Screening of Geraniol-rich Flavor Hop and Interesting Behavior of beta-Citronellol During Fermentation under Various Hop-Addition Timings

Article *in* Journal of the American Society of Brewing Chemists · January 2014

DOI: 10.1094/ASBCJ-2014-0116-01

Kiyoshi Takoi
Sapporo Breweries

Yutaka Itoga
Sapporo Breweries

Junji Takayanagi
Sapporo Breweries

Takayuki Kosugi
Sapporo Breweries

Screening of Geraniol-rich Flavor Hop and Interesting Behavior of β-Citronellol During Fermentation under Various Hop-Addition Timings[1]

Kiyoshi Takoi,[2] *Product & Technology Innovation Department, Sapporo Breweries Ltd., 10 Okatohme, Yaizu, Shizuoka, 425-0013, Japan;* **Yutaka Itoga,** *Bioresources Research & Development Department, Sapporo Breweries Ltd., 3-5-25 Kamifurano-cho Motomachi, Sorachi-gun, Hokkaido, 071-0551, Japan;* **Junji Takayanagi,** *Frontier Laboratories of Value Creation, Sapporo Breweries Ltd.;* **Takayuki Kosugi, Toru Shioi, and Takeshi Nakamura,** *Product & Technology Innovation Department, Sapporo Breweries Ltd.;* **and Junji Watari,** *Value Creation Division, Sapporo Breweries Ltd., 4-20-1 Ebisu, Shibuya, Tokyo, 150-8522*

ABSTRACT

J. Am. Soc. Brew. Chem. 72(1):22-29, 2014

In a previous study, we have focused on biotransformation of hop-derived monoterpene alcohols (linalool, geraniol, β-citronellol, nerol, and α-terpineol) and their contribution to the flavor of hopped beer. Geraniol showed a drastic decrease during the growth phase and a part of geraniol was converted to β-citronellol by yeast. β-Citronellol was almost absent in hop and wort and gradually increased during the total fermentation period. The concentrations of geraniol and β-citronellol in finished beer could be enriched depending on the initial geraniol content in the wort by using a geraniol-rich hop. As a result of sensory evaluation, there was an additive effect among linalool, geraniol, and β-citronellol, and the flavor impression became lime-like by coexistence of these three monoterpene alcohols. In this study, we compared the composition of monoterpene alcohols in various hops, including new flavor hop varieties. Of all screened hops, most of the U.S. hop varieties contained relatively large amount of geraniol. Cascade, Bravo, and Mosaic were screened as geraniol-rich flavor hops. By using these varieties, we brewed test beers under various hop-addition timings to compare the composition of three monoterpene alcohols and total hop flavor profiles in the finished beers. As a result, the content of geraniol in finished beer could increase by delaying the timing of hop addition, while the yeast growth phase was avoided. On the other hand, the β-citronellol contents in the finished beers made with the same hop variety (except Bravo) were present at almost the same levels, regardless of the timings of the hop addition, because of active generation of β-citronellol during the storage period. In addition, interesting behaviors of monoterpene alcohols, especially β-citronellol, observed in this study, and the relationship between the timing of hop addition and the profile of various flavor compounds in beer are discussed.

Keywords: Beers, Biotransformation, β-Citronellol, Flavor, Geraniol, Hop-addition timing, Hops, Linalool, Monoterpene alcohols

RESUMEN

En un estudio previo nos habíamos concentrado en la biotransformación de alcoholes monoterpénicos derivados del lúpulo (linalol, geraniol, β-limonelol, nerol y α-terpinol) y su contribución al sabor de cerveza lúpulado. Se observó una disminución drástica de la concentración del geraniol durante la fase de crecimiento; una parte del geraniol se convirtió en β-limonelol por la levadura. El β-limonelol estuvo prácticamente ausente en el lúpulo y en el mosto pero gradualmente fue aumentando durante la fermentación. La concentración de geraniol y de β-limonelol en la cerveza terminada pudo ser enriquecida, dependiendo de la concentración inicial del geraniol en el mosto, utilizando un lúpulo rico en geraniol. Una evaluación sensorial demostró que se tiene un efecto aditivo entre linalol, geraniol y β-limonelol; se obtuvo un sabor parecido al de la lima por coexistir estos tres alcoholes monoterpénicos. En este estudio se comparó la composición de alcoholes monoterpénicos de diferentes lúpulos, incluyendo nuevas variedades de lúpulo de sabor nuevo. De todos los lúpulos estudiados, la mayoría de las variedades de lúpulo de los EE.UU. contenían una cantidad relativamente alta de geraniol. Se encontró que Cascade, Bravo y Mosaic eran ricos en geraniol. Se utilizaron estas tres variedades para elaborar cervezas de prueba usando diferentes tiempos de adición del lúpulo para comparar la composición de tres alcoholes monoterpénicos y el perfil sensorial total del lúpulo en las cervezas terminadas. El contenido de geraniol en la cerveza terminada pudo ser aumentado atrasando el momento de su adición a la cerveza, evitando la fase de reproducción de la levadura. El contenido de β-limonelol en las cervezas terminadas elaboradas con la misma variedad de lúpulo (a excepción de Bravo) estuvo presente a casi el mismo nivel sin importar el momento de la adición del lúpulo, debido a la generación activa de β-limonelol durante la maduración. También se comenta el comportamiento interesante de los alcoholes monoterpénicos, especialmente el β-limonelol, observado en este estudio, además de la relación entre el momento de la adición del lúpulo y el perfil de varios compuestos sensoriales en la cerveza terminada.

Palabras claves: Alcoholes monoterpénicos, Biotransformación, Cerveza, Geraniol, β-Limonelol, Linalol, Lúpulo, Momento de adicionar el lúpulo, Sabor

In recent years, many craft brewers have been brewing unique beers, made with various characteristic hops, all around the world. These hops (for example, Cascade, Nelson Sauvin [NNS], Citra, and so on) give citrus or exotic fruit-like flavors to finished beer. Now, a group of such characteristic hops are categorized as "flavor hop" (18,30). A beer brewed with such a flavor hop has very unique "varietal aroma." However, key flavor compounds contributing to the varietal aroma of each flavor hop have not been fully investigated yet, because most such hops are newer varieties than traditional aroma hop or bitter hop varieties.

Cascade is a relatively old hop variety among flavor hop varieties. In the 1980s, Peacock et al (23) and Lam et al (17) proposed floral or fruity monoterpene alcohols (linalool, geraniol, and β-citronellol) and their derivatives (geranyl acetate and geranyl isobutyrate) as key flavor compounds for the variety-specific aroma of Cascade. Recently, Steinhaus et al (32,33), Kishimoto et al (12,15), and Morimoto et al (19) have reported that 4-methyl-4-sulfanylpentane-2-one (4MSP) was detected in Cascade hop and beer. In addition, Kishimoto et al (13) and Gros et al (8,9) pointed out that Cascade contained a relatively high amount of 3-sulfanylhexan-1-ol (3SH). Volatile thiols 4MSP (box tree-like odor) and 3SH (grapefruit-like odor) have very low odor thresholds and are well-known as key compounds contributing to the varietal aroma of Sauvignon Blanc wine (4,29,39,40).

[1] A preliminary report of some of this work was given at the World Brewing Congress 2012, Portland, OR, July–August 2012.

