		nonenal potential	
	-H	-D	-H	-D
wort at the end of the 36 °C mashing stage	26.8		8.0	
wort after 5 min of the 63 °C mashing stage	9.6	6.1		
wort after filtration[b]	1.6	1.1	5.2	7.2
wort before fermentation[c]			2.2	1.1
fresh beer[d]	<0.03	<0.03	1.2	0.5
beer after a 5-day accelerated aging at 40 °C	0.37	0.15		

[a] (*E*)-2-Nonenal concentrations are expressed for an equivalent 12 °P wort. [b] T_{inh} = 42.3 min. [c] T_{inh} = 40.9 min with T_{inh} equal to the inhibition time of the AAPH-induced linoleic acid oxidation assay as described by Liégeois et al. (*16*). Linoleic acid (0.16 mM) was incubated with 2 mM AAPH in 50 mM potassium phosphate buffer, pH 7.4, at 37 °C under air, in the presence of an equivalent 12 °P wort diluted 400 times in the assay. [d] Less than 1 mg/L sulfite was measured by the ASBC procedure (*38*).

However, as expected (*7*), labeled (*E*)-2-nonenal was able, to some extent, to form labeled nonenal potential in the wort: 7.2 μg/L was measured in the filtered wort, compared to 5.2 μg/L unlabeled nonenal potential (**Table 1**). Despite intense losses through steam distillation, hydration, or oxidation in the kettle, 1.1 μg/L deuterated nonenal potential persisted in the clarified wort. The apparent higher stability of unlabeled nonenal potential in the kettle (2.2 μg/L in the pitching wort) is attributable to additional synthesis by chemical autoxidation at this step of the process.

To avoid excessive excretion of sulfites liable to mask alkenals (*37*), a top fermentation yeast was used. During fermentation, free (*E*)-2-nonenal and the nonenal potential were strongly reduced, so that no free fraction was detectable in the fresh beer. On the other hand, traces of labeled and unlabeled nonenal potential were clearly evidenced, as depicted in **Table 1**. This first proof that malt lipoxygenase activity can increase the nonenal potential of fresh beer was further corroborated by the level of free deuterated (*E*)-2-nonenal measured in the aged beer (0.15 μg/L after 5 days at 40 °C). Of course, this value represents only 2% of the total level of precursors in the unboiled wort (7.2 μg/L). Taking into account this percentage, the unlabeled nonenal potential before boiling (5.2 μg/L) should contribute 0.11 μg/L of the (*E*)-2-nonenal released during aging. This suggests that boiling contributed ~70% ([0.37−0.11]/0.37 × 100) of this free (*E*)-2-nonenal, whereas mashing contributed only ~30%. Our findings clearly differ from those of Noël et al. (*7*), who found no trace of labeled (*E*)-2-nonenal after aging. This discrepancy is probably due to the high intrinsic antioxidant potential of our malt, maintained throughout the process thanks to flour deaeration and nitrogen bubbling in the wort [our wort was characterized by a very long inhibition time in the antioxidant assay (*16*), T_{inh} = 42.3 min; usual values for industrial worts are equal to 30−48 min].

Influence of Protection against Oxygen. The same variety of malt was used in a second experiment to assess the impact of flour deaeration and nitrogen bubbling (**Table 2**).

For the productions with deoxygenated malt flour, 24.1 μg/L (*E*)-2-nonenal (expressed for an equivalent 12 °P wort) was measured in the water−flour mixture. Whatever the nature of the gas used for bubbling, the (*E*)-2-nonenal concentration measured at the 36 °C mashing stage was about the same (25.4 and 22.6 μg/L). We conclude that the (*E*)-2-nonenal content present at this stage of mashing derives integrally from malt. During mashing and filtration, the free (*E*)-2-nonenal content decreased dramatically, even in the absence of oxygen (non-oxidative degradation pathway). In the presence of oxygen, the residual (*E*)-2-nonenal content in the filtered wort was only slightly higher (2.7 μg/L as compared to 1.8 μg/L) due to the balance between higher lipoxygenase-catalyzed synthesis and increased oxidative degradation.

