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ARTICLE

Biotransformation of Hop-Derived Monoterpene Alcohols by Lager Yeast and Their Contribution to the Flavor of Hopped Beer

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It is well-known that various beers contain many flavor compounds derived from barley malts, hops, yeast fermentation, and other raw materials. Among these flavor compounds, terpenoids are mainly derived from hops. Linalool, one of the monoterpene alcohols, has been found in various beers and been regarded as an important factor for a hop-derived beer flavor. We focus on contributions of other monoterpene alcohols (geraniol, β-citronellol, nerol, and α-terpineol) to hopped beer flavor. Several researchers have reported that monoterpene alcohols are biotransformed by yeast and that geraniol is mainly transformed to β-citronellol during the first 2−4 days in model fermentation. In this study, we investigated the biotransformation of monoterpene alcohols during fermentation of hopped beer by using various hop cultivars. As a result, geraniol drastically decreased during the first 3 days. β-Citronellol was almost absent in wort and gently increased during the total fermentation period. The concentrations of geraniol and β-citronellol in finished beer increased, depending on the initial concentration of geraniol in the wort. The continuous increase of β-citronellol did not correspond to the fast decrease of geraniol. This increase of β-citronellol might be partly explained by an occurrence of glycosidically bound flavor precursor and a glucoside hydrolase activity secreted from lager yeast. In addition, we examined flavor characteristics of monoterpene alcohols and found that there was an additive effect among linalool, geraniol, and β-citronellol and that only 5 μg/L of geraniol and β-citronellol were enough for this effect. Therefore, it is suggested that not only linalool but also geraniol and β-citronellol might contribute to hopped beer flavor at lower levels, at which OAVs of these compounds become below 1.0.

KEYWORDS: Beers; hops; flavor; monoterpene alcohols; biotransformation; linalool; geraniol; β-citronellol; additive effect

INTRODUCTION

It is well-known that various beers contain many flavor compounds derived from barley malts, hops, yeast fermentation and other raw materials. Among these flavor compounds, terpenoids are mainly derived from hops. Hops (*Humulus lupulus* L.) include various compounds, for example terpenoids, polyphenols, and resins, and give a characteristic flavor and bitterness to beers. Beer researchers have regarded hop terpenoids as important components for the flavor characteristics of beers. β-Myrcene, α-humulene, β-caryophyllene, and β-farnesene have been well-known as main components of hop oil, and the ratio of these terpenoids has been used for the classification of hop cultivars (*1*). Steinhaus and Schieberle have proposed that β-myrcene and linalool are the most potent odorants in the hop cones (*2*). However, it has been confirmed that most of the hydrophobic terpene hydrocarbons, including β-myrcene and α-humulene, would not remain from hop cones or pellets as part of finished beer during beer production (*3*−*5*). Terpene alcohols are more hydrophilic and easier to retain in wort and beer than terpene hydrocarbons. Especially linalool has been found in various beers, and it has been regarded as an important factor for a hop-derived beer flavor (*3*, *6*, *7*). However, contributions of other monoterpene alcohols (geraniol, β-citronellol, nerol, and α-terpineol) to beer flavor have not been completely clarified.

Several researchers have pointed out that Cascade, one of the U.S. hop cultivars, contains not only linalool but also geraniol in the hop cone, pellet, hop oil, and finished beer (*8*−*11*). Geraniol has been considered to be more cultivar-specific than linalool (*8*, *9*). On the other hand, Seaton et al. have investigated the refinement of hop flavor by yeast and have proposed the transformation of geraniol to β-citronellol by yeast metabolism (*6*). Lam et al. have also reported that β-citronellol was absent in wort and became detectable during fermentation. They have also pointed out that all of these terpene alcohols might contribute to the flavor of beer brewed with the Cascade hop (*12*).

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Table 1. Conditions of Hop Water Extraction and Test Brewing

			hop water extraction	test brewing		
					late hopping	
hop cultivar	BU[a]	α acid[a] (%)	hop dosage (g/200 mL)	kettle hopping hop dosage[b] (g/L)	hop dosage 1[b] (g/L)	hop dosage 2[c] (g/L)
HHT	36.0	4.5	4.62	1.19	0.34	1.38
9702A	50.3	7.7	2.70	0.86	0.28	1.12
9803A	44.3	6.0	3.47	0.97	0.32	1.28
Cascade	45.6	5.3			0.29	1.17
Nugget	93.9	12.7			0.14	0.55
Millennium	104	14.5			0.12	0.50

[a] Measured according to Analytica-EBC (*15*). [b] Hop was added at the beginning of wort boiling. [c] Hop was added at 5 min before the end of wort boiling.

Recently, King et al. have reported the biotransformation of monoterpene alcohols by yeast metabolism (*13*, *14*). They have proposed metabolism cascades of monoterpene alcohols in various yeasts by using model fermentations containing each monoterpene alcohol and confirmed that geraniol was mainly transformed to β-citronellol by both lager and ale yeast (*14*). However, biotransformation of monoterpene alcohols has been reported not in model fermentation but in fermentation of hopped beer. We now examine the behavior of monoterpene alcohols during beer fermentation and evaluate the contribution of these monoterpene alcohols to beer flavor by using sensory techniques.

MATERIALS AND METHODS

Hop Raw Materials. Hallertauer Tradition (HHT) was grown in Germany. 9702A and 9803A were bred and grown in Japan (Bioresources Research & Development Department, Sapporo Breweries, Ltd.). Cascade, Nugget, and Millennium were grown in the U.S. All hops were harvested in 2007.

Hop Extraction with Hot Water. For analysis of the flavor compounds, all hops were extracted with hot water. Hop pellets (approximately 3−5 g, see **Table 1**) were added to 200 mL of water and autoclaved at 105 °C for 5 min. After it was cooled on ice−water, the mixture was filtered by using filter paper and the eluant was obtained as the hop water extract. Hop dosage was adjusted according to the α acid (*15*) content of each hop.

