Research article

Institute of Brewing & Distilling

Received: 25 September 2013 Revised: 18 December 2013 Accepted: 18 December 2013 Published online in Wiley Online Library: 28 January 2014

(wileyonlinelibrary.com) DOI 10.1002/jib.122

The influence of LOX-less barley malt on the flavour stability of wort and beer

Junhong Yu,* Shuxia Huang, Jianjun Dong, Wei Fan, Shuli Huang, Jia Liu, Zongming Chang, Yuhong Tian, Junguang Hao and Shumin Hu

The aim of this study was to investigate the influence of lipoxygenase-less (LOX-less) barley malt on the quality of wort and beer, with the main focus on beer flavour stability. In the current study, pilot-scale (1000 L) brewing trials were conducted with a control barley malt AC Metcalfe and a LOX-less barley malt, PolarStar. The results clearly indicated that the LOX-less barley malt showed less nonenal potential than the control, although LOX activities in both barley malts were relatively low. The beer brewed from the LOX-less barley malt contained much lower concentrations of *trans*-2-nonenal (T2N) and gamma-nonalactone, especially after the (forced or natural) aging of the beer, compared with the beer brewed under the same conditions using the control malt. The sensory panel evaluation indicated similar results in the general flavour profile. The freshness scores of beer brewed from the LOX-less malt were higher than those from the control malt, and this was more pronounced after forced aging. In addition, the beer brewed from LOX-less malt had a much better foam stability, almost 30 s (NIBEM test). These results confirm that the use of the LOX-less barley malt was beneficial to beer flavour stability and foam stability.

Keywords: lipoxygenase-less (LOX-less); flavour stability; nonenal potential (NP); *trans*-2-nonenal (T2N); freshness score

Introduction

Although numerous research studies have been made on flavour stability over the past few years, it is still one of the most difficult and important topics in the brewing industry. For pale lager beers, cardboard flavour (*trans*-2-nonenal) has received particular attention owing to its low flavour threshold (0.1 ppb). Furthermore, cardboard flavour in beer increases quickly during storage, even before the suggested expiry date, which significantly decreases the real shelf-life of the beer, potentially negatively impacting the beer brand image. It is well known that the typical cardboard stale flavour comes from *trans*-2-nonenal (T2N), which is the main degradation and oxidation product of polyunsaturated fatty acids by malt lipoxygenase (LOX) during the malting and mashing process *(1)*. Lipoxygenases (EC 1.13.11.12) are a group of enzymes that catalyse the oxygenation of unsaturated fatty acids containing a *cis*, *cis*-1,4-pentadiene system to form conjugated hydroperoxides. Finally, hydroperoxides are degraded to T2N, the cardboard off-flavour in stale beer. Fickert and Schieberle *(2)* suggested that lipid oxidation and Strecker degradation are two key flavour-generating reactions during the malting process. Gamma-nonalactone is another primary product of lipid degradation, in addition to T2N. Suzuki *et al*. *(3)* identified that gamma-nonalactone aggravated the degree of staling flavour when combined with T2N. Van Waesberghe *(4)* proved that all of the aldehydes found in beer as aging indicators were already present in the malt. T2N exists in the form of an adduct by combining with carbonyl from amino acids and the proteins in malt and wort, which protect the T2N from yeast reduction, but T2N can also be released by acidic hydrolysis *(5)*.

Malting and mashing parameters have been optimized to reduce the malt LOX activity and the T2N levels in the finished beer, thus decreasing the production of cardboard flavour. The LOX activity of malt under a low germination temperature was found to be lower than that under a high germination temperature *(6)*. Owing to the relatively low thermostability of LOX, kilning parameters can severely affect LOX activity; for example, increasing the kilning temperature and extending the kilning time was found to reduce LOX activity *(7)*. In addition, malt nonenal potential (NP) had a significant correlation with the T2N level in stale beer, and was considered as a good predictor of determining the levels of T2N released during storage, which had a great influence on the sensory score *(8–10)*. Thus, lowering the malt LOX activity and NP content was an effective approach to improving beer flavour stability. It has also been reported that increasing the boiling evaporation rate and decreasing the pH of the wort was of benefit for decreasing the NP value of wort *(11,12)*. Kageyama *(13)* decreased stale flavour compounds and improved the freshness score of a storage beer by using the malt that was polished or treated with sub-critical H_2O.

