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Analysis of Hop-Derived Terpenoids in Beer and Evaluation of Their Behavior Using the Stir Bar–Sorptive Extraction Method with GC-MS

Toru Kishimoto,* Akira Wanikawa, Noboru Kagami, and
Katsuyuki Kawatsura

Brewing Research and Development Laboratory, Asahi Breweries Ltd., 1-21, Midori 1-chome,
Moriya-shi, Ibaraki, 302-0106, Japan

Hop aroma components, which mainly comprise terpenoids, contribute to the character of beers. However, pretreatments are necessary before analyzing these components because of their trace levels and complicated matrixes. Here, the stir bar–sorptive extraction (SBSE) method was used to detect and quantify many terpenoids simultaneously from small samples. This simple technique showed low coefficients of variation, high accuracy, and low detection limits. An investigation of the behavior of terpenoids identified two distinct patterns of decreasing concentration during wort boiling. The first, which was seen in myrcene and linalool, involved a rapid decrease that was best fitted by a quadratic curve. The second, which was observed in β-eudesmol, humulene, humulene epoxide I, β-farnesene, caryophyllene, and geraniol, involved a gentle linear decrease. Conversely, the concentration of β-damascenone increased after boiling. As the aroma composition depended on the hop variety, we also examined the relationship between terpenoid content and sensory analysis in beer.

KEYWORDS: Behavior of terpenoids; boiling; GC-MS; hop aroma; LogKow; SBSE; stir bar-sorptive extraction

INTRODUCTION

During the brewing process, hops are used to provide a characteristic bitter taste and distinctive aroma. Generally, hops are added either during or after the wort boiling process. This step is necessary not only for the extraction and transformation of hop components but also for the formation and precipitation of protein–polyphenol complexes, wort sterilization, and the evaporation of off-flavors. However, hop aroma components also evaporate as boiling progresses. The control of hop aroma is therefore of great importance during the brewing process.

Investigating and controlling the hop aroma requires detailed knowledge of its components, which include hydrocarbons, terpenoids, aldehydes, ketones, esters, acids, alcohols, and sulfur compounds. Terpenoids, some of which are derived only from hops, have been the focus of several previous reports on hop aroma.

Modern studies have used gas chromatography coupled with mass spectrometry (GC-MS) as an indispensable tool for sensitive quantification on the basis of the monitoring of selected ion fragments. Various sample pretreatments (such as extraction, concentration, and cleanup steps) are necessary for the analysis of hop terpenoids by GC-MS because of the trace levels and complicated matrixes that are present. A range of extraction and concentration methods have been developed for the analysis of terpenoids, which include steam distillation (*1*) or extraction with a conventional solvent (*2*), supercritical-fluid CO_2 extraction (*3*), and column chromatography (*4*). However, these techniques require raw hops, large sample volumes, or beers with rich hop aromas to extract the hop-derived terpenoids. Thus, for the analysis and investigation of beers with low-intensity hop aromas, such as Japanese beers, a more sensitive analytical method is needed.

Recently, Baltussen et al.[5] described a new extraction technique, known as the stir bar–sorptive extraction (SBSE) method, which is based on the partition coefficient between poly(dimethylsiloxane) (PDMS) and water. This approach uses magnetic stir bars coated with 50–300 μL of PDMS and is sensitive and easy to use. Additional studies have evaluated this method to detect and quantify with trace volatiles in beers (*6, 7*) and with malt whisky (*8*). In the present study, we used the SBSE approach to analyze the terpenoid contents of beers, and we examined their association with sensory characteristics. We also investigated the behavior of terpenoids during the boiling process.

MATERIALS AND METHODS

Reagents. Linalool, geraniol, myrcene, caryophyllene, α-humulene, β-damascenone, and β-damascone were purchased from Fluka (Stein-

* Author to whom correspondence should be addressed. E-mail: toru.kishimoto@asahibeer.co.jp.

