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Specific Flavor Compounds Derived from Nelson Sauvin Hop and Synergy of these Compounds

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Nelson Sauvin (NS) is a unique hop cultivar that gives a specific flavor (exotic fruit-like, Sauvignon Blanc wine-like) to finished beers. We have attempted to identify the specific flavor compounds derived from NS. First, isobutyric esters, including 2-methylbutyl isobutyrate (2MIB), have been found in the beers used with NS (NS product). These compounds had a green apple-like and/or apricot-like flavor. We next focused on certain volatile thiols that are well known to contribute to wine flavors, especially Sauvignon Blanc, and identified two new volatile thiols, 3-sulfanyl-4-methylpentan-1-ol (3S4MP) and 3-sulfanyl-4-methylpentyl acetate (3S4MPA), having a grapefruit-like and/or rhubarb odor, similar to that of Sauvignon Blanc. Among these compounds, 3S4MP was contained twice of its thresholds and 3S4MPA and 2MIB were contained below their threshold, in the NS product. However, it was confirmed that 3S4MP enhanced the flavors of 3S4MPA and 2MIB by synergy. In addition, we also confirmed that 3S4MP enhanced the flavors of terpene alcohols (linalool and geraniol). Therefore, we concluded that 3S4MP might contribute to the specific flavor of the NS product as a key compound having two roles, its own characteristic flavor and its function as a flavor enhancer.

Descriptors: beer aroma, hop, Nelson Sauvin, flavor, isobutyric esters, thiols, synergy

1 Introduction

In general, 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 hop cone. Several terpenoids (myrcene, humulene, linalool, etc.), esters, aldehydes and ketones were well known as the major flavor compounds in the hop oil. However, 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. Therefore, new techniques for the evaluation of the hop flavor characteristics are needed.

Recently, several researchers have focused on volatile thiols having very low threshold as characteristic flavor compounds in

beer. Among various volatile thiols, beer researchers have mainly focused on 3-methyl-2-butene-1-thiol (3MBT), as a light-struck 'skunky' off flavor [1]. On the other hand, wine researchers have revealed the contribution of volatile thiols to cultivar-specific 'fruity' flavors of certain white wines [2, 3, 4, 5]. For example, 4-methyl-4-sulfanylpentan-2-one (4MSP) and 3-sulfanylhexan-1-ol (3SH) were contributed to the cultivar-specific flavor of Sauvignon Blanc wines [2, 3, 4]. 4MSP had a box tree-like flavor and its threshold was about 0.8 ng/L. 3SH had a grapefruit-like flavor and its threshold was about 60 ng/L. In addition, 3-sulfanylpentan-1-ol (3SP) and 3-sulfanylheptan-1-ol, having grapefruit-like flavor similar to the one of 3SH, were also identified in botrytized sweet wines [5]. These compounds were present in very small amounts in the wines, but they contributed to the specific flavor of the wines [2, 3, 4, 5]. C. Vermeulen et al. reported the occurrence of a very small amount of volatile thiols, including 3MBT, 4MSP and 3SH, in various commercial beers [6]. However, most of these thiols have negative flavors for beer, like onion, burnt, meaty, roasted, etc., except for 3SH and 4MSP, and the amounts of 3SH and 4MSP in the beers that they analyzed were both at trace levels [6]. M. Steinhaus et al. proposed that 4MSP was one of the key compounds contributed to the cultivar-specific flavor of Cascade hop [7, 8]. T. Kishimoto et al. compared the concentrations of 4MSP among various hop cultivars and found that hop cultivars grown in US, including Cascade, contained larger amount of 4MSP than those grown in Europe [9]. They also analyzed 3SH and 3-sulfanylhexyl acetate (3SHA) during beer brewing process and suggested that these thiols increased during beer fermentation as well as wine fermentation [10].

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Tables and figures see Appendix

Nelson Sauvin (NS) is a unique hop variety that is bred and grown in New Zealand. This is a triploid variety developed at Hort Research, Riwaka Research Centre, from a smoothcone 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. We have focused on the NS hop and previously reported that isobutyric esters, including 2-methylbutyl isobutyrate (2MIB), and new volatile thiols, 3-sulfanyl-4-methylpentan-1-ol (3S4MP) and 3-sulfanyl-4-methylpentyl acetate (3S4MPA), were identified in the NS hop and the beers made with NS hop (Fig. 1) [13, 14]. Now, we studied how these compounds contribute to the characteristic flavor of the NS product by using sensory evaluation techniques.

2 Materials and methods

2.1 Hop raw materials

All the hop cultivars that we used were commercial ones. Hallertauer Tradition (HHT), Hallertauer Magnum (HHM) and Hallertauer Nugget (HNU) were grown in Germany. Saaz (CSA) was grown in the Czech Republic. Pacific Hallertau (NPH) and Nelson Sauvin (NS) were grown in New Zealand. (Since 2008, Pacific Hallertau has been renamed to Pacifica)

2.2 Hop extraction with water

For analysis of the flavor compounds, all hops were extracted with water. Fifteen g 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 the hop water extract.