Although cardboard flavour could be partly reduced by performing some process optimization, malt LOX activity has not been eliminated from the barley. If LOX could be eliminated from the source, for example, breeding a new barley variety with less LOX, it could be used to produce more stable and fresh beer for maltsters and brewers. The process would be easier to control in malting and brewing, and the final beer would be fresher, as well as retaining a longer shelf-life, which would benefit

* Correspondence to: J. Yu, State Key Laboratory of Biological Fermentation Engineering of Beer, Tsingtao Brewery Co., Ltd, Qingdao, China. E-mail: yujh@tsingtao.com.cn

State Key Laboratory of Biological Fermentation Engineering of Beer, Tsingtao Brewery Co., Ltd, Qingdao, China

the consumer. Fortunately, this concept is now approaching commercial adoption with the development of LOX-less malting barley varieties. For example, researchers *(14–16)* have shown that beer brewed with malt produced from a LOX-less barley line has a lower T2N level and better foam stability. In 2001, Carlsberg collaborated with Heineken to breed a new barley variety that lacked the LOX-1 gene, followed by another barley line in 2010 lacking both the LOX-1 and the LOX-2 genes *(15,16)*. The LOX-less barley named PolarStar is a barley germplasm with a completely deleted LOX-1 protein, which was developed by a joint breeding programme between Sapporo Breweries, Prairie Malt Limited and the University of Saskatchewan. The aim of the work in this paper was to investigate various malt quality parameters of PolarStar barley malt, as well as the malt's influence on the wort and beer quality, particularly flavour and foam stability, using pilot brewing trials.

Materials and methods

Barley malt samples

The LOX-less barley malt used for the pilot brewing trial was PolarStar, provided by Cargill Malt and Prairie Malt Limited. The AC Metcalfe barley malt was malted by Dalian Cofco Malt Co., Ltd (China National Cereals, Oils and Foodstuffs Corporation) and was used as a control. The PolarStar and AC Metcalfe barley were both grown in Saskatchewan, Canada.

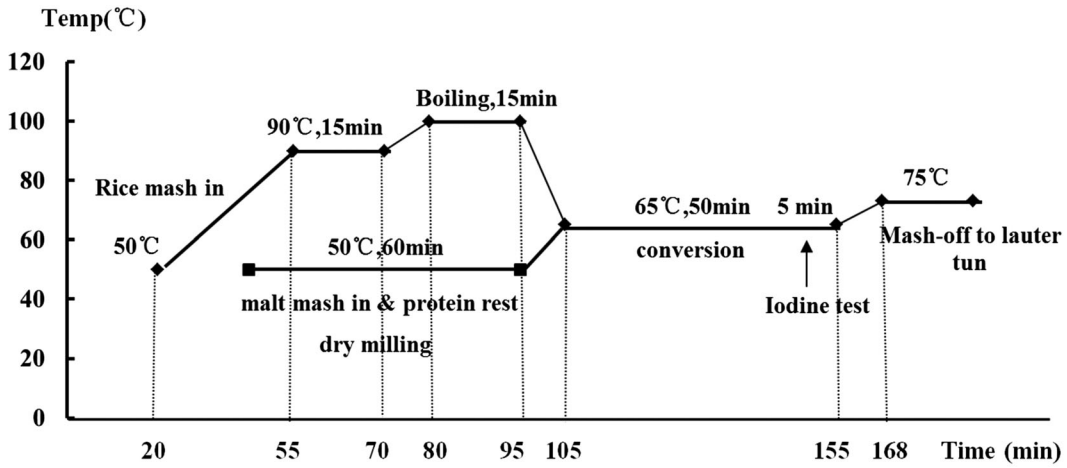

Figure 1. The mashing procedure for the pilot brew.

