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Identification of the Compounds Responsible for the Sweat-Like Odor in Hop (*Humulus lupulus* L.) Volatile Oil

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Abstract

The aroma of hop volatile oil contains a sweat-like odor. We studied the odorous volatile compounds responsible for the sweat-like odor in the volatile oil extracted from Hallertau Perle hop (*Humulus lupulus* L.) pellets. The combined use of gas chromatography-mass spectrometry/olfactometry (GC-MS/O), aroma extract dilution analysis (AEDA) by GC-MS/O (an odor dilution technique), and heart-cut multidimensional GC-MS (heart-cut MDGC-MS) equipped with the polar (1D) and apolar (2D) capillary columns revealed seven sweat-like odor producing compounds: methyl-branched saturated and unsaturated aliphatic acids, such as 3-methylbutanoic acid (sweaty/rancid/cheese-like), 2-methylbutanoic acid (sweaty/rancid/cheese-like), 4-methyl-3-pentenoic acid (sweaty/urine-like/malodor in laundry), and (*E*)-4-methyl-3-hexenoic acid (sweaty/urine-like/malodor in laundry), as well as others, such as an unknown compound (sweaty), methyl (*E*)-4-methyl-3-hexenoate (sweaty/malodor in laundry/fruity), and *S*-methyl (*E*)-4-methyl-3-hexenethioate (sweaty/rubber). The reference substances were synthesized stereoselectively using for the identification procedures. In this study, (*E*)-4-methyl-3-hexenoic acid, methyl (*E*)-4-methyl-3-hexenoate, and *S*-methyl (*E*)-4-methyl-3-hexenethioate were identified for the first time in hop volatile oil.

Keywords: hop (*Humulus lupulus* L.), sweaty odor, gas chromatography-mass spectrometry/olfactometry (GC-MS/O), aroma extract dilution analysis (AEDA), multidimensional gas chromatography-mass spectrometry (MDGC-MS), organic synthesis

Abbreviations: AEDA: aroma extract dilution analysis; 1D: first dimension; 2D: second dimension; FID: flame ionization detector; FD: flavor dilution; FPD: flame photometric detector; GC: gas chromatography; GC-MS/O: gas chromatography-mass spectrometry/olfactometry; GC-O: gas chromatography-olfactometry; heart-cut MDGC-MS: heart-cut multidimensional gas chromatography-mass spectrometry; IR: Infrared; NMR: nuclear magnetic resonance; RI: retention index; ^{t}R: retention time.

1. Introduction

Hop (*Humulu slupulus* L.) is essential material for beer production along with water, malted barley, and yeast, which act the characteristic hop aroma and bitter taste. To date, 429 distinct hop volatiles have been reported (Nijssen, Ingen-Visscher, & Donders, 2013). The odor-active compounds contributing to the overall aroma of hops have been found by means of aroma extract dilution analysis (AEDA) (Ullrich & Grosch, 1987), an odor dilution technique for screening using gas chromatography-olfactometry (GC-O) (Steinhaus & Schieberle, 2000; Lermusieau, Bulens, & Collin, 2001; Steinhaus, Wilhelm, & Schieberle, 2007). Steinhaus and Schieberle (2000) determined 38 and 31 odor-active compounds of fresh and dried hop cones (Spalter Select), respectively. Lermusieau et al. (2001) reported 56 odor-active components in fresh hop pellets (Challenger and Saaz). Later, Steinhaus et al. (2007) characterized 38 odor-active compounds in five different hop varieties (Hallertau Perle, Hallertau Hersbrucker Spät, Slowenian Golding, Hallertau Smaragd, and US Cascade). In addition, by another dilution technique, many odor-active compounds have been identified in hops (Eyres & Dufour, 2009); however, not all odorous compounds of hops have been identified.

The hop aroma contains a sweat-like odor (Furukawa, Murakami, & Ichii, 2012). Other than brief descriptions (Steinhaus & Schieberle, 2000; Lermusieau et al., 2001; Steinhaus et al., 2007), there are no detailed reports regarding the aroma components responsible for the sweat-like odor in hops, most of which may contribute to the overall aroma. Based on our previous study (Miyazato, Hashimoto, & Hayashi, 2013), we predict that the

sweat-like odor perceived in hop volatile oil might be associated with volatile carboxylic acids.