[2] Corresponding author. Phone: +81 54 629 7989; Fax: +81 54 629 3144; E-mail: kiyoshi.takoi@sapporobeer.co.jp

http://dx.doi.org/10.1094/ASBCJ-2014-0116-01

The hop NNS (1,7) has been bred in New Zealand and released in 2000. This hop imparts Sauvignon Blanc wine-like flavor to finished beer. Our group has found two novel volatile thiols, 3-sulfanyl-4-methylpentan-1-ol (3S4MP) and 3-sulfanyl-4-methylpantyl acetate (3S4MPA), present in NNS hop and beer (35,36). These two thiols have grapefruit-like odor, similar to that of 3SH. In contrast to Cascade, 3S4MP was much more dominant than 3SH in NNS hop and beer (8,9,35,36). It has been also reported that 3S4MP could enhance the flavor intensity of other hop-derived flavor compounds—for example, 3S4MPA, isobutyric ester (2-methylbutyl isobutyrate), linalool, and geraniol—and that 3S4MP might contribute to NNS varietal aroma as a key compound having two roles: its own characteristic flavor and its function as a flavor enhancer (35).

Citra (26,28) was bred in the United States and released in 2007. The beer made with this hop has a complicated fruity flavor, somewhat reminiscent of lime, lychee, gooseberry, and others. We have found that Citra contained very high amounts of geraniol (37).

In previous studies, we focused on biotransformation of hop-derived monoterpene alcohols (linalool, geraniol, β-citronellol, nerol and α-terpineol) (Fig. 1) and their contribution to the flavor of hopped beer. As a result, the β-citronellol was almost absent in hop and wort and gently increased during fermentation, because of the biotransformation from geraniol to β-citronellol by brewing yeast. Though, in general, the geraniol drastically decreases during the first 3–4 days of fermentation, the concentration of geraniol and β-citronellol in beer could be enriched depending on the initial geraniol content in the wort; for example, by using geraniol-rich hop Citra (37,38).

In addition, we found that there was an additive effect among linalool, geraniol, and β-citronellol (38) and that the flavor impression became lime-like by coexistence of these three monoterpene alcohols (37). Therefore, we proposed that the lime-like character of Citra beer might be due to synergy among linalool, geraniol, and β-citronellol (37).

In this study, we compared the composition of monoterpene alcohols in various hops, including new flavor hop varieties, and brewed test beers under various hop-addition timings with selected geraniol-rich flavor hop varieties.

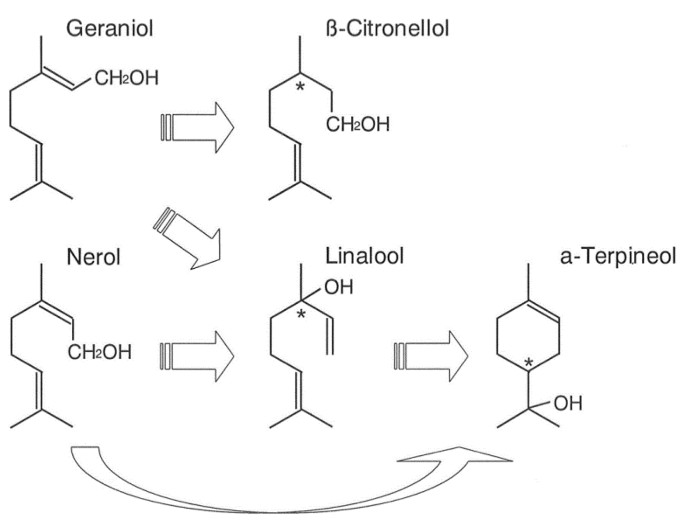

Fig. 1. Metabolism cascade of monoterpene alcohols by lager and ale yeast proposed by King et al (10,11). The asterisk indicate a chiral center. (This figure was previously shown by Takoi et al [37,38]).

MATERIAL AND METHODS

Hop Raw Materials

Hallertauer Tradition (HHT) was grown and pelletized in Germany in 2007 and 2009 (type 90 pellet). Hallertauer Magnum (HHM) was grown and harvested in Germany in 2007 (type 90 pellet). Saaz (CSA) was grown and harvested in the Czech Republic in 2007 and 2009 (type 90 pellet). Hops 9702A and 9803A were bred and grown in Japan (Bioresources Research & Development Department, Sapporo Breweries, Ltd.) in 2007 (hop powder). Pacifica (named Pacific Hallertau until 2007) and NNS were harvested and pelletized in New Zealand in 2007 (type 90 pellet). Nugget and Millennium were grown in the United States in 2007 (type 90 pellet). Cascade was harvested in the United States in 2007 (type 90 pellet) and 2008 (hop powder). Citra was bred and grown in the United States in 2007 and 2008 (type 90 pellet). Amarillo, Apollo, Bravo, Chinook, Glacier, Mosaic, Mt. Hood, Palisade, Simcoe, and Willamette were grown and harvested in the United States in 2008 (hop powder).

Pilot-Scale Brewing

Beers from the Cascade hop, the Bravo hop, or the Mosaic hop were made 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-L pilot-scale apparatus. Boiling period was 90 min. For prevention of over boiling, HHT hop was added at the beginning of boiling (hop at 0.2 g/L). Cooled wort was collected to fermentation tanks (30 L/tank) and media bottles (900 mL/bottle). For each hop flavoring, 24.8 g of hop was added to each bottle (sealed with aluminum foil) and autoclaved at 105°C for 5 min. After cooling, the hop-flavored wort sample was mixed with 30 L of wort in each fermentation tank. This condition corresponded to that of the late-hopping, with hop at 0.8 g/L. Subsequently, the fermentation was started by adding lager yeast (brewery-collected *Saccharomyces pastorianus*) at 15.0×10^6 cells/mL to the wort (timing 1). For timing 2 and 3, the fermentation was started without addition of hop-flavored wort. The temperature of the fermentation was maintained at 10–12°C (primary fermentation) for 7 days. After fermentation, the fermented wort was transferred to another storage tank under a CO_2 atmosphere and the maturation was carried out at 13°C for 8 days, then at 0°C for 2–3 weeks. For timing 2 and 3, the hop-flavored sample wort was added to each fermentation tank in the third day of the primary fermentation period and at the end of primary fermentation, respectively. Filtration and bottling were done using the pilot-scale equipment under air-free conditions.

No malt beers using the CSA hop were made following the recipe of Japanese third-category beer. Briefly, the wort was prepared using syrup and hop in a 400-L pilot-scale apparatus. Boiling period was 60 min. The CSA hop was added at the beginning of boiling (0.5 g/L) and 5 min before the end of boiling (0.5 g/L). After cooling, the fermentation was started by adding lager yeast at 30.0×10^6 cells/mL to the wort as a control. For the test, the fermentation was started with four-fifths volume of the wort prepared by the same recipe, except for the hopping condition, in which CSA hop (0.5 g/L) was added only at the beginning of boiling. The temperature of the fermentation was maintained at 15°C. In the fifth day of primary fermentation, another one-fifth volume of a late-hopped wort (CSA hop at 2.5 g/L was added at the end of boiling) was mixed with the previous four-fifths volume of the test-fermenting wort. After fermentation, the fermented wort was transferred to another storage tank under a CO_2 atmosphere and the maturation was carried out at 15°C for 2 days, then at 0°C for 2–3 weeks. Filtration and bottling were done using the pilot-scale equipment under air-free 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. Geraniol (98%) was purchased from Aldrich Chemical Company Inc.. Isobutyl isobutyrate (>98%), isoamyl isobutyrate (>98%), isobutyric acid (>98%), and 2-methylbutan-1-ol (>97%) were purchased from Wako Pure Chemical Industries, Ltd. 2-Methylbutyl isobutyrate (>97%) was synthesized by esterification of isobutyric acid and 2-methylbutan-1-ol, as previously described (35). Ethyl isobutyrate (>98%) and ethyl isovalerate (>97%) were purchased from Wako Pure Chemical Industries, Ltd. Ethyl 2-methylbutyrate (>98%, racemic mixture) was purchased from Tokyo Chemical Industry Co., Ltd.

