

## Identification and Characteristics of New Volatile Thiols Derived from the Hop (*Humulus lupulus* L.) Cultivar Nelson Sauvin<sup>†</sup>

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Nelson Sauvin (NS) is a unique hop cultivar that was bred and grown in New Zealand. This hop gives a specific flavor (exotic fruit-like, white wine-like) to finished beers. However, the key compounds of this flavor have not yet been identified. We have attempted to identify the specific flavor compounds derived from NS. We focused on certain volatile thiols that are well-known to contribute to wine flavors, especially Sauvignon Blanc. The product made from NS (NS product) lost its specific flavor by contact with copper. Copper is well-known as an absorber of thiols in the field of wine flavor investigations. Therefore, it might point to the existence of thiols. We analyzed the NS product by GC-FPD, GC-olfactometry and GC-MS, and identified two new volatile thiols, 3-sulfanyl-4-methylpentan-1-ol (3S4MP), and 3-sulfanyl-4-methylpentyl acetate (3S4MPA). These compounds have a grapefruit-like and/or rhubarb-like odor, similar to that of Sauvignon Blanc. We quantified these compounds in the NS products and determined their thresholds. As a result, 3S4MP contained about 2-fold of its threshold in beers, and 3S4MPA was included below its threshold. However, it was confirmed that 3S4MP enhanced the flavors of 3S4MPA by synergy. Therefore, we concluded that both of the new volatile thiols would contribute to the specific odor of beers produced with NS.

**KEYWORDS:** Beers; hops; Nelson Sauvin; flavor; thiols; 3-sulfanyl-4-methylpentan-1-ol; 3-sulfanyl-4-methylpentyl acetate; synergy

### INTRODUCTION

It is well-known that hops (*Humulus lupulus* L.) give a characteristic flavor and bitterness to beers. Hop flavor compounds are important for the flavor characteristics of beers; therefore, quality control in the brewing process is indispensable. Various hop cultivars have different aroma characters, and beers brewed with certain hops have cultivar-specific flavors. The flavor compounds of hops are mainly derived from the hop oil included in the lupulin gland of the hop cone. Several terpenoids

( $\alpha$ -humulene,  $\beta$ -myrcene, linalool, etc.), esters, aldehydes, and ketones were well-known as the major flavor compounds in hop oil, and several classification methods of the hop cultivars based on the analyses of these compounds have already been proposed (1–3). Among these terpenoids,  $\beta$ -myrcene and linalool have been reported as the most potent odorants in hop cones (4). However, several previous studies (5–7) suggested that most of the hydrophobic terpenoids would not remain from hop cones or pellets to finished beer. Therefore, the key compounds that contributed to the cultivar-specific flavors of the various hops, especially the flavor of beer using a certain hop, have not been completely clarified.

Among many hop cultivars, several researchers have focused on Cascade (7–10), originally bred in the United States of America. It has been well known that this hop gives a very floral aroma to finished beer. Linalool and geraniol have been mainly proposed as the flavor compounds contributing to the specific flavor of Cascade (7–10). However, the key flavor compounds derived from many other hop cultivars have not been well-studied.

<sup>†</sup> This work is dedicated to the memory of Dr. Takatoshi Tominaga, who tragically passed away on June 8th, 2008. He initiated the research of wine flavor compounds, especially volatile thiols, and will be remembered for his passionate interests in all aspects of flavor science and enology.

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Nelson Sauvignon (NS) is a unique hop cultivar that is bred and grown in New Zealand. This is a triploid cultivar developed at Hort Research, Riwaka Research Centre, from a Smoothcone (female) and New Zealand male cross, and first released in 2000. This hop belongs to the high  $\alpha$ -type hop ( $\alpha$ -acids: 12.0–14.0% w/w), but it is said to impart a very characteristic grape-like flavor to finished beer (11, 12). The hop growers in New Zealand have represented the specific flavor of NS as white wine-like, especially Sauvignon Blanc. However, the key compounds of this cultivar-specific flavor have not yet been identified.

Tominaga et al. (13) reported that several volatile thiols, including 3-sulfanylhexan-1-ol (3SH) and 4-methyl-4-sulfanylpentan-2-one (4MSP), contributed to the cultivar-specific flavor of the Sauvignon Blanc wines. Recently, these volatile thiols with very low thresholds have been focused on in the field of beer flavor investigation. Vermeulen et al. (14) reported the occurrence of several thiols in fresh lager beers. Steinhaus et al. (15) and Kishimoto et al. (16) reported the occurrence of 4MSP in the Cascade hop cultivar and its contribution to the characteristic flavor of this hop. However, there has never been a report that suggested the existence of these volatile thiols in the NS hop and/or the beer brewed with this hop. In this study, we have attempted to identify the unknown flavor compounds derived from the NS hop and have studied how these compounds contribute to the characteristic flavor.

## MATERIALS AND METHODS

**Hop Raw Materials.** All of the hop varieties that we used were commercial ones. Hallertau Tradition (HHT), Hallertau Magnum (HHM), and Nugget (HNU) were grown in Germany. Saaz (CSA) was grown in the Czech Republic. Pacific Hallertau (NPH) and Nelson Sauvignon (NS) were grown in New Zealand.

**Hop Extraction with Water.** For analysis of the flavor compounds, all hops were extracted with water. Fifteen grams of hop pellets was added to 500 mL of water at 25 °C and stirred by a magnetic stirrer for 45 min. The mixture was filtered by using filter paper, and the eluant was obtained as a hop water extract.

In addition, NS hop pellets were compared between different extraction temperatures (2 °C, 25 °C, 50 °C, and 90 °C) at the same conditions except for temperature.

**Pilot-Scale Brewing.** Beer, Hoppo-shu (Japanese low-tax beer using less than 24% malted barley (malt)) and Japanese third category (a beer-like alcohol beverage using no malt) were made from the CSA hop or the NS 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 malts and/or syrups and hops in a 400-L or 5000-L scale pilot plant. The worts of normal beer were made with only malt. Those of Hoppo-shu were made with 24% malt and 76% syrups, and those of the Japanese third category were made with syrups, using no malt. Then 0.4 g/L of hops was added at 5 min before the end of wort boiling. Boiling periods were 90 min for beer and 60 min for Hoppo-shu and the third category. After cooling, the fermentation was started by adding  $15.0 \times 10^6$ – $30.0 \times 10^6$  cells/mL lager yeast (brewery collected) to the cooled wort. The temperature of the fermentation was maintained at 10–15 °C. After transferring the fermented wort to another lagering tank under a CO<sub>2</sub> atmosphere, the maturation was carried out at 13 °C for 3–8 days, then at 0 °C for 2–3 weeks. Filtration and bottling were done using the pilot-scale equipment under antioxidative conditions.

