The Effect of Mash-In Temperature on the Characteristics and Flavor Stability of Pilsner-Type Beer

タンパク質休止温度（マッシュイン温度）はビールの香りと味・酸化耐久性にどう影響するのか？

Abstract in Japanese

【内容】35℃, 50℃, 65℃のマッシュイン温度でビールを醸造し, その香味プロファイルと安定性を検討した。
● 35℃と 50℃でマッシュインを行ったビールは,「コクのある味わい」と「エステル香が高い」という官能評価結果を示し, エステルやアミノ酸の濃度が高くなった。さらに麦汁のエキス収得率(%)が高くなった。デンプン粒子は麦芽の組織中でタンパク質, β グルカンに囲まれて存在する。それらの分解に最適な温度である 35℃と 50℃でマッシュインした麦汁では, デンプン粒子の切出しと遊離が良くなる。また, これらの温度帯では限界デキストリナーゼも働きやすい。そのためエキス収得率が高くなったと考えられる。
● 一方で, 65℃のマッシュインを行ったビールは, 官能評価にて,「味が軽快」「キレが良い」そして「トーストの香り」がエステルの香りよりも優勢であった。65℃でマッシュインを行ったために, 高分子タンパク質が分解されずに残存し, その高分子物質はロイター濾過工程で除去され, さらに煮沸工程にて凝集沈殿される。そのため最終的にビール中に, 味に寄与する成分が残存しなかったと考えられる。
●「タンパク質休止工程（アインマイシェ）は必要か？」という点では, アミノ態窒素含有量から考えると, 酵母に栄養を与えるという観点では, 麦芽 100%のビールの仕込みであれば不要と思われた。
● ビールを 30℃で 1 ヶ月間保存すると, マッシュイン温度が 35℃と 50℃のビールではカードボード臭が優勢となり, 一方でマッシュイン温度が 65℃のビールでは, カードボード臭（(E)-2-ノネナール）の生成が低くなったために,「蜂蜜とシェリー」の香りが優勢になった。ピルスナービールの劣化臭に寄与する 14 成分の推移を測定したところ, (E)-2-ノネナール濃度のみがマッシュイン温度と相関し, それ以外の物質では, ストレッカーアルデヒドを含めても, マッシュイン温度と相関がなかった。

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The Effect of Mash-In Temperature on the Characteristics and Flavor Stability of Pilsner-Type Beer

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ABSTRACT

Beers were brewed at mash-in temperatures of 35 °C, 50 °C, and 65 °C, and their flavor profiles and stability were examined. Beers with mash-ins at 35 °C and 50 °C exhibited full-bodied taste and estery sensory qualities with higher concentrations of esters and amino acids. Higher extract yields were obtained in wort with mash-ins at 35 °C and 50 °C, the optimal temperatures for enzymes to break down the tissue where starch granules are located. In contrast, the beer with a 65 °C mash-in temperature had a light taste and a toast-like aroma. In terms of providing nutrition for the yeast, the proteolytic process to produce amino acids seemed unnecessary for brewing beer made exclusively from malt. Following a month of storage at 30 °C, cardboard flavors predominated in beers with mash-in temperatures of 35 °C and 50 °C, while honey and sherry-like scents predominated in beers with a mash-in temperature of 65 °C. The concentrations of the 14 components contributing to the stale flavor of Pilsner-type beers, including Strecker aldehydes, did not correlate with mash-in temperature, with the exception of the concentration of (*E*)-2-nonenal, even though the mash-in temperatures were predicted to affect the formation of Strecker aldehydes during beer storage.

KEYWORDS

Mash-in temperature; protease; Strecker aldehyde; (*E*)-2-nonenal; extract yield

Introduction

The mashing process of beer production consists of proteolytic, saccharification, wort filtration, and boiling processes. If the specifications of these processes are inappropriate, yeast nutrition, extract yield, and foam stability are negatively affected.[1]

The temperature of mash-in, the start of the mashing process, is defined by the optimum temperature of the enzymes that should be activated first and can range depending on the brewery (e.g., from about 35 °C to about 65 °C).[1] Even though different enzymes function at different temperatures, the mash-in temperature is generally set for proteolysis and maintained to produce amino acids. Variations in the mash-in temperature can affect the flavor of the beer. Proteolytic enzymes such as endopeptidase, carboxypeptidase, aminopeptidase, and dipeptidase function at optimum temperatures of 45 °C–50 °C.[1] Supplementation of the yeast catabolic Ehrlich pathway with amino acids has been demonstrated to increase the concentrations of higher alcohols and esters in beer.[2–10] The amounts of sugars influence the changes in the aromatic profile of the final beer.[2]