Table 1. Comparison of regular malt parameters, lipoxygenase (LOX) activity and nonenal potential

Sample	Moisture (%)	Extract fine (% d.b.)	α-N (mg/100 g)	DP (WK)	Colour (EBC)	Viscosity (mPa·s)	β-Glucan (mg/100 g)	Total nitrogen (g/100 g)	Kolbach index (%)	LOX activity (U/g)	Nonenal potential (μg/L)
PolarStar	4.4	81.0	139.9	315.8	4.2	1.49	160.8	1.69	42.8	2.44	45.8
AC Metcalfe	4.3	83.1	166.3	267.2	5.0	1.43	26.0	1.70	45.1	6.40	63.1

Table 2. Sensory evaluation of malt samples of PolarStar and AC-Metcalfe

Sample	Sensory score[a]	Ranking	Malty[b]	Acetaldehyde	Astringent	Fermented	Earthy	Grainy
PolarStar	2.83	3	2	6	1	0	1	0
AC Metcalfe	2.33	1	5	1	2	0	0	2
PolarStar (90°C,3 h)	2.92	4	3	0	1	3	0	0
PolarStar: AC Metcalfe 1:1	2.50	2	4	1	1	0	0	0

[a]The average score from each trained panellist's score; the lower the value, the better the malt quality. If the sensory score was $<$3.0, then the malt sample was regarded as acceptable.
[b]Other flavour data were the sum of numbers of trained panellists who identified the flavour feature.

Pilot brewing trials

Pilot brewing trials were carried out on a 1000 L scale at the Tsingtao Brewery Co., Ltd in China. The original wort concentration was 10°P. The proportion of malt and rice was 65:35. Hop pellets were added in three aliquots. Filtered beers were bottled followed by hand crown sealing and brewery pasteurization. The entire brewing process of trial and control were monitored as much as possible for consistency. The mashing procedure for the pilot brew is shown in Fig. 1.

Determination of the malt LOX activity

LOX activity was determined based on the improved method described by Li and Schwarz *(17)* and Yang and Schwarz *(18)*. A 5 g aliquot of finely ground malt was added to 5 mL of acetate buffer (pH 5.0), and incubated in ice water for 15 min, and then centrifuged at 10,000 rpm for 10 min. The supernatant was used as a crude enzyme extract. The linoleic acid substrate solution was prepared by the addition of 250 μL Tween 20, 250 μL linoleic acid and 650 μL of 1 M NaOH solution into 5 mL of 0.5 M borate buffer (pH 9.0). The solution was stored at 4°C and used within 1 week. The reaction components were incubated at 25°C in a water bath, containing 50 μL linoleic acid substrate solution, 50 μL crude enzyme extract and 2.9 mL phosphate buffer (pH 6.8). The optical density was measured by UV spectrophotometer at 234 nm. A LOX activity unit (U/g) was defined as the rate of optical density change per minute.

Determination of *trans*-2-nonenal

The T2N was determined based on the method of Vesely *et al. (19)*. T2N content was analysed using a GC-MS (PerkinElmer Clarus600) equipped with an Headspace Solid-phase Microextraction (HS-SPME) autosampler and a 65 μm Polydimethylsiloxane-Divinylbenzene (PDMS-DVB) fibre. Pentafluorobenzylhydroxylamine acted as the derivation agent. A 0.5 mL wort aliquot was transferred to a sample bottle, and 4.5 mL deionized water (which had been boiled for 30 min and cooled) was added. Internal standard (50 μL fluorobenzaldehyde) and NaCl (2 g) were added.

Determination of malt nonenal potential

Nonenal potential was determined according to the method of Drost *et al. (1)*. Pretreatment techniques were as follows. The wort was prepared using the AC Metcalfe or PolarStar malt, according to the Tsingtao mashing procedure (Fig. 1). The pH of wort was adjusted to 4.0 with phosphoric acid. After being purged with argon gas to reduce the oxygen level, 50 μL of internal standard and 2 g NaCl were added, and then the bottle was crown sealed. The sample was heated at 100°C in water bath for 2 h, and cooled for 1 h. The T2N content of the samples was analysed according to the T2N determination method. Finally, the NP was calculated from the corresponding T2N content.

Determination of gamma-nonalactone of beer

Gamma-nonalactone was determined based on the method of Suzuki *et al. (3)*. It was analysed using a GC-MS (PerkinElmer Clarus600) equipped with an HS-SPME autosampler and a 65 μm DVB/Carboxen/PDMS fibre. Fluorobenzaldehyde acted as the internal standard.