**Chemicals.**  $\alpha$ -Humulene,  $\beta$ -myrcene, and linalool were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Sodium *p*-hydroxymercuribenzoate, 5,5'-dithio-bis-(2-nitrobenzoic acid), 4-methyl-3-penten-1-ol (97%), and thioacetic acid (96%) were purchased from Sigma-Aldrich Chemicals (L'Isle d'Abeau, France). 4-Methoxy-2-methylbutane-2-thiol was purchased from Oxford Chemicals (Grasse, France). 4-Methyl-4-sulfanylpentan-2-one was supplied by Interchim

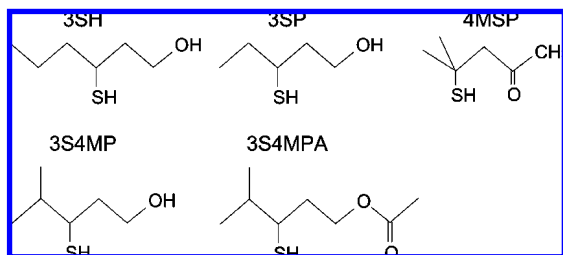
(Montluçon, France). 3-Sulfanylhexan-1-ol (>95%) was obtained from Lancaster (Bischheim, France). 3-Sulfanylpentan-1-ol was synthesized as previously described (17).

**Quantification of Terpenoids 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 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. An 8 mL sample of each beer was put into a 20 mL glass vial including 3 g of sodium chloride at 0 °C, and the vial 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  $\mu$ m film thickness, Supelco, Bellefonte, PA, USA] 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  $\mu$ m film thickness]. For all of 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,  $\alpha$ -humulene,  $\beta$ -myrcene, and linalool were quantified in the SIM mode, selecting the following ions: *m/z* 204, 93, and 109. Calibration curves were determined using water (including 5% ethanol) containing these terpenoids at final concentrations ranging from 0 to 10  $\mu$ g/L. All calibrations produced a linear response with an *R*<sup>2</sup> value >0.98, over the concentration range analyzed.

**Purification of Volatile Thiols.** The volatile thiols were specifically extracted by reversible combination of the thiols with sodium *p*-hydroxymercuribenzoate (*p*-HMB) as described by Tominaga et al. (13).

A 500 mL sample of the hop water extract or beer, containing 2.5 nmol of 4-methoxy-2-methylbutane-2-thiol as the internal standard, was extracted with two successive additions of 100 mL of dichloromethane in a 1 L flask with magnetic stirring for 10 min each time. The combined organic phases were centrifuged at 4000g for 15 min to break the emulsion and separated in a separating funnel. The organic phase obtained was then extracted with two successive additions of 20 mL *p*-HMB solution [1 mM in Trizma base [2-amino-2-(hydroxymethyl)-1,3-propanediol] at 0.2 M] for 10 min each time. The two aqueous phases were combined and then loaded on a strongly basic anion-exchange column (1.5  $\times$  3 cm) (Dowex 1–1  $\times$  2–100). The column was then washed with 50 mL of sodium acetate buffer (0.1 M, pH 7). The volatile thiols were released from the thiol-*p*-HMB complex fixed on the column by percolating with 60 mL of cysteine solution (10 g/L) adjusted to pH 7 with NaOH (10 M). The eluate containing the volatile thiols was collected in a 100 mL flask, and 0.5 mL of ethyl acetate was added. The eluate was extracted twice with dichloromethane (4 and 3 mL, respectively) for 10 min each time, under magnetic stirring. The two organic phases were combined, dried over anhydrous sodium sulfate, and then concentrated under nitrogen flow in a 10 mL graduated tube to approximately 200  $\mu$ L. The concentrate was then transferred to a 1 mL vial and concentrated to 25  $\mu$ L.

**Gas Chromatography Coupled Simultaneously with Olfactometry (GC-O) and Flame Photometric Detection (GC-FPD).** The olfactory analyses were carried out using a Hewlett-Packard 5890 gas chromatograph (Agilent Technologies) equipped with a flame photometric detector (from Agilent technologies) and a sniffing-port (ODO-1 from SGE, Ringbow, Australia). Three microliters of each concentrated extract was injected by a splitless injector (230 °C; purge time = 1 min; purge flow = 50 mL/min) at oven temperature (45 °C) onto a type BP20 capillary column [SGE, 50 m, 0.32 mm i.d., 0.25  $\mu$ m film thickness]. For all of the analyses, the temperature program was as follows: 45 °C for 10 min, raised at 3 °C/min to 230 °C, followed by a 20 min isotherm. The flame photometric detector was held at 230 °C. The carrier gas was hydrogen with a column-head pressure of 22 psi at a flow rate of 1 mL/min. Each GC-O analysis was performed by three experienced judges.



**Figure 1.** Structures of 3-sulfanylhexan-1-ol (3SH), 3-sulfanypentan-1-ol (3SP), 4-methyl-4-sulfanypentan-2-one (4MSP), 3-sulfanyl-4-methylpentan-1-ol (3S4MP), and 3-sulfanyl-4-methylpentyl acetate (3S4MPA).

#### Identification and Quantification of Volatile Thiols by GC-MS.

A 3  $\mu$ L sample of each concentrated extract was analyzed on a 6890N gas chromatograph (Agilent Technologies) under the conditions described above. 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 250  $^{\circ}$ C. The mass spectra were taken over the  $m/z$  40–300 range. Volatile thiols were identified on the basis of their linear retention indices and a comparison of the MS fragmentation patterns obtained in the SCAN mode on the BP20 capillary column [SGE, 50 m, 0.25 mm i.d., 0.25  $\mu$ m film thickness] to those of the previously reported reference compounds.

The two new thiols, 3-sulfanyl-4-methylpentan-1-ol (3S4MP, see **Figure 1**) and 3-sulfanyl-4-methylpentyl acetate (3S4MPA, see **Figure 1**), as well as 3-sulfanylhexan-1-ol (3SH), 3-sulfanypentan-1-ol (3SP), 4-methyl-4-sulfanypentan-2-one (4MSP), were quantified in the SIM mode, selecting the following ions:  $m/z$  134, 116, and 100 for 3SH,  $m/z$  120, 102, and 86 for 3SP,  $m/z$  132 and 75 for 4MSP,  $m/z$  134 and 100 for 3S4MP, and  $m/z$  116 and 101 for 3S4MPA. The internal standard, 4-methoxy-2-methylbutane-2-thiol, was detected by the  $m/z$  134 and 100 ions. Calibration curves were determined using the hop water extracts or beers containing 3SH, 3SP, 4MSP, 3S4MP, and 3S4MPA, at final concentrations ranging from 0 to 400 ng/L. The concentrations of the volatile thiol standards were previously determined according to Ellmans's method (18) using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). For each concentration, the volatile thiols were extracted from standard solutions according to the process described above. All calibrations produced a linear response with an  $R^2$  value  $>0.98$ , over the concentration range analyzed.