The flavor stability is also impacted by the differing mash-in temperatures. According to Dalgliesh's assessment of sensory changes,[11] the cardboard flavor and sweet and toffee-like aroma increase with an increase in the aging period. It was reported that barley lipoxygenase, the cardboard-flavored (*E*)-2-nonenal producing enzyme, which has an optimum temperature of 47 °C, is stable up to 50 °C but becomes completely inactivated at 65 °C.[12] The formation of Strecker aldehydes in the presence of amino acids has also been observed.[13] Lehnhardt et al. conducted brewing using three proteolytic malt modifications and reported that beers with higher amounts of soluble nitrogen resulted in flavor instability.[14] Vanderhaegen et al. summarized 15 volatile compounds formed during beer storage[15,16], including aldehydes, ketones, cyclic acetals, heterocyclic compounds, ethyl esters, lactones, and sulfur compounds. Saison et al. investigated the effect of 26 staling compounds on the aged flavor of a Belgian lager beer and concluded that (*E*)-2-nonenal, methional, 3-methylbutanal, 2-furfuryl ethyl ether, β-damascenone, and acetaldehyde were the key contributors to the aged flavor.[17] In a previous study, the contributors to the stale flavor in aged Pilsner-type beer were examined through sensory analysis using construction and omission testing of 40 aroma compounds.[18] It was concluded that at least nine volatiles, (*E*)-2-nonenal, γ-nonalactone, dimethyl trisulfide, methional, (*E*)-β-damascenone, ethyl 2-methylpropionate, ethyl 2-methylbutyrate, sotolon, and 3-methyl-2-butene-1-thiol were necessary for the construction of the odor, and Strecker aldehydes including 2-methylbutanal, 3-methylbutanal, benzaldehyde, and 2-phenylacetaldehyde were also important contributors.

In this study, the effects of the mash-in temperature on the characteristics and flavor stability of Pilsner-type beer were examined. Beers with different mash-in temperatures

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of 35 °C, 50 °C, and 65 °C were prepared. The fresh beers were sensorially evaluated and then analyzed for components that might have contributed to the characteristics. The beers were then stored at 30 °C for a month, and the components that may contribute to the stale flavor of beer were analyzed. [18]

Experimental

Materials for brewing

A Canadian Pilsner-type malt with a moisture content of 4.3%, a Kolbach index (KI) of 39%, and a diastatic power of 333°WK was purchased from Rahr Malting Co. (MN, U.S.A.). Bottom-fermenting sludge yeast was purchased from Chuetsu Yeast Co., Ltd. (Niigata, Japan).

Reagents

Sotolon-$^{13}C_2$, ethyl 2-methyl-d_3-butyrate, (*E*)-2-nonenal-d_2, 3-(methylthio)propionaldehyde-d_3, benzaldehyde-d_5, phenyl-d_5-acetaldehyde, 3-sulfanyl-1-hexanol-d_5, and dimethyltrisulfide-d_6 were purchased from AromaLAB AG (Freising, Germany). (*E*)-β-damascenone, (*E*)-β – damascone, sotolon, ethyl 2-methylbutyrate, (*E*)-2-nonenal, and methional were obtained from Merck KGaA (Darmstadt, Germany). The 3-methyl-2-butene-1-thiol was purchased from BOC Sciences (NY, U.S.A.). The O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine Hydrochloride, dimethyl trisulfide, ethyl 2-methylpropionate, 2-methylbutanal, 3-methylbutanal, benzaldehyde, and 2-phenylacetaldehyde were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Furfural, pentanal, γ-nonalactone, and 3-mercaptohexanol were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The beer filtration apparatus and the filters (0.45 µm and 5.0 µm) were purchased from Toyo Roshi Kaisha, Ltd. (Tokyo, Japan).

Beer brewing processes

To 14 L of distilled water, 2394 g of milled Pilsner malt was added. The 5.6 pH mash was then kept at 35 °C, 50 °C, and 65 °C for 30 min to allow proteolysis. The temperature of the mash was raised and allowed to saccharify at 65 °C for 30 min. It was then held at 78 °C for 5 min before filtering. The extract remaining in the spent grains was rinsed with water at 76 °C, and the volume of wort before boiling was adjusted to 14 L. The concentration of the extract in the wort (i.e., the extract yield) was analyzed. Ten grams of Hallertauer Magnum (14.0% alpha-acids; Germany) and 10 g of Saazer (3.0% alpha-acids; Czech Republic) were added to the wort and boiled for 60 min with a boiling intensity of 7.9–8.5%. The wort was then kept for 20 min until the trub settled and the supernatant was obtained. Water was added to prepare the wort at an extract concentration of 12.0% and then cooled to 4 °C. Sludgy bottom-fermenting yeast was added to 5 L of wort to achieve $15–20 \times 10^6$ cells/mL and fermented for 7 days at 10 °C with stirring at a speed of 700 rpm. Following the 7 days of fermentation, the beer was maturated for 5 days at 10 °C and maintained at 4 °C for 6 days. The original extract of beer was then adjusted to 11.9%. The beer was filtered through a pressurized filter equipped with 5.0 µm filter, followed by 0.45 µm filter. The beers were bottled in 500 mL beer bottles and carbonated at 0.23 MPa. The bottled beer was aged at 30 °C for one month and then used for analysis.