Quantification of Flavor Compounds by GC-MS

GC-MS analyses were carried out using a 6890N gas chromatograph (Agilent Technologies). The carrier gas was helium, with a column-head pressure of 15 psi and 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 hop, 20 mg of ground hop was directly put into a 20-mL glass vial. For analysis of wort, fermenting beer, and finished beer, 8 mL of each sample was put 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). After preincubation, an SPME fiber (polydimethylsiloxane, 100-μm film thickness; Supelco) 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 (30 m, 0.25-mm i.d., 1.0-μm film thickness) (Agilent Technologies). 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. Ethyl isobutyrate, ethyl isovalerate, and ethyl 2-methylbutyrate were quantified in the SIM mode, selecting *m/z* 116, 88, and 102, respectively. Calibration curves were determined using water (including 5% ethanol) containing these terpenoids and esters at final concentrations ranging from 0 to 10 μg/L. All calibration produced a linear response with an R^2 value >0.98 over the concentration range analyzed.

RESULTS

Screening of Geraniol-rich Flavor Hops

We have previously pointed out that geraniol might be more variety specific than linalool (37,38). German traditional aroma hop varieties and CSA hop contained very low amounts of geraniol and Cascade, one of the U.S. hops, contained relatively large amounts of geraniol (15,32,33). In a previous report, we screened various hops, including German hops, American hops, and Japanese hops, and picked up not only Cascade but also Citra, released in 2007 by the Hop Breeding Company in the United States (26,28), as geraniol-rich hops (37).

In recent years, craft brewers have used various flavor hops (for example, Cascade, Citra, and newly bred hop varieties) to impart unique fruity flavors to the finished beers. Such hops have been bred and grown in the United States, New Zealand, and Australia.

We demonstrated (Fig. 2) that European hop varieties (HHT, HHM, and CSA) contained very small amounts of geraniol. New Zealand hop varieties (NNS and Pacifica) contained relatively low levels of geraniol. On the other hand, most American hop varieties, including Cascade, Citra, Mt. Hood, Mosaic, Chinook, Apollo, Amarillo, and others, contained relatively large amounts of geraniol. In particular, the geraniol level in Bravo and Mosaic were higher than that in Cascade and Citra.

Although the geraniol contents in Cascade and Citra varied between different crop years, the geraniol-rich hop varieties selected still contained relatively high amounts of geraniol regardless of the crop year.

Effect of Hopping Condition on the Behavior of Monoterpene Alcohols during Fermentation

Of the geraniol-rich hop varieties screened in Figure 2, we selected Cascade, Bravo, and Mosaic for evaluating the effect of hopping condition on the behavior of monoterpene alcohols during fermentation. Cascade is one of the popular flavor hop varieties. Bravo is found as the most geraniol-rich hop in Figure 2. Mosaic is the newest flavor hop variety released in 2012 (27). In previous articles, we reported the behavior of monoterpene alcohols during fermentation by using geraniol-rich hop, Citra (37). In this study, we expanded to Cascade, Bravo, and Mosaic and observed the behavior of monoterpene alcohols during fermentation with varying initial geraniol contents. We also reported that the

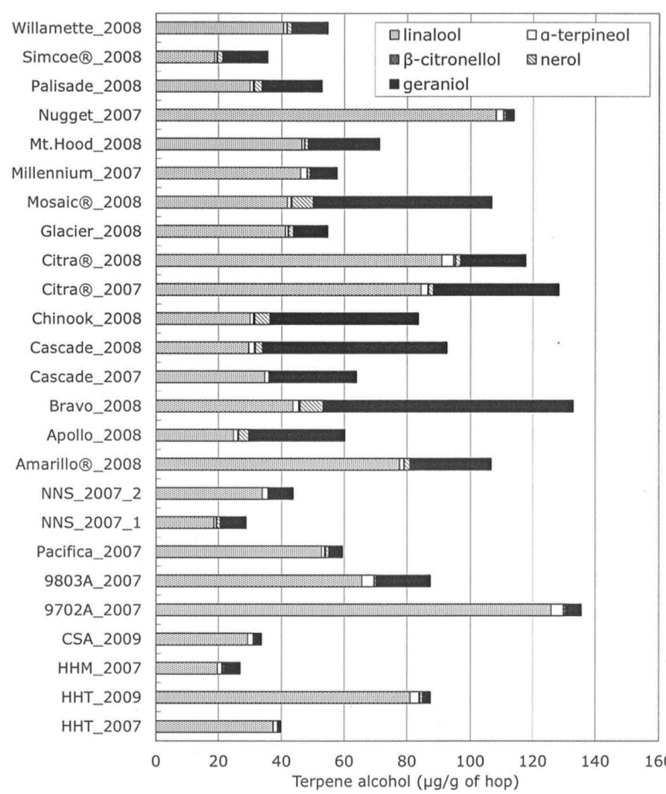

Fig. 2. Composition of monoterpene alcohols (μg/g of hop) in various hops: HHT, Hallertauer Tradition; HHM, Hallertauer Magnum; CSA, Czech Saaz; NNS, New Zealand Nelson Sauvin. (Data of the HHT_2007, 9702A_2007, and 9803A_2007 were previously reported by Takoi et al [37]. Data of the HHM_2007, Cascade_2007, Millennium_2007, Nugget_2007, and Citra_2008 were previously reported by Takoi et al [38]).

geraniol content in wort showed a drastic decrease in first 3 days of the fermentation (37,38). Therefore, we set out to compare the behavior of monoterpene alcohols among various timings of hop flavoring.

The behavior of linalool, β-citronellol, and geraniol during fermentation with Cascade_2008 is shown in Figure 3. Linalool gradually decreased from wort to finished beer. The behaviors of linalool during the storage period were nearly indistinguishable between different hop-addition timings. The initial linalool levels in timing 2 (the third day of fermentation) and timing 3 (the end of primary fermentation) were lower than the level in the wort of timing 1, even though the same amount of Cascade hop was added. The initial geraniol content in timing 1 was 65 µg/L. The initial geraniol levels in timing 2 and timing 3 were not at the same level in the wort but were higher than that in timing 1. During the storage period, geraniol slightly increased and the geraniol contents in the finished beer reached 31 µg/L in timing 1, 37 µg/L in timing 2, and 53 µg/L in timing 3. The contents of β-citronellol just after hop addition were at trace levels in all timings and gradually increased. Interestingly, the levels of β-citronellol in the finished beers were all at relatively the same levels among all hop-addition timings.