**Syntheses of New Volatile Thiols.** 3-Sulfanyl-4-methylpentyl acetate and 3-sulfanyl-4-methylpentan-1-ol were synthesized in two steps (**Figure 2a**).

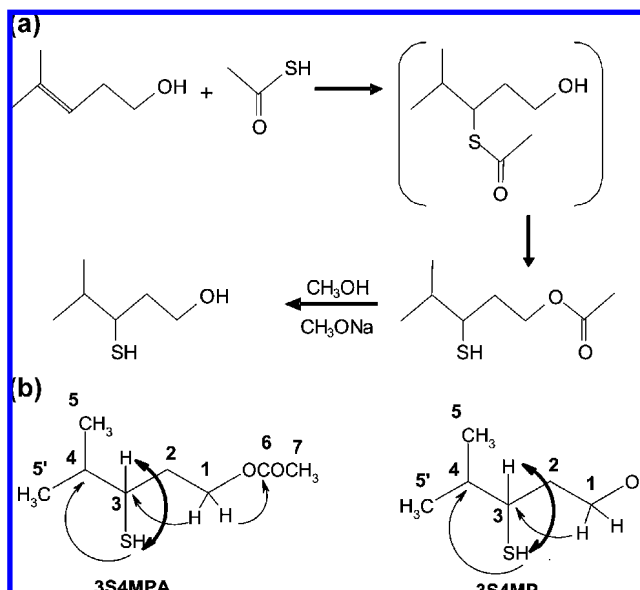
**Experimental Equipment.**  $^1$ H and  $^{13}$ C NMR spectra were recorded with a Bruker AC-300 FT ( $^1$ H, 300 MHz;  $^{13}$ C, 75 MHz), using TMS as an internal standard. Chemical shifts ( $\delta$ ) and coupling constants ( $J$ ) are expressed in parts per million (ppm) and hertz (Hz), respectively. Standard Bruker pulse sequences were used for homonuclear and heteronuclear correlation experiments (COSY, HMQC, and HMBC).

**Elementary Analysis.** Compounds with no known CAS Registry Number (CAS RN) were submitted to elementary analysis at the Service Central d'Analyse du CNRS, Service Central d'Analyse, Vernaison, France.

**3-Sulfanyl-4-methylpentyl Acetate (3S4MPA).** Freshly distilled (bp 100  $^{\circ}$ C/760 mmHg) thioacetic acid (490 mg, 6 mmol), was added to 4-methyl-3-penten-1-ol (620 mg, 6 mmol). The reaction mixture was heated to 100  $^{\circ}$ C for 50 min. After cooling to room temperature, 3-sulfanyl-4-methylpentyl acetate was obtained as a colorless oil (98%; 1 g).

$^1$ H NMR (CDCl<sub>3</sub>)  $\delta$  0.87 (d,  $^3J = 6.7$  Hz, 3H, C(CH<sub>3</sub>)<sub>2</sub>), 0.94 (d,  $^3J = 6.7$  Hz, 3H, C(CH<sub>3</sub>)<sub>2</sub>), 1.14 (d,  $^3J = 8.3$  Hz, 1H, SH), 1.57–1.65 (m, 1H, -CH<sub>2</sub>-CH<sub>2</sub>-O-), 1.78–1.82 (m, 1H, CH(CH<sub>3</sub>)<sub>2</sub>), 1.92–1.99 (m, 1H, -CH<sub>2</sub>-CH<sub>2</sub>-O-), 1.98 (s, 3H, C(O)CH<sub>3</sub>), 2.75–2.79 (m, 1H, CHSH), 4.18–4.24 (m, 2H, CH<sub>2</sub>OC(O)CH<sub>3</sub>).  $^{13}$ C NMR (CDCl<sub>3</sub>)  $\delta$  17.31 and 20.17 (C5, C5'), 20.97 (C7), 33.87 (C4), 35.24 (C2), 44.15 (C3), 62.72 (C1), 171.08 (C6).

MS (EI, 70 eV):  $m/z = 176$  [M]<sup>+</sup>;  $m/z = 116$  [M-CH<sub>3</sub>COOH]<sup>+</sup>;  $m/z = 101$  [M-CH<sub>3</sub>COOH-CH<sub>3</sub>]<sup>+</sup>;  $m/z = 73$  [M-(CH<sub>3</sub>)<sub>2</sub>CHCH(SH)CH<sub>2</sub>]<sup>+</sup>.



**Figure 2.** (a) Synthetic pathway from 4-methyl-3-penten-1-ol to 3-sulfanyl-4-methylpentyl acetate and 3-sulfanyl-4-methylpentan-1-ol. (b) HMBC ( $\leftrightarrow$ ) and COSY ( $\leftrightarrow$ ) correlations observed for 3S4MPA and 3S4MP, respectively.

Anal. Calcd for C<sub>8</sub>H<sub>16</sub>O<sub>2</sub>S: C, 54.51; H, 9.15; O, 18.15; S, 18.19. Found: C, 54.55; H, 9.08; O, 18.21; S, 18.15.

**3-Sulfanyl-4-methylpentan-1-ol (3S4MP).** 3-Sulfanyl-4-methylpentyl acetate was added to absolute methanol (16 mL) and stirred with sodium methoxide [solution of sodium (14 mg) in methanol (20 mg)] for 16 h, at room temperature and under a nitrogen atmosphere. After elimination of the methanol by distillation, water (10 mL) was added to the reaction mixture and extracted with diethylether (3  $\times$  10 mL). The organic phase was washed with water and dried over anhydrous sodium sulfate. After elimination of the diethylether by distillation, the reaction mixture was purified by silica gel chromatography (Merck silica gel 60, 70–230 mesh). This column was eluted with pentane/diethylether = 48/52 as the eluant. After elimination of the eluant, 3-sulfanyl-4-methylpentan-1-ol was obtained in liquid form ( $R_f = 0.54$ ; 60%; 480 mg).

$^1$ H NMR (CDCl<sub>3</sub>)  $\delta$  0.84 (d,  $^3J = 6.7$  Hz, 3H, C(CH<sub>3</sub>)<sub>2</sub>), 0.92 (d,  $^3J = 6.7$  Hz, 3H, C(CH<sub>3</sub>)<sub>2</sub>), 1.15 (d,  $^3J = 8.3$  Hz, 1H, SH), 1.54–1.60 (m, 1H, CH<sub>2</sub>-CH<sub>2</sub>OH), 1.78–1.86 (m, 2H, -CH<sub>2</sub>-CH<sub>2</sub>OH and CH(CH<sub>3</sub>)<sub>2</sub>), 2.80–2.84 (m, 1H, CHSH), 3.75 (t,  $^3J = 6.4$  Hz, 2H, CH<sub>2</sub>OH).  $^{13}$ C NMR (CDCl<sub>3</sub>)  $\delta$  17.45 and 20.21 (C5, C5'), 34.18 (C4), 38.80 (C2), 44.53 (C3), 60.88 (C1).