Table 2. (*E*)-2-Nonenal and Nonenal Potential (Micrograms per Liter) of Wort Samples in Relation to the Oxygen Level during Mashing[a]

malt flour: mashing under:	O_2-free N_2 bubbling	O_2-free air bubbling	non-O_2-free air bubbling
(*E*)-2-nonenal			
wort at the end of the 36 °C mashing stage	25.4	22.6	6.6
filtered wort	1.8	2.7	
nonenal potential			
wort at the end of the 36 °C mashing stage	9.6	9.7	5.5
filtered wort	5.7[b]	14.8[c]	6.8
boiled wort	2.0	6.7	5.2
clarified wort	2.2	8.1	6.2

[a] (*E*)-2-Nonenal concentrations are expressed for an equivalent 12 °P wort. [b] T_{inh} = 50.5 min. [c] T_{inh} = 41.5 min with T_{inh} equal to the inhibition time of the AAPH-induced linoleic acid oxidation assay as described by Liégeois et al. (*16*). Linoleic acid (0.16 mM) was incubated with 2 mM AAPH in 50 mM potassium phosphate buffer, pH 7.4, at 37 °C under air, in the presence of an equivalent 12 °P wort diluted 400 times in the assay.

The nonenal potential, on the other hand, was much higher with air bubbling than with nitrogen bubbling (14.8 versus 5.7 μg/L). This result, expected on the basis of the experiments of Collin et al. (*14*), is attributable to the higher lipoxygenase efficiency resulting from air bubbling.

During boiling, the synthesis of nonenal potential through autoxidation failed to balance the losses due to steam distillation, hydration, and/or oxidation in the kettle. In the wort produced under oxygen bubbling, the nonenal potential decreased by 55%, from 14.8 to 6.7 μg/L. In the wort produced under nitrogen bubbling, a 65% decrease was observed, from 5.7 to 2.0 μg/L. The higher antioxidant activity of the wort produced under nitrogen bubbling (50.5 min as compared to 41.5 min for the wort produced under oxygen bubbling) may explain this higher decrease.

When non-deoxygenated malt flour was used, we assume that the oxygen contained in the flour degraded much of the (*E*)-2-nonenal to nonenoic acid (*14*) (only 6.6 μg/L remained after 15 min of mashing at 36 °C, as opposed to 22.6 μg/L for the malt produced with deoxygenated malt flour). Due to air bubbling, an intense lipoxygenase activity logically occurred at the 50 °C mashing stage. As depicted in **Table 2**, the nonenal potential detected after filtration remains lower in the case of non-oxygen-free flour (6.8 versus 14.8 μg/L for the oxygen-free flour). However, intensified autoxidation in this less protected medium may explain why the boiling step drastically reduced the difference (6.2 versus 8.1 μg/L).

Contribution of Lipoxygenase Activity. As suggested by the first experiment with labeled (*E*)-2-nonenal, lipoxygenase activity contributes only a small fraction of the precursors. It is therefore not surprising that the nonenal potentials of clarified worts obtained with malts having very different LOX-1 activities

Table 3. (*E*)-2-Nonenal and Nonenal Potential (Micrograms per Liter) in Wort Samples in Relation to the Malt Lipoxygenase Activity[a]

	LOX activity (nkat/mg of proteins)		(*E*)-2-nonenal (μg/L)	nonenal potential (μg/L)	
variety	total LOX	LOX-1	wort at the end of the 45 °C mashing stage	filtered wort	clarified wort
Clipper	0.82	0.62	4.1	6.7	7.8
Maud	1.98	1.32	10.5	9.2	8.3
Cork	2.15	1.58	18.9	10.8	9.6

[a] (*E*)-2-Nonenal concentrations are given for an equivalent 12 °P wort.

differed by only 2 μg/L (**Table 3**). Big differences were measured between worts after the 45 °C mashing stage (18.9 versus 4.1 μg/L), but alkenal instability in the unprotected worts (no nitrogen bubbling) led to a difference of only 4 μg/L after filtration. Although no beer was fermented from these worts, we can assume that the resulting aged beers would have differed by <0.1 μg/L (2% of 4 μg/L). These results confirm those previously obtained by Drost et al. (*5*), who compared four malts with very different total lipoxygenase activities.

Practical Implications for the Brewing Process. Although unstable, deuterated (*E*)-2-nonenal nitrogen adducts created during mashing can in some cases partially persist in the pitching wort, to release deuterated (*E*)-2-nonenal during beer aging. Oxygen appears to have a dual impact, enhancing (*E*)-2-nonenal synthesis during mashing (nitrogen bubbling is therefore advisable) but intensifying (*E*)-2-nonenal degradation to nonenoic acid (deaeration of the flour is not advisable when mashing is performed under unprotected atmosphere). To a lesser extent, low malt lipoxygenase activity emerges as another way to minimize the nonenal potential of the pitching wort.