The behavior of monoterpene alcohols during fermentation with Bravo hop, which is the geraniol-richest hop in Figure 2, is shown in Figure 4. The initial linalool content in timing 2 and 3 were slightly lower than that in timing 1. However, the behaviors of linalool during the storage period and the finished beers were nearly indistinguishable in all hop-addition timings. The initial geraniol content in timing 1 was approx. 270 µg/L. The initial geraniol levels in timing 2 and 3 were lower than that in the wort in timing 1. However, the geraniol levels in timing 2 and 3 became higher than those in timing 1 after fermentation. During the storage period, the geraniol increased and reached a content of geraniol of 76 µg/L in timing 1, 132 µg/L in timing 2, and 170 µg/L in timing 3 in the finished beers. The contents of β-citronellol just after hop addition were at trace levels in all timings and gradually increased. The β-citronellol in the finished beers reached almost the same levels, approx. 50 µg/L in timing 1 and 2 but 27 µg/L in timing 3.

The behavior of monoterpene alcohols during fermentation with Mosaic hop, which is a new flavor hop variety released in 2012 in the United States (27), is shown in Figure 5. This hop imparts very comprehensive fruity flavor to finished beer, perceived as rose-like, lime-like, gooseberry-like, mango-like, and others (27). This variety was formally called HBC 369 and the 2008 crop was used for this study. Because the amount of sample hop was limited, hop was used at 0.53 instead of 0.8 g/L for tim-

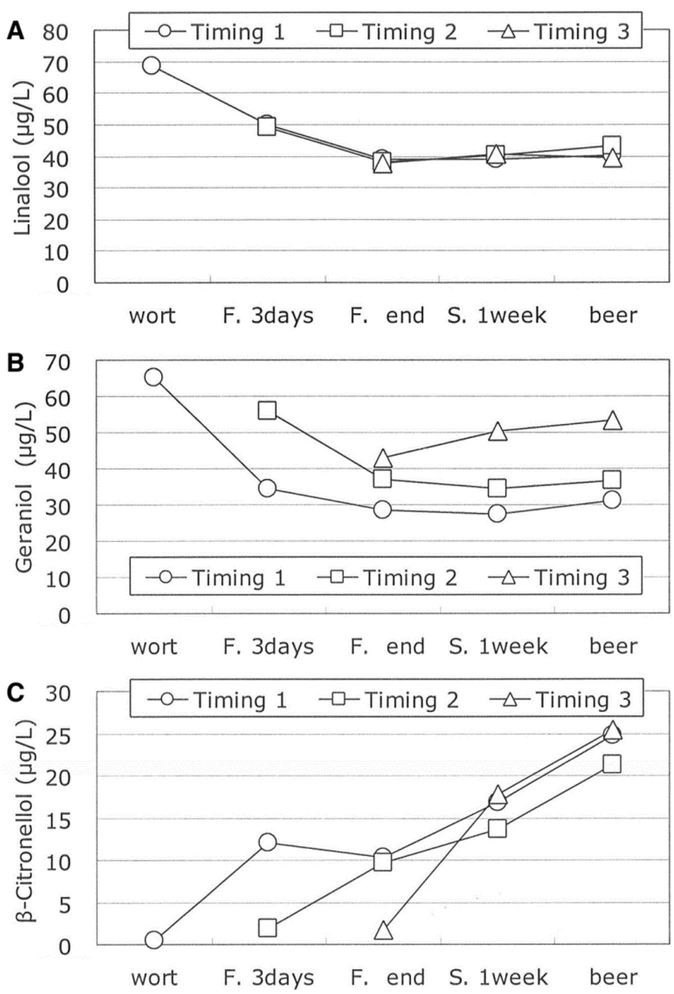

Fig. 3. Comparison of monoterpene alcohols (µg/L) during fermentation by using Cascade hop under various hop-addition timings: F., fermentation; S., Storage; Timing 1, hop-addition before yeast pitching; Timing 2, hop-addition at the third day of fermentation; Timing 3, hop-addition at the end of fermentation; **A,** linalool; **B,** geraniol; **C,** β-citronellol.

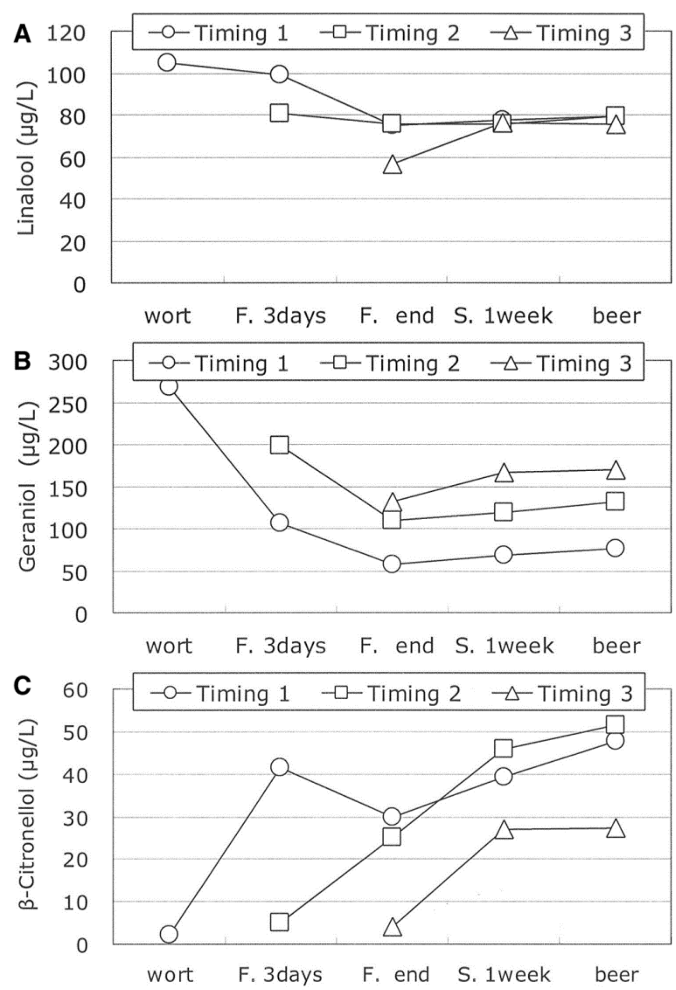

Fig. 4. Comparison of monoterpene alcohols (µg/L) during fermentation by using Bravo hop under various hop-addition timings; **A,** linalool; **B,** geraniol; **C,** β-citronellol.

ing 3. Therefore, the data obtained from timing 3 were adjusted according to the amount of other hop additions. The behaviors of linalool were nearly indistinguishable among different hop-addition timings. The difference in linalool contents between the wort and the finished beer was less than that in the case of other two varieties. The initial geraniol content in timing 1 was approx. 150 μg/L but decreased significantly during the rest of the stages. During the storage period, the geraniol increased slightly in timing 2 and 3. The geraniol contents in the finished beers reached 55 μg/L in timing 1, 78 μg/L in timing 2, and 127 μg/L in timing 3. The contents of β-citronellol right after hop additions were at trace and low levels in all timings and gradually increased after the fermentation. The levels of β-citronellol in the finished beers reached to nearly the same levels, approx. 30 μg/L, among all hop-addition timings, similar to the case of Cascade.