2.3 Pilot-scale brewing

Beer, 'Happo-shu' (Japanese low-tax beer using less than 24% malt) and 'Japanese third category' (a beer-like alcohol beverage using no malt) were made from the Saaz hop (CSA) or the Nelson Sauvin hop (NS) 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 Happo-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 5min before the end of wort boiling. Boiling periods were 90 min for beer and 60 min for Happo-shu and the third category. The temperature of the fermentation was maintained at 10–15°C. After transferring the fermented wort to another lagering tank under a CO₂ 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 a CO₂ atmosphere.

2.4 Standard products

Humulene, myrcene, linalool and citronellol were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Geraniol was purchased from Aldrich Chemical Company Inc. (Milwaukee, US). Isobutyl isobutyrate, isoamyl isobutyrate, isobutyric acid and 2-methylbutan-1-ol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, 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 [5].

2.5 Syntheses of standard products

Isobutyric acid and 2-methylbutan-1-ol were esterified and 2-methylbutyl isobutyrate was obtained in the liquid form [13].

Freshly distilled thioacetic acid (b.p. 100 °C / 760 mm Hg) (490 mg, 6 mmols), was added to 4-methyl-3-penten-1-ol (620 mg, 6 mmols). The reaction mixture was heated to 100°C for 50 min. After cooling to room temperature, 3-sulfanyl-4-methylpentyl acetate was obtained as a colorless oil (98%; 1 g). This sulfanylester was added to absolute methanol (16 mL) and stirred with sodium methoxide [solution of sodium (14 mg) in methanol (20 mg)] for 16 hours, 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 x 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 chromatograph (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 the liquid form (60 %; 480 mg) [13, 14].

2.6 Quantification of terpenoids and isobutyric esters by Gas Chromatography-Mass spectrometry (GC-MS)

GC-MS analyses were carried out using a 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA). The carrier gas was helium with a column-head pressure of 15 psi and 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 μm 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 µm film thickness]. For all the analyses, the temperature program was as follows: 50 °C for 1 min, raised at 5 °C/min to 250 °C, followed by a 1 min isotherm. The terpenoids, myrcene, humulene, linalool, citronellol and geraniol were quantified in the SIM mode, selecting the following ions: *m/z* 93 (for myrcene and geraniol), 109 (for linalool and citronellol), 204 (for humulene). The isobutyric esters, isobutyl isobutyrate (IBIB), isoamyl isobutyrate (IAIB) and 2-methylbutyl isobutyrate (2MIB), were quantified in the SIM mode, selecting the following ions: *m/z* 71 and 87. Calibration curves were determined using water (including 5 % ethanol) containing these terpenoids and isobutyric esters at final concentrations ranging from 0 to 10 µg/L. All calibration produced a linear response with an R² value > 0.98, over the concentration range analyzed.

2.7 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. [4].

From a 500ml 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 and purified. Finally, 25 µL of the concentrated extract (in dichloromethane) was obtained.

2.8 Quantification of volatile thiols by GC-MS

GC-MS analyses were carried out using a 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA). A 3 µL 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, 0.25 mm i.d., 0.25 µm film thickness]. For all 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 carrier gas was hydrogen with a column-head pressure of 22 psi at the flow rate of 1 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 250 °C. The two new thiols, 3-sulfanyl-4-methylpentan-1-ol (3S4MP) and 3-sulfanyl-4-methylpentyl acetate (3S4MPA), as well as 3-sulfanylnhexan-1-ol (3SH), 3-sulfanylpentan-1-ol (3SP), 4-methyl-4-sulfanylpentan-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 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 calibration produced a linear response with an R² value > 0.98, over the concentration range analyzed [4, 5, 14].

2.9 Sensory evaluation of the flavor compounds

2.9.1 Determining Odor Thresholds

Each sensory evaluation was performed by 10-13 well-trained panelists. Perception thresholds of the synthesized 3S4MP, 3S4MPA, and 2MIB were assessed by a forced-choice ascending concentration series method of limits [15]. Briefly, the directional triangular tests of six increasing concentrations in model carbonated dilute alcohol solution (5 % v/v ethanol, 2.5 kg/cm² pressure), or in Japanese commercial beer (belonging to the Japanese third category). The 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. In addition, group thresholds of linalool, citronellol, and geraniol were assessed by same method.

2.9.2 Study of the Synergy among the Volatile Thiols

In order to assess synergy between the volatile thiols and 2MIB, 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) was compared with test solutions containing the same concentration of 3S4MP together with 20 ng/L of 3S4MPA and/or 5 µg/L of 2MIB. The 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 test solutions containing same concentration of 3S4MP together with 20 ng/L of 3S4MPA and/or 5 µg/L of 2MIB were prepared. The 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.

Furthermore, in order to assess synergy between 3S4MP and terpene alcohols, triangular tests were carried out in model carbonated dilute alcohol solution (5 % v/v ethanol, carbonated), as follows. A control solution containing the estimated threshold concentration of 3S4MP (40 ng/L) was compared with test solutions containing the same concentration of 3S4MP and a certain terpene alcohol (3 µg/L of linalool, 5 µg/L of citronellol, or 5 µg/L of geraniol). The 50 mL of each sample solution was presented in plastic cups. The significance of the results was determined according to the binominal law.