MS (EI, 70 eV):  $m/z = 134$  [M]<sup>+</sup>;  $m/z = 100$  [M-H<sub>2</sub>S]<sup>+</sup>;  $m/z = 91$  [M-CH(CH<sub>3</sub>)<sub>2</sub>]<sup>+</sup>;  $m/z = 73$  [M-CH(CH<sub>3</sub>)<sub>2</sub>-H<sub>2</sub>O]<sup>+</sup>;  $m/z = 61$  [M-CH<sub>3</sub>CH=CH<sub>2</sub>-CH<sub>2</sub>OH]<sup>+</sup>.

Anal. Calcd for C<sub>6</sub>H<sub>14</sub>OS: C, 53.68; H, 10.51; O, 11.92; S, 23.89. Found: C, 53.73; H, 10.48; O, 12.01; S, 23.86.

**Sensory Evaluation of the Identified Flavor Compounds.** *Determining Odor Thresholds.* Each sensory evaluation was performed by 10–13 well-trained panelists. Perception thresholds of the synthesized 3S4MP and 3S4MPA were assessed by a forced-choice ascending concentration series method of limits (19). Briefly, the directional triangular tests of six increasing concentrations in model carbonated dilute alcohol solution (5% v/v ethanol, 2.5 kg/cm<sup>2</sup> pressure) or in Japanese commercial beer (belonging to the Japanese third category). Then 50 mL 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 the Synergy among the Volatile Thiols.** In order to assess synergy among the volatile thiols identified, triangular tests were carried out in model carbonated dilute alcohol solution (5% v/v ethanol), as follows. A control solution containing the estimated threshold concentration of 3S4MP (40 ng/L) and a test solution containing the same



**Table 1.** Comparison of the Concentrations of Typical Terpenoids ( $\mu\text{g/L}$ ) in Pilot Brewing Samples<sup>a</sup>

beer type	beer		Happo-shu		third category	
	CSA	NS	CSA	NS	CSA	NS
hop cultivar						
$\alpha$ -humulene	tr	tr	0.2	0.4	tr	tr
$\beta$ -myrcene	0.2	0.5	2.1	3.1	2.7	5.1
linalool	14.1	12.5	6.8	10.2	9.5	10.6

<sup>a</sup> tr, trace.

concentration of 3S4MP and 20 ng/L of 3S4MPA were prepared. Then 50 mL of each sample solution was presented in plastic cups. The significance of the results was determined according to the binominal law.

In addition, a change of flavor characters by synergy of these thiols were assessed in model solution (5% v/v ethanol, carbonated), as follows. A control solution containing 120 ng/L of 3S4MP and a test solution containing the same concentration of 3S4MP and 20 ng/L of 3S4MPA were prepared. Then 50 mL of each sample solution was presented in plastic cups, and five flavor characters (flowery, fruity, green, spicy, and smoky) of each sample were scored from 0 (no flavor) to 3 (strong flavor) at intervals of 0.5.

## RESULTS AND DISCUSSION

**Comparison of Typical Terpenoids in Pilot Brewing Samples.** First, in order to confirm the brewing character of the Nelson Sauvin (NS) hop, we brewed the various types of beers using our pilot brewing apparatus. Normal beers were made with only malt. The Japanese low-tax beers, the so-called Happo-shu, were made with 24% malt. The Japanese third category was made using no malt. We used Saaz (CSA) hop, belonging to the traditional fine aroma hop, as the control, and both hops were added to the boiling worts according to late-hopping for giving hop flavors to finished beers.

Well-trained panelists evaluated these beers and represented the flavors of the beers using NS (NS Product) as fruity, citrus-like, exotic fruit-like, rhubarb-like, white wine-like, and so on. However, the flavors of the beers using CSA (CSA product) were represented as the normal hop flavor.

**Table 1** shows a comparison of the concentrations of typical terpenoids ( $\alpha$ -Humulene,  $\beta$ -myrcene, and linalool) in the pilot-brewing samples. Similar to the results in previous reports (5–7), hydrophobic terpenoids ( $\alpha$ -Humulene and  $\beta$ -myrcene) were trace in most samples. Although 6.8 to 14.1  $\mu\text{g/L}$  of linalool was detected in these samples, there is no significant difference in the concentrations of this compound between the CSA products and the NS products. Therefore, we decided that these terpenoids did not contribute to the specific flavor of the NS products.

**Identification of Two New Volatile Thiols in the NS Product and NS Hop Water Extract.** We next focused on the very small amount of volatile thiols, which have been mainly studied in wine flavors, especially Sauvignon Blanc, because the hop growers in New Zealand have represented the specific flavor of NS as Sauvignon Blanc wine-like. Tominaga et al. (13, 20, 21) reported that several volatile thiols contributed to the cultivar-specific flavor of the Sauvignon Blanc wines. 3-Sulfanylhexan-1-ol (3SH) had a grapefruit-like flavor, and its threshold was about 60 ng/L. 4-Methyl-4-sulfanylpentan-2-one (4MSP) had a box tree-like flavor, and its threshold was about 0.8 ng/L. These compounds were present in very small amounts in the wines, but they contributed to the specific flavor of the Sauvignon Blanc wines (13, 20, 21). In the field of wine flavor investigations, copper is well-known as an absorber of thiols. We tried to apply this method to our NS product. As a result of