Measurement of beer properties

Apparent extracts and alcohol contents were obtained using an Alcolyzer ME system equipped with the DMA5000M (Anton Paar GmbH, Graz, Austria). Amino acid analysis was performed using an amino acid analyzer (LA8080 AminoSAAYA, Hitachi High-Tech Corporation, Tokyo, Japan). The free amino nitrogen (FAN) in beer and wort was analyzed according to an authorized method.[19]

Sensory evaluation

Beers produced at mash-in temperatures of 35 °C, 50 °C, and 65 °C and stored at 30 °C for one month, underwent sensory evaluation by seven trained panelists. To determine the attributes that represent the characteristics of these beers, characteristics that differed among the samples were selected through careful discussion among the panelists. Consequently, estery, toast-like aroma, body, and light taste were selected as attributes for the evaluation of the fresh beer. The attributes of cardboard, honey, and sherry-like aroma were selected for the aged beer. The sensory panel participants then assessed the intensity of the beer odor attributes for each beer. The respective odor intensities were rated on the following scale (using 0.5-interval steps): 0 = not perceivable; 1 = very weak; 2 = weak; 3 = normal; 4 = strong; 5 = very strong. The characteristics of the varieties were compared by calculating the mean intensity values of the scores. Sensory data between panelists for a single observation were averaged and analyzed using 2-way ANOVA that assessed variance attributed to the panelist and the samples.

Quantification of 3-methyl-2-butene-1-thiol in beer

The extraction of 3-methyl-2-butene-1-thiol from beer was performed using a purge-and-trap apparatus, including a coiled condenser (GL Science, Tokyo, Japan). According to a previously published report, an eggplant-shaped flask was silanized with trimethylsilyl chloride.[20] An eggplant-shaped flask containing 100 mL of beer, 2 mg/L of dimethyltrisulfide-d_6 as an internal standard, and 20 µL of *n*-octyl alcohol as antifoam was set at the bottom of the purge-and-trap apparatus and then incubated at 0 °C using propylene glycol. Nitrogen gas was purged into the flask to bubble the beer at a flow rate of 100 mL/min for 60 min to collect aroma substances in a glass liner packed with 110 mg of Tenax GR, which was set at the top of the purge-and-trap apparatus. The coiled

condenser between the flask and the glass liner was cooled to 0 °C to remove water vapor. The glass liner was then applied to a thermal desorption unit (TDU, Gerstel, Germany), and the trapped aroma substances were thermally desorbed from Tenax GR at 200 °C and cryoforced at −150 °C using liquid nitrogen in the programmable temperature-vaporization inlet (CIS4; Gerstel). Aroma substances were injected from a CIS4 inlet programmed from −150 °C up to 200 °C, and separated using the Agilent 8890 GC system (Agilent Technologies, CA, U.S.A.) equipped with DB-FFAP capillary column (30 m length x 0.25 mm i.d.; film thickness = 0.5 µm; Agilent Technologies). Ionization was performed using an MS Triple Quad system (Agilent 7000D, Agilent Technologies) operated in electron ionization (EI) and multiple reaction monitoring (MRM) modes, with the precursor ion to the product ion set at m/z 102 to 69 for 3-methyl-2-butene-1-thiol with a collision energy of 5 eV and m/z 132 to 82 for dimethyltrisulfide-d_6 with a collision energy of 15 eV. The data shows the mean value of the duplicate analysis.

Quantification of dimethyl trisulfide in beer

Dimethyl trisulfide was extracted from 50 mL of beer using the solvent-assisted stir bar sorptive extraction (SA-SBSE, Gerstel, Germany) method with reference to an existing study,[21] followed by the separation using an Agilent 8890 GC system equipped with a DB-FFAP capillary column (30 m length × 0.25 mm i.d.; film thickness = 0.5 µm; Agilent Technologies). The ionization was performed using an Agilent 7000D MS Triple Quad system (Agilent Technologies) operated in EI and MRM modes, with the precursor ion to the product ion set at m/z 126 to 79 for dimethyl trisulfide and m/z 132 to 82 for dimethyltrisulfide-d_6, with a collision energy of 15 eV. The data shows the mean value of the duplicate analysis.