3 Results and discussions

3.1 Comparison of typical terpenoids

We brewed the various types of beers using the NS hop or the CSA hop (as the control; traditional fine aroma hop) with our pilot

brewing apparatus. Normal beers were made with only malt. The Japanese low-tax beers, the so-called 'Happo-shu', were almost made with 24 % malt. The Japanese third category was made using no malt. Both hops were added to the boiling worts according to late-hopping, as described above. Well-trained panelists evaluated these beers and represented the specific flavors of the beers using NS (NS Product) as fruity, citrus-like, exotic fruit-like, white wine-like, and so on. On the other hand, the flavors of the beers using CSA (CSA product) were represented as the normal hop flavor.

Figure 2 shows a comparison of the concentrations of typical terpenoids (myrcene, humulene, linalool, citronellol, and geraniol) in the pilot-brewing beers. This result suggested that there is no difference in the concentrations of these terpenoids between the CSA products and the NS products. Therefore, we decided that these compounds did not directly contribute to the specific flavor of the NS products.

3.2 Comparison of isobutyric esters

Subsequently, we have compared the GC-MS peaks, obtained from the SCAN mode analysis of the SPME-GC-MS, between the CSA products and the NS products, and found three specific peaks from the NS products. These peaks have been identified as isobutyl isobutyrate (IBIB), isoamyl isobutyrate (IAIB) and 2-methylbutyl isobutyrate (2MIB) [13]. Based on our sensory evaluation, the standard products of these isobutyric esters have shown fruity flavors like green apple, apricot, and so on [13]. Figure 3 shows a comparison of the concentrations of the isobutyric esters of the pilot-brewing beers. In the NS products, IBIB and IAIB were detected at about 1 µg/L, and 2MIB was at about 5–7 µg/L. On the other hand, these compounds were only slightly detected in the CSA products. We have next analyzed hop water extracts of various hop cultivars (HHT: Hallertau Tradition, CSA: Czech Saaz, HHM: Hallertau Magnum, HNU: Hallertau Nugget, NPH: NZ Pacific Hallertau, NS: Nelson Sauvin) and compared the concentrations of these isobutyric esters among various hop cultivars (Fig. 4). As a result, CSA and NPH included few of these compounds. However, not only NS but also HHT, HHM and HNU included the close levels of these compounds.

These isobutyric esters had been previously found in traditional bitter hops and modern high alpha-type hops, for example, Northern Brewer, Target, Challenger, Nugget, Galena, etc. [16]. Among traditional aroma hops, Hallertau included these compounds, on the other hand, Saaz and Lublin included few of these compounds [16]. Therefore, it is reasonable that these compounds were detected in the NS products, because NS hop was bred as the high alpha-type hop. These compounds had also found in the late hopping beers and dry hopping beers [17, 18, 19]. We have previously reported that Japanese commercial beers, almost made with kettle hopping, had included few of these compounds [13]. J. C. Seaton et al. reported that isobutyric esters could be unstable during boiling and fermentation [19]. We have also confirmed that the concentrations of isobutyric esters in late hopping worts decreased to about one third during fermentation and that these compounds were not remained in kettle hopping worts and beers (data not shown). In general, bitter hops and high alpha-type hops are not significantly used for late-hopping beers, except for English ales and ale-type

craft beers. Because of this, few of these compounds might be included in most commercial beers. J. C. Seaton et al. also suggested that the hop fraction containing myrcene and 2MIB had a citrus-like flavor [19]. Therefore, we expect that these isobutyric esters contribute to part of the NS specific flavor, because of their fruity flavors. However, the flavor characters of these compounds were different from the total flavor impression of the NS products. We speculated that the specific flavor of the NS products could not be completely explained only by these compounds.

3.3 Comparison of volatile thiols

We next focused on the very small amount of volatile thiols that 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. In the field of wine flavor investigations, copper is well known as an absorber of volatile thiols. Therefore, we have added copper to our NS product, and confirmed that the NS product lost its specific flavor as a result of contact with copper. Subsequently, we have tried to add 3SH to the beer made by using the CSA hop 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 occurrence of volatile thiols in the NS product. Therefore, we have tried to detect the unknown thiols from the NS hop and the NS products, and identified two new volatile thiols, 3-sulfanyl-4-methylpentan-1-ol (3S4MP) and 3-sulfanyl-4-methylpentyl acetate (3S4MPA), as specific compounds derived from the NS hop [14]. Standard products of 3S4MP and 3S4MPA were synthesized by the scheme described above and the structure of the resulting product was confirmed by an NMR analysis [14]. These standard products had the grapefruit-like flavor similar to the one of 3SH. 3S4MP and 3S4MPA are novel compounds that have never been discovered in wines or other foods.

Figure 5 shows a comparison of the concentrations of the volatile thiols (4MSP, 3S4MPA, 3SP, 3S4MP and 3SH) of the pilot-brewing beers. As described above, 4MSP, 3SP and 3SH have been mainly reported in the field of wine investigation [2, 3, 4, 5], and 4MSP has also focused on as the key flavor compound of certain hop cultivars, for example Cascade and several US hop cultivars [7, 8, 9]. As a result of our analysis, only 3S4MP existed in all NS products at the highest level among all thiols. 3S4MPA was detected at 0–25 ng/L in the NS products, not almost detected in the CSA products. 3S4MPA might probably be generated from 3S4MP during fermentation. On the other hand, 4MSP, 3SP and 3SH were not necessarily specific in all NS products. In figure 5, 4MSP was detected at trace levels in all products. 3SP and 3SH were contained at larger levels in the all-malt beer than in the Happo-shu and the third category. T. Kishimoto et al. suggested that 3SH was derived from not only hops but also malts, because of the occurrence of 3SH in the unhopped beer [10]. From our results, 3SP and 3SH seemed to be derived from both hops and malts. Therefore, we decided that two new volatile thiols, 3S4MP and 3S4MPA, were cultivar-specific compounds derived from NS hop.