The focus of this study is on the novel sweat-like-odor active compounds in Hallertau Perlehop volatile oil. The oil was extracted from the pelletized hop by simultaneous steam distillation–extraction. In order to concentrate the extracted hop oil, the following three procedures were conducted: chemical treatment by an alkaline solution to separate the acidic and neutral/basic volatile fractions, fractionation by silica gel column chromatography, and separation by preparative GC. The concentrated volatile fractions were analyzed by gas chromatography-mass spectrometry/olfactometry (GC-MS/O), AEDA, and heart-cut multidimensional gas chromatography-mass spectrometry (heart-cut MDGC-MS) to identify target active compounds with a sweat-like odor by comparison with synthesized reference substances.

2. Materials and Methods

2.1 Materials

Hallertau Perle hop pellets (product name: Hallertau Perle Pellets 90) were cultivated in Germany in 2007. The sample was encased in a hermetic dark package and stored at −15 °C prior to use.

2.2 Chemicals

4-Methyl-3-pentenoic acid was synthesized according to the literature (Mikolajczak & Smith Jr., 1978). (*E*)-4-Methyl-3-hexenoic acid was synthesized stereoselectively in two steps, according to the literature (Miyazato et al., 2013). Methyl (*E*)-4-methyl-3-hexenoate was synthesized via boron trifluoride-catalyzed methyl esterification of (*E*)-4-methyl-3-hexenoic acid starting from (*E*)-4-methyl-3-hexen-1-ol. *S*-Methyl (*E*)-4-methyl-3-hexenethioate was synthesized via the direct thioesterification of (*E*)-4-methyl-3-hexenoic acid with methyl chlorothiolformate according to the literature (Khan et al., 1999), starting from (*E*)-4-methyl-3-hexen-1-ol.

The starting substances for the ester or thioester synthesis were commercially available. Boron trifluoride-methanol solution (14%) and methyl chlorothiolformate were purchased from Sigma Aldrich Japan (Tokyo, Japan). *tert*-Butyl methyl ether, dichloromethane, 4-dimethylaminopyridine, and sodium chloride were purchased from Wako Pure Chemical Industries (Osaka, Japan). Acetone, pentane, 2-propanol, hexane, ethyl acetate, diethyl ether, hydrochloric acid (35%), triethylamine, sodium hydrogen carbonate, anhydrous sodium sulfate, anhydrous magnesium sulfate, and silica gel (Ultra Pure Silica Gel 230-400 mesh, SiliCycle Inc., Quebec City, Canada) were purchased from Nacalai Tesque (Kyoto, Japan). Jones reagent was prepared according to the literature (Miyazato et al., 2013).

2.3 Syntheses

2.3.1 Methyl (*E*)-4-methyl-3-hexenoate

(*E*)-4-Methyl-3-hexen-1-ol (300 mg) was dissolved in acetone (25 mL) at 0 °C. The Jones reagent (400 μL) was added to the solution and the mixture was stirred at 0 °C for 5 min. 2-Propanol (300 μL) and sodium hydrogen carbonate (150 mg) was added and then the mixture was filtered by a filter paper (Grade No.2, ADVANTEC, Tokyo, Japan). The organic phase was dried over anhydrous magnesium sulfate, filtered by a filter paper, and then concentrated using a rotary evaporator Rotavapor R-200 (Nihon Büchi K.K., Tokyo, Japan)(30 mmHg, 40 °C) to give a green product, which was mixed with boron trifluoride-methanol (2 mL) and then heated at 100 °C for 2 min. After cooling at room temperature, the mixture was shaken with brine (4 mL) for 2 min. The aqueous phase was extracted with pentane (1 × 10 mL). The organic phase was dried over anhydrous magnesium sulfate, filtered by a filter paper, and then concentrated using a rotary evaporator (760 mmHg, 40 °C) to afford a crude product, which was then purified by flash column chromatography on silica gel (hexane/ethyl acetate = 98/2, v/v) to give methyl (*E*)-4-methyl-3-hexenoate (110 mg; impurities: 19% by GC), which was further purified by preparative GC under the conditions shown in Table 1. Final purification level was 87% by GC. The spectral data except for mass spectrum are shown below.