Quantification of sotolon in beer

The odorants, including internal analytical standards, were extracted using CH_2Cl_2 and concentrated as described previously,[22] followed by the separation using an Agilent 8890 GC system equipped with an InertCap Pure-WAX capillary column (30 m length x 0.25 mm i.d.; film thickness = 0.25 µm, GL Sciences, Japan). Ionization was performed using an Agilent 7000D MS Triple Quad system (Agilent Technologies) operated in EI and MRM modes, with the precursor ion to the product ion set at m/z 128 to 83 for sotolon and m/z 130 to 85 for sotolon-$^{13}C_2$ with a collision energy of 2 eV. The data shows the mean value of the duplicate analysis.

Quantification of ethyl 2-methylbutyrate and ethyl 2-methylpropionate in beer

Ethyl 2-methylbutyrate and ethyl 2-methylpropionate were extracted using the solid-phase microextraction (SPME) method with reference to an existing study, [23] followed by separation using an Agilent 8890 GC system equipped with a DB-FFAP capillary column (30 m length x 0.25 mm i.d.; film thickness = 0.5 µm). The ionization was performed using an Agilent 7000D MS Triple Quad system (Agilent Technologies) operated in EI and MRM modes, with the precursor to the product ion set at m/z 116 to 88 for ethyl 2-methylpropionate with a collision energy of 1.0 eV, m/z 102 to 74 for ethyl 2-methylbutyrate with a collision energy of 5.0 eV, and m/z 105 to 77 for ethyl 2-methyl-d_3-butyrate as the internal standard with a collision energy of 5.0 eV. The data shows the mean value of the duplicate analysis.

Quantification of Strecker aldehydes in beer

The extraction and quantification of Strecker aldehydes were conducted with reference to an existing study,[24] using solid-phase microextraction (SPME) with an on-fiber derivatization system using o-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBOA), followed by the separation using an Agilent 8890 GC system equipped with an HP-5MS UI capillary column (30 m length × 0.25 mm i.d.; film thickness = 0.25 µm). Ionization was performed using an Agilent 7000D MS Triple Quad system (Agilent Technologies) operating in EI and MRM modes, with the precursor to the product ion set at m/z 266 to 181 for 2-methylbutanal and 3-methylbutanal, m/z 239 to 181 for pentanal, m/z 291 to 181 for furfural, m/z 299 to 181 for 3-(methylthio)propionaldehyde, m/z 302 to 181 for 3-(methylthio)propionaldehyde-d_3, m/z 301 to 181 for benzaldehyde, m/z 306 to 181 for benzaldehyde-d_5, m/z 315 to 181 for 2-phenylacetaldehyde, and m/z 320 to 181 for phenyl-d_5-acetaldehyde with a collision energy of 10 eV. The internal standard pentanal was used for the quantification of 2-methylbutanal and 3-methylbutanal, 3-(methylthio) propionaldehyde-d_3 for the quantification of 3-(methylthio) propionaldehyde, phenyl-d_5-acetaldehyde for the quantification of 2-phenylacetaldehyde, and benzaldehyde-d_5 for the quantification of furfural and benzaldehyde. The data shows the mean value of the duplicate analysis.

Quantification of (E)-2-nonenal in beer

The extraction of (*E*)-2-nonenal was conducted with reference to an existing study,[22] using SBSE with in situ derivatization of PFBOA, followed by separation using an Agilent 8890 GC system equipped with an HP-5MS UI capillary column (30 m length x 0.25 mm i.d.; film thickness = 0.25 µm; Agilent Technologies). Ionization was performed using an Agilent 7000D MS Triple Quad system (Agilent Technologies) operated in EI and MRM modes, with the precursor to the product ion set at m/z 335 to 250 for (*E*)-2-nonenal with a collision energy of 2.5 eV and m/z 337 to 252 for the internal standard, (*E*)-2-nonenal-d_2 with a collision energy of 2.5 eV. The data shows the mean value of the duplicate analysis.