Pilot-Scale Brewing. Beers were made from the HHT hop, the 9702A hop, the 9803A hop, the Cascade hop, the Nugget hop, or the Millennium hop with the same recipe according to the standard method of the Production & Technology Development Center, Sapporo Breweries, Ltd. Briefly, the wort was prepared using commercially available 67% malts, 33% adjuncts (starch, corn, and rice), and hops in a 400 or 5000 L scale pilot apparatus. The boiling period was 90 min. For kettle hopping, hops were added to worts at the beginning of boiling. For late hopping, hops were added at the beginning of boiling (20% of total dosage) and at 5 min before the end of boiling (80% of total dosage). The dosage of each hop was determined by adjusting the bitterness unit (BU (*15*)) of finished beer to approximately 20−22 (**Table 1**). After cooling, the fermentation was started by adding 15.0×10^6 cells/mL of lager yeast (brewery collected; *Saccharomyces pastorianus*) to the cooled wort. The temperature of the fermentation was maintained at 10−12 °C. After the fermented wort was transferred to another storage tank under a CO_2 atmosphere, the maturation was carried out at 13 °C for 6−8 days and then at 0 °C for 2−3 weeks. Filtration and bottling were done using the pilot-scale equipment under antioxidative conditions.

Chemicals. α-Humulene (>93%), β-myrcene (>70%), linalool (>98%, racemic mixture), α-terpineol (>95%, racemic mixture), nerol (>98%), and β-citronellol (>92%, racemic mixture) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Geraniol (98%) was purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI). Isobutyl isobutyrate (>98%), isoamyl isobutyrate (>98%), isobutyric acid (>98%), and 2-methylbutan-1-ol (>97%) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 2-Methylbutyl isobutyrate (>97%) was synthesized by esterification of isobutyric acid and 2-methylbutan-1-ol as previously described (*16*). *p*-Nitrophenol (>99%) and *p*-nitrophenyl-β-glucopyranoside were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sumizyme BGA (β-1,4-glucosidase [EC 3.2.1.21], derived from *Aspergillus niger*, 2000 U/g (pH 4.0)) was obtained from Shin Nihon Chemical Co., Ltd. (Aichi, Japan).

Quantification of Terpenoids and Isobutyric Esters by Gas Chromatography−Mass Spectrometry (GC-MS). GC-MS analyses were carried out using a 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA). The carrier gas was helium with a column-head pressure of 15 psi and a flow rate of 1.8 mL/min. The detector was a mass spectrometer (MS 5973, Agilent Technologies) functioning in the EI mode (70 eV) and was connected to the GC by a transfer line heated to 280 °C. For analysis of raw hops, 20 mg of ground hops was directly placed into a 20 mL glass vial. For analysis of hop water extract, wort, fermenting beer, and finished beer, 8 mL of each sample was placed into a 20 mL glass vial including 3 g of sodium chloride at 0 °C. The vial, including a sample, was sealed with a magnet cap. The vial was preincubated with stirring at 40 °C for 15 min using a Combi-PAL autosampler (CTC Analytics, Zwingen, Switzerland). After preincubation, an SPME fiber (PDMS (polydimethylsiloxane), 100 μm film thickness, Supelco, Bellefonte, PA) was inserted into the head space of the vial and adsorption was carried out for 15 min. After the adsorption, the SPME fiber was injected into a splitless injector (260 °C; purge time 3 min, purge flow 20 mL/min) at oven temperature (50 °C) onto a type HP-1MS capillary column (Agilent Technologies, 30 m, 0.25 mm internal diameter (i.d.), 1.0 μm film thickness). For all the analyses, the temperature program was as follows: 50 °C for 1 min, raised at 5 °C/min to 250 °C, followed by a 1 min isotherm. The terpenoids (α-humulene, β-myrcene, linalool, α-terpineol, nerol, β-citronellol, and geraniol) were quantified in the SIM mode, selecting the following ions: *m*/*z* 93 (for β-myrcene, α-terpineol, nerol and geraniol), 109 (for linalool and β-citronellol), and 204 (for α-humulene). The isobutyric esters isobutyl isobutyrate, isoamyl isobutyrate, and 2-methylbutyl isobutyrate were quantified in the SIM mode, selecting the following ions: *m*/*z* 71 and 87. Calibration curves were determined using water (including 5% ethanol) containing these terpenoids and isobutyric esters at final concentrations ranging from 0 to 10 μg/L. All calibrations produced a linear response with an R^2 value >0.98, over the concentration range analyzed.

Glucoside Hydrolase Activity. The assay for glucoside hydrolase activity is based on the enzymatic hydrolysis of *p*-nitrophenyl-β-glucopyranoside (pNP-β-Glc) at pH 4.0 and 40 °C. One unit (U) of activity is defined as the quantity of enzyme that will liberate 1 μmol of *p*-nitrophenol (pNP) from pNP-β-Glc per minute under the conditions of this assay. Fermenting beer was directly used as a sample solution, and 0.05 mol/L acetate buffer (pH 4.0) was used as a reaction buffer. For the substrate solution, 172.0 mg of pNP-β-Glc was dissolved in 100 mL of 0.05 mol/L acetate buffer (pH 4.0). The reaction-stop agent was 1 mol/L sodium carbonate solution. For enzyme reactions, a test tube containing 0.7 mL of substrate solution was preincubated at 40 °C for 5 min. Then, 0.1 mL of sample solution was added into this test tube. After exactly 180 min, 0.2 mL of 1 mol/L sodium carbonate was added and mixed. For the reaction blank, 0.2 mL of 1 mol/L sodium carbonate was mixed before adding a sample solution. The absorbance of the reaction mixture was determined by using a spectrophotometer at 405 nm. The difference in absorbance between a reaction mixture and a corresponding blank mixture was regarded as the absorbance obtained by glucoside hydrolase activity in a sample solution. pNP standard solutions (containing 0.02,

0.04, 0.06, and 0.08 μmol/mL of pNP, respectively) were prepared, and their absorbance at 405 nm was measured. Glucoside hydrolase activity (U/L) of each sample solution was calculated from an absorbance of the reaction mixture and the absorbance of pNP standard solutions.

Estimation of Glycosidically Bound Flavor Potential. In order to estimate the glycosidically bound flavor potential of fermenting beer, an excess of the β-glucosidase Sumizyme BGA was added into beer for liberation of flavor compounds from their glycosidically bound precursors. For removal of yeast, the beer sample was centrifuged at 5000 rpm for 10 min. An 8 mL sample of centrifuged beer was placed into a 20 mL glass vial, and 80 mg of Sumizyme BGA was added to this beer at 0 °C. For the blank, no Sumizyme BGA was added. This vial was sealed with a silicone rubber septum and an aluminum cap and was incubated at 50 °C for 60 min. After incubation, this vial was cooled on ice−water and was decapped. A 3 g portion of sodium chloride was added to the beer, and the vial was resealed with a magnet cap. Subsequently, the measurement of terpene alcohols was carried out by using GC-MS as described above. The liberation of each terpene alcohol was calculated by subtracting the concentration of each terpene alcohol in a blank from that in an enzyme-reacted sample. This liberation was regarded as the glycosidically bound flavor potential.