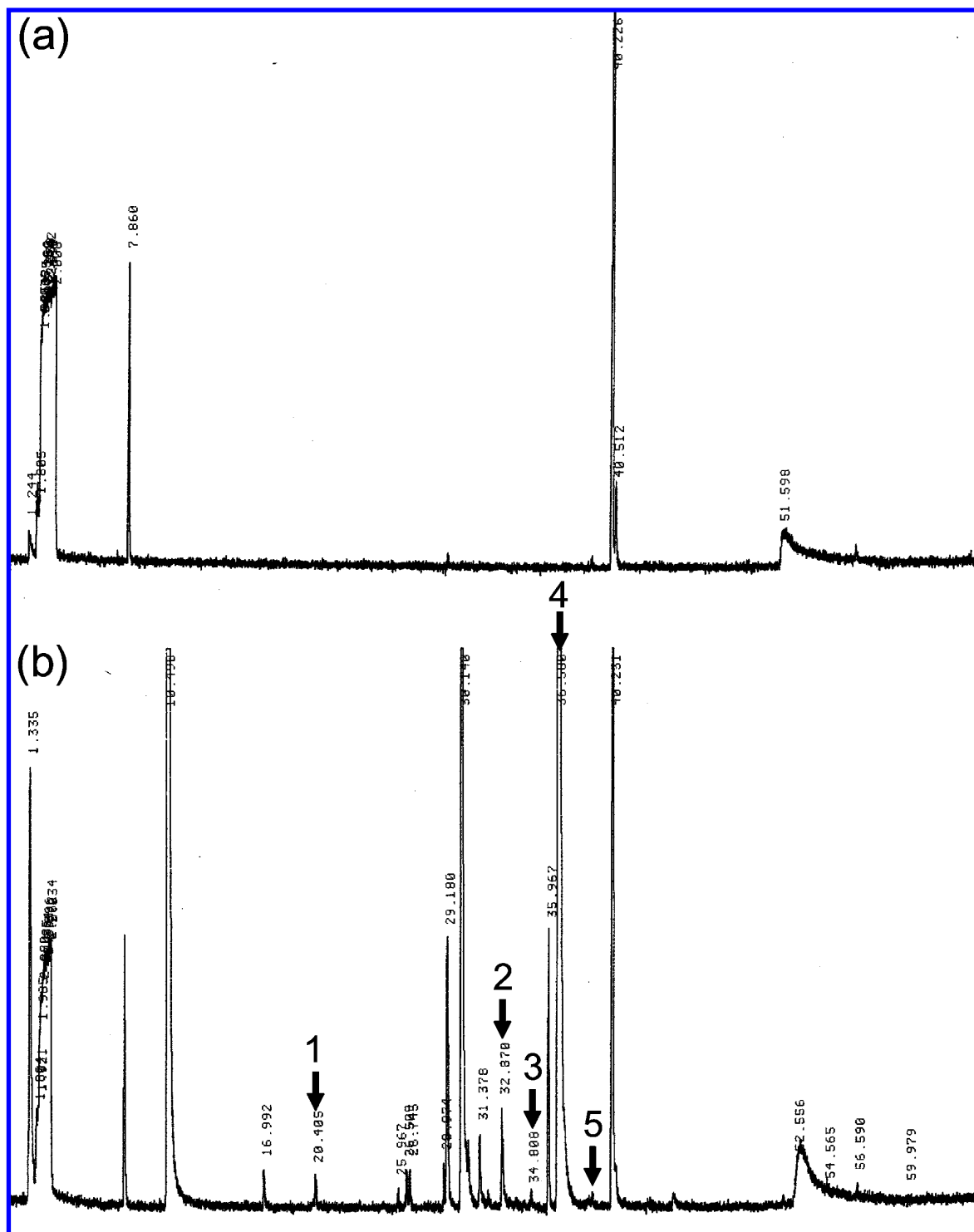
contact with copper, the NS product lost its specific flavor (data not shown). Subsequently, we tried to add 3SH to the normal commercial beer and confirmed that the flavor impression of the beer including 3SH was similar to that of the NS product (data not shown). These results could suggest the existence of thiols in the NS product. Therefore, we tried to detect the unknown thiols from the NS hop.

First, we purified the volatile thiols from the CSA product and the NS product according to the method by Tominaga et al. (13). We next extracted all the hop pellets, Hallertau Tradition (HHT), Saaz (CSA), Hallertau Magnum (HHM), Nugget (HNU), Pacific Hallertau (NPH), and Nelson Sauvin (NS), with water under the conditions described above, and the volatile thiols were purified from these water extracts. All purified samples including thiols were analyzed using GC-O, coupling with GC-FPD. From comparison between the brewing products, we found out many thiol peaks in the NS product. However, only a few thiols were detected in the CSA product. Similarly, many thiols were detected in the NS hop water extract, though thiol peaks were weak and limited in the other five hop water extracts.

**Figure 3** shows the GC-FPD analyses results of the hop water extracts of CSA and NS. Although few sulfur-containing compound peaks were detected in the CSA extract, many peaks were detected in the NS extract. The results of other hop water extracts also included few peaks (data not shown). Among these sulfur-containing compound peaks detected in the NS product and NS hop water extract, 5 odorous zones (OZ), corresponding to volatile thiols (**Table 2**), were focused on because of the odorous intensities and characters. Three of them (OZ 1, 3, and 5) had already been identified in wines (17, 20, 21) and been detected in beers and/or hops (7, 14–16). OZ 1, with a strong box tree odor, corresponds to 4-methyl-4-sulfanylpentan-2-one (4MSP) (20). OZ 3, reminiscent of citrus zest and grapefruit, corresponds to 3-sulfanylpentan-1-ol (3SP) (17). Finally, OZ 5, with a grapefruit aroma, corresponds to 3-sulfanylhexan-1-ol (3SH) (21).

The two unknown odorous zones were reminiscent of citrus zest, grapefruit, and rhubarb (OZ 2 and 4). Especially, OZ 4 was the highest GC-FPD peak in all sulfur-containing compound peaks of the NS product and the NS hop water extract. However, these two peaks have not ever been detected in any wines and beers. Therefore, we tried to identify these unknown compounds. GC-MS on the BP20 column was used to obtain two mass spectra corresponding to OZ 2 and 4. From these profiles of mass spectra, OZ 2 and 4 were assumed to be sulfanyl ester and sulfanyl primary alcohol, respectively. However, these two mass spectra did not coincide with previously reported mass spectra of sulfanyl esters and sulfanyl primary alcohols (22, 23). These profiles of mass spectra (**Figure 4**) were assumed to be 3-sulfanyl-4-methylpentyl acetate and 3-sulfanyl-4-methylpentan-1-ol [see assignments in Syntheses of New Volatile Thiols (MS 3S4MPA and MS 3S4MP)].