The results using the geraniol-rich flavor hops (Cascade, Bravo, and Mosaic) suggested that the content of geraniol in the finished beer could be increased by delaying the timing of hop addition, while the contents of linalool and β-citronellol could be kept.

On the other hand, the geraniol content in CSA hop is very low (Fig. 2). We then investigated the effect of hop-addition timings on the behavior of monoterpene alcohols during fermentation with CSA hop. As described in Materials and Methods for the malt-free beer, the flavoring hop was added at the end of boiling as "Control" and in the sixth day of fermentation as "Test". As a result (Fig. 6), all three monoterpene alcohols were present at lower levels with three hop addition timings than those in Figures 3–5 and also showed an insignificant difference between Control and Test. The initial geraniol content in the wort was at approx. 3 μg/L. Geraniol decreased in the third day and slightly increased during the storage period. The β-citronellol was at trace levels in the wort and gradually increased. The β-citronellol content reached 7 μg/L in the finished beers. According to the previous study (38), when the initial geraniol level was present at approx. 3 μg/L, the β-citronellol level in the finished beer might be expected to appear at 1–2 μg/L. In other evidence, we have found that approx. 5 μg/L of glycosidically bound geraniol potential (38) remained in the Japanese commercial beer made with CSA hop (*data not shown*). Therefore, we assumed that this geraniol potential in CSA hop could contribute to the increase of β-citronellol during fermentation.

This result of using the CSA hop demonstrated that delaying the timing of the hop addition had less effect on the behavior of monoterpene alcohols during fermentation in the case of geraniol-poor hops.

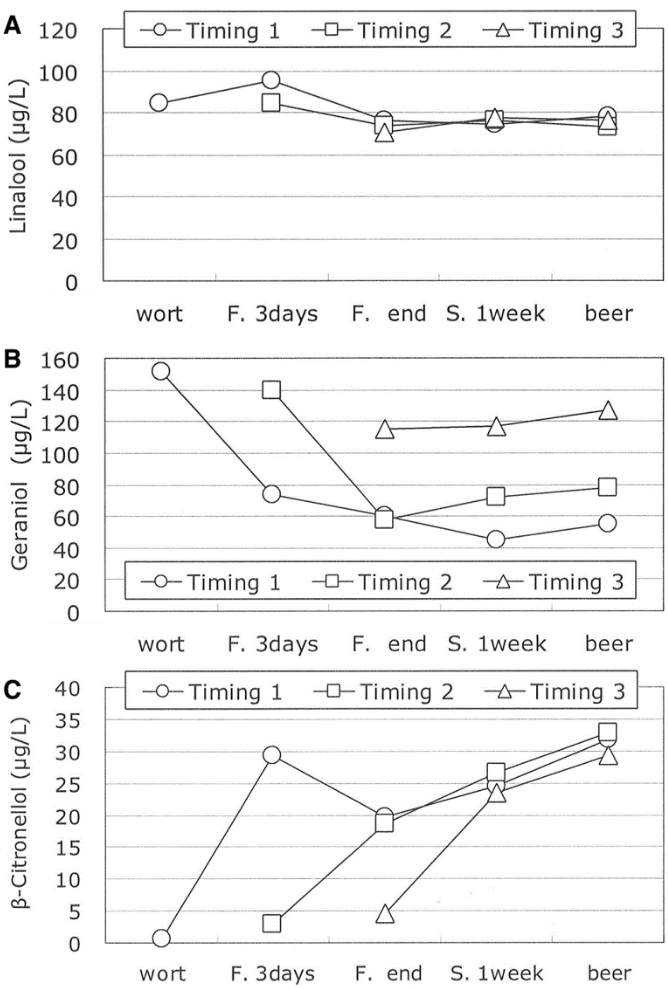

Fig. 5. Comparison of monoterpene alcohols (μg/L) during fermentation by using Mosaic hop under various hop-addition timings; **A,** linalool; **B,** geraniol; **C,** β-citronellol.

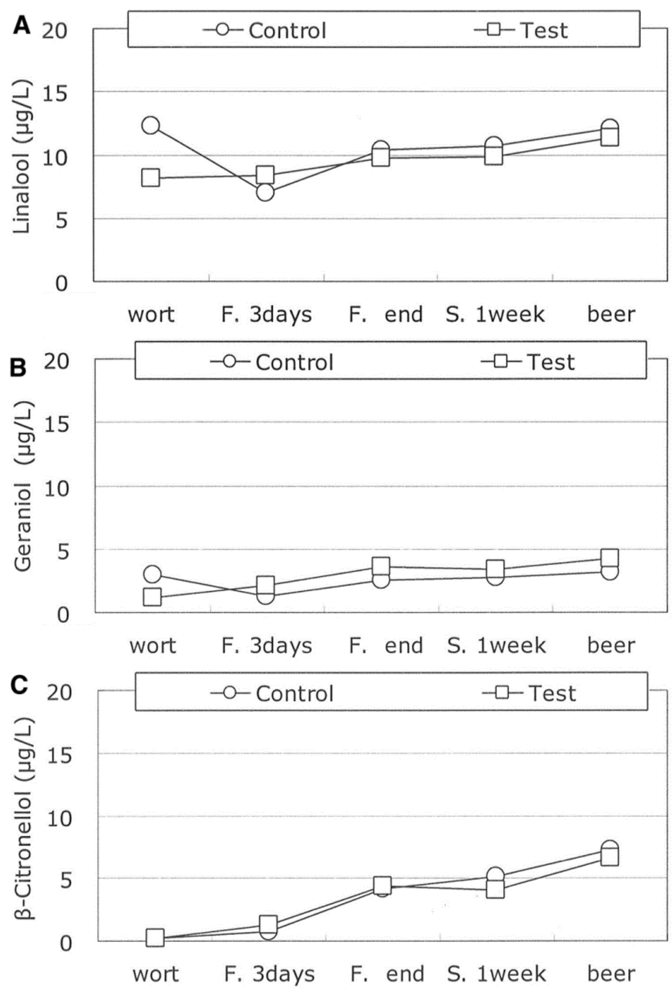

Fig. 6. Comparison of monoterpene alcohols (μg/L) during fermentation by using CSA hop under various hop-addition timings: Control, hop-added at the end of boiling; Test, hop-added at the fifth day of fermentation; **A,** linalool; **B,** geraniol; **C,** β-citronellol.

The Profile of Flavor Compounds in Beer made with Various Hop-Addition Timings

As described above, the variation in the timing of the hop addition could affect the composition of three monoterpene alcohols in the finished beer. On the other hand, it is expected that the time variation of the hop addition could also change the profile of other flavor compounds in beer. Therefore, we analyzed the total profiles of various flavor compounds in all test-brewed beers, including three monoterpene alcohols, terpene hydrocarbons (humulene and myrcene), isobutyric esters (isobutyl isobutyrate, isoamyl isobutyrate, and 2-methylbutyl isobutyrate), and ethyl esters of branched short-chain fatty acids (ethyl isobutyrate, ethyl 2-methylbutyrate, and ethyl isovalerate).