Table 1. Concentration of Terpenoids (ppb) in Japanese Commercial Beers[a]

	SBSE method		extraction with dichloromethane	
	Japanese beer A	Japanese beer B	Japanese beer A	Japanese beer B
linalool	1.31	4.57	1.16	4.97
geraniol	2.44	3.49	2.25	3.45
β-citronellol	3.01	4.26	ND	ND
myrcene	0.47	0.34	ND	ND
β-caryophyllene	0.19	ND	ND	ND
α-humulene	0.73	0.23	ND	ND
humulene epoxide I	0.10	0.53	ND	ND
β-eudesmol	1.27	0.43	1.07	0.50
β-farnesene	0.36	0.54	ND	ND
β-damascenone	1.38	2.23	ND	ND

[a] Terpenoids were extracted by dichloromethane from 350 mL beer and by the SBSE method from 6 mL of beer. ND, not detected.

heim, Switzerland). β-Citronellol and *cis*-3-hepten-1-ol were obtained from Sigma-Aldrich (St. Louis, MO) and Avocado Research Chemicals Ltd. (Lancashire, U.K.), respectively. Eudesmol, β-farnesene, and synthesized humulene epoxide were purchased from Wako (Osaka, Japan). All reagents were analytical grade.

Preparation of Volatiles by Liquid Extraction. A 350-mL sample of beer containing 5 μg of *cis*-3-hepten-1-ol (internal standard) was extracted with 150 mL of dichloromethane for 3 h at room temperature. The dichloromethane layer was then separated and dried over anhydrous sodium sulfate for 30 min. The extract was concentrated to approximately 1 mL at 750 hPa using a rotary evaporator at 40 °C.

GC-MS Conditions for the Liquid-Extraction Method. Separation of the extract was performed with an Agilent 6890 gas chromatograph coupled to an MSD5973N quadrupole mass spectrometer (Agilent Technologies, CA) equipped with a DB-WAX capillary column (60 m length × 0.25 mm i.d.; film thickness = 0.25 μm; Agilent Technologies) using pulsed splitless injection with helium carrier gas (1 mL/min). The inlet temperature was set at 250 °C, and the oven temperature was programmed from 40 °C (held for 5 min) up to 240 °C (held for 20 min) at a rate of 3 °C/min. A 1-μL sample of concentrated volatile was injected into the GC-MS apparatus, which was set up to detect ions with a mass-to-charge ratio (m/z) of between 30 and 350 and was operated in the electron-impact mode at 70 eV. All compounds were identified on the basis of their mass spectra and retention time by comparison with authentic compounds.

Preparation of Volatiles using the SBSE Method. Stir bars (length = 20 mm) coated with 47 μL of PDMS (Twister; Gerstel, Mulheim a/d Ruhr, Germany) were conditioned for 1 h at 300 °C in a stream of helium gas before use. β-Damascone was added to the beer or wort sample at a final concentration of 0.1 ppb as an internal standard. A 30-mL sample diluted with four volumes of distilled water was transferred into a vial and a PDMS-coated stir bar was added. After the vial was capped, the PDMS-coated bar was stirred in a water bath set at 40 °C for 2 h to extract the aroma substances. The stir bar was then withdrawn from the vial and washed with distilled water. After drying with a lint-free tissue, the stir bar was thermally desorbed into a GC-MS system via a Twister desorption unit (TDU; Gerstel) and a programmable temperature-vaporization inlet (CIS4; Gerstel). Quantification was carried out in the selected ion-monitoring mode at the following m/z values: 69 (geraniol), 80 (α-humulene), 85 (humulenol II), 93 (linalool, myrcene, β-caryophyllene, and β-farnesene), 123 (β-citronellol and humulene epoxide I), 149 (β-eudesmol), 177 (β-damascenone; internal standard), and 190 (β-damascenone).