These substances were synthesized according to the pathway in **Figure 2a**. The 3S4MPA was obtained by reaction of thioacetic acid with the unsaturated alcohol (4-methyl-3-penten-1-ol) after trans-esterification of the thioacetate intermediate. Saponification reaction under MeOH/MeONa conditions, gave 3S4MP. The structures of the final compounds were confirmed by NMR analyses (<sup>1</sup>H and <sup>13</sup>C). The 2D NMR spectra (**Figure 2b**), including the analysis of <sup>1</sup>H–<sup>1</sup>H COSY as well as the heteronuclear multiple quantum correlations (HMQC) and the heteronuclear multiple bond correlation (HMBC) spectra allowed the assignment of all proton and carbon signals. The <sup>1</sup>H–<sup>1</sup>H COSY correlations H-S-C3-H and the HMBC correla-



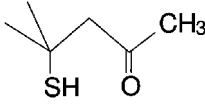
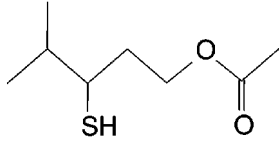
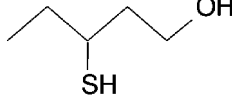
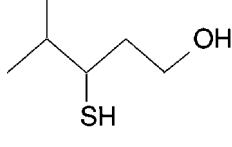
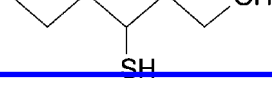
**Figure 3.** Comparison of GC-SPD peaks obtained from the analyses of the hop water extracts: (a) CSA extract; (b) NS extract; 1, 4-methyl-4-sulfanylpentan-2-one (4MSP); 2, 3-sulfanyl-4-methylpentyl acetate (3S4MPA); 3, 3-sulfanylpentan-1-ol (3SP); 4, 3-sulfanyl-4-methylpentan-1-ol (3S4MP); 5, 3-sulfanylhexas-1-ol (3SH).

tions H-S to C4 confirmed the position of the sulfanyl group for both compounds 3S4MPA and 3S4MP. The HMBC correlations, H-C1 to C6 and H-C1 to C3 (compound 3S4MPA) and H-C1 to C3 (compound 3S4MP) established the position of the acetyl or hydroxyl group.

**Figure 4** shows mass spectra of these two compounds. On the basis of the comparison of mass spectra and linear retention indices of OZ 2 and 4 with those of synthetic substances, we

concluded that OZ 2 was 3-sulfanyl-4-methylpentyl acetate (3S4MPA) and that OZ 4 was 3-sulfanyl-4-methylpentan-1-ol (3S4MP). Like 3-sulfanylhexas-1-ol (3SH), 4-methyl-4-sulfanylpentan-2-one (4MSP), and 3-sulfanylpentan-1-ol (3SP), these two new volatile thiols are strong odorants, with the O and S functions in the 1,3-position. To our knowledge, none of these two volatile thiols had ever been described in wines and other foods before.

**Table 2.** Odorous Zone (OZ) Corresponding to Volatile Thiols Detected in the NS Product

LRI <sup>a</sup>				
OZ	BP20	Descriptors	Compound	Structure
1	1389	box tree	4-methyl-4-sulfanylpentan-2-one <sup>b,c</sup>	
2	1704	grapefruit	3-sulfanyl-4-methylpentyl acetate <sup>b,c</sup>	
3	1772	grapefruit	3-sulfanylpentan-1-ol <sup>b,c</sup>	
4	1822	grapefruit	3-sulfanyl-4-methylpentan-1-ol <sup>b,c</sup>	
5	1857	grapefruit	3-sulfanylhexan-1-ol <sup>b,c</sup>	

<sup>a</sup> LRI, linear retention index calculated on the BP20 capillary column. <sup>b</sup> Coincidence of LRI and odors on the BP20 capillary column. <sup>c</sup> The mass spectrum is in agreement with the spectrum of the synthesized substance.

The standard substances of 3S4MPA and 3S4MP had the citrus zest-like, grapefruit-like, and/or rhubarb-like flavors similar to that of the NS product. Therefore, they were thought to contribute to the cultivar-specific flavor of the NS product.

**Comparison of Volatile Thiols in Pilot Brewing Samples and Hop Water Extracts.** 4MSP (OZ 1), 3S4MPA (OZ 2), 3SP (OZ 3), 3S4MP (OZ 4), and 3SH (OZ 5) were all quantified. **Table 3** shows concentrations of these thiols in the brewing products, and **Table 4** shows amounts of those in the hop pellets calculated from concentrations of each hop water extract.

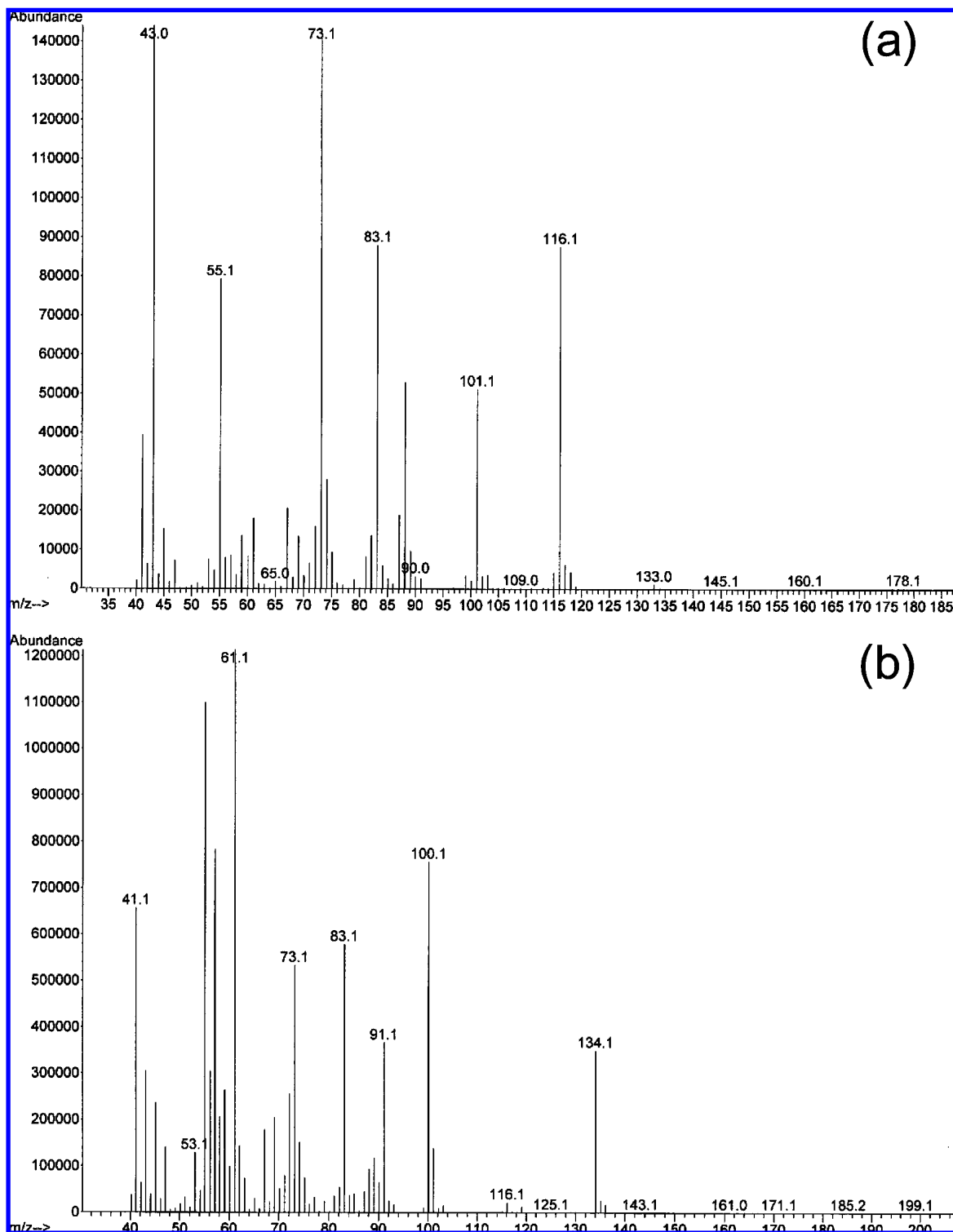
In normal beer samples (**Table 3**), 4MSP was trace in both samples (the CSA product and the NS product), and 3SP and 3SH were similar levels in both samples. However, 3S4MPA and 3S4MP were specific in the NS product. In Happo-shu samples, 4MSP, 3SP, 3S4MP, and 3SH were at quantifiable and/or significant levels, and 3S4MPA was trace in the NS product. However, 4MSP, 3SP, and 3S4MPA were not detected, 3S4MP was much smaller than that of the NS product, and only 3SH existed at quantifiable levels in the CSA product. In the third category samples, all thiols were detected in the NS product, though 4MSP, 3S4MPA, and 3S4MP were not detected, and only 3SP and 3SH were at quantifiable levels in the CSA product. From these results, 4MSP, 3SP, and 3SH were not necessarily specific in all NS products. Only 3S4MP existed in all NS products at the highest level. 3S4MPA might probably be generated from 3S4MP by esterification during fermentation. Therefore, we concluded that two new volatile thiols, 3S4MPA and 3S4MP, were cultivar-specific compounds derived from NS hop.