Sensory Evaluation of Terpene Alcohols. *Determination of Flavor Thresholds*. Each sensory evaluation was performed by 10−13 well-trained panelists. Perception thresholds of linalool, α-terpineol, β-citronellol, nerol, and geraniol were assessed by a forced-choice ascending concentration series method of limits (*17*). Briefly, directional triangular tests of six increasing concentrations in model carbonated dilute alcohol solution (5% v/v ethanol) or in Japanese commercial beer made with kettle hopping were carried out. A 50 mL portion of each sample solution was presented in plastic cups. The best estimate threshold was calculated for each panelist as the geometric mean of the highest concentration missed and the next highest concentration. The group threshold was calculated as the geometric mean of the best estimate thresholds of the panelists.

Study of Additive Effect among Terpene Alcohols. Evaluation of an additive effect was performed according to the methods previously described (*18*). Namely, in order to assess an additive effect among three terpene alcohols (linalool, geraniol, and β-citronellol), triangular tests were carried out in model carbonated dilute alcohol solution (5% v/v ethanol) and Japanese commercial beer, as follows. A control solution containing the estimated threshold concentration of linalool (3 μg/L for the model solution and 5 μg/L for beer) was compared with test solutions containing the same concentration of linalool together with 5 μg/L of geraniol and/or 5 μg/L of β-citronellol. A 50 mL portion of each sample solution was presented in plastic cups. The significance of the results was determined according to the binomial law.

RESULTS AND DISCUSSION

Behavior of Terpenoids and Isobutyric Esters during Boiling. We focused not only on terpenoids, including terpene hydrocarbons (α-humulene and β-myrcene) and terpene alcohols (linalool, α-terpineol, β-citronellol, nerol, and geraniol), but also on isobutyric esters (isobutyl isobutyrate, isoamyl isobutyrate, and 2-methylbutyl isobutyrate) as major flavor compounds of hops because isobutyric esters have been found in various hops, late-hopped beers, and dry-hopped beers (*6*, *16*, *19*−*21*). Hallertauer Tradition (HHT) was selected as a typical German aroma hop. The beer brewed with HHT hops has a normal "spicy" hop flavor. 9702A and 9803A were bred and grown in Japan. The beers made from these hops have "floral" and/or "fruity'" flavor. We have confirmed that the HHT beer mainly contained linalool and that the 9702A beer and the 9803A beer contained not only linalool but also geraniol (data not shown). Therefore, we selected these cultivars for comparison of terpene alcohols.

In order to confirm the effect of boiling on hop flavor compounds, we compared the behavior of flavor compounds before and after boiling. The content of flavor compounds in ground hops was regarded as the content before boiling, and that in a hot water extract of hops (autoclaved at 105 °C for 5 min; this condition corresponded to that of late-hopping) as the content after boiling. For a comparison, all of these contents were converted into the content in 1 g of each hop (μg/g of hop). As a result (**Table 2**), terpene hydrocarbons and isobutyric esters decreased drastically by boiling. Especially terpene hydrocarbons remained below 1 μg/g of hop after boiling, corresponding to below 1% of thier contents in hops before boiling. Several researchers have also reported that most of the hydrophobic terpene hydrocarbons would not remain from hop to finished beer during beer production (*3*−*5*) and that isobutyric esters could be unstable during boiling and fermentation (*6*). On the other hand, terpene alcohols remained at levels higher than those of terpene hydrocarbons and isobutyric esters. Linalool was contained in all cultivars at significant levels before and after boiling. α-Terpineol and geraniol were found in all cultivars at quantifiable levels. β-Citronellol and nerol were almost at trace levels.

Table 2. Comparison of the Contents of Volatile Compounds (μg/g of hop) during Boiling[a]

	HHT		9702A		9803A	
volatile compd	before boiling	after boiling	before boiling	after boiling	before boiling	after boiling
α-humulene	50.9	0.1	5.1	tr	36.1	0.1
β-myrcene	127	0.1	153	0.8	134	0.2
isobutyl isobutyrate	4.5	0.2	3.8	0.3	2.4	0.1
isoamyl isobutyrate	1.1	tr	4.1	0.1	1.3	tr
2-methylbutyl isobutyrate	12.2	0.2	16.4	0.4	6.6	0.1
linalool	37.4	6.9	126	42.3	65.6	17.4
α-terpineol	1.3	0.4	4.0	2.4	3.9	1.7
β-citoronellol	tr	tr	tr	tr	tr	tr
nerol	0.1	tr	0.4	0.2	0.5	0.2
geraniol	0.8	0.2	5.2	2.0	17.2	4.7

[a] Legend: before boiling, calculated from measurement of ground hop; after boiling, calculated from measurement of hop water extract (autoclaved at 105 °C for 5 min); tr, trace.

Figure 1 shows the composition of five monoterpene alcohols before and after boiling. In the HHT hop, the total amount of monoterpene alcohols was smallest among all three cultivars and only linalool was a main component, especially after boiling. In the 9702A hop, the total amount of monoterpene alcohols was largest and not only linalool but also α-terpineol and geraniol remained after boiling. The 9803A hop contained the largest amount of geraniol before and after boiling. From these results, we confirmed that these three cultivars (HHT, 9702A, and 9803A) might be suitable for a comparison of monoterpene alcohols.

Behavior of Monoterpene Alcohols during Fermentation of Hopped Beer. Figure 2 shows the metabolism cascade of monoterpene alcohols by lager and ale yeast. King et al. have proposed this cascade from the results of model fermentation containing each monoterpene alcohol (*13*, *14*). Geraniol could be mainly transformed to β-citronellol and adjunctively to linalool. Nerol could be converted to linalool and α-terpineol. A part of linalool could be cyclized to α-terpineol. Especially biotransformation of geraniol to β-citronellol was observed to be fast within the first 2−4 days of model fermentation. A decrease of geraniol and a corresponding increase of β-citronellol occurred drastically during this period (*13*, *14*). We tried to confirm the behavior of monoterpene alcohols in the fermentation of hopped beer made by using selected hop cultivars (HHT, 9702A, and 9803A).