We also analyzed volatile thiols in water extracts of various commercial hop cultivars (Fig. 6). As a result, the water extract

of the NS hop contained large amounts of 4MSP, 3SP, 3S4MP and 3SH. Few of these volatile thiols were contained in those of other hop cultivars. In contrast with the case of isobutyric esters, all volatile thiols were very specific in only NS hop. Among these thiols, 3S4MP was at the highest level in this hop. Because the NPH hop contained few of all thiols, the flavor composition of the NS hop might depend on the cultivar, not on the grown region. 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.

3.4 Sensory impact of 3S4MP, 3S4MPA and 2MIB on the Beer brewed with NS hop

Figure 1 shows the specific flavor compounds found in the NS hop and the NS products (3S4MP, 3S4MPA and 2MIB). In order to confirm the olfactory impact of these compounds on the NS products, we firstly determined their thresholds in model carbonated dilute alcohol solution (5 % v/v ethanol), or in Japanese commercial beer. Table 1 lists olfactory descriptions of these compounds, their perception thresholds, their concentrations in our test-brewing beers, and their odor activity values (OAVs: ratio of concentration to odor threshold) in beer. The aroma of 3S4MP was somewhat reminiscent of grapefruit and/or rhubarb, and its threshold is slightly lower (40 ng/L in model solution, and 70 ng/L in beer) than that of 3SH (60 ng/L in model solution [3, 4, 5]). 3S4MPA had a grapefruit-like, peach-like and/or rhubarb-like odor, though its threshold is higher (120 ng/L in model solution and 160 ng/L in beer) than that of 3S4MP and 3SH. 2MIB had a green apple-like and/or apricot-like odor and its threshold was much higher (57 µg/L in model solution and 78 µg/L in beer) than that of volatile thiols. 3S4MP was included at about twice of its threshold (93–138 ng/L) in the NS products. OAVs of 3S4MP in test-brewing beers were calculated 1.3–2.0. These OAVs clearly indicated the contribution of 3S4MP to the specific flavor of the NS product. On the other hand, OAVs of 3S4MPA are below 1.0 and those of 2MIB are below 0.1. However, we expected that 3S4MPA and 2MIB might contribute to the flavor of the NS product, because of the specific occurrence of these compounds in the NS product and their fruity flavors. Several researchers reported that some volatile thiols, which belong to the same class of compounds and have the similar odors, demonstrated an additive effect [5, 20, 21]. E. Sarrazin et al. reported that the threshold of 3SP was much higher than that of 3SH and 3-sulfanylheptan-1-ol. However, additive effect was observed by coexistence of 3SH, 3SP and 3-sulfanylheptan-1-ol [5]. In this case, 3SH and/or 3-sulfanylheptan-1-ol, having lower thresholds, seemed to functionate as a key compound of observed additive effect. Therefore, we assumed that 3S4MP might functionate as a similar key compound and assessed a possible synergy among these compounds (3S4MP, 3S4MPA and 2MIB) by triangular tests, as shown in table 2.

A control model solution (5 % v/v ethanol) containing 40 ng/L of 3S4MP (perception threshold of 3S4MP) was compared with test solutions containing 40 ng/L of 3S4MP together with 20 ng/L of 3S4MPA and/or 5 µg/L of 2MIB. These concentrations corresponded to possible occurrence levels of 3S4MPA and 2MIB in

beer made with the NS hop and were much lower than thresholds of these compounds in the model solution. However, there were significant differences with risks of below 5 % between the control solution and the test solution, in all triangular tests (Table 2). These results suggested that odors of small amounts of 3S4MPA and 2MIB, much lower than their thresholds, might be enhanced by an occurrence of 3S4MP at a threshold level. Surprisingly, the flavor of 2MIB was enhanced by coexistence with 3S4MP, though 2MIB is not thiol and OAV of 2MIB is below 0.1. It has not been reported that certain volatile thiol enhance not only the flavors of another thiol belonging to the same class of compounds, but also the one of ester belonging to the other class of compounds.

We next assessed changes of flavor character by synergy of these compounds. Figure 7 shows five flavor characters (flowery, fruity, green, spicy and smoky) of four model solutions. A control solution contained 120 ng/L of 3S4MP, and test solutions contained the same concentration of 3S4MP together with 20 ng/L of 3S4MPA and/or 5 µg/L of 2MIB. These concentrations corresponded to possible occurrence levels of these compounds in beer made from the NS hop. As a result, the average scores of 'flowery' and 'fruity' characters were increased by adding 20 ng/L of 3S4MPA or 5 µg/L of 2MIB (Fig. 7, B and C). When all of these compounds coexisted, the change of flavor impression was most drastic. The average score of 'flowery' character was increased and those of 'green' and 'smoky' were decreased (Fig. 7, D). These results demonstrated that 3S4MPA and 2MIB could change a flavor impression of 3S4MP despite their small amount below thresholds. Therefore, we decided that 3S4MP, 3S4MPA and 2MIB, which were specific in the NS product, might have a considerable impact on the overall flavor of beers brewed by using the NS hop.