In hop samples (**Table 4**), 4MSP, 3SP, 3S4MP, and 3SH were detected at quantifiable and/or significant levels, and 3S4MPA was trace in the NS hop. Among all thiols, 3S4MP was at the highest level in this hop. In HHT hop, only 3SH was detected. In CSA and HNU hop, 3S4MP and 3SH were found. In HHM hop, 4MSP, 3SP, 3S4MP, and 3SH were detected at quantifiable levels, though the levels of 4MSP, 3S4MP, and 3SP were much lower than those in NS hop. In NPH hop, only 4MSP was found. From a comparison between NS hop and NPH hop, it was seemed that NS hop was a very unique cultivar in New Zealand. From these results, it is suggested that NS hop originally includes various volatile thiols at much higher levels than those in other hop cultivars and that 3S4MP is the main component among all thiols and very specific in this hop cultivar.

#### Comparison of the Amounts of 3S4MP between Various Extraction Temperatures and during Beer Fermentation.

**Table 5** shows a comparison of the amounts of volatile thiols in the NS hop water extracts between various extraction temperatures, 5 °C, 25 °C, 50 °C, and 90 °C. As a result, the higher the extraction temperature was, the more all volatile thiols extracted. In beer brewing, hop pellets are added to boiling wort. Therefore, we thought that this process (wort boiling) might have a positive effect on extracting volatile thiols from NS hop to wort.

We next compared the amounts of volatile thiols in wort with those in fermented beer. The potential amounts of thiols in boiling wort were calculated from the amounts of those in the NS hop water extract at 90 °C. This potential thiol amounts in wort was compared with the thiol amounts in the NS product



**Figure 4.** Mass spectra of 3-sulfanyl-4-methylpentyl acetate (OZ 2, 3S4MPA) (a) and 3-sulfanyl-4-methylpentan-1-ol (OZ 4, 3S4MP) (b) (synthesized substances).

of the third category (Table 6). Although 4MSP was at similar levels during fermentation, 3SP, 3S4MP, and 3SH increased at 5–6-fold after fermentation. These results suggested that the concentrations of 3SP, 3S4MP, and 3SH were increasing during beer fermentation and that there were unknown precursors of these thiols in wort, before fermentation.

It is well-known that *S*-cysteine-conjugates were precursors of volatile thiols in wines (24–26). We tried to identify the precursor of 3S4MP using an assaying method of *S*-cysteine-

conjugates (24–26). However, the *S*-cysteine-conjugate of 3S4MP was not found in the NS hop water extract (data not shown). It is probable that an unknown precursor of 3S4MP exists in the NS hop.

**Concentrations of 3S4MP and 3S4MPA in Commercial Beers.** In order to confirm the specificity of 3S4MP and 3S4MPA, volatile thiols in three Japanese commercial beers were isolated and analyzed by GC-MS (Table 7). In all beers, 3S4MP and 4MSP were traces, 3S4MPA was not detected, and

**Table 3.** Comparison of the Concentrations of Volatile Thiols (ng/L) in Pilot Brewing Samples<sup>a</sup>

beer type	beer		Happo-shu		third category		
	hop cultivar	CSA	NS	CSA	NS	CSA	NS
4MSP	tr	tr	n.d.	4.3	tr	3.0	
3S4MPA	n.d.	25.0	n.d.	tr	tr	10.0	
3SP	68.2	99.3	n.d.	19.8	10.9	27.0	
3S4MP	1.8	92.5	6.5	97.0	tr	138	
3SH	34.4	26.6	42.8	14.2	14.9	5.4	

<sup>a</sup> tr, trace; n.d., not detected.**Table 4.** Comparison of the Concentrations of Volatile Thiols (ng/L) in Hop Water Extracts (30g Hop/L Water)<sup>a</sup>

hop cultivar	HHT	CSA	HHM	HNU	NPH	NS
4MSP	n.d.	n.d.	1.8	n.d.	0.9	149
3S4MPA	n.d.	n.d.	n.d.	n.d.	n.d.	tr
3SP	n.d.	n.d.	6.4	n.d.	n.d.	125
3S4MP	n.d.	1.6	20.5	7.9	n.d.	848
3SH	6.0	2.3	10.0	3.0	n.d.	8.8

<sup>a</sup> tr, trace; n.d., not detected.**Table 5.** Comparison of the Concentrations of Volatile Thiols (ng/L) in NS Hop Water Extracts (30g Hop/L Water) between Various Extraction Temperature

extraction temperature	5 °C	25 °C	50 °C	90 °C
4MSP	57.5	113	141	160
3SP	46.6	78.6	166	326
3S4MP	411	597	1054	1922
3SH	5.6	6.7	13.6	70.6

**Table 6.** Comparison of the Concentrations of Volatile Thiols (ng/L) during Fermentation

	before fermentation	after fermentation	ratio
4MSP	2.1	3.0	1.4
3SP	4.3	27.0	6.2
3S4MP	25.6	138	5.4
3SH	0.9	5.4	5.7

**Table 7.** Comparison of the Concentrations of Volatile Thiols (ng/L) in Japanese Commercial Beers<sup>a</sup>

beer brand	A	B	C
4MSP	tr	tr	tr
3S4MPA	n.d.	n.d.	n.d.
3SP	37.0	37.0	28.0
3S4MP	tr	tr	tr
3SH	34.0	28.0	37.0

<sup>a</sup> tr, trace; n.d., not detected.