Thermal Desorption−GC-MS Conditions. Thermal desorption of the trapped aroma substances from the PDMS-coated stir bar was carried out in the TDU (Gerstel), which was programmed from 25 °C (held for 0 min) up to 240 °C (held for 5 min) at a rate of 2.5 °C/s in a splitless mode. The desorbed substances from the TDU were cryofocused in the CIS4 inlet at −100 °C using liquid nitrogen. The CIS4 inlet program for injecting the substances into the GC-MS column was started concomitantly with the initiation of the GC-MS program. The CIS4 inlet was programmed from −100 °C (held for 0 min) up to 240 °C (held for 5 min) at a rate of 2 °C/s in a splitless mode. The GC-MS

conditions, with the exception of the inlet, were similar to those used in the preparation of volatiles by liquid extraction.

Brewing Processes. To investigate the terpenoid contents of different hop varieties, Saaz (4.0% α-acid pellets; Czech Republic), Tettnang (7.0% α-acid pellets; Germany), and Hersbrucker (4.7% α-acid pellets; Germany) were brewed in 3000-L volumes. During each brewing, 67% of the total hops (on the basis of the α-acid contents) was added at the beginning of the boiling process and the remaining 33% was added 15 min before the end of this stage.

An additional 3000-L brew was performed to evaluate the behavior of terpenoids during and after boiling. Hersbrucker (4.7% α-acid pellets) was added at the beginning of wort boiling and the behavior of the terpenoids was traced during the process.

Sensory Analysis. Flavor-profile analyses of Saaz, Tettnang, and Hersbrucker beers were performed using a modified version of the Lermusieau (9) and Engel (10) methods. A panel comprising 19 trained individuals was asked to select from a list of attributes (hop pelletlike, resinous, green, floral, citrus, estery, muscatlike, or spicy) after tasting to describe the character of the hop aroma. The samples were served in a random order to each panelist.

RESULTS AND DISCUSSION

Several previous reports on hop aroma have analyzed the terpenoid content of beers. Lam et al. (*11*) extracted aroma components with Celite from 2 L of beer and identified linalool, geraniol, and β-citronellol as responsible for citrus and floral notes. Steinhaus and Schieberle (*2*) directly extracted components from hop cones and identified linalool and myrcene, among others, as potent odorants. De Keukeleire et al. (*3*) investigated differences in aromas between several varieties of hops using supercritical-fluid CO_2 extraction and identified myrcene, β-caryophyllene, α-humulene, and β-farnesene as marker compounds. Irwin (*4*) and Goiris et al. (*12*) prepared volatiles from Hersbrucker beer using column chromatography and showed that the oxygenated sesquiterpenoid fraction (which included eudesmol and humulene epoxides, humulenol II, and others) contributed to its spicy hop character. Lermusieau et al. (*9*) identified β-damascenone and linalool as odor-active constituents using XAD-2 resin chromatography. However, the methods used in these studies required large sample volumes or were labor intensive, making them unsuitable for frequent or wide-ranging analyses.