In the case of kettle hopping (**Figure 3**), not only terpene hydrocarbons but also most of the monoterpene alcohols were

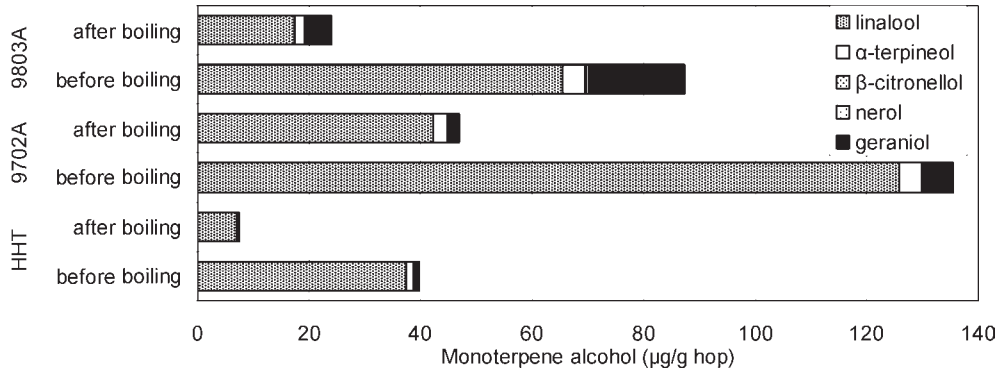

Figure 1. Composition of monoterpene alcohols (μg/g of hop) before and after boiling (HHT, 9702A, and 9803A).

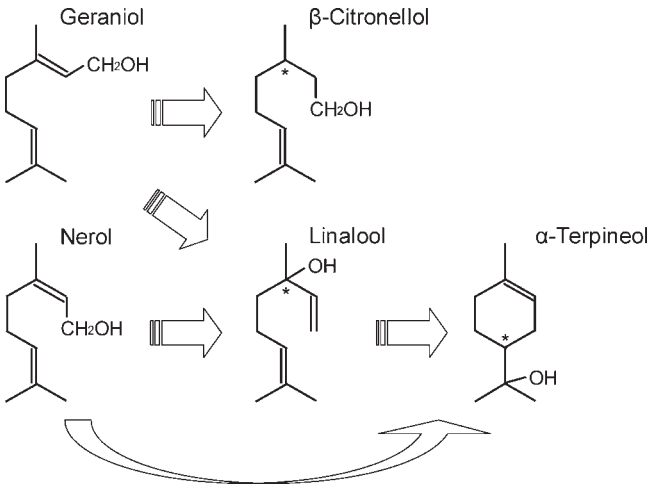

Figure 2. Metabolism cascade of monoterpene alcohols by lager and ale yeast (proposed by King et al. (*13*, *14*)). An asterisk indicates a chiral center.

lost by boiling. However, differences in monoterpene alcohols among hop cultivars were also observed in worts produced under these conditions. During the total fermentation period (primary fermentation and storage (secondary fermentation) periods), linalool and α-terpineol gradually decreased. The contents of linalool and α-terpineol in finished beers became approximately two-thirds of those in worts (**Figure 3A**,**B**). β-Citronellol was not detected in worts and increased up to approximately 1.5 μg/L (**Figure 3C**). Nerol was at a trace level in worts and gradually increased up to approximately 0.5 μg/L (**Figure 3D**). The difference of geraniol content in worts among hop cultivars corresponded to that in each hop. However, the difference in geraniol contents decreased after 3 days of fermentation (**Figure 3E**). At a relatively higher level of geraniol (wort made from the 9803A hop; 3 μg/L), a fast decrease of geraniol was observed. At the lowest level (wort made from the HHT hop; below 1 μg/L), geraniol slightly increased during fermentation. Hanke et al. have also reported that an increase of geraniol was observed during fermentation at lower levels of this compound (*22*).

In the case of late hopping (**Figure 4**), monoterpene alcohols remained at higher levels and differences in these monoterpene alcohols among hop cultivars were just corresponding to those in hops and hop water extracts. The contents of linalool and α-terpineol in worts were at levels more significant than those under kettle-hopping conditions. The contents of these compounds in finished beers were approximately two-thirds of those in worts (**Figure 4A**,**B**). β-Citronellol was at a trace level in the HHT wort and increased up to approximately 1.5 μg/L during the total fermentation period, as well as in the case of kettle hopping. On the other hand, this compound increased up to approximately 4−5 μg/L during fermentation by using the 9702A hop and the 9803A hop (**Figure 4C**). Nerol was detected at a quantifiable level (1−6 μg/L) in worts. However, this compound showed a fast decrease after 3 days of fermentation and a slight increase during the storage period (**Figure 4D**). In the HHT wort, the geraniol content was at a low level (2−3 μg/L) and was almost steady during fermentation. In the 9702A wort and the 9803A wort, geraniol content was at a more significant level (approximately 10−30 μg/L) and the drastic decrease of geraniol during first 3 days of fermentation was also observed at this level. However, approximately 5 μg/L of geraniol remained in the 9702A beer and the 9803A beer (**Figure 4E**). These results suggested that the contents of β-citronellol and geraniol in finished beer might depend on the initial content of geraniol in wort. Surprisingly, in our hopped beer fermentation, the increase of β-citronellol was gently continuing during the total fermentation period and did not correspond to a fast decrease of geraniol. It is suggested that this β-citronellol generation might be dependent on some unknown mechanism, different from simple transformation from geraniol to β-citronellol in model fermentation (*13*, *14*).

Behavior of Monoterpene Alcohols during Fermentation by Using U.S. Commercial Hop Cultivars. We next tried to confirm the behavior of monoterpene alcohols during fermentation by using commercially available hops, which were grown in the U.S. (Cascade, Nugget, and Millennium). We carried out test brewing with these hops according to the late hopping method. The conditions of hop dosage are shown in **Table 1**. **Figure 5** shows the behavior of monoterpene alcohols during fermentation by using these hops. In the Nugget beer and the Millennium beer, the behaviors of monoterpene alcohols were similar to those in the 9702A beer and the 9803A beer. In the Cascade hop, the amount of geraniol in the wort was distinguished (approximately 160 μg/L). A fast decrease of this compound was also observed at this level. However, approximately 20 μg/L of geraniol remained in the finished beer. On the other hand, β-citronellol increased up to approximately 20 μg/L during the total fermentation period. These results suggested that the contents of geraniol and β-citronellol in finished beer might be able to be increased by enriching the initial content of geraniol in the wort: for example, by using a geraniol-rich hop such as Cascade.