3SP and 3SH were found at a level of about 30 ng/L. 3SP and 3SH were not specific in the NS products, as described above (Table 3). Therefore, these thiols might not be derived from a certain hop.

3S4MP was specific for the NS hop. Because of this, 3S4MP and 3S4MPA might not exist in most commercial beers unless the NS hop is used. In the field of beer flavor investigations, 3-methyl-2-butene-1-thiol (3MBT) was well known as a light-struck off flavor. Recently, C. Vermerlen et al. reported the occurrence of a very small amount of volatile thiols, including 3MBT, 3SH, and 4MSP, in various commercial beers (14). However, most of these thiols have negative flavors for beer, such as onion, burnt, meaty, roasted, and so forth, except for

**Table 8.** Olfactory Descriptions and Perception Thresholds of the Volatile Thiols (3S4MP and 3S4MPA) Identified in the NS Products (ng/L) in the Model Solution (5% v/v Ethanol, Carbonated) and Commercial Beer

ref compd	olfactory descriptions	olfactory perception threshold	
		model solution	beer
3S4MPA	grapefruit, rhubarb	120	160
3S4MP	grapefruit, rhubarb	40	70

**Table 9.** Triangular Test Involving 10 Panelists

test solution	control solution	correct answers/ total answers	<i>p</i>
40 ng/L 3S4MP + 20 ng/L 3S4MPA	40 ng/L 3S4MP	8/10	0.01

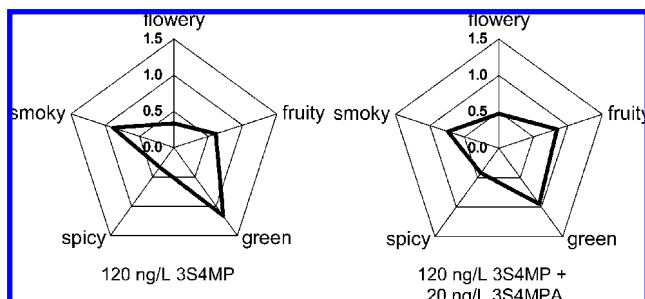
3SH and 4MSP. The amounts of 3SH and 4MSP in the beers were found to be at trace levels, only detected by GC-O, and not at quantifiable levels (14). However, we showed that significant and/or quantifiable levels of volatile thiols existed in the NS products, as described above. These thiols might give positive fruity flavors to finished beers. Recently, Kishimoto et al. reported the occurrence of quantifiable levels (5–70 ng/L) of 3SH and 3-sulfanylhexyl acetate (3SHA) in test-brewing beers (27). They also reported that 3SH was derived from not only hops but also malts. It has not been reported that volatile thiols having positive fruity flavors are derived from only a certain hop and are detected at levels of 10–130 ng/L in finished beers.

**Sensory Impact of 3S4MPA and 3S4MP on the Beer Brewed with NS Hop.** The olfactory impact of these new volatile thiols on the overall aroma of the NS products was examined by determining their odor threshold, in model carbonated dilute alcohol solution (5% v/v ethanol), or in Japanese commercial beer (belonging to the Japanese third category). Table 8 lists olfactory descriptions of these two new compounds and their perception thresholds. The aroma of 3S4MP is somewhat reminiscent of grapefruit and/or rhubarb, and its threshold is slightly lower (40 ng/L in model solution and 70 ng/L in commercial beer) than that of 3SH (60 ng/L in model solution 13, 17, 21). 3S4MPA has a grapefruit-like, peach-like, and/or rhubarb-like odor, though its threshold is much higher (120 ng/L in model solution and 160 ng/L in commercial beer) than that of 3S4MP and 3SH. 3S4MP included about 2-fold of its threshold (93–138 ng/L) and 3S4MPA was below its thresholds (10–25 ng/L) in the NS products. However, as both 3S4MP and 3S4MPA are reminiscent of citrus, possible synergy was assessed by a triangular test, as shown in Table 9. Several researchers reported that some volatile thiols, which belong to the same class of compounds and have the same odors, demonstrated an additive effect (17, 28, 29).

A control model carbonated dilute alcohol solution (5%v/v ethanol) containing 40 ng/L of 3S4MP (perception threshold of 3S4MP) was compared with a test solution containing 40 ng/L of 3S4MP, together with 20 ng/L of 3S4MPA. This concentration corresponded to a possible occurrence level of 3S4MPA in beer made from NS hop and was much lower than the threshold of 3S4MPA in the model solution. However, there was a significant difference with a risk of 1% between the control solution and the test solution (Table 9). This demonstrated that an odor of small amounts of 3S4MPA (much lower than its threshold) might be enhanced by an occurrence of 3S4MP at a threshold level.

In addition, we assessed a change of flavor character by synergy of two volatile thiols. Figure 5 shows five flavor





**Figure 5.** Flavor profile of model solutions containing 3S4MP and 3S4MPA.

characters (flowery, fruity, green, spicy, and smoky) of two solutions. A control solution contained 120 ng/L of 3S4MP, and a test solution contained the same concentration of 3S4MP and 20 ng/L of 3S4MPA. These concentrations corresponded to possible occurrence levels of these thiols in beer made from NS hop. As a result, the average scores of flowery and fruity characters were increased by adding 20 ng/L of 3S4MPA. This demonstrated that 3S4MPA could change a flavor impression of 3S4MP despite its small amount below threshold. Therefore, we concluded that both 3S4MP and 3S4MPA, which were specific in the NS product, might have a considerable impact on the overall flavor of beers brewed by using the NS hop.

**Abbreviations Used.** HHT, Hallertauer tradition; HHM, Hallertauer magnum; HNU, Nugget; CSA, Saaz; NPH; Pacific Hallertau; NS, Nelson Sauvign; 3SH, 3-sulfanylhexan-1-ol; 3SP, 3-sulfanylpentan-1-ol; 4MSP, 4-methyl-4-sulfanylpentan-2-one; 3S4MP, 3-sulfanyl-4-methylpentan-1-ol; 3S4MPA, 3-sulfanyl-4-methylpentyl acetate; GC-MS, gas chromatography–mass spectrometry; GC-O, gas chromatography–olfactometry; GC-FPD, gas chromatography–flame photometry detection; p-HMB, sodium-*p*-hydroxymercuribenzoate.

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