LITERATURE CITED

(1) Meilgaard, M. C. Stale flavor carbonyls in brewing. *Brewers Dig.* **1972**, *47* (4), 48−57.

(2) Hashimoto, N. Flavor stability of packaged beer. In *Brewing Science*; Pollock, J. R. A., Ed.; Academic Press: San Diego, CA, 1981; pp 347−405.

(3) Dupire, S. Evolution des composés identifiables au cours du vieillissement de la bière: les esters et les produits carbonylés. *Louvain Brewing Lett.* **1990**, *1*, 41−50.

(4) Wang, P. S.; Siebert, K. J. Determination of *trans*-2-nonenal in beer. *MBAA Tech. Q.* **1974**, *11* (2), 110−117.

(5) Drost, B. W.; Van den Berg, R.; Freijee, F. J. M.; van der Velde, E. G.; Hollemans, M. Flavor stability. *J. Am. Soc. Brew. Chem.* **1990**, *48*, 124−131.

(6) Lermusieau, G.; Noël, S.; Liégeois, C.; Collin, S. Nonoxidative mechanism for development of *trans*-2-nonenal in beer. *J. Am. Soc. Brew. Chem.* **1999**, *57*, 29−33.

(7) Noël, S.; Liégeois, C.; Lermusieau, G.; Bodart, E.; Badot, C.; Collin, S. Release of deuterated nonenal during beer aging from labeled precursors synthesized in the boiling kettle. *J. Agric. Food Chem.* **1999**, *47*, 4323−4326.

(8) Gijs, L.; Perpète, P.; Timmermans, A.; Collin, S. 3-Methylthiopropionaldehyde as precursor of dimethyl trisulfide in aged beers. *J. Agric. Food Chem.* **2000**, *48*, 6196−6199.

(9) Chevance, F.; Guyot-Declerck, C.; Dupont, J.; Collin, S. Investigation of the β-damascenone level in fresh and aged commercial beers. *J. Agric. Food Chem.* **2002**, *50*, 3818−3821.

(10) Gijs, L.; Chevance, F.; Jerkovic, V.; Collin, S. How low pH can intensify beer staling through aging. *J. Agric. Food Chem.* **2002**, *50*, 5612−5616.

(11) Meilgaard, M. C. Individual difference in sensory threshold for aroma chemicals added to beer. *Food Qual. Preference* **1993**, *4*, 153−167.

(12) Schieberle, P.; Komarek, D. Changes in key aroma compounds during natural beer aging; *Abstracts of Papers*, 222nd ACS National Meeting, Chicago, IL; American Chemical Society: Washington, DC, 2001.

(13) Noël, S.; Collin, S. *Trans*-2-nonenal degradation products during mashing. *European Brewery Convention*; IRL Press, Oxford University Press: Oxford, U.K., 1995; pp 483−490.

(14) Collin, S.; Noël, S.; Bonte, S.; Metais, N.; Bodart, E.; Peladan, F.; Dupire, S. The use of $^{18}O_2$ in appraising the impact of oxidation processes during mashing and beer storage. *European Brewery Convention*; IRL Press, Oxford University Press: Oxford, U.K., 1997; pp 535−544.

(15) Noël, S.; Metais, N.; Bonte, S.; Bodart, E.; Peladan, F.; Dupire, S.; Collin, S. The use of oxygen 18 in appraising the impact of oxidation process during beer storage. *J. Inst. Brew.* **1999**, *105*, 269−274.

(16) Liégeois, C.; Lermusieau, G.; Collin, S. Measuring antioxidant efficiency of wort, malt and hops against the 2,2′-azobis(2-amidinopropane) dihydrochloride-induced oxidation of an aqueous dispersion of linoleic acid. *J. Agric. Food Chem.* **2000**, *48*, 1129−1134.

(17) Lermusieau, G.; Liégeois, C.; Collin, S. Reducing power of hop cultivars and beer aging. *Food Chem.* **2001**, *72*, 413−418.

(18) Kaneda, H.; Kano, Y.; Osawa, T.; Ramarathnam, N.; Kawakishi, S.; Kamada, K. Detection of free radicals in beer oxidation. *J. Food Sci.* **1988**, *53*, 885−888.