We measured the amounts of terpenoids in Japanese commercial beers using both the conventional method (extraction with dichloromethane) employed in previous studies and the novel SBSE method. The amounts of compounds present were calculated from the ratio of the area of each sample to that of an internal standard compound. **Table 1** shows the amounts of terpenoids detected using the two different analytical methods in beers A and B, which were Japanese commercial beers with
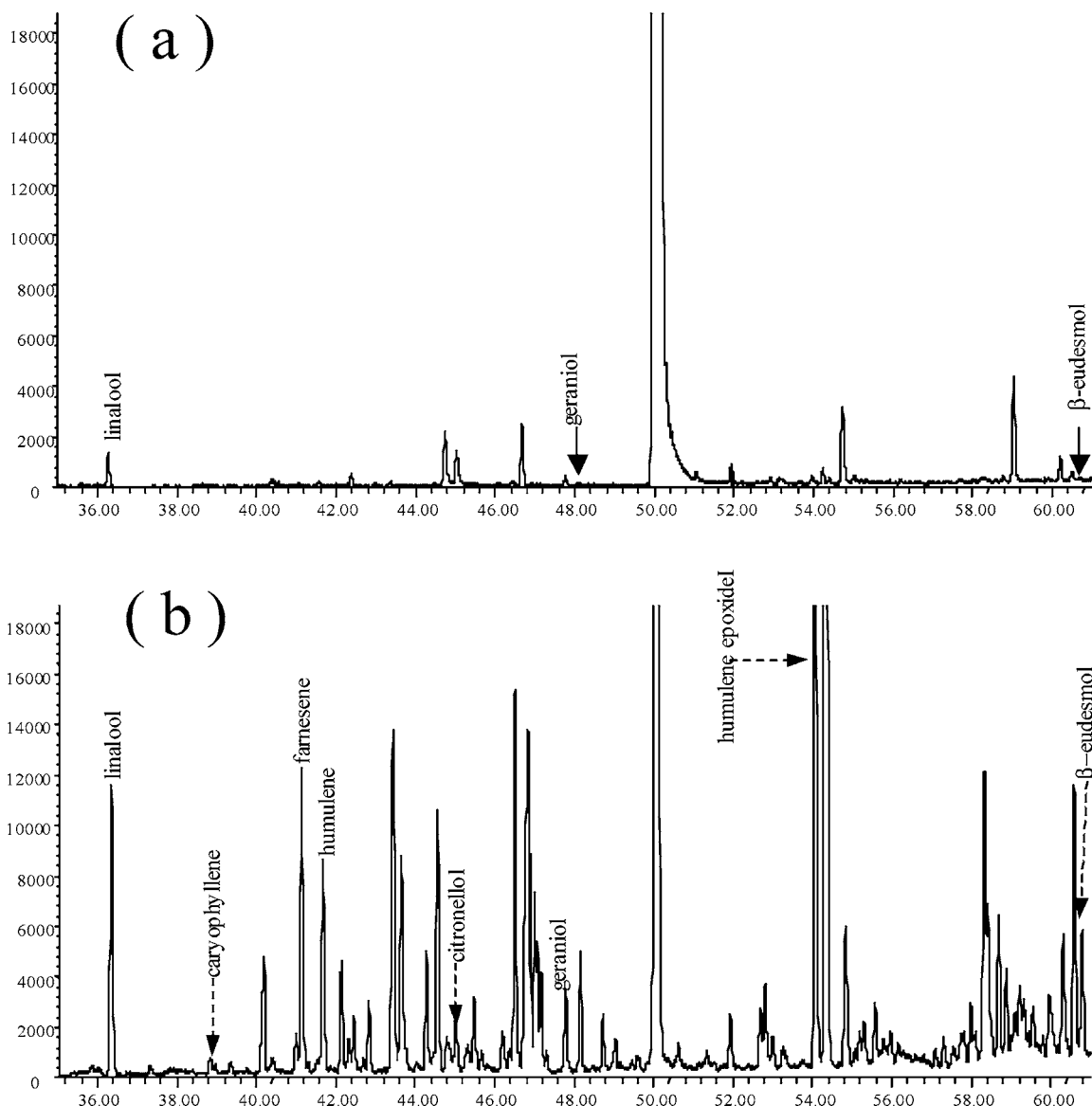
Figure 1. Selected ion chromatogram (*m/z* 93) of volatiles prepared by dichloromethane extraction (**a**) and by the SBSE method (**b**) from Japanese commercial beer B.
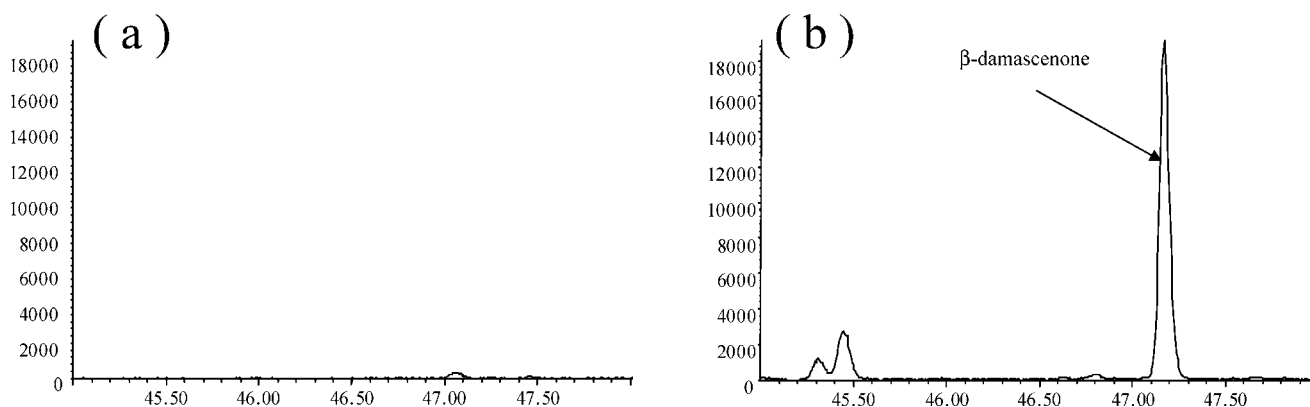