IR (neat, cm^{-1}): 2966 m, 1743 s, 1436 m, 1365 w, 1314 w, 1259 w, 1199 w, 1160 m, 1110 w, 1016 m, 836 w. ^{1}HNMR (401.3 MHz, $CDCl_3$, δ ppm): 5.30 (1H, t, J = 7.4 Hz, C=C$\underline{H}CH_2$), 3.68 (3H, s, O$C\underline{H}_3$), 3.05 (2H, d, J = 6.9 Hz, =CH$C\underline{H}_2$C=O), 2.03 (2H, q, J = 7.4 Hz, $CH_3C\underline{H}_2$C=), 1.63 (3H, s, $C\underline{H}_3$C=), 1.00 (3H, t, J = 7.4 Hz, $C\underline{H}_3CH_2$). ^{13}C NMR (100.9 MHz, $CDCl_3$, δ ppm): 173.19 ($\underline{C}OOCH_3$), 141.06 (=$\underline{C}$=), 114.37 (=$\underline{C}HCH_2$), 51.88 (O$\underline{C}H_3$), 33.66 (CH$\underline{C}H_2COOCH_3$), 32.37 ($CH_3\underline{C}H_2$C=), 16.39 ($\underline{C}H_3$C=), 12.60 ($\underline{C}H_3CH_2$).

2.3.2 *S*-Methyl (*E*)-4-methyl-3-hexenethioate

The crude product containing the corresponding acid, obtained from (*E*)-4-methyl-3-hexen-1-ol (300 mg) in the same manner as the above ester synthesis, was mixed with 4-dimethylaminopyridine (1.4 mg) and triethylamine

(2 mL) in dichloromethane (60 mL). After cooling at 0 °C, methyl chlorothiolformate (480 μL) was added and the mixture was stirred at 0 °C for 1 h followed by incubating at room temperature for 4 h. After adding 1 N hydrochloric acid (20 mL), the mixture was extracted with *tert*-butyl methyl ether (3 × 50 mL). The combined organic phases were washed with saturated sodium hydrogen carbonate solution (3 × 20 mL) and brine (3 × 20 mL), dried over anhydrous magnesium sulfate, filtered via the filter paper, and then concentrated using a rotary evaporator (30 mmHg, 40 °C) to afford a crude product, which was then purified by flash column chromatography on silica gel (hexane/ethyl acetate = 98/2, v/v) to give *S*-methyl (*E*)-4-methyl-3-hexenethioate (157 mg; impurities: 80% by GC), which was further purified by preparative GC under the conditions shown in Table 1. Final purification level was 66% by GC. The spectral data except for mass spectrum are shown below.

IR (neat, cm^{-1}): 2965 m, 2929 m, 1693 s, 1656 m, 1408 w, 1379 w, 1249 w, 1102 m, 1071 m, 1016 m, 854 w. ^{1}H NMR (401.3 MHz, $CDCl_3$, δ ppm): 5.30 (1H, dt, *J* = 1.4, 7.4 Hz, C=C$\underline{H}$CH$_2$), 3.26 (2H, d, *J* = 7.4 Hz, =CHC$\underline{H}_2$C=O), 2.27 (3H, s, SC$\underline{H}_3$), 2.06 (2H, q, *J* = 7.4 Hz, CH$_3$C$\underline{H}_2$C=), 1.66 (3H, s, C$\underline{H}_3$C=), 1.02 (3H, t, *J* = 7.4 Hz, C$\underline{H}_3$CH$_2$).^{13}C NMR (100.9 MHz, $CDCl_3$, δ ppm): 199.31 (C(=O)SCH$_3$), 142.81 (=C=), 114.12 (=CHCH$_2$), 43.28 (=CHCH$_2$C(=O)), 32.47 (CH$_3$CH$_2$C=), 16.50 (CH$_3$C=), 12.51 (CH$_3$CH$_2$), 11.82 (SCH$_3$).