(19) Uchida, M.; Ono, M. Improvement for oxidative flavor stability of beer—Role of OH-radical in beer oxidation. *J. Am. Soc. Brew. Chem.* **1996**, *54*, 198−204.

(20) Uchida, M.; Suga, S.; Ono, M. Improvement for oxidative flavor stability of beer—Rapid prediction method for beer flavor stability by electron spin resonance spectroscopy. *J. Am. Soc. Brew. Chem.* **1996**, *54*, 198−204.

(21) Uchida, M.; Ono, M. Technological approach to improve beer flavor stability: analysis of the effect of brewing processes on beer flavor stability by the electron spin resonance method. *J. Am. Soc. Brew. Chem.* **2000**, *58*, 8−13.

(22) Kaneda, H.; Kobayashi, N.; Furusho, S.; Sahara, H.; Koshino, S. Chemical evaluation of beer flavor stability. *MBAA Tech. Q.* **1995**, *32*, 76−80.

(23) Kaneda, H.; Kobayashi, N.; Furusho, S.; Sahara, H.; Koshino, S. Reducing activity and flavor stability of beer. *MBAA Tech. Q.* **1995**, *32*, 90−94.

(24) Kaneda, H.; Kano, Y.; Kamimura, M.; Osawa, T.; Kawakishi, S. Detection of chemiluminescence produced during beer oxidation. *J. Food Sci.* **1990**, *55*, 881−882.

(25) Kaneda, H.; Kano, Y.; Kamimura, M.; Osawa, T.; Kawakishi, S. Evaluation of beer deterioration by chemiluminescence. *J. Food Sci.* **1990**, *55*, 1361−1364.

(26) Kaneda, H.; Kobayashi, N.; Tsuchiya, Y.; Munekata, M.; Koshino, S. Some applications of chemiluminescence analysis to brewing. *J. Am. Soc. Brew. Chem.* **1994**, *52*, 163−168.

(27) De Buck, A.; Aerts, G.; Bonte, S.; Dupire, S.; Van den Eynde, E. Rapid evaluation between lipoxygenase extraction during brewing, reducing capacity of the wort and the organoleptical stability of beer. *European Brewery Convention*; IRL Press, Oxford University Press: Oxford, U.K., 1997; pp 333−340.

(28) Yabuuchi, S. Occurrence of a new lipoxygenase isoenzyme in germinating barley embryos. *Agric. Biol. Chem.* **1976**, *40*, 1987−1999.

(29) Kobayashi, N.; Kaneda, H.; Kano, Y.; Koshino, S. Behavior of lipid hydroperoxides during mashing. *J. Am. Soc. Brew. Chem.* **1994**, *52*, 141−145.

(30) Yang, G.; Schwarz, P. B.; Vick, B. A. Purification and characterization of lipoxygenase isoenzymes in germinating barley. *Cereal Chem.* **1993**, *70*, 589−595.

(31) Yang, G.; Schwarz, P. B. Activity of lipoxygenase isozymes during malting and mashing. *J. Am. Soc. Brew. Chem.* **1995**, *53*, 45−49.

(32) Walker, M. D.; Hughes, P. S.; Simpson, W. J. Use of chemiluminescence HPLC for measurement of positional isomers of hydroperoxy fatty acids in malting and the protein rest stage of mashing. *J. Sci. Food Agric.* **1996**, *70*, 341−346.
(33) Visser, M. K.; Lindsay, R. C. Ester precursors of aldehydes associated with staling of beer. *MBAA Tech. Q.* **1971**, *8* (3), 123−128.
(34) Tressl, R.; Bahri, D.; Silwar, R. Formation of aldehydes by oxidation of lipids and their importance as "off-flavour" components in beer. *European Brewery Convention*; IRL Press, Oxford University Press: Oxford, U.K., 1979; pp 27−41.
(35) Surrey, K. Spectrophotometric method for determination of lipoxidase activity. *Plant Physiol.* **1964**, *39*, 65−70.
(36) Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(37) Nyborg, M.; Outtrup, H.; Dreyer, T. Investigations of the protective mechanism of sulfite against beer staling and formation of adducts with *trans*-2-nonenal. *J. Am. Soc. Brew. Chem.* **1999**, *57*, 24−28.
(38) ASBC procedure. Beer-21: Total sulfur dioxide. Issued 1992.

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