Table 3. Comparison of stale flavour compounds and their potential in the filtered wort

Sample	Stale flavour	2-Methyl propanal (μg/L)	2-Methyl butanal (μg/L)	3-Methyl butanal (μg/L)	Pentanal (μg/L)	Hexanal (μg/L)	Furfural (μg/L)	Methional (μg/L)	Phenyl acetaldehyde (μg/L)	*Trans*-2-nonenal (μg/L)
PolarStar	compound	564.4	206.6	562.9	104.7	211.8	2391.6	723.6	426.9	3.6
AC Metcalfe	compound	413.6	211.3	765.5	167.9	241.1	1996.4	910.7	845.2	9.3
PolarStar	potential	104.6	69.2	86.3	20.5	32.8	51.6	56.3	87.4	1.3
AC Metcalfe	potential	146.5	140.3	134.1	36.4	47.4	85.0	114.4	154.2	1.6

Sensory evaluation for freshness

Sensory evaluation for freshness of the beer was conducted by nine trained sensory panellists from the Tsingtao Brewery Co., Ltd. The beer samples were kept in an incubator at 35°C for 7 days and for 1 day at 4°C, and then tasted at 15°C. The freshness score was ranked on a scale of 0–10. In general, the higher the freshness score, the fresher the beer.

Natural and forced aging test

The packaged beers were stored for 2 months at room temperature (about 20–25°C) for the natural aging test. Packaged beers were stored for one week at 35°C for the forced aging test.

Determination of high molecular weight proteins

The high molecular weight proteins of wort or beer were determined based on the Bradford Coomassie blue binding method *(20)*. The wort was filtered with filter paper and the beer was degassed before use. Bovine serum albumin solution (0.1 mg/mL) was used for the protein for the standard curve.

Results and discussion

Analysis of malt quality

The PolarStar and the AC Metcalfe barley malts were analysed for the various malt quality parameters as shown in Table 1. The Kolbach index of the PolarStar malt was slightly lower than that of the AC Metcalfe (43 vs 45), although total nitrogen was similar (1.69–1.70 g/100 g). The corresponding Free Amino Nitrogen (FAN) was lower for the PolarStar malt (139.9 vs 166.3 mg/100 g). The Diastatic Power (DP) of the PolarStar was higher than that of the control (315.8 vs 267.2 WK). Furthermore, in terms of β-glucan content, there was a significant difference between the PolarStar (160.8 mg/100 g) and the AC Metcalf (26.0 mg/100 g). To ensure an efficient mashing and lautering processes, most of Chinese brewers request that the β-glucan content in malt should be $<$100 mg/100 g. Thus, β-glucanase (Ultraflo XL) from Novozymes was added during the mashing process to avoid filtration problems.

The malt LOX activities are affected by the barley variety and the malting recipe. As shown in Table 1, the LOX activities in both malts were relatively low, lower than 10 U/g. The PolarStar malt had a slightly lower nonenal potential level than that of the AC Metcalfe.

Malt flavour was evaluated using six trained panellists to taste the corresponding Congress worts (Table 2). The results shown in Table 3 are the averaged data. The sensory score of the AC Metcalfe malt sample was better than that of the PolarStar. All six trained panellists identified an 'acetaldehyde' flavour from the PolarStar, while only one panellist identified the same flavour from the AC Metcalfe. Two panellists identified a 'malty' flavour from the PolarStar, while five panellists identified it from the AC Metcalfe. Thus, the 'acetaldehyde' flavour of the PolarStar was heavier than that of AC Metcalfe. The 'malty' of the PolarStar was not as good as that of the AC Metcalfe. These results were in part owing to differences in the kilning intensity. In terms of the last curing cycle, the PolarStar malt was kilned at 84°C for 2.5 h, while the AC Metcalfe was kilned at 85°C for 3 h. To confirm that the issue was kilning intensity, the PolarStar malt was heat-treated at 90°C for an additional 3 h and then was evaluated by the same sensory panel using the same methodology. They found that the 'acetaldehyde' flavour of the PolarStar had disappeared, and the 'malty' flavour increased slightly; however, a 'fermented' flavour had appeared. Acetaldehyde, which is an immature beer flavour, had possibly been eliminated by the high heat-treatment. The increase in 'malty' flavour was probably due to the formation of Maillard reaction products, by the additional heating at 90°C for 3 h. Those compounds not only give a malty flavour, but also have antioxidant properties, which may have inhibited some lipid oxidation.