Quantification of (E)-β-damascenone and γ-nonalactone in beer

The extraction of (*E*)-β-damascenone and γ-nonalactone was conducted according to the modified method of the previous study,[25] using the stir-bar sorptive extraction

(SBSE, Gerstel, Germany) method, followed by the separation using an Agilent 8890 GC system equipped with a DB-WAX UI capillary column (30 m length x 0.25 mm i.d.; film thickness = 0.25 µm; Agilent Technologies). Ionization was performed using an Agilent 7000D MS Triple Quad system (Agilent Technologies) operated in EI and MRM modes, with the precursor to the product ion set at m/z 190 to 121 for (*E*)-β-damascenone, m/z 156 to 101 for γ-nonalactone, and m/z 192 to 177 for the internal standard, (*E*)-β-damascone. The data shows the mean value of the duplicate analysis.

Results and discussion

Sensory evaluation of fresh bottled beer

As shown in Figure 1 and Table 1, full-bodied and ester aroma characteristics were dominant, and their averaged scores were significantly higher ($p < 0.05$) in the beer with mash-ins at 35 °C and 50 °C. The light taste and toast-like aroma were dominant in the beer with a 65 °C mash-in and their averaged score was significantly higher ($p < 0.05$). The mash-ins at 35 °C and 50 °C contributed to increased activities of enzymes in malt, which then resulted in the full-bodied taste, increased concentrations of esters, and a higher alcohol content in the beers. Higher alcohols have also been reported to contribute to the beer's body as well as being precursors to the corresponding esters.[26]

Components contributing to the characteristics of fresh bottled beer

The ester and amino acid concentrations are shown in Tables 2 and 3, respectively. The concentration of amino acids in wort affects the formation of higher alcohols during fermentation and, hence, the formation of esters.[2,9,10] Beers with mashed-in temperatures of 35 °C and 50 °C had higher concentrations of amino acids, esters, and higher alcohols, which is consistent with the results of the sensory evaluation.

High-molecular components such as proteins and sugars remained in the mash when degradative enzyme activity was reduced at the mash-in temperature of 65 °C, and they were easily removed during the wort filtration and boiling processes.[1] While lower molecular weight amino acids were easily dissolved in wort and then contributed to the full-bodied characteristics of the beer, the components that contributed to taste did not remain in the beer with mash-in at 65 °C. This resulted in the light-tasting characteristics of the beer.

Yeast consumes at least 100–140 mg/L of FAN in the wort to use as nutrients required for fermentation.[1] In this experiment, a FAN of more than 270 mg/L was contained even in the wort at a mash-in temperature of 65 °C. In another experiment using Australian malt with a Kohlbach index of 38% and a diastatic power of 295°WK, 255 mg/L of FAN was contained in the wort at a mash-in temperature of 65 °C. This suggests that the preparation of a proteolytic process is not necessary when brewing beer made exclusively from malt in terms of providing nutrition to the yeast.

As shown in Table 2, the extract yield after adjusting the kettle-full volume to 14 L before boiling was higher in the wort with mash-ins at 35 °C and 50 °C. The starch granules in the malt grist are surrounded by a protein matrix that binds to the hemicellulose and β-glucans of the endosperm cell wall.[1,27–29] Maintaining the mash at the optimum temperature for enzymes that break its structure, i.e., proteases, β-glucanases, and limit dextrinases that cleave the 1,6- and 1,4-linkages of amylopectin, resulted in the higher extract yields.

Sensory evaluation of beer stored at 30 °C for 1 month

After a month of beer storage at 30 °C, the highest possible temperature under general warehouse and home storage conditions, the sensory evaluation was conducted. The honey and sherry-like aroma (i.e., the sweet and toffee-like aroma proposed by Dalgliesh)[11] was dominant ($p < 0.05$) in the sensory evaluation of beer with a mash-in temperature of 65 °C, as shown in Figure 2 and Table 1. In the beers with mash-ins at 35 °C and 50 °C, the cardboard flavor predominated ($p < 0.05$) over honey and sherry-like aromas, and the concentration of (*E*)-2-nonenal (Table 4) was consistent with the results of the sensory evaluation. The higher lipoxygenase activity at 35 °C and 50 °C mash-ins, which resulted in a higher concentration of (*E*)-2-nonenal, was thought to be responsible for these results.[12]