3.5 Flavor enhancement of terpene alcohols by occurrence of 3S4MP

In addition, we assumed that 3S4MP might enhance other flavor compounds, because 3S4MP enhanced the flavor of 2MIB not belonging to thiol. We focused on terpene alcohols (linalool, citronellol and geraniol; see Fig. 2) mainly derived from hops, because of these floral and/or fruity flavors. In order to evaluate synergy of these compounds exactly, we firstly determined group thresholds of these terpene alcohols in model solution (5 % v/v ethanol) or in Japanese commercial beer, by using our panelists. Table 3 lists olfactory descriptions of these terpene alcohols, their group thresholds, their concentrations in our test-brewing beers, and their OAVs in beer. The aroma of linalool was reminiscent of lavender, and its threshold was estimated at 3 µg/L in model solution, and at 5 µg/L in beer. Citronellol had a lemon-like and/or lime-like odor, and its threshold was estimated at 9 µg/L in model solution, and at 8 µg/L in beer. Geraniol had a rose-like odor and its threshold was estimated at 6 µg/L in both model solution and beer. These group thresholds were at similar levels in comparison with previously reported ones (linalool; approx. 1–2 µg/L [22, 23, 24], citronellol; 8 µg/L [23] and geraniol; 4–5 µg/L [23, 24]). Linalool was included at about twice of its threshold (10–13 µg/L) in the NS products. OAVs of linalool in test-brewing beers were calculated 2.0–2.6. On the other hand, OAVs of citronellol and geraniol were below 1.0. In order to assess a possible synergy

among 3S4MP and these terpene alcohols, triangular tests were designed and carried out, as shown in Table 4.

A control model solution (5 % v/v ethanol) containing 40 ng/L of 3S4MP (perception threshold of 3S4MP) was compared with test solutions containing 40 ng/L of 3S4MP and a certain terpene alcohol (3 µg/L of linalool, 5 µg/L of citronellol, or 5 µg/L of geraniol). The concentrations of citronellol and geraniol corresponded to possible occurrence levels of these compounds in beer made with the NS hop and were lower than their thresholds in the model solution. The concentration of linalool was designed at estimated threshold level, not at possible occurrence level (10 µg/L), because 10 µg/L of linalool was expected to be enough to recognize with a significant difference for our panelists, regardless of an occurrence of 3S4MP. As a result, there were significant differences with risks of below 5 % between the control solution and the test solution containing linalool or geraniol, and there was no significant difference between the control solution and the test solution containing citronellol (Table 4). These results suggested that odors of linalool and geraniol might be enhanced by an occurrence of 3S4MP at a threshold level, as well as in the case of 2MIB. Therefore, we concluded that 3S4MP might functionate as a flavor enhancer for various compounds, including not only same class of volatile thiols, but also the other class of compounds, for example isobutyric esters and terpene alcohols.

4 Conclusions

We found some flavor compounds that were specific to the Nelson Sauvin (NS) hop and beers brewed using the NS hop (NS product). Isobutyric esters, including 2-methylbutyl isobutyrate (2MIB), had a green apple-like and/or apricot-like fruity flavor. Novel thiols, 3-sulfanyl-4-methylpentan-1-ol (3S4MP) and 3-sulfanyl-4-methylpentyl acetate (3S4MPA), had a grapefruit-like and/or rhubarb-like flavor similar to that of the Sauvignon Blanc wines. The NS hop pellet included a high concentration of 3S4MP. By comparison of various hop cultivars, 3S4MP was specific for NS. Among these compounds, 3S4MP was contained twice of its threshold and 3S4MPA and 2MIB were contained below their thresholds, in the NS product. However, it was confirmed that 3S4MP enhanced the flavors of 3S4MPA and 2MIB by synergy. These results suggested that all of these compounds (3S4MP, 3S4MPA and 2MIB) would contribute to the specific, citrus-like, exotic fruit-like and Sauvignon Blanc wine-like flavor of the NS product. In addition, we confirmed that 3S4MP enhanced the flavors of terpene alcohols, for example linalool and geraniol. From these results, it is suggested that 3S4MP, the novel volatile thiol derived from the NS hop, might contribute to the specific flavor of the NS product as a key compound having two roles, its own characteristic flavor and its function as a flavor enhancer.

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Appendix

Table 1 Olfactory descriptions, perception thresholds (in model solution [5 % v/v Ethanol, carbonated] and commercial beer), concentrations in test-brewing beers and OAV (in beer) of 3M4MP, 3M4MPA and 2MIB

ref compd	olfactory descriptions	olfactory perception threshold		Concentrations in test-brewing beers	OAV ^c in beer
		model solution ^a	beer ^b		
3S4MP (ng/L)	grapefruit, rhubarb	40 ^d	70 ^d	93–138	1.3–2.0
3S4MPA (ng/L)	grapefruit, rhubarb	120 ^d	160 ^d	0–25	< 1
2MIB (µg/L)	green apple, apricot	57	78	2.3–5.4	< 0.1

^aModel carbonated dilute ethanol solution (5 % v/v Ethanol, Carbonated).