Figure 2. Selected ion chromatogram (*m/z* 190) of volatiles prepared by dichloromethane extraction (**a**) and by the SBSE method (**b**) from Japanese commercial beer B.

a poor and rich aroma, respectively. Only three compounds (linalool, geraniol, and β-eudesmol) were detected by the conventional dichloromethane extraction. This was presumably due to the interference of the high matrix content (including proteins, amino acids, and polyphenols) in the beers. By contrast, the SBSE method identified several additional substances.

Figure 1 shows selected ion *m/z* 93 chromatograms for general terpenoids. **Figure 2** shows *m/z* 190 chromatograms for β-damascenone from a Japanese commercial beer prepared using the dichloromethane extraction and SBSE methods. These results clearly illustrate that the SBSE method identified several substances that were not detected by the conventional dichlo-

Table 2. CVs, Detection Limits, and r^2 Values for Terpenoids Analyzed by the SBSE Method

	Log Kow	CV (%)[a]	detection limit (ppb)[b]	$r^{2\ c}$	boiling point (°C) at 760 mmHg
linalool	3.38	4.3	0.049	0.997	194
geraniol	3.47	5.7	0.099	0.997	229
β-citronellol	3.56	6.8	0.278	0.999	
myrcene	4.88	7.4	0.001	0.999	167
β-caryophyllene	6.30	8.2	0.031	0.999	262
α-humulene	6.95	4.2	0.035	0.999	266
humulene epoxide I	5.55	2.1	0.044	0.994	
β-eudesmol	4.88	5.5	0.013	1.000	
β-farnesene	7.10	6.7	0.023	1.000	260
β-damascenone	4.21	2.0	0.019	1.000	
β-damascenone (IS)[d]	4.42				

[a] CVs were calculated from concentrations obtained by injections between 6 days. [b] The concentration when the signal/noise was 3. [c] The correlation between the internal standard and the observed concentrations. [d] IS, internal standard.

romethane extraction. Among the hundreds of substances that can potentially be detected by the SBSE method, we focused on those introduced as potent odorants or marker compounds to the beers (as discussed above).

The theory behind the SBSE method is straightforward (*5*). A PDMS-coated stir bar is introduced into the beer or wort sample and the extraction occurs during the stirring process as the hydrophobic substances are absorbed into the PDMS. The logarithm of the PDMS−water partition coefficient is roughly equivalent to the logarithm octanol−water partition coefficient (LogKow); Kow is a physical parameter that is commonly used to describe the hydrophilic or hydrophobic properties of chemicals (*13*), and logarithm of that (LogKow) is generally used to characterize its value. The LogKow values of the terpenoids calculated by SRC-KOWWIN software (Syracuse Research, Syracuse, NY) are shown in **Table 2**. A high LogKow value indicates high hydrophobicity and most likely a high recovery by the PDMS-coated stir bar. The PDMS−water partition attains equilibrium rapidly at higher temperatures; our observations indicated that a temperature of 40 °C was appropriate for a 2-h extraction. The LogKow values of the terpenoids ranged between 3.38 and 7.10. β-Damascone was selected as the internal standard compound as its LogKow value (4.42) fell within this range, and strong correlations between the areas of β-damascenone and each terpenoid were observed.