2.4 Methods

The sample preparation is summarized in a flowchart (Figure 1).

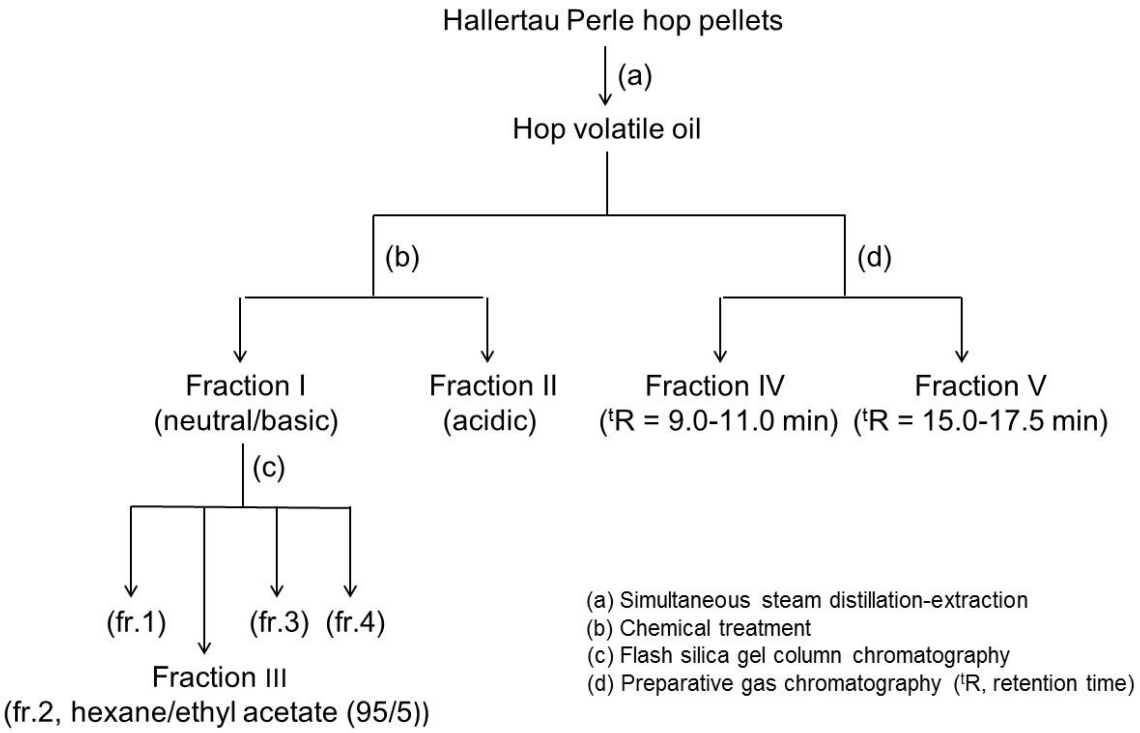

Figure 1. Sample preparation flowchart

2.4.1 Isolation of the Hop Volatile Oil

The hop pellet (100.6 g) oil extraction was performed using simultaneous steam distillation–extraction with a Likens-Nickerson extractor (Likens & Nickerson, 1964) and diethyl ether (200 mL) for 1.5 h. The extract was dried over anhydrous sodium sulfate, filtered via the filter paper, and then concentrated using a rotary evaporator (760 mm Hg, 40 °C) to produce the hop volatile oil (~1.2 g).