^bJapanese commercial beer.

^cOAVs were calculated by dividing the concentrations by the respective thresholds in beer.

^dPreviously reported in ref 14.

Table 2 Synergy among 3S4MP, 3S4MPA and 2MIB

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 ^a	0.01
40 ng/L 3S4MP + 5 µg/L 2MIB	40 ng/L 3S4MP	7/10 ^a	0.05
40 ng/L 3S4MP + 20 ng/L 3S4MPA + 5 µg/L 2MIB	40 ng/L 3S4MP	7/10	0.05

^aPreviously reported in ref 14.**Table 3 Olfactory descriptions, perception thresholds (in model solution [5 % v/v Ethanol, carbonated] and commercial beer), concentrations in test-brewing beers, and OAVs (in beer) of terpene alcohols (linalool, citronellol and geraniol)**

ref compd	olfactory descriptions	olfactory perception threshold		Concentrations in test-brewing beers	OAV ^c in beer
		model solution ^a	beer ^b		
Linalool (µg/L)	lavender	3	5	10–13	2.0–2.6
Citronellol (µg/L)	lemon, lime	9	8	4–5	< 1
Geraniol (µg/L)	rose	6	6	5–6	< 1

^aModel carbonated dilute ethanol solution (5 % v/v Ethanol, Carbonated).^bJapanese commercial beer.^cOAVs were calculated by dividing the concentrations by the respective thresholds in beer.**Table 4 Synergy among 3S4MP and terpene alcohols (linalool, citronellol and geraniol)**

test solution	control solution	correct answers/ total answers	<i>p</i>
40 µg/L 3S4MP + 3 µg/L linalool	40 ng/L 3S4MP	8/13	0.05
40 ng/L 3S4MP + 5 µg/L citronellol	40 ng/L 3S4MP	6/13	–
40 ng/L 3S4MP + 5 µg/L geraniol	40 ng/L 3S4MP	9/13	0.01

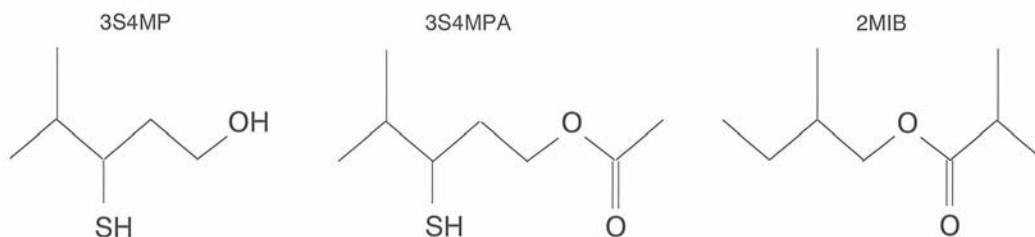


Fig. 1 Specific flavor compounds derived from NS hop

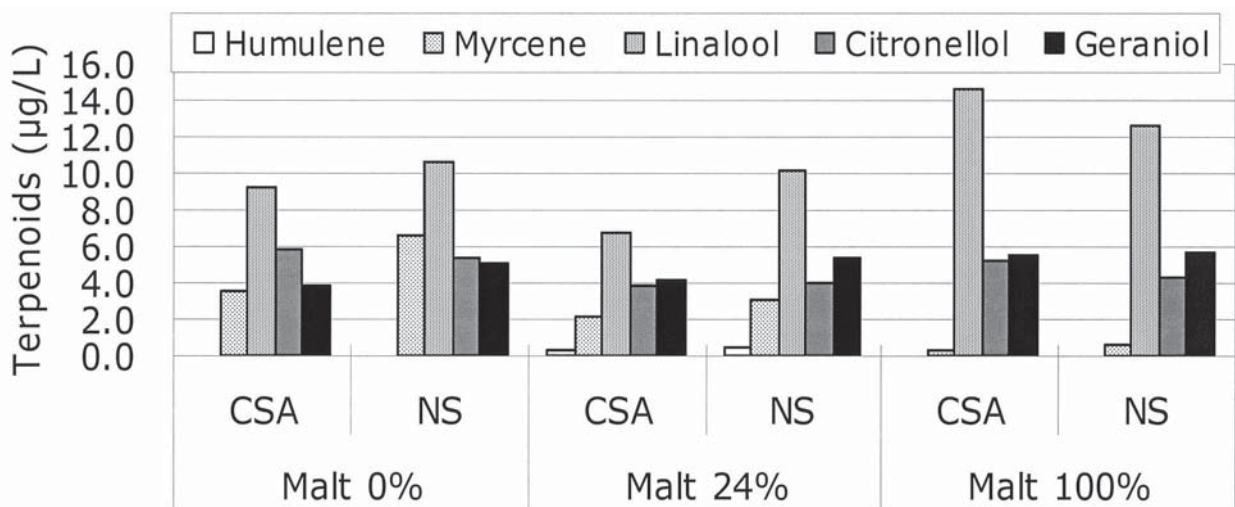


Fig. 2 Typical terpenoid concentrations in the pilot-brewing beers: CSA, Czech Saaz; NS, Nelson Sauvin; Malt 100 %,all-malt beer; malt 24 %, Japanese ‘Happo-shu’; malt 0 %, Japanese third category (the concentrations of humulene, myrcene and linalool were previously reported in ref 13, 14).