Analytical data of hop-derived flavor compounds in test-brewed beers made with Cascade, Bravo, and Mosaic are shown in Table I. Of the terpene hydrocarbons, the contents of humulene were not significantly changed among various hop-addition timings. The contents of myrcene were at relatively the same levels between timings 1 and 2 but drastically increased in timing 3 in the finished beers, as expected. The contents of isobutyric esters present in the finished beers gradually increased from timing 1 to timing 3. The compositions of isobutyric esters widely varied depending on the hop varieties. The Mosaic beer in timing 3 contained the largest amount of these esters. The compositions of ethyl esters of branched short-chain fatty acids in the finished beers were low and insignificantly different among various hop-addition timings in Table I.

DISCUSSION

The Relationship between Geraniol Metabolism by Yeast and the Timing of Hop Addition

As our goals, we focused on biotransformation of hop-derived monoterpene alcohols (Fig. 1) in the finished beers, with three different hop addition times. It has been established that the geraniol present in the initial wort was drastically decreased during first 3–4 days of fermentation, whereas the β-citronellol was almost absent in hop and wort but gradually increased during fermentation, because of the conversion of geraniol to β-citronellol by brewing yeast. The concentration of geraniol and β-citronellol in the finished beer could be enriched, depending on the initial geraniol content in the wort, by using the geraniol-rich hops (37,38).

These results indicated that the content of geraniol in the finished beer could be increased by delaying the timing of the hop addition. Therefore, we have screened and selected several geraniol-rich flavor hop varieties (Fig. 2) and brewed test beers to study the effect under various hop-addition timings (Figs. 3–6).

The Behavior of Linalool under Varying Timings of Hop Addition

Linalool contents during primary fermentation were slightly different among hop-addition timings (Figs. 3–5). The initial linalool levels in timing 2 (the third day of fermentation) and timing 3 (the end of primary fermentation) were lower than that in the wort of timing 1. In this study, the hop-flavored wort was added to the cooled wort before the yeast pitching for timing 1, and added to the fermenting wort containing active yeast for timings 2 and 3. Regardless, the fermenting yeast was quickly removed from each sample wort by centrifugation; however, a contact of wort with yeast might have an effect on certain flavor compounds; for example, adsorption on the yeast surface or consumption by the yeast metabolism. Nevertheless, the linalool contents during the storage period as well as in the finished beer were present at relatively the same levels among all timings in all tests.

In addition, the linalool behaved differently among three hop varieties. The linalool contents in the finished Cascade beers were reduced by approx. 60% based on the concentration in the initial wort (Fig. 3) whereas, in the Bravo beers, the linalool contents were decreased by approx. 20% (Fig. 4). Unexpectedly, the linalool contents in Mosaic beers were not varied significantly in all stages (Fig. 5). King et al have reported that the total amount of monoterpene alcohols was decreased during a model fermentation (10,11). We have also showed that the amount of linalool decreased during fermentation of hopped beer brewed with several hop varieties (38). It has been reported that the occurrence of glycosidically bound flavor precursors in hops (2,5,6,16,21) and hydrolysis by the secretion of the hydrolyzing enzymes (glucoside hydrolases) by yeast (3,34,38). The balance between the linalool consumption by yeast and the linalool generation from a glycosidically bound precursor might contribute to the variety specificity of the behavior of linalool during the fermentation.

The Behavior of Geraniol under Varying Timings of Hop Addition

The initial geraniol levels in timing 2 and timing 3 were relatively lower than that in the wort of timing 1 (Figs. 3–5). Similar to the case of linalool, a part of the geraniols might be adsorbed on the yeast surface or consumed by yeast metabolism. In general, the geraniol could be used for ergosterol biosynthesis by yeast (41) and showed a drastic decrease during the growth phase (during the first 2–4 days of fermentation) (10,11,37,38). There-

TABLE I
Comparison of the Contents of Volatile Compounds (μg/L) in the Finished Beer Among Various Hop-Addition Timings

	Cascade			Bravo			Mosaic		
	Timing 1	Timing 2	Timing 3	Timing 1	Timing 2	Timing 3	Timing 1	Timing 2	Timing 3
humulene (μg/L)	0.5	0.5	0.8	1.0	0.7	0.8	1.2	0.8	1.8
myrcene (μg/L)	6.0	6.3	32.1	52.2	93.0	165	41.2	55.1	272
linalool (μg/L)	40.1	43.2	39.4	79.4	79.2	75.8	78.0	73.2	76.3
α-terpineol (μg/L)	3.3	3.1	3.2	1.7	2.0	2.2	1.6	1.6	2.7
β-citronellol (μg/L)	24.7	21.3	25.3	47.8	51.7	27.4	31.9	32.8	29.3
nerol (μg/L)	2.2	2.4	2.5	14.3	17.7	17.3	11.1	12.9	16.9
geraniol (μg/L)	31.1	36.6	53.2	76.0	132	170	55.2	78.1	127
isobutyl isobutyrate (μg/L)	3.2	3.9	5.2	9.7	11.3	13.0	16.9	18.4	24.9
isoamyl isobutyrate (μg/L)	3.4	4.2	5.8	10.9	13.6	14.1	22.1	23.8	35.6
2-methylbutyl isobutyrate (μg/L)	11.1	12.9	18.0	65.7	77.2	78.7	52.8	55.4	81.6
ethyl isobutyrate (μg/L)	-	-	-	5.7	6.6	4.9	5.7	6.5	7.8
ethyl 2-methylbutyrate (μg/L)	-	-	-	1.0	1.1	1.0	0.7	1.0	1.0
ethyl isovalerate (μg/L)	-	-	-	0.7	0.7	0.7	0.6	0.6	0.9

fore, we assumed that geraniol could be increased by avoiding the hop addition in the growth phase. In this study, the results indicated that the content of geraniol in the finished beer was increased by delaying the timing of the hop addition. Greater levels of hop-derived geraniol and later hop additions (at the end of fermentation) had the most effect on increasing the geraniol in the finished beer (Figs. 3–5). In the storage period, the geraniol contents were relatively steady or slightly increased. Such increase of geraniol might be contributed by the occurrence of hop-derived glycosidically bound geraniol precursor and yeast-derived glucoside hydrolase activity (38) or by the occurrence of geranyl esters (geranyl acetate and geranyl isobutyrate) and yeast-derived esterase (17).

The Behavior of β-Citronellol under Varying the Timing of Hop addition

King et al reported that the biotransformation of geraniol to β-citronellol took place rapidly within the first 2–4 days of a model fermentation. A decrease of geraniol with a corresponding increase of β-citronellol occurred drastically during this period (10,11). In the case of the hopped beer fermentation, the β-citronellol was gradually increased during the total fermentation period and did not correlate to the rate of decreasing geraniol (37,38). It was suggested that this β-citronellol generation might involve some unknown mechanisms, rather than a simple transformation of geraniol to β-citronellol in the model fermentation (10,11). Furthermore, it seemed that the formation of β-citronellol during fermentation of the hopped wort was slow. Therefore, it is expected that delaying the hop-addition time might reduce the formation of the β-citronellol in the finished beer. However, in this study, the β-citronellol contents in finished beers made with the same hop variety and three different addition times were present at almost the same levels (except for Bravo-timing 3 beer), because of the active generation of β-citronellol during the storage period (Figs. 3–5).