Another method to improve malt flavour is the use of blending malts. Generally, two or three malt varieties are blended by Chinese brewers. Thus, the PolarStar malt was mixed with the AC Metcalfe at a proportion of 1:1. The results of the sensory score of the mixed malt sample were better than that of single PolarStar, with 'malty' being increased and 'acetaldehyde' being less distinct.

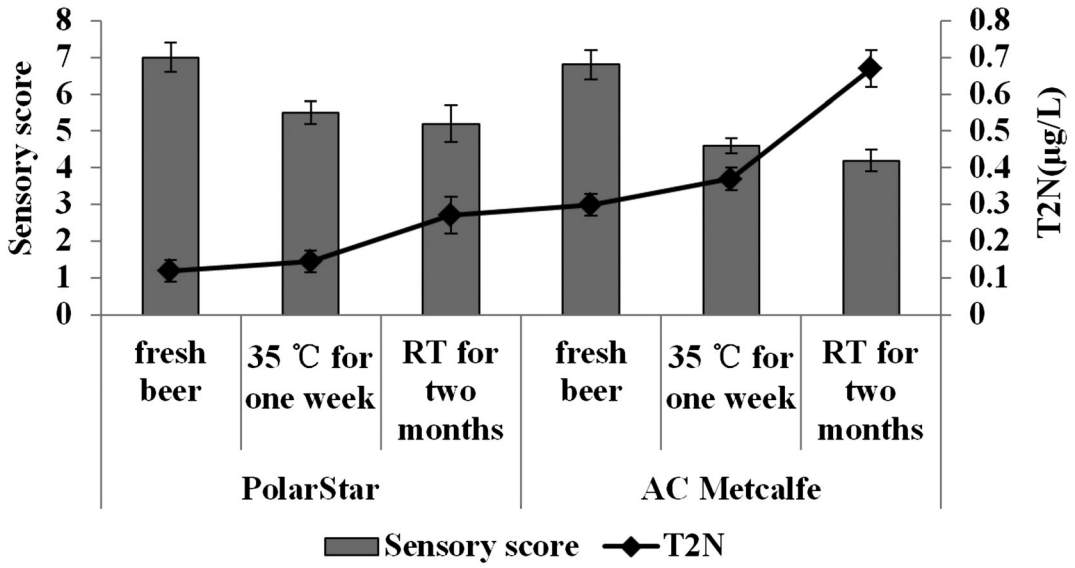

Figure 2. Comparison of sensory score and *trans*-2-nonenal content of beer.

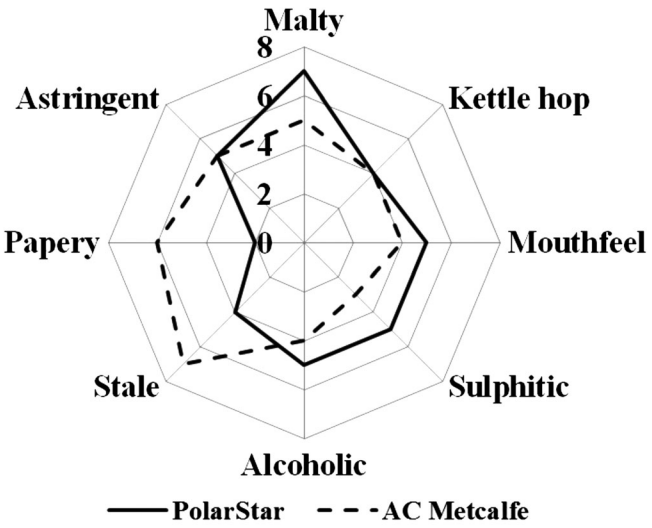

Figure 3. Comparison of the sensory profiles in the fresh beer.

Analysis of wort

As mentioned above, 30 mL/10 hL mash of β-glucanase (Ultraflo XL) from Novozymes was added to PolarStar malt mash to avoid filtration problems, owing to the higher malt β-glucan content. The control mash did not receive this additional exogenous β-glucanase treatment, owing to the low β-glucan in that malt. Other mashing conditions were kept identical. There were no significant differences between the two worts for some of the regular wort parameters. Almost all of the stale flavour compounds, except 2-methyl propanal, in the trial wort were lower than those of the control. Similar results were obtained for the stale flavour compounds potential (Table 3). The data demonstrated that the worts brewed from PolarStar malt not only had less stale flavour compounds, but also a lower stale flavour potential.