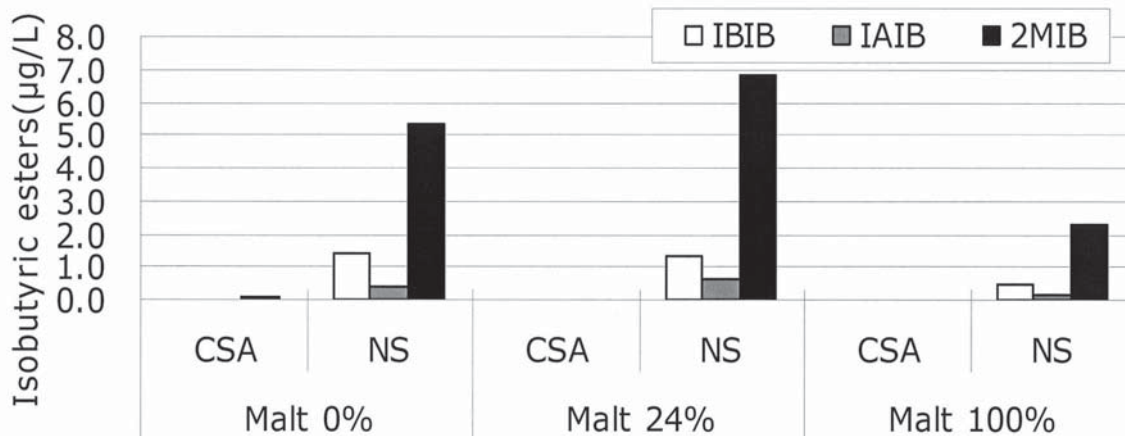


Fig. 3 Isobutyric ester concentrations in the pilot-brewing beers: IBIB, isobutyl isobutyrate; IAIB, isoamyl isobutyrate; 2MIB, 2-methylbutyl isobutyrate (previously reported in ref 13).

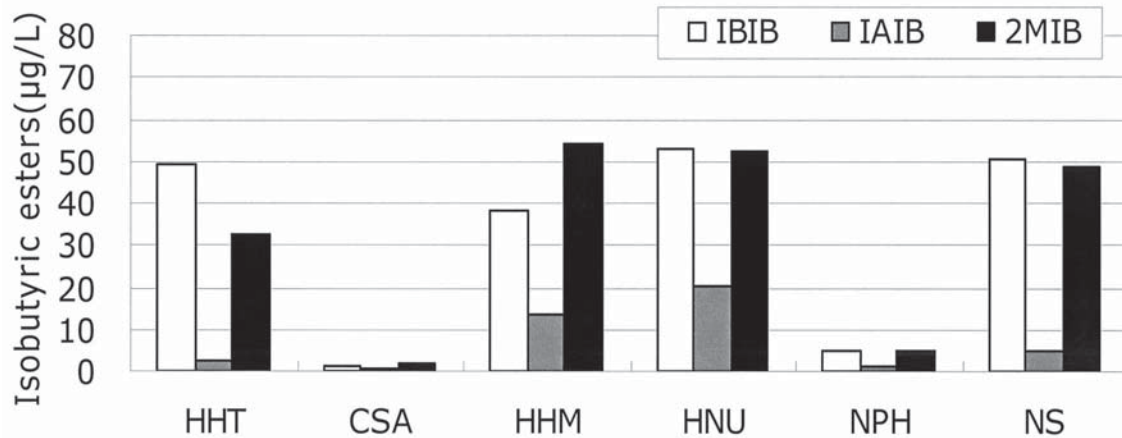


Fig. 4 Isobutyric ester concentrations in various hop water extracts (30g hop/L): HHT, Hallertau Tradition; CSA, Czech Saaz; HHM, Hallertau Magnum; HNU, Hallertau Nugget; NPH, NZ Pacific Hallertau; NS, Nelson Sauvin.