Table 2 shows the coefficients of variation (CVs), detection limits, and correlations (r^2) between the internal standard ratio and the observed amounts according to the SBSE method. This technique showed high sensitivity coupled with low detection limits (0.001−0.28 ppb), low CVs (less than 10%), and high correlations between the internal standard compound and the sample concentrations (greater than 0.99).

The simplicity of the SBSE method, which requires small sample volumes and is less labor intensive, allowed us to trace the behavior of hop terpenoids throughout the wort boiling process (**Figure 3**). Caryophyllene, humulene, and β-farnesene are generally present at high concentrations in hop pellets but at relatively low concentrations during the boiling process. These facts confirm their poor solubility in wort. β-Citronellol was not detected in the wort samples (data not shown), as it was transformed from geraniol during fermentation (*14*).

Two distinct patterns of decrease were detected among the terpenoids. The first was seen in myrcene and linalool, the levels of which fell rapidly during the boiling process in a pattern corresponding to a quadratic curve. This was partly due to their low boiling points (**Table 2**), which were reflected in various aspects of their chemical structure (for example, the number of carbons, the types of functional groups, and the positions of the double bonds). Hops must therefore be added toward the end or after the boiling process to retain higher concentrations of these terpenoids. The second pattern was observed in β-eudesmol, humulene, humulene epoxide I, β-farnesene, caryophyllene, and geraniol, all of which have higher boiling points (**Table 2**); the concentrations of these components decreased gently and linearly throughout the boiling process. These two distinct patterns supported our observation that the hop aroma characters of beers depend on the time at which the hops are added.

Interestingly, an alternative pattern was seen in β-damascenone, the concentration of which increased after boiling. Isoe et al. (*15*) suggested that β-damascenone could be formed by the acid-catalyzed conversion of polyols (enyne diols or allene triols) resulting from enzymatic transformations of the carotenoid neoxanthin. Chevance et al. (*16*) also proposed that β-damascenone was released by the acidic hydrolysis of glycosides during the aging of beers. Conversely, Kotseridis et al. (*17*) reported increased β-damascenone levels in wine after heat treatment. We also observed β-damascenone formation both during and after boiling; however, the β-damascenone that was formed during this process evaporated immediately, so the increase could only be detected after boiling.

Next, we studied the differences between the hop varieties using the SBSE method. **Table 3** shows the amounts of terpenoids in beers brewed using different varieties of hops. Hersbrucker beer contained greater amounts of linalool, myrcene, humulene epoxide I, humulenol II, and β-eudesmol compared with the other two varieties, even when the α-acid contents were taken into consideration. Humulene epoxides reflect deterioration of the hops (*8, 17*). We therefore used fresh cold-stored

Table 3. Concentration of Terpenoids (ppb) in Beers Brewed Using Different Hop Varieties[a]

	Saaz	Tettnang	Hersbrucker	thresholds (ppb)	characteristics
linalool	4.8	4.0	10.5	27[a]	floral, citrus[g]
geraniol	3.5	3.4	3.8	36[a]	floral, citrus,[g] roselike[b]
β-citronellol	3.0	2.0	2.2	11 (in water)[b]	roselike,[b] floral, citrus[g]
myrcene	0.7	0.4	1.1	30–200[c]	resinous[i]
β-caryophyllene	0.3	0.2	0.1	450[c]	clove, turpentine[b]
α-humulene	0.3	0.7	0.2	450[c]	
humulene epoxide I	5.5	5.3	7.1	10 (in water)[d]	haylike[g]
humulenol II	4.1	3.5	6.5	2500[a]	sagebrushlike[g]
β-eudesmol	0.6	0.6	17.5	>10 000[e]	contained in spicy fraction[e]
β-farnesene	1.4	1.4	1.6	550[c]	
β-damascenone	0.9	0.8	0.6	0.02–0.09[f] (in water)	apple, peach,[h] fruity[f]