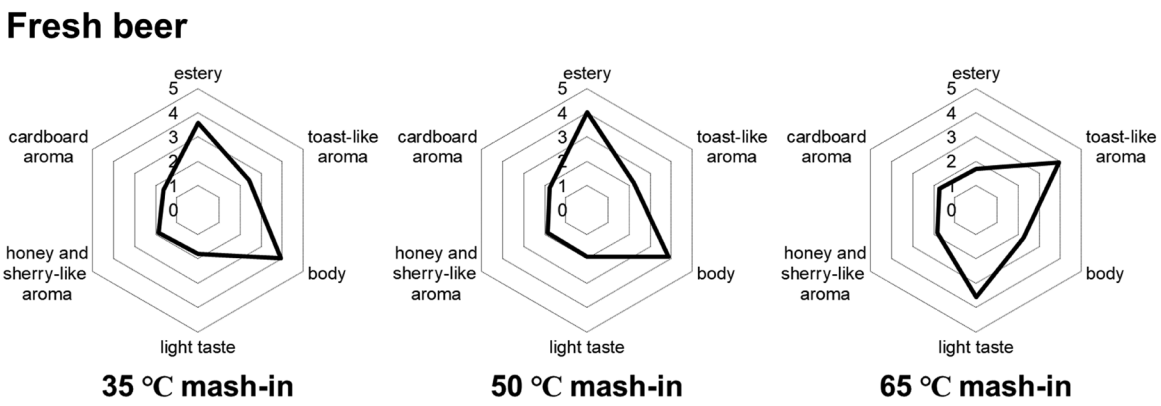

Figure 1. Sensory evaluation of fresh bottled beer.

Table 1. The highlight of the differences in flavor characteristics at lower and higher mash-in temperatures.

	Lower mash-in temperature 35 °C or 50 °C	Higher mash-in temperature 65 °C
Fresh beer before storage	Full-bodied, estery characteristics	Light taste with a toast-like aroma
After storage at 30 °C for 1 month	Predominance of cardboard flavor over honey and sherry-like aroma	Dominance of honey and sherry-like aroma

Table 2. Concentration of esters and higher alcohols in beer.

	Unit	35 °C mash-in		50 °C mash-in		65 °C mash-in	
Original extract before boiling	% Plato	12.0	(±0.3)	12.1	(±0.3)	11.0	(±0.3)
Original extract of wort	% Plato	12.0	(±0.3)	11.9	(±0.3)	12.1	(±0.3)
Original extract of beer	% Plato	11.9	(±0.3)	11.9	(±0.3)	11.9	(±0.3)
Alcohol	% w/w	5.3	(±0.2)	5.2	(±0.2)	5.3	(±0.2)
Color of beer	°EBC	2.8	(±0.1)	2.7	(±0.1)	2.6	(±0.1)
Bitter unit of beer	B.U.	16.0	(±2.0)	17.0	(±2.1)	17.0	(±2.1)
Ethyl acetate	mg/L	22.9	(±1.7)	19.1	(±1.5)	17	(±1.3)
Isoamyl acetate	mg/L	1.3	(±0.1)	0.9	(±0.1)	0.6	(±0.1)
Ethyl hexanoate	mg/L	0.2	(±0.01)	0.2	(±0.01)	0.1	(±0.01)
Ethyl n-octanoate	mg/L	0.5	(±0.04)	0.4	(±0.03)	0.2	(±0.01)
2-Methyl-1-propanol	mg/L	31.2	(±1.7)	28.5	(±1.5)	24.2	(±1.3)
3-Methyl-1-butanol	mg/L	184.8	(±9.4)	169.8	(±8.7)	144.3	(±7.4)
2-Phenylethyl alcohol	mg/L	39.1	(±3.6)	38	(±3.5)	34.5	(±3.1)

Table 3. Concentration of amino acid and free amino nitrogen (FAN) in wort and beer.