Glucoside Hydrolase Activity and Glycosidically Bound Flavor Potential in Fermenting Beer. As described above, the increase of β-citronellol did not correspond to the fast decrease of geraniol and continued during the total fermentation period. We assumed that an occurrence of glycosidically bound flavor precursor might contribute to this increase of β-citronellol. Glycosidically bound flavor compounds are odorless and nonvolatile molecules, which

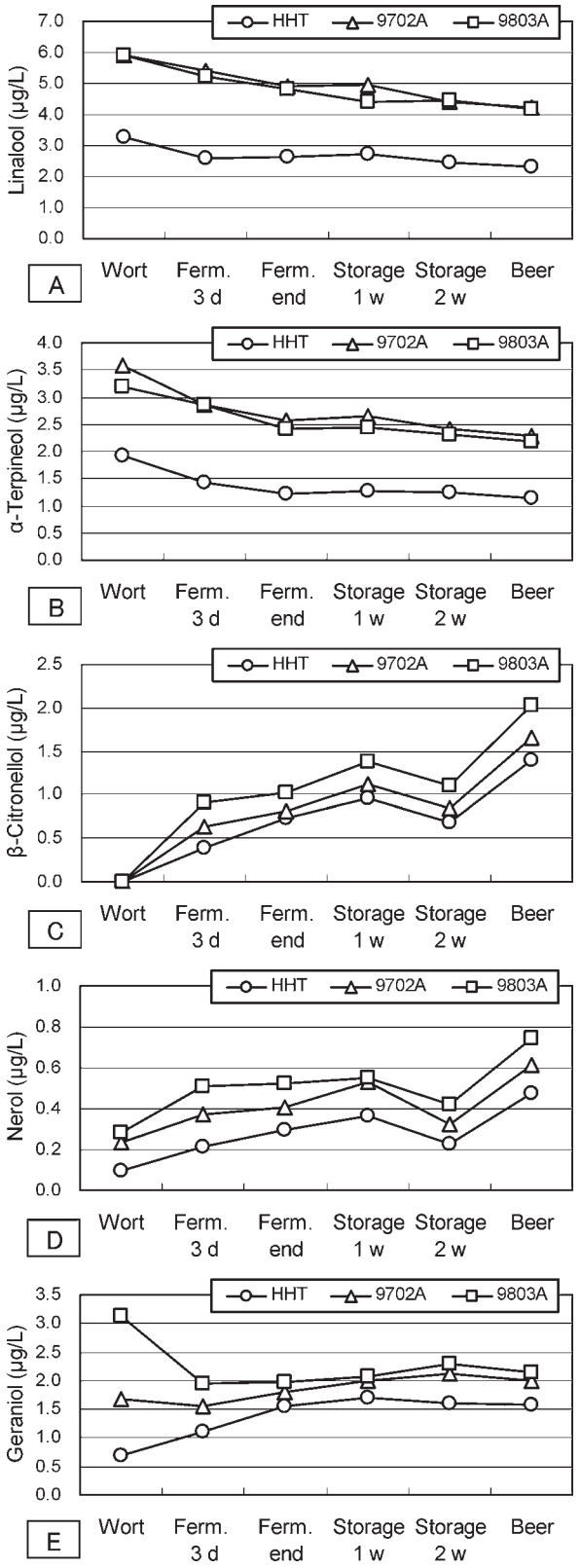

Figure 3. Comparison of monoterpene alcohols (μg/L) during fermentation of kettle-hopped beer (HHT, Hallertauer Tradition; Ferm., fermentation; 3 d, 3 days; 1 w, 1 week; 2 w, 2 weeks): (**A**) linalool; (**B**) α-terpineol; (**C**) β-citronellol; (**D**) nerol; (**E**) geraniol.

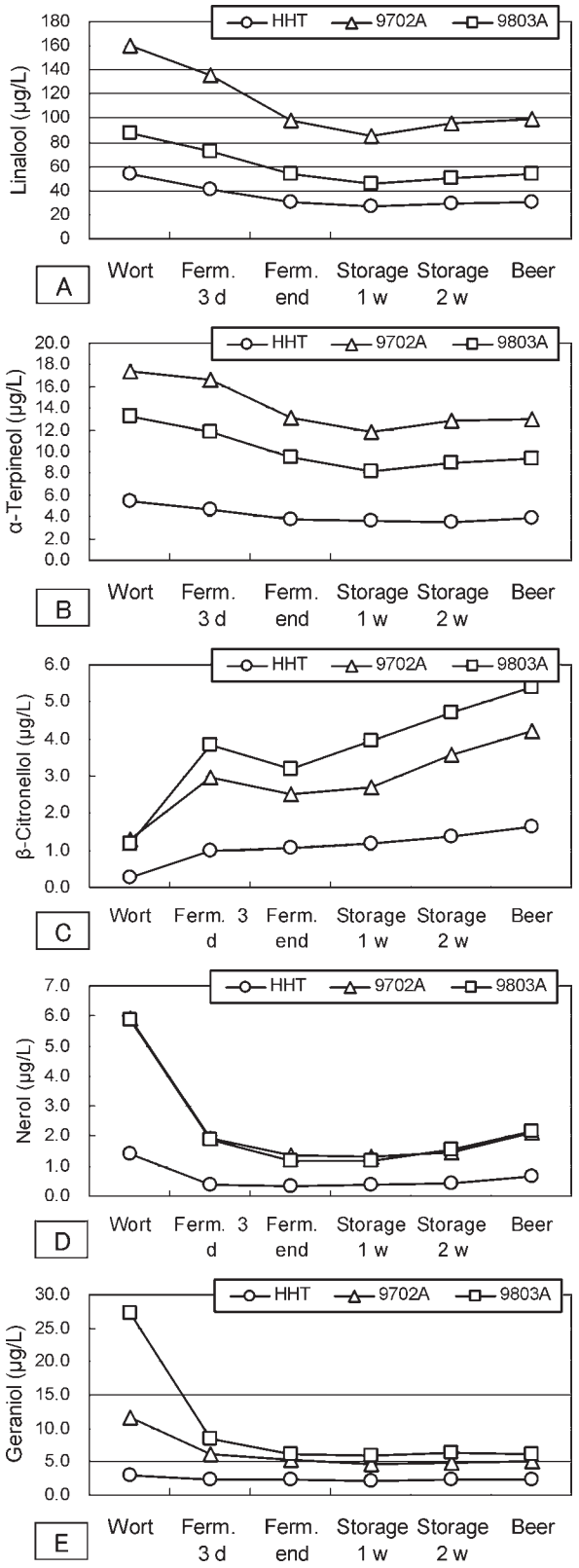

Figure 4. Comparison of monoterpene alcohols (μg/L) during fermentation of late-hopped beer (HHT, 9702A, and 9803A).