[a] The values are shown relative to the threshold in beer (*21*),[a] water (*21*),[b] beer (*22*),[c] water (*18*),[d] beer (*4*),[e] beer (*16*),[f] beer (*11*),[g] beer (*23*),[h] and beer (*24*).[i]

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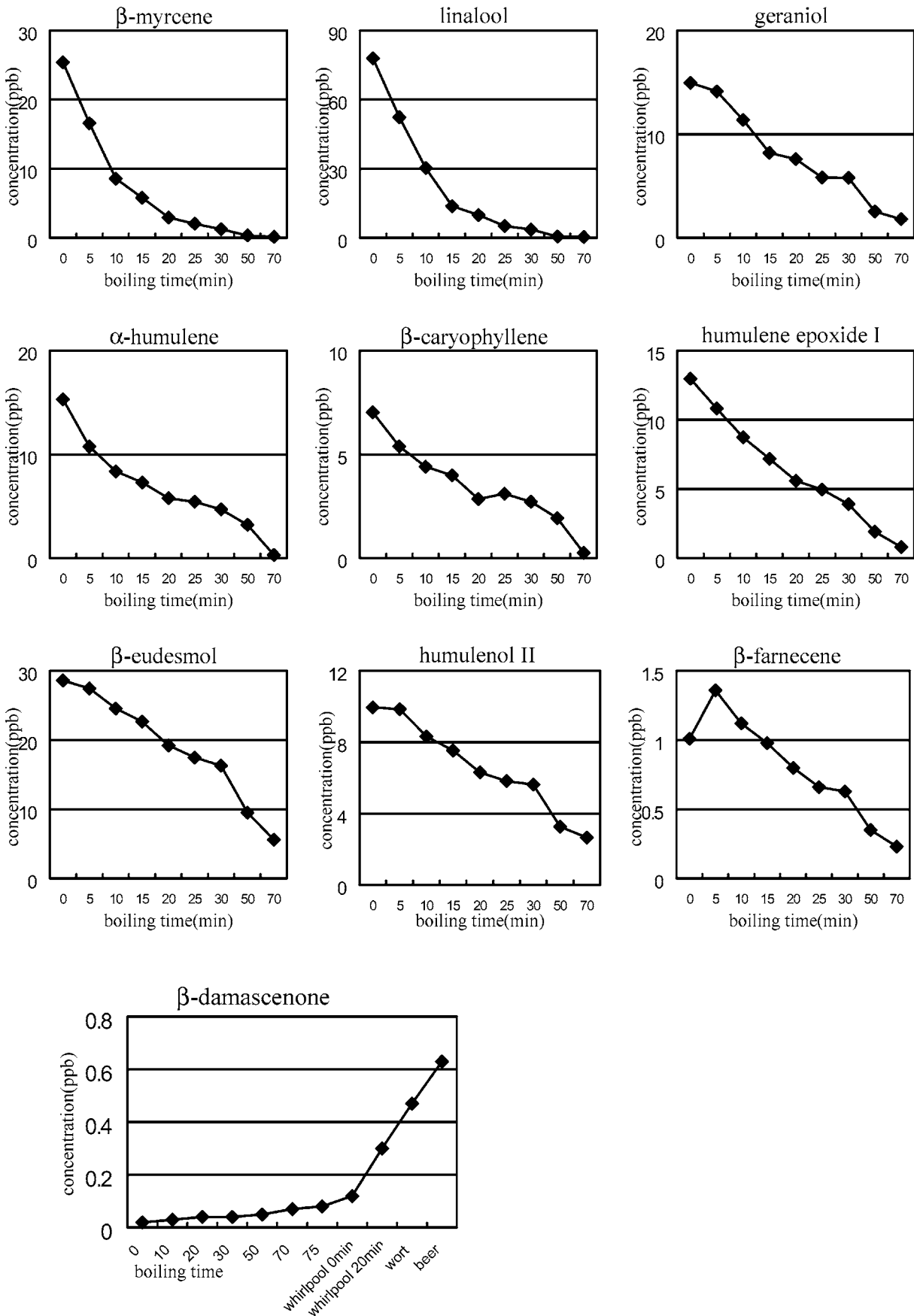*J. Agric. Food Chem.*, Vol. 53, No. 12, 2005 **4705**