	Concentration in wort (mg/L)						Concentration in bottled fresh beer (mg/L)					
	35 °C mash-in		50 °C mash-in		65 °C mash-in		35 °C mash-in		50 °C mash-in		65 °C mash-in	
Free amino nitrogen in wort	358	(±15)	344	(±14)	271	(±11)	231	(±10)	214	(±9)	170	(±7)
Glutamic acid	25.4	(±1.6)	41.5	(±2.7)	35.0	(±2.3)	1.5	(±0.1)	2.1	(±0.1)	1.4	(±0.1)
Aspartic acid	50.4	(±4.5)	58.5	(±5.2)	36.9	(±3.3)	13.3	(±1.2)	13.7	(±1.2)	11.2	(±1.0)
Serine	57.5	(±5.2)	62.7	(±5.7)	37.4	(±3.4)	n.d.		n.d.		n.d.	
Threonine	47.2	(±3.8)	51.4	(±4.1)	30.4	(±2.4)	0.7	(±0.1)	0.9	(±0.1)	0.6	(±0.1)
Lysine	55.7	(±4.1)	58.6	(±4.3)	33.3	(±2.4)	0.2	(±0.01)	0.5	(±0.04)	0.1	(±0.01)
Arginine	78.8	(±4.4)	88.8	(±5.0)	54.7	(±3.1)	0.4	(±0.02)	0.9	(±0.05)	0.2	(±0.01)
Valine	86.3	(±7.9)	93.5	(±8.6)	55.8	(±5.1)	0.2	(±0.02)	0.2	(±0.02)	0.1	(±0.01)
Methionine	21.6	(±1.7)	22.2	(±1.8)	12.0	(±1.0)	1.5	(±0.1)	1.6	(±0.1)	1.3	(±0.1)
Leucine	106.7	(±7.9)	113.7	(±8.4)	66.6	(±4.9)	1.0	(±0.1)	0.9	(±0.1)	0.8	(±0.1)
Isoleucine	52.6	(±3.5)	56.7	(±3.7)	33.2	(±2.2)	4.3	(±0.3)	4.3	(±0.3)	4.0	(±0.3)
Histidine	36.1	(±2.1)	40.7	(±2.4)	25.9	(±1.5)	0.1	(±0.01)	0.2	(±0.01)	0.1	(±0.01)
Glycine	27.6	(±2.5)	28.0	(±2.5)	15.3	(±1.4)	2.0	(±0.2)	4.1	(±0.4)	1.5	(±0.1)
Phenylalanine	95.7	(±7.0)	106.0	(±7.7)	64.8	(±4.7)	7.0	(±0.5)	7.6	(±0.6)	6.4	(±0.5)
Tyrosine	64.9	(±5.3)	72.0	(±5.8)	43.7	(±3.5)	0.4	(±0.03)	0.5	(±0.04)	0.4	(±0.03)
Alanine	89.3	(±8.4)	99.8	(±9.4)	61.8	(±5.8)	1.2	(±0.1)	2.5	(±0.2)	0.6	(±0.1)
Tryptophan	31.3	(±2.0)	35.3	(±2.3)	22.0	(±1.4)	0.2	(±0.01)	0.1	(±0.01)	0.1	(±0.01)
Proline	386.0	(±26.2)	399.9	(±27.2)	261.3	(±17.8)	352.3	(±24.0)	357.2	(±24.3)	328.1	(±22.3)

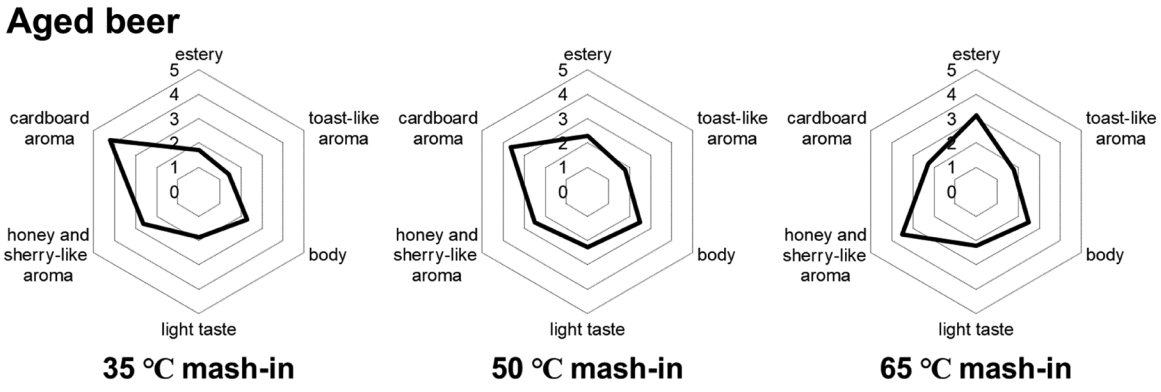

Figure 2. Sensory evaluation of beer stored at 30 °C for 1 month.

Concentration of the components contributing to the stale flavor of beer

Table 4 shows the concentrations of components that have been reported to contribute to the stale flavor of Pilsner-type beer.[18] Each component increased significantly after a month of storage at 30 °C; they contributed to the honey and sherry-like aroma of the beer at 65 °C mash-in. Beyond the results, (*E*)-2-nonenal increased significantly higher than the threshold in the beers with 35 °C and 50 °C mash-ins, and

Table 4. Concentration of components contributing to the stale flavor of beer.