Flavour stability of beer

Trans-2-nonenal formation was studied in fresh and stored beer (including forced and naturally aged beer). It was found that the beer brewed from the PolarStar malt contained much lower concentrations of T2N in the fresh stage compared with those brewed under the same conditions from the control malt, AC Metcalfe. After the forced (35°C, a week) and natural aging (room temperature, 2 months), the T2N content of both beers increased quickly. However, the control beer increased more rapidly (Fig. 2). The results demonstrated that the beer brewed with PolarStar malt had much lower levels of T2N in the forced and naturally aged beer. Gamma-nonalactone has been reported to be another stale flavour substance in beer strengthening stale flavour perception, especially in the presence of T2N *(3)*. The results suggested that the gamma-nonalactone level in the beer brewed from the PolarStar (25.03 μg/L) was much lower than that from the control (64.86 μg/L). Other stale flavour compounds were also analysed (data not shown) and the results were similar.

The specially trained sensory panel indicated similar results for general flavour profile of beer. The freshness scores of the trial beer were higher than those of control beer, especially beer that was forced aged. The higher score represented beers considered to be much fresher. In addition, detailed flavour descriptions of

Table 4. Comparison of foam stability and other standard parameters

Sample	High Molecular Weight proteins content (mg/L)	Foam stability (S)	Sensitive protein (EBC)	Sensitive polyphenols (mg/L PVP)	Alcohol (% v/v)	pH	Real degree of fermentation (%)
PolarStar	163.29	283	4.2	10.7	4.20	4.69	67.8
AC Metcalfe	129.61	258	2.4	10.6	3.59	4.84	67.2

the beers are recorded in Fig. 3. Some beer flavour evaluations such as malty and mouthfeel were more positive in the trial beers than in the control beers. Negative evaluations such as stale and papery were found less often in the trial beer.

Foam stability of beer

The trial beer was found to have a much better foam stability by almost 30 s (NIBEM test) (Table 4), which could in part be attributed to a higher content of high molecular weight proteins in the trial beer. Another likely reason was that the trial malt, with lower LOX activity, produced less trihydroxy-octadecenoic acid (THOD), which is known to be detrimental to foam stability *(12)*.

Colloidal stability of beer

Besides beer flavour stability, colloidal stability is another important parameter used to judge beer quality. The trial beer had a slightly higher content of sensitive protein and sensitive polyphenols than the control; however, both were low values (Table 4).

Other beer quality parameters

Additional parameters in the beers were analysed such as alcohol, pH and real degree of fermentation, but no large differences between the two were observed (Table 4).

Conclusions

A LOX-less barley variety named PolarStar was investigated in a 1000 L pilot-scale brewing trial for its influence on beer flavour stability and other beer quality aspects. The results indicated that the use of a LOX-less barley malt was beneficial to beer flavour stability and foam stability. To further evaluate the benefits of the PolarStar barley malt for brewing, industrial-scale trials must be performed in the future.

Acknowledgements

The authors wish to thank Cargill Malt and Prairie Malt Limited for providing samples of PolarStar barley malt, and especially thank Dr Xiangsheng Yin for support. This research was supported by a grant from the National Basic Research Program of China (973 Program, grant no. 2012CB723707) and another grant from the National High Technology Research and Development Program of China (863 Program, grant no. 2013AA102109).