consist of an aglycone and a sugar moiety. The aglycone is the volatile compound with flavor activity and includes at least one hydroxyl group. In general, the aglycone and the sugar moiety bind as the *O*-β-D-glucoside or *O*-diglucoside and could be mainly cleaved by β-1,4-glucosidase. Recently, several researchers have reported an occurrence of glycosidically bound flavor compounds, including some monoterpene alcohols as the aglycones, in hops and hopped beers (*23*−*27*). Daenen et al. have reported the variation of glucoside hydrolase activity in various yeasts, including brewing yeasts (belonging to *Saccharomyces cerevisiae*

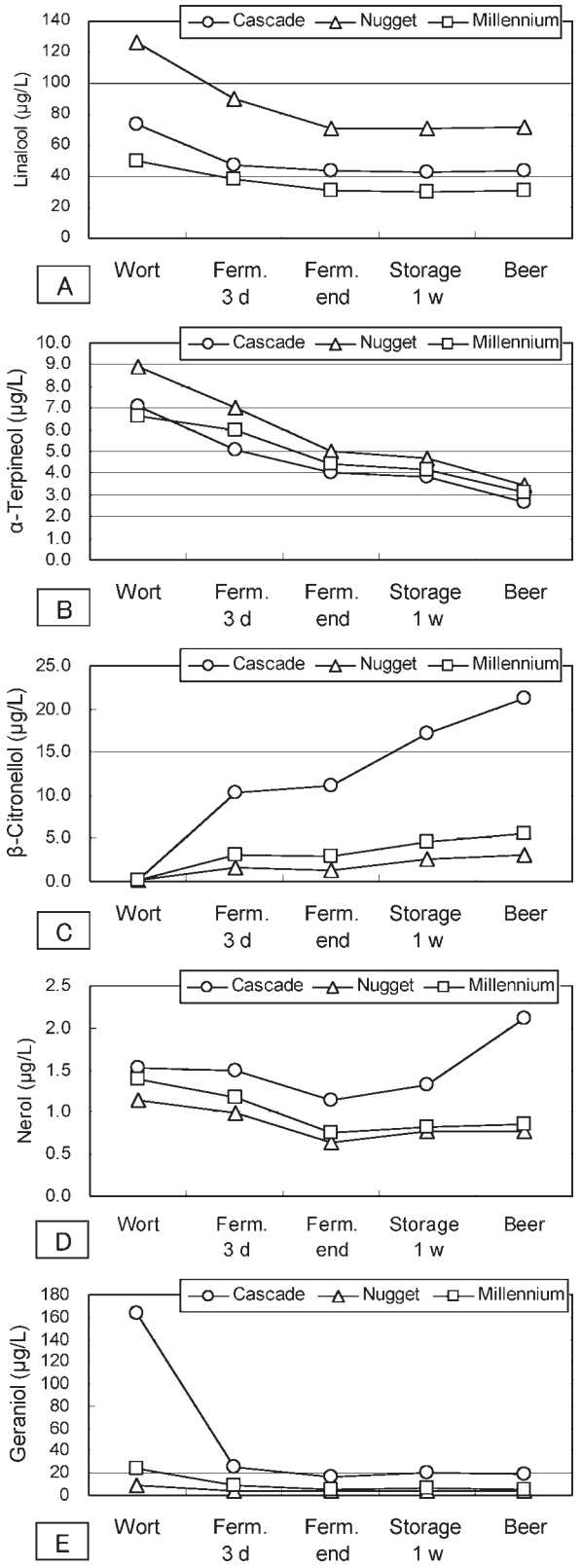

Figure 5. Comparison of monoterpene alcohols (μg/L) during fermentation of late-hopped beer (Cascade, Nugget, and Millennium).

and *Saccharomyces pastorianus*), wine yeasts and *Brettanomyces* yeasts (*28*). As their result, the examined brewing yeasts had no β-1,4-glucosidase. However, these brewing yeasts showed glucoside hydrolase activity depending on *exo*-β-1,3-glucanase. Suzuki et al. have reported that *exo*-β-1,3-glucanase originating from *Saccharomyces cerevisiae* showed broad substrate specificity and

Table 3. Comparison of Glucoside Hydrolase Activity (U/L) during Fermentation

	late hopping					
	HHT	9702A	9803A	Cascade	Nugget	Millennium
ferm 3 days	0.8	0.7	0.8	0.6	0.5	0.5
ferm end	0.8	0.8	0.7	0.8	0.8	0.7
storage 1 week	0.7	0.8	0.7	0.8	0.7	0.7
storage 2 weeks	0.6	0.6	0.6			
beer	0.4	0.4	0.4			

Table 4. Comparison of the Glycosidically Bound Flavor Potentials (μg/L) in Fermenting Beers after Storage for 1 Week[a]

	late hopping					
	HHT	9702A	9803A	Cascade	Nugget	Millennium
linalool	3.7	16.5	8.9	1.0	5.4	0.2
α-terpineol	5.7	8.5	6.1	1.8	1.2	1.4
β-citronellol	0.4	0.9	0.8	0.8	0.1	tr
nerol	0.2	0.4	0.6	tr	tr	tr
geraniol	1.1	1.3	1.5	5.2	0.8	1.2

[a] The glycosidically bound flavor potential is the liberation of each monoterpene alcohol by exogenous β-glucosidase (Sumizyme BGA) for a fermenting beer sample, with late-hopped beer made with each cultivar after storage for 1 week.

could cleave not only β-1,3-glucane but also *p*-nitrophenyl-β-glucopyranoside (pNP-β-Glc), which is one of the synthesized substrates for β-1,4-glucosidase (*29*). It is well-known that the genome of lager yeast (*Saccharomyces pastorianus*) originated from both *Saccharomyces cerevisiae* and *Saccharomyces bayanus*. Therefore, it is considered that lager yeast commonly shows weak glucoside hydrolase activity depending on *exo*-β-1,3-glucanase.