When the hop-addition time is delayed, the conversion of geraniol to β-citronellol during the storage period should be increased as an increase of free geraniol. However, in many cases in our test fermentations, although the contents of free geraniol were increased (Figs. 3–5), we observed that the β-citronellol formation was not accounted for in the increase of free geraniol. It is suggested that the same amount of free geraniol and geraniol precursor could exist in the hop-flavored wort made with the same amount of the same hop variety in all hop-addition timings. Both free geraniol and geraniol precursor added in fermenting wort could contribute not only to an increase of free geraniol but also to a generation of β-citronellol. In Bravo beers, the generation of β-citronellol during primary fermentation was very active in timings 1 and 2, because of a significantly high amount of the hop-derived geraniol present, and the increasing amounts of β-citronellol during the storage period were at nearly the same levels among all timings (Fig. 4). A nearly constant difference of the β-citronellol contents between timings 1 and 2 and timing 3 lasted from the end of fermentation to the storage period.

In addition, in the case of the geraniol-rich hops, the β-citronellol first increased in the third day of the fermentation, slightly decreased in the end of the fermentation, and gradually increased again during the storage period (Figs. 3–5, timing 1). A similar behavior of the β-citronellol has also been observed in our previous study (37). We thought that the comprehensive behavior of β-citronellol shown in this study was commonly observed in fermentation by using the geraniol-rich hop varieties. An explanation of an increase of β-citronellol during the storage period is described above. However, a model which could totally explain a biotransformation of monoterpene alcohols in a fermentation of hopped beer has not been established yet. Further investigation is needed for understanding the behavior of hop-derived flavor compounds during fermentation.

Relationship Between the Timing of Hop Addition and the Profile of Flavor Compounds in Beer

It is well known that terpene hydrocarbons drastically decrease by evaporation or absorption in foam during primary fermentation because of their high hydrophobicity. Therefore, the delaying of hop-addition timing might be effective for retaining terpene hydrocarbons. It was observed that myrcene contents increased by the delaying of hop-addition timings. However, humulene contents were not significantly changed. The chain length of humulene is longer than that of myrcene, and the hydrophobicity of humulene is higher than that of myrcene. Therefore, the decreases of humulene were significant in all conditions. It has been reported that an odor threshold of myrcene was 13–36 µg/L in water (25). Therefore, myrcene (>40 µg/L in Table I) might be an important contributor to beer flavor in the test beers.

Isobutyric esters are widely present in German aroma hops, bitter or high α hops, and newly bred flavor hops (24,35). These compounds have also been found to be present in late-hopped beers and dry-hopped beers (20,22,31). It reported that isobutyric esters could be unstable during boiling and fermentation (31). These esters were perceived as having fruity, green apple-like, and apricot-like flavor (35). Seaton et al suggested that the hop fraction containing myrcene and 2-methylbutyl isobutyrate had a citrus-like flavor (31). Therefore, these isobutyric esters are expected to contribute to a part of the specific flavor in strongly hopped IPA-type craft beers. An odor threshold of 2-methylbutyl isobutyrate was 78 µg/L in beer (35). Therefore, isobutyric esters might contribute to the flavor of Bravo beers and Mosaic beers (Table I).

Ethyl esters of branched short-chain fatty acids were mainly formed by esterification of isobutyric acid, 2-methylbutyric acid, and isovaleric acid derived from hop or yeast fermentation. These esters provided fruity, apple-like, and pineapple-like flavor and their thresholds were 6.3 µg/L for ethyl isobutyrate, 0.7–1.0 µg/L for ethyl 2-methylbutyrate, and 2.0 µg/L for ethyl isovalerate (14). In our test-brewed beers, the hop-addition timing had no significant effect on the compositions of these esters in the finished beers.

In this study, the relationship among the comprehensive behaviors of hop-derived flavor compounds during the primary fermentation and the hop-addition timings was partly understood. These results are expected to help in understanding the phenomena occurring during dry-hopped fermentation and for developing new products in craft brewing.

ACKNOWLEDGMENTS

We thank Yakima Chief, Inc., John I. Haas, Inc., Hop Breeding Company, LLC, and S. S. Steiner, Inc. for supplying the U.S. hop samples; all panelists at the Product & Technology Innovation Department and the Frontier Laboratories of Value Creation for their sensory work; R. Miyamoto at the Frontier Laboratories of Value Creation for technical assistance; and A. Inaba, N. Suda, and K. Koie at the Bioresources Research & Development Department and T. Oshima and T. Shigyo at the Frontier Laboratories of Value Creation for their kind help.

LITERATURE CITED

1. Beatson, R. A., Ansell, K. A., and Graham, L. T. Breeding, development, and characteristics of the hop (*Humulus lupulus*) cultivar 'Nelson Sauvin'. *N. Z. J. Crop Hortic. Sci.* 31:303-309, 2003.
2. Biendl, M., Kollmannsberger, H., and Nitz, S. Occurrence of glycosidically bound flavour compounds in different hop products. *Proc. Congr. Eur. Brew. Conv.* 29:252-258, 2003.