References

1. Drost, B.W., van den Berg, R., Freijee, F.J.M., van der Velde, E.G., and Hollemans, M. (1990) Flavor stability, *J. Am. Soc. Brew. Chem. 48*, 124–131.
2. Fickert, B. and Schieberle, P. (1998) Identification of the key odorants in barley malt using GC/MS techniques and odour dilution analyses, *Food Nahrung 42*(6), 371–375.
3. Suzuki, M., Wanikawa A., Kono, K., and Shibata, K. (2006) Factors affecting the formation of gamma-nonalactone and its contribution to the flavor and aroma of aging beer, in *Institute of Brewing and Distilling Meeting – Asia Pacific Section*. Available from: www.ibdasiapac.com.au/asia-pacific…/Suzuki%20Miho%20Paper.pdf
4. Van Waesberghe, J.W.M. (2002) Flavour stability starts with malt and in the brewhouse: a survey of research results, *Brau. Int. 20*, 375–378.
5. Lermusieau, G., Noel, S., Liegeois, C., and Collin, S. (1999) Non-oxidative mechanism for development of *trans*-2-nonenal in beer, *J. Am. Soc. Brew. Chem. 57*, 29–33.
6. Yang, G. and Schwarz, P.B. (1995) Activity of lipoxygenase isoenzymes during malting and mashing, *J. Am. Soc. Brew. Chem. 53*(2), 45–49.
7. Dumoulin, M. and Boivin, P. (2001) Industrial kilning technologies and their influence on organoleptic quality of malt, in *Proc. Eur. Brew. Conv. Congr., Budapest*, pp. 200–209, Fachverlag Hans Carl, Nürnberg.
8. Ueda, T., Sasaki, K., Inomoto, K., Kono, K., Kagami, N., Shibata, K., and Eto, M. (2001) Development of novel malt evaluation method for improving beer flavor stability, in *Proc. Eur. Brew. Conv. Congr., Budapest*, pp. 885–889, Fachverlag Hans Carl: Nürnberg.
9. Liegeois, C., Meurens, N., Badot, C., and Collin, S. (2002) Release of deuterated (*E*)-2-nonenal during beer aging from labeled precursors synthesized before boiling, *J. Agric. Food Chem. 50*(26), 7634–7638.
10. De Buck, A., Aerts, G., Bonte, S., Dupire, S., and Van den Eynde, E. (1997) Rapid evaluation between lipoxygenase extraction during brewing, reducing capacity of the wort and the organoleptical stability of wort, in *Proc. Eur. Brew. Conv. Congr., Maastricht*, pp. 333–340, IRL Press, Oxford.
11. Yano, M., Morikawa, M., Yasui, T., Ogawa, Y., and Ohkochi, M. (2004) Influence of wort boiling and wort clarification conditions on cardboard flavor in beer, *Tech. Q. Master Brew. Assoc. Am. 41*, 317–320.
12. Kuroda, H., Hirota, N., Kaneda, H., Kobayashi, N., Takeda, K., Ito, K., and Takashio, M. (2004) Lipid oxidation during mashing and its impact on beer quality, in *World Brewing Congress 2004*.
13. Kageyama, N. (2008) Newest, breakthrough technologies on malt processing for improvement of beer quality, in *World Brewing Congress 2008*.
14. Hirota, N., Kuroda, H., Takoi, K., Kaneko, T., Kaneda, H., Yoshida, I., Takashio, M., Ito, K., and Takeda, K. (2006) Development of novel barley with improved beer foam and flavor stability – the impact of lipoxygenase-1-less barley in brewing industry, *Tech. Q. Master Brew. Assoc. Am. 43*, 131–135.
15. Douma, A.C., Doderer, A., Cameron-Mills, V., Skadhage, B., Bech, L.M., Schmitt, N., Heistek, J.C. and Van Mechelen, J.R. (2002) Low Lipoxygenase 1 barley, patent no. *WO2002053721 A1*.
16. Breddam, K., Olsen, O., Skadhauge, B., Lok, F., Knudsen, S., and Bech, L. (2005) Barley for production of flavor-stable beverage, patent no. *WO2005087934 A3*.
17. Li, Y. and Schwarz, P.B. (2012) Use of a ferrous oxidation-xylenol orange (FOX) assay to determine lipoxygenase activity in barley and malt, *J. Am. Soc. Brew. Chem. 70*(4), 287–289.
18. Yang, G. and Schwarz, P.B. (1995) Activity of lipoxygenase isoenzymes during malting and mashing, *J. Am. Soc. Brew. Chem. 53*(2), 45–49.
19. Vesely, P., Lusk, L.T., Basarova, G., Seabrooks, J.R., and Ryder, D.S., (2003) Analysis of aldehydes in beer using solid-phase microextraction with on-fiber derivatization and gas chromatography/mass spectrometry, *J. Agric. Food Chem. 51*, 6941–6944.
20. Walker, J.M. (ed.) (2009) The Bradford method for protein quantitation, in *The Protein Protocols Handbook*, 3rd edn, Springer: New York.