Figure 3. Behavior of hop-derived terpenoids during wort boiling.

hops in which no such changes were observed before the brewing process. We believe that the high concentrations detected in Hersbrucker beer reflected high concentrations in the original hop pellets. In particular, the β-eudesmol concentration in Hersbrucker was extremely high, which is a characteristic of this hop variety (*19*). The hop aroma character of beer is

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Table 4. Sensory Evaluation of Beers Brewed Using Different Hop Varieties[a]

	Saaz	Tettnang	Hersbrucker
hop pelletlike	2	3	5
resinous	6	2	3
green	5	2	7
floral	4	5	9
citrus	3	2	3
estery	4	5	4
muscatlike	1	1	3
spicy	2	1	1

[a] The data in the table indicate the number of panelists ($n = 19$) who selected each attribute.

reported to depend on the hop variety that is used. This observation was partially supported by the differences in hop oil composition described here.

Finally, we investigated the relationship between the terpenoid content and sensory analysis. **Table 4** shows the results of the sensory evaluation of beers that were brewed using different hop varieties; the data indicate the numbers of panelists who selected each attribute. **Table 3** shows the concentrations of terpenoids in beers brewed using different hop varieties, along with the detection thresholds and characteristics of each terpenoid.

The number of panelists who selected the terms "floral", "hop pelletlike", and "green" to describe Hersbrucker beer was higher than for the other two types of beer. Previous reports (4, 9) indicated that the sesquiterpenoid fraction (containing eudesmol, humulene epoxides, and humulenol II) contributed to the spicy hop character of Hersbrucker beer. Another study[11] suggested that linalool, geraniol, and β-citronellol contributed to the citrus and floral notes. In addition, myrcene, β-caryophyllene, α-humulene, and β-farnesene have been identified as marker compounds between the different hop varieties (3). In the present study, all of the terpenoids measured were lower than the threshold values. Among these, the floral and hop pelletlike characteristics might partly correspond to increased amounts of linalool and humulene epoxide I, respectively, because the concentrations of these terpenoids were closest to the threshold values. However, the "spicy" character was not reflected by the concentrations of sesquiterpenoid. The number of panelists who selected the term "resinous" to describe Saaz beer was larger than those for the other two beers. The concentration of myrcene, which has a resinous character, was far lower than the threshold value and did not contribute independently to the character. Thus, other resinous compounds should be investigated in future studies. Not all of the sensory characteristics could be explained from our results, particularly in terms of the higher threshold substances, and relatively small amounts of substances might have contributed to these qualities.

In conclusion, changes in the concentrations of hop-derived terpenoids during the boiling process showed three distinct patterns. Most terpenoid concentrations decreased during boiling as a result of evaporation, and the patterns were either linear or followed a rapid quadratic curve depending upon the boiling point. By contrast, the β-damascenone concentration increased slowly during the boiling process and rose dramatically thereafter during the relatively cool whirlpool-processing step. Some associations between the terpenoid contents and sensory analyses of beers were detected, but it was difficult to explain the relationships clearly, particularly in terms of the higher threshold substances.

ABBREVIATIONS USED

GC-MS, gas chromatography coupled with quadrupole-mass spectrometry; PDMS, poly(dimethylsiloxane); SBSE, stir bar−sorptive extraction; TDU, Twister desorption unit

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