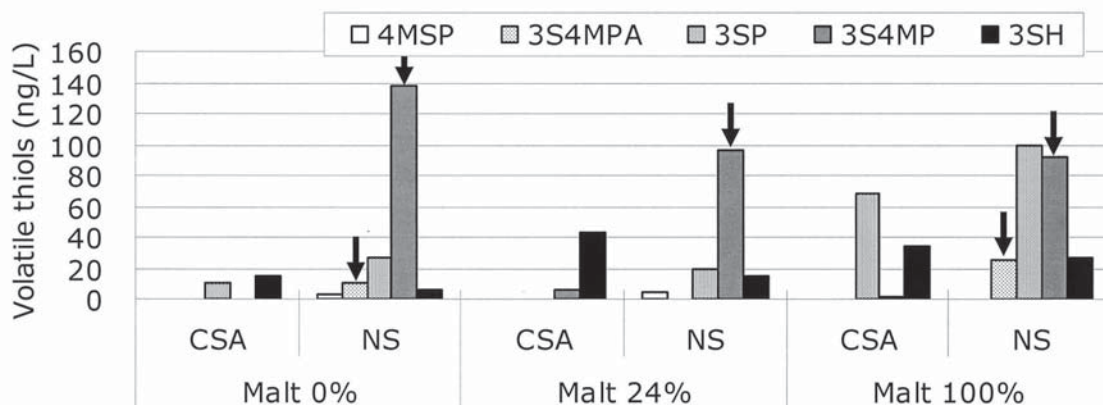


Fig. 5 Volatile thiol concentrations in the pilot-brewing beers: 4MSP, 4-methyl-4-sulfanyl-pentan-2-one; 3S4MPA, 3-sulfanyl-4-methyl-pentyl acetate; 3SP, 3-sulfanyl-pentan-1-ol; 3S4MP, 3-sulfanyl-4-methyl-pentan-1-ol; 3SH, 3-sulfanyl-hexan-1-ol (previously reported in ref 14)

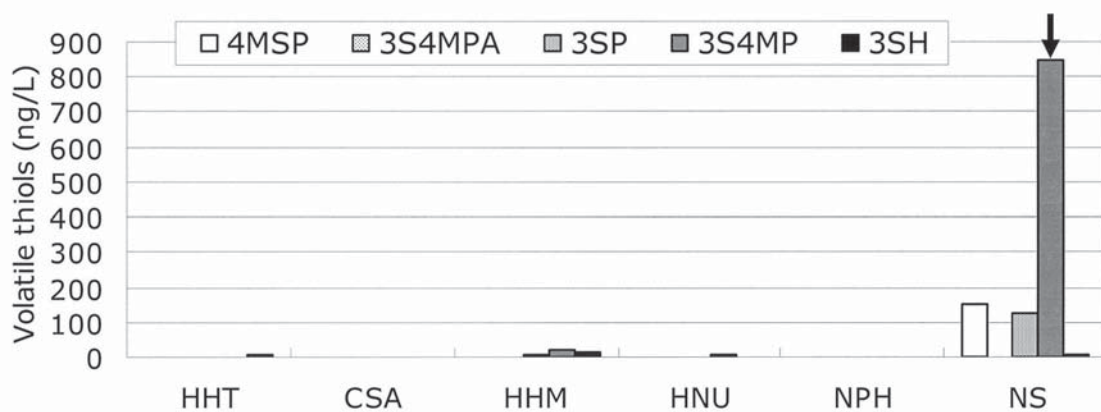


Fig. 6 Volatile thiol concentrations in various hop water extracts (30 g hop/L) (previously reported in ref 14)

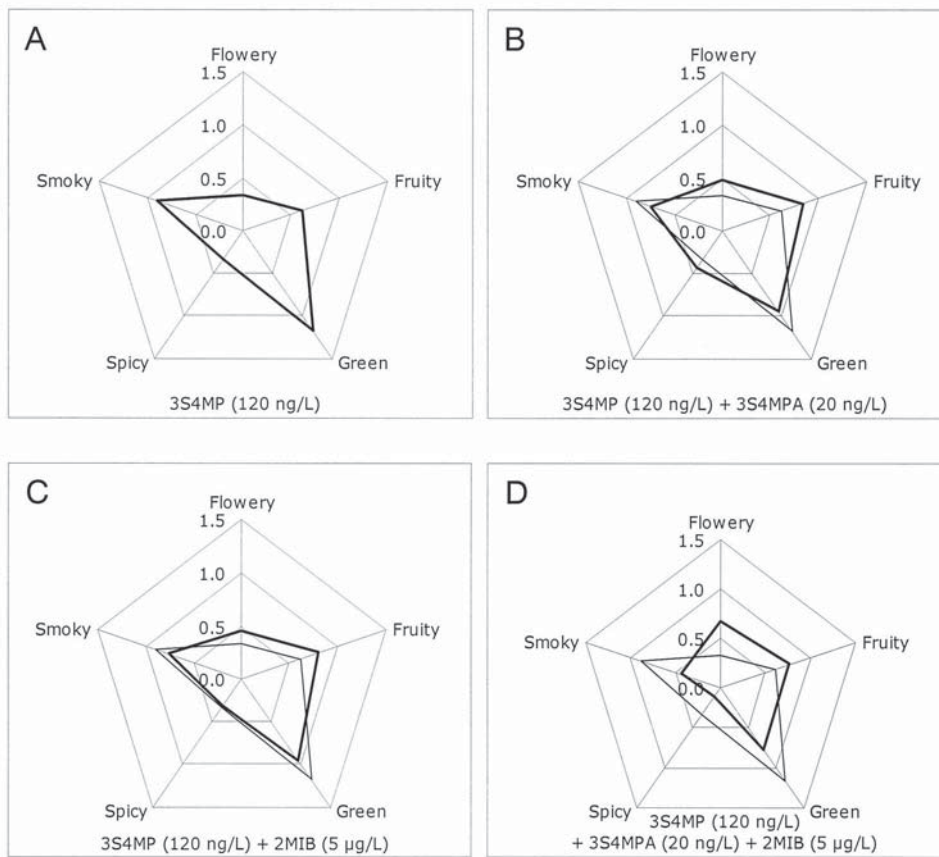


Fig. 7 Flavor profile of model solutions containing 3S4MP, 3S4MPA and 2MIB (A and B were previously reported in ref 14)