	Concentration in bottled fresh beer (μg/L)						Concentration in beer stored at 30 °C for 1 month (μg/L)						Differential threshold	Possible
	35 °C mash-in		50 °C mash-in		65 °C mash-in		35 °C mash-in		50 °C mash-in		65 °C mash-in		value in beer (μg/L)	precursors
(*E*)-2-nonenal	0.10	(±0.01)	0.07	(±0.01)	0.07	(±0.01)	0.46	(±0.04)	0.45	(±0.04)	0.21	(±0.02)	0.10[22]	Linoleic acid[15]
2-methylbutanal	1.3	(±0.1)	1.9	(±0.2)	2.0	(±0.2)	3.2	(±0.3)	2.9	(±0.2)	3.4	(±0.3)	86[17]	isoleucine[13]
3-methylbutanal	6.2	(±0.5)	8	(±0.7)	8.4	(±0.7)	14.5	(±1.2)	14.1	(±1.2)	15.3	(±1.3)	9.6[22]	leucine[13]
Furfural	7.6	(±0.9)	5.6	(±0.7)	7.9	(±0.9)	533.1	(±62.4)	502.3	(±58.8)	476.2	(±55.7)	15157[17]	pentose[15]
Methional	1.0	(±0.1)	1.2	(±0.1)	1.1	(±0.1)	1.8	(±0.2)	1.8	(±0.2)	1.5	(±0.1)	1.8[22]	methionine[13]
Benzaldehyde	2.5	(±0.4)	2.5	(±0.4)	3.3	(±0.5)	4.0	(±0.6)	4.2	(±0.6)	4.9	(±0.7)	515[17]	phenylalanine[13]
Phenylacetaldehyde	38.9	(±5.1)	47.6	(±6.3)	52.6	(±6.9)	107	(±14.1)	104	(±13.7)	107.2	(±14.2)	39.7[22]	phenylalanine[13]
Sotolon	0.6	(±0.05)	0.6	(±0.05)	0.6	(±0.05)	1.0	(±0.08)	0.9	(±0.07)	1.2	(±0.09)	0.54[22]	threonine[30]
Dimethyltrisulfide	0.008	(±0.0005)	0.007	(±0.0004)	0.008	(±0.0005)	0.082	(±0.0048)	0.066	(±0.0038)	0.072	(±0.0042)	0.016[22]	methionine[13]
(*E*)-β-damascenone	1.1	(±0.05)	1.2	(±0.05)	1.2	(±0.05)	3.4	(±0.14)	2.9	(±0.12)	3.1	(±0.13)	2.5[22]	
γ-Nonalactone	39.7	(±1.9)	36.9	(±1.7)	33.4	(±1.6)	90	(±4.2)	90.6	(±4.3)	77.5	(±3.6)	11.2[22]	linoleic acid[31]
Ethyl 2-methylpropionate	0.8	(±0.1)	0.6	(±0.0)	0.7	(±0.1)	1.1	(±0.1)	1.1	(±0.1)	1.1	(±0.1)	6.30[22]	iso-alpha acids[32]
Ethyl 2-methylbutyrate	0.7	(±0.1)	0.6	(±0.0)	0.8	(±0.1)	1.2	(±0.1)	1.2	(±0.1)	1.4	(±0.1)	1.10[22]	iso-alpha acids[32]
3-Methyl-2-butene-1-thiol	0.003	(±0.0004)	0.003	(±0.0004)	0.003	(±0.0004)	0.01	(±0.0012)	0.011	(±0.0013)	0.01	(±0.0012)	0.007[22]	iso-alpha acids[32]

the cardboard flavor predominated over honey and sherry-like aroma. When the mash-in temperature is reduced to produce beer with full-bodied and ester characteristics, the aged flavors are predominantly cardboard flavor. To yield amino acids while avoiding the formation of cardboard flavors, the use of malt with a higher Kohlbach index and the adoption of a high mash-in temperature may be more effective.

As shown in Table 3, the concentrations of precursor amino acids of Strecker aldehydes such as leucine, isoleucine, methionine, and phenylalanine differed greatly in wort depending on the mash-in temperature; however, after fermentation (i.e., after consumption by yeast) the difference in concentration of each amino acid in beer was minimal. These slight differences in each amino acid did not result in a difference in the concentration of each Strecker aldehyde (2-methylbutanal, 3-methylbutanal, furfural, methional, benzaldehyde, and phenylacetaldehyde) after storage for a month at 30 °C, as shown in Table 4. No differences proportional to the slight differences in amino acid content in the beer were found in the concentrations of other stale odor components, such as dimethyltrisulfide, (*E*)-β-damascenone, ethyl 2-methylpropionate, ethyl 2-methylbutyrate, sotolon, and 3-methyl-2-butene-1-thiol.

In the treatment after fermentation, the samples were bottled with great care to avoid involving oxygen and were carbonated to 0.23 MPa. All samples were handled and treated in the same manner. There should be no difference in the amount of dissolved oxygen, though the amount of dissolved oxygen was not measured. The relationship between the amount of dissolved oxygen and the concentration of components contributing to the stale flavor should be investigated in future studies.

Disclosure statement

No potential competing interest was reported by the authors.

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