First, in order to confirm the occurrence of glucoside hydrolase activity in our test-brewing beers, we tried to assay glucoside hydrolase activity in fermenting beers by using pNP-β-Glc as a substrate. As a result, 0.4−0.8 U/L of glucoside hydrolase activities was detected in all fermenting beers (**Table 3**). We next tried to evaluate glycosidically bound flavor potential in fermenting beer. It is assumed that the flavor compounds could be released from glycosidically bound precursors with an excess of β-1,4-glucosidase. As described in Materials and Methods, Sumizyme BGA, which is a Japanese commercial enzyme used for flavor enhancement of wines and green teas, was used as the β-1,4-glucosidase. We have confirmed the steady release of monoterpene alcohols at 50 °C for 60−90 min (data not shown). Therefore, we regarded a release of monoterpene alcohols at 50 °C for 60 min as a flavor potential in a sample. We selected the fermenting beer after storage for 1 week as a sample for evaluation of glycosidically bound flavor potential, because the increase of β-citronellol continued from storage for 1 week to the finished beer. As a result, there were flavor potentials of monoterpene alcohols in fermenting beers after storage for 1 week and these potentials varied depending on the hop cultivar (**Table 4**). From these results, β-citronellol could be generated from geraniol, which was released from the glycosidically bound precursor, by yeast metabolism. We think that this mechanism could partly contribute to the gentle increase of β-citronellol.

Perception Thresholds and Characteristics of Monoterpene Alcohols. In the field of brewing science, linalool has been regarded as a major component in hopped beer, and the sensory contribution of this compound has been well investigated. Recently, the threshold of linalool has been estimated at approximately 1−2 μg/L, including the thresholds for (R)-linalool and racemic mixture (30−32). However, the sensory impact of other monoterpene alcohols (α-terpineol, β-citronellol, nerol, and geraniol)

Table 5. Olfactory Descriptions and Perception Thresholds of the Monoterpene Alcohols (μg/L) in Model Solution (5% v/v Ethanol, Carbonated) and Commercial Beer

		olfactory perception threshold[a]			
ref compd	olfactory description	model soln[b]	beer[c]	concn in test-brewing beers[d]	OAV[e] in beer
linalool	lavender	3[f]	5[f]	54−99	11−20
α-terpineol	lilac	450[f]		9−13	
β-citronellol	lemon, lime	9[f]	8[f]	4−5	<1
nerol	rose, citrus	80		2	
geraniol	rose	7	6	5−6	<1

[a] Flavor threshold determined by 10−13 panelists. [b] Model carbonated dilute ethanol solution (5% v/v ethanol, carbonated). [c] Japanese commercial beer. [d] Test-brewing beers made by using 9702A or 9803A (late hopping). [e] OAVs were calculated by dividing the concentrations by the respective thresholds in beer. [f] Determined using racemic mixture.

on hopped beer flavor had not sufficiently been focused on, because thresholds of these compounds were much higher than that of linalool. In previous papers, thresholds of these four monoterpene alcohols had been reported as follows; 330 μg/L for α-terpineol (*33*), 40 μg/L for β-citronellol (*34*), 300 μg/L for nerol (*34*), and 40 μg/L for geraniol (*35*). However, thresholds of β-citronellol and geraniol have been recently estimated at lower levels; 8 μg/L for β-citronellol (*31*) and 4−5 μg/L for geraniol (*31*, *32*). As described above, Lam et al. have speculated that linalool, geraniol, and β-citronellol might contribute to the floral and/or citrus flavor of the beer brewed with late hopping of the Cascade hop (*12*). In our results (**Figure 5**), the late-hopped Cascade beer contained approximately 80 μg/L of linalool, approximately 20 μg/L of geraniol, and approximately 20 μg/L of β-citronellol, and this beer had a floral and/or fruity flavor. In comparison with recently estimated thresholds of linalool, geraniol, and β-citronellol, it seems that these monoterpene alcohols might sufficiently contribute to a fruity flavor of this Cascade beer. However, our panelists recognized that the late-hopped beers made with the 9702A hop and the 9803A hop had not only floral "linalool-like" flavor, but also fruity "citrus" flavor (data not shown). The 9702A beer and the 9803A beer contained only approximately 5 μg/L of geraniol and β-citronellol (**Figure 4**). Therefore, we tried to evaluate contributions of all monoterpene alcohols to hopped beer flavor in detail.

In order to evaluate flavor characteristics of monoterpene alcohols exactly, we determined group thresholds of five monoterpene alcohols in model solution (5% v/v ethanol, carbonated) or in Japanese commercial beer, by using our own panelists. **Table 5** gives olfactory descriptions of terpene alcohols, their group thresholds, their concentrations in our test-brewing beers (the 9702A beer and the 9803A beer, with late hopping), and their odor activity values (OAVs: ratio of concentration to odor threshold) in these beers. In the model solution, group thresholds of monoterpene alcohols were estimated as follows: 3 μg/L for linalool, 450 μg/L for α-terpineol, 9 μg/L for β-citronellol, 80 μg/L for nerol, and 7 μg/L for geraniol (**Table 5**; the thresholds of linalool, α-terpineol, and β-citronellol were determined by using a racemic mixture). The thresholds of α-terpineol and nerol were much higher than those of other three monoterpene alcohols. As described above, the concentrations of α-terpineol and nerol in test-brewing beers were almost lower than those of linalool, β-citronellol, and geraniol (**Figures 3−5**). Therefore, we decided that α-terpineol and nerol might not contribute to hopped beer flavor. The thresholds of linalool, geraniol, and β-citronellol were at similar levels in comparison with recently estimated levels. In beer, group thresholds of these three monoterpene alcohols were estimated by our panelists as follows: 5 μg/L for linalool, 8 μg/L for β-citronellol, and 6 μg/L for geraniol (**Table 5**). It seemed that there might be no masking effect for these monoterpene alcohols with the occurrence of other flavor compounds in beer.