3. Daenen, L., Saison, D., Sterckx, F., Delvaux, F. R., Verachtert, H., and Derdelinckx, G. Screening and evaluation of the glucoside hydrolase activity in *Saccharomyces* and *Brettanomyces* brewing yeasts. *J. Appl. Microbiol.* 104:478-488, 2008.
4. Darriet, P., Tominaga, T., Lavigne, V., Boidron, J. N., and Dubourdieu, D. Identification of a powerful aromatic component of *Vitis vinifera* L. var. Sauvignon wines: 4-mercapto-4-methylpentan-2-one. *Flavour Fragr. J.* 10:385-392, 1995.
5. Goldstein H., Ting, P., Navarro, A., and Ryder, D. Water-soluble hop flavor precursors and their role in beer flavor. *Proc. Congr. Eur. Brew. Conv.* 27:53-62, 1999.
6. Goldstein H., Ting, P. L., Schulze, W. G., Murakami, A. A., Lusk, L. T., and Young, V. D. Methods of making and using purified kettle hop flavorants. US patent 2003/0138546 A1, 2003.
7. Graves, I. R., Brier, M. B., Chandra, G. S., and Alspach, P. A. Kettle hop flavour of from New Zealand hop (*Humulus lupulus* L.) cultivars. *Proc. Conv. Inst. Brew. Asia-Pac. Sect.* 27:17-22, 2002.
8. Gros, J., Nizet, S., and Collin, S. Occurrence of odorant polyfunctional thiols in the super alpha Tomahawk hop cultivar. Comparison with the thiol-rich Nelson Sauvin bitter variety. *J. Agric. Food Chem.* 59:8853-8865, 2011.
9. Gros, J., Peeters, F., and Collin, S. Occurrence of odorant polyfunctional thiols in beers hopped with different cultivars. first evidence of an S-cysteine conjugate in hop (*Humulus lupulus* L.). *J. Agric. Food Chem.* 60:7805-7816, 2012.
10. King, A., and Dickinson, J. R. Biotransformation of monoterpene alcohols by *Saccharomyces cerevisiae, Torulaspora delbrueckii* and *Kluyveromyces lactis*. *Yeast* 16:499-506, 2000.
11. King, A., and Dickinson, J. R. Biotransformation of hop aroma terpenoids by ale and lager yeasts. *FEMS Yeast Res.* 3:53-62, 2003.
12. Kishimoto, T., Kobayashi, M., Yako, N., Iida, A., and Wanikawa, A. Comparison of 4-mercapto-4-methylpentan-2-one contents in hop cultivars from different growing regions. *J. Agric. Food Chem.* 56:1051-1057, 2008.
13. Kishimoto, T., Morimoto, M., Kobayashi, M., Yako, N., and Wanikawa, A. Behaviors of 3-mercaptohexan-1-ol and 3-mercaptohexyl acetate during brewing processes. *J. Am. Soc. Brew. Chem.* 66:192-196, 2008.
14. Kishimoto, T., Wanikawa, A., Kono, K., and Aoki, K. Odorants comprising hop aroma of beer: hop-derived odorants increased in the beer hopped with aged hops. *Proc. Congr. Eur. Brew. Conv.* 31:226-235, 2007.
15. Kishimoto, T., Wanikawa, A., Kono, K., and Shibata, K. Comparison of the odor-active compounds in unhopped beer and beers hopped with different hop varieties. *J. Agric. Food Chem.* 54:8855-8861, 2006.
16. Kollmannsberger, H., Biendl, M., and Nitz, S. Occurrence of glycosidically bound flavour compounds in hops, hop products and beer. *Monatsschr. Brauwiss.* 59:83-89, 2006.
17. Lam, K. C., Foster, R. T., II, and Deinzer, M. L. Aging of hops and their contribution to beer flavor. *J. Agric. Food Chem.* 34:763-770, 1986.
18. Lutz, A., Kammhuber, K., and Seigner, E. New trend in hop breeding at the hop research center Huell. *BrewingScience* 65:24-32, 2012.
19. Morimoto, M., Kishimoto, T., Kobayashi, M., Yako, N., and Wanikawa, A. Effects of Bordeaux mixture (copper sulfate) treatment on blackcurrant/muscat-like odors in hops and beer. *J. Am. Soc. Brew. Chem.* 68:192-196, 2010.
20. Murakami, A., Chicoye, E., and Goldstein H. Hop flavor constituents in beer by headspace analysis. *J. Am. Soc. Brew. Chem.* 45:19-23, 1987.
21. Murakami, A. A., Navarro, A., Ryder, D. S., and Goldstein H. Use of glycosides extracted from hop plant parts to flavor malt beverages. US patent 2003/0211788 A1, 2003.
22. Murakami, A. A., Rader S., Chicoye, E., and Goldstein H. Effect of hopping on the headspace volatile composition of beer. *J. Am. Soc. Brew. Chem.* 47:35-42, 1989.
23. Peacock, V. E., Deinzer, M. L., Likens, S. T., Nickerson, G. B., and McGill, L. A. Floral hop aroma in beer. *J. Agric. Food Chem.* 29:1265-1269, 1981.
24. Perpéte, P., Mélotte, L., Dupire, S., and Collin, S. Varietal discrimination of hop pellets by essential oil analysis I. Comparison of fresh samples. *J. Am. Soc. Brew. Chem.* 56:104-108, 1998.
25. Plotto, A., Margaría, C. A., Goodner, K. L., Goodrich, R., and Baldwin, E. A. Odour and flavour thresholds for key aroma components in an orange juice matrix: terpenes and aldehydes. *Flavour Fragr. J.* 19:491-498, 2004.
26. Probasco, G., Perrault J., and Varnum, S. Citra—a new special aroma hop variety. *Proc. World Brew. Congr.* Poster 115, 2008.
27. Probasco, G., Perrault J., and Varnum, S. Mosaic HBC 369—a new flavor hop variety. Online. http://www.worldbrewingcongress.org/2012/Abstracts/AbstractsDetail.cfm?AbstractID=343 Proc. World Brew. Congr. Poster 147, 2012.
28. Probasco, G., Varnum, S., Perrault J., and Hysert, D. Citra—a new special aroma hop variety. *MBAA Tech. Q.* 47:17-22, 2010.
29. Roland, A., Schneider, R., Razungles, A., and Cavelier, F. Varietal thiols in wine: discovery, analysis and applications. *Chem. Rev.* 111:7355-7376, 2011.
30. Schönberger, C. Unnachahmliche Noten. Flavour Hops—Herausstellungsmerkmal Hopfen. *Brauindustrie* 97:28-30, 2012.
31. Seaton, J. C., Moir, M., and Sugget, A. The refinement of hop flavour by yeast action. *Proc. Conv. Inst. Brew. Aust. N. Z. Sect.* 17:117-124, 1982.
32. Steinhaus, M., and Schieberle, P. Transfer of the potent hop odorants linalool, geraniol and 4-methyl-4-sulfanyl-2-pentanone from hops into beer. *Proc. Congr. Eur. Brew. Conv.* 31:1004-1011, 2007.
33. Steinhaus, M., Wilhelm, W., and Schieberle, P. Comparison of the most odor-active volatiles in different hop varieties by application of a comparative aroma extract dilution analysis. *Eur. Food. Res. Technol.* 226:45-55, 2007.
34. Suzuki, K., Yabe, T., Maruyama, Y., Abe, K., and Nakajima, T. Characterization of recombinant yeast exo-β-1,3-glucanase (Exg 1p) expressed in *Escherichia coli* cells. *Biosci. Biotechnol. Biochem.* 65:1310-1314, 2001.
35. Takoi, K., Degueil, M., Shinkaruk, S., Thibon, C., Kurihara, T., Toyoshima, K., Ito, K., Bennetau, B., Dubourdieu, D., and Tominaga, T. Specific flavor compounds derived from Nelson Sauvin hop and synergy of these compounds. *BrewingScience* 62:108-118, 2009.
36. Takoi, K., Degueil, M., Shinkaruk, S., Thibon, C., Maeda, K., Ito, K., Bennetau, B., Dubourdieu, D., and Tominaga, T. Identification and characteristics of new volatile thiols derived from the hop (*Humulus lupulus* L.) cultivar Nelson Sauvin. *J. Agric. Food Chem.* 57:2493-2502, 2009.
37. Takoi, K., Itoga, Y., Koie, K., Kosugi, T., Shimase, M., Katayama, K., Nakayama, Y., and Watari, J. Contribution of geraniol metabolism to citrus flavour of beer: synergy of geraniol and β-citronellol under coexistence with excess linalool. *J. Inst. Brew.* 116:251-260, 2010.
38. Takoi, K., Koie, K., Itoga, Y., Katayama, K., Shimase, M., Nakayama, Y., and Watari, J. Biotransformation of hop-derived monoterpene alcohols by lager yeast and their contribution to the flavor of hopped beer. *J. Agric. Food Chem.* 58:5050-5058, 2010.
39. Tominaga, T., Furrer, A., Henry, R., and Dubourdieu, D. Identification of new volatile thiols in the aroma of *Vitis vinifera* L. var. Sauvignon Blanc wines. *Flavour Fragr. J.* 13:159-162, 1998.
40. Tominaga, T., Murat, M.-L., and Dubourdieu, D. Development of a method for analyzing the volatile thiols involved in the characteristic aroma of wines made from *Vitis vinifera* L. cv. Sauvignon Blanc. *J. Agric. Food Chem.* 46:1044-1048, 1998.
41. Vaudano, E., Moruno, E. G., and Stefano, R. D. Modulation of geraniol metabolism during alcohol fermentation. *J. Inst. Brew.* 110:213-219, 2004.