Additive Effect among Linalool, Geraniol, and β-Citronellol. In the 9702A beer and the 9803A beer made with late hopping, linalool was present at a much higher level (54−99 μg/L) than its threshold, and the OAVs of linalool in these beers were calculated at 11−20. On the other hand, the concentrations of β-citronellol and geraniol were approximately 5 μg/L, and the OAVs of these compounds were below 1.0 (**Table 5**). Several researchers reported that certain flavor compounds, which belong to the same class of compounds and have similar odors, demonstrated an additive effect (*36*, *37*). For example, Sarrazin et al. reported that the threshold of 3-sulfanylpentan-1-ol was much higher than those of 3-sulfanylhexan-1-ol and 3-sulfanylheptan-1-ol. However, an additive effect was observed with the coexistence of these three compounds (*37*). In this case, 3-sulfanylhexan-1-ol and/or 3-sulfanylheptan-1-ol, having lower thresholds, seemed to function as a key compound of the observed additive effect. Therefore, we assumed that linalool, having the lowest threshold among monoterpene alcohols, might function as a similar key compound. In order to assess a possible additive effect among linalool, geraniol, and β-citronellol, triangular tests were designed and carried out, as shown in **Tables 6** and **7**.

A control model solution (5% v/v ethanol, carbonated) containing 3 μg/L of linalool (perception threshold of linalool in model solution) was compared with test solutions containing 3 μg/L of linalool together with 5 μg/L of geraniol and/or 5 μg/L of β-citronellol. For evaluation in beer, 5 μg/L of linalool (perception threshold of linalool in beer) was used. The concentrations of geraniol and β-citronellol corresponded to possible occurrence levels of these compounds in the late-hopped test-brewing beers and were lower than their thresholds. In model solution, there were significant differences with risks of 0.1% between the control solution and the test solution containing geraniol and with risks of 1% between the control solution and the test solution containing both geraniol and β-citronellol, and there was no significant difference between the control solution and the test solution containing β-citronellol (**Table 6**). In beer, there were significant differences with risks of 5% between the control solution and the test solution containing β-citronellol and with risks of 5% between the control solution and the test solution containing both geraniol and β-citronellol, and there was no significant difference between the control solution and the test solution containing geraniol (**Table 7**). These results suggested that odors of geraniol and β-citronellol might be enhanced by the occurrence of linalool at a threshold level and that coexistence of geraniol and β-citronellol might be more effective. Therefore, we concluded that there might be an additive effect among three monoterpene alcohols (linalool, geraniol, and β-citronellol) and that only 5 μg/L of geraniol and β-citronellol were enough for this effect. It is suggested that not only linalool but also geraniol and β-citronellol might contribute to hopped beer flavor at lower levels, at which the OAVs of these compounds become below 1.0.

In this study, we have used a commercial racemic mixture of linalool and β-citronellol for sensory evaluation. However, the stereochemistry of these compounds has recently been investigated. In the viewpoint of flavor chemistry, the threshold of (*R*)-linalool was lower than that of the *S* isomer and racemic mixture (*30*, *32*). Kaltner et al. have reported that (*R*)-linalool dominated in various raw hops and that the ratio of *R* isomer was almost above 90% (*38*). The racemization of linalool has been

Table 6. Triangular Test Involving 12 Panelists (in Model Solution (5% v/v Ethanol, Carbonated))

test soln	control soln	correct answers/total answers	*p*
3 μg/L linalool + 5 μg/L geraniol	3 μg/L linalool	10/12	0.001
3 μg/L linalool + 5 μg/L β-citronellol	3 μg/L linalool	5/12	
3 μg/L linalool + 5 μg/L geraniol + 5 μg/L β-citronellol	3 μg/L linalool	9/12	0.01

Table 7. Triangular Test Involving 12 Panelists (in Commercial Beer)

test soln	control soln	correct answers/total answers	*p*
5 μg/L linalool + 5 μg/L geraniol	5 μg/L linalool	5/12	
5 μg/L linalool + 5 μg/L β-citronellol	5 μg/L linalool	8/12	0.05
5 μg/L linalool + 5 μg/L geraniol + 5 μg/L β-citronellol	5 μg/L linalool	8/12	0.05

Table 8. Comparison of the Concentrations of Monoterpene Alcohols (μg/L) in Japanese Commercial Beers

	brand A	brand B	brand C	brand D	brand E
linalool	23.8	28.2	10.8	26.1	69.3
α-terpineol	7.6	10.6	9.3	10.0	8.7
β-citronellol	13.2	20.3	6.4	18.4	45.2
nerol	2.3	2.4	0.7	1.4	6.1
geraniol	7.6	9.0	1.9	4.4	107

observed during brewing process and beer staling. In kettle-hopped beer, *R*/*S* ratios have been near to the racemate. In late-hopped and dry-hopped beers, (*R*)-linalool has dominated (*30*, *32*, *38*). On the other hand, Gramatica et al. have reported that stereospecific reduction of geraniol to (*R*)-β-citronellol occurred with *Saccharomyces cerevisiae* (*39*). From these previous reports, enantiomeric ratios of linalool and β-citronellol in beers are expected to change, depending on the brewing process and the beer staling. The effect of *R*/*S* ratios on the additive effect among monoterpene alcohols should be revealed in the future.

Concentrations of Monoterpene Alcohols in Commercial Beers. **Table 8** shows the concentrations of five monoterpene alcohols in Japanese commercial beers, which were advertised as having "fruity" hop flavor. In all beers, all monoterpene alcohols were at quantifiable and/or significant levels. From our results, linalool, geraniol, and β-citronellol in these beers were expected to contribute to part of the fruity flavor by exceeding their individual threshold and/or by the additive effect among these compounds. Especially brand E, one of the Japanese commercial craft beers, contained a very large amount of geraniol and β-citronellol. The beer type of brand E belongs to IPA (India Pale Ale). This beer is strongly hopped by using the Cascade hop and has a characteristic citrus flavor. We have previously reported that certain volatile thiols, having a grapefruit-like flavor, contributed to the citrus flavor of the beer made with the Nelson Sauvin hop, which is one of the New Zealand hop cultivars (*16*, *18*). However, brand E contained a very low amount of volatile thiols (data not shown). Several researchers have pointed out the occurrence of 4-methyl-4-sulfanylpentan-2-one (4MSP) in the beers made with the Cascade hop as a contributor to cultivar-specific flavor of this hop (*5*, *9*). 4MSP had a box tree like flavor and its threshold was approximately 0.8 ng/L (*40*, *41*). Though the threshold of 4MSP was very low, its flavor character was not citrus. Therefore, we supposed that the citrus flavor derived from the Cascade hop could be partly explained by the coexistence of linalool, geraniol, and β-citronellol.

ABBREVIATIONS USED

HHT, Hallertauer Tradition; GC-MS, gas chromatography−mass spectrometry; U, units; pNP, *p*-nitrophenol; pNP-β-Glc, *p*-nitrophenyl-β-glucopyranoside.

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