2.4.2 Separation of Acidic and Neutral/Basic Fractions by Chemical Treatment

The hop volatile oil (1.01 g) was dissolved with diethyl ether (30 mL). Sodium hydrogen carbonate (10%) aqueous solution (55 mL) was added, and the mixture was stirred at room temperature for 16 h. After separation of the basic aqueous phase from the ethereal phase, the aqueous phase was washed with diethyl ether (2 × 20

mL). The combined organic phases were dried over anhydrous sodium sulfate, filtered via the filter paper, and then concentrated using a rotary evaporator (760 mmHg, 40 °C) to give the neutral/basic fraction I (~0.89 g). To generate the acidic fraction, the basic aqueous phase was acidified with 50% hydrochloric acid and then the solution was extracted with diethyl ether (3 × 30 mL). The combined organic phases were washed with brine (4 × 30 mL) and then dried over anhydrous sodium sulfate. After filtered via the filter paper, the filtrate was concentrated to yield the acidic fraction II (~0.03 g) to analyze by GC-MS/O and heart-cut MDGC-MS.

2.4.3 Flash Silica Gel Column Chromatography

The neutral/basic fraction I (0.89 g) was loaded onto a hexane-filled silica gel column (25.3 g, 7.5 cm long × 3.4 cm in diameter). The fraction was subjected to flash column chromatography. Elution was performed by 200 mL hexane (100%, fraction [fr.] 1), 200 mL hexane/ethyl acetate (95/5, v/v, fr. 2), 200 mL hexane/ethyl acetate (80/20, v/v, fr. 3), and finally 200 mL ethyl acetate (100%, fr. 4). The eluate (fr. 2) was concentrated using a rotary evaporator (30 mm Hg, 40 °C) to yield the concentrated fraction III (~0.20 g) to analyze by GC-MS/O.

2.4.4 Preparative Gas Chromatography (GC)

Preparative GC was conducted using a Shimadzu GC-14B gas chromatograph connected with a thermal conductivity detector (TCD) (Shimadzu, Kyoto, Japan). The chromatograph was equipped with an InertCap WAX (polyethylene glycol) fused silica capillary column (30 m × 0.53 mm i.d.; film thickness, 1.00 μm) (GL Sciences Inc.,Tokyo, Japan). The flow rate of the carrier gas, helium, was 2.5 mL/min. The oven temperature was programmed from 50 °C (maintained for 5 min) to 230 °C (maintained for 60 min) at a rising rate of 5.0 °C/min. The injector and the TCD temperatures were maintained at 250 °C. The exit of the chromatograph (TCD vent) was equipped with an amputated DB-WAX (polyethylene glycol) capillary column (18 cm × 0.53 mm i.d.; film thickness, 1.00 μm) (J & W Scientific Inc., Tokyo, Japan), which was used to trap the eluted fractions. The samples were introduced in the direct injection mode. The separation conditions are summarized in Table 1. The hop oil was separated by repeating the procedure 10 times and 50 times to obtain the concentrated fractions IV and V, respectively, to analyze by heart-cut MDGC-MS.

Table 1. Preparative GC analysis conditions

Sample	Cutting time span (min)	Injection volume (μL)	Rate of temperature increase (°C/min)
Hop volatile oil[a), b)]	9.0–11.0 (for **C**) 15.0–17.5 (for **D**)	10.0	5
Methyl (*E*)-4-methyl-3-hexenoate[c)]	13.0–15.5	5.0	5
S-Methyl (*E*)-4-methyl-3-hexenethioate[d)]	19.0–20.5	5.0	5

a) Repeated 10 times for **C**.

b) Repeated 50 times for **D**.

c) Repeated 5 times.

d) Repeated 15 times.

2.5 Instrumental Analysis

2.5.1 Gas Chromatography-Mass Spectrometry/Olfactometry (GC-MS/O)

GC-MS/O analysis was carried out using a GC-17A gas chromatograph (Shimadzu) connected with a mass spectrometer (GCMS-QP5050, Shimadzu) and coupled with an olfactory port (OP275, GL Sciences Inc.). The chromatograph was equipped with an InertCap 1 (100% methylpolysiloxane) fused silica capillary column (60 m × 0.25 mm i.d.; film thickness, 0.25 μm) (GL Sciences Inc.) or with an InertCap WAX capillary column (60 m × 0.32 mm i.d.; film thickness, 0.25 μm) (GL Sciences Inc.), which were interfaced with both the mass spectrometer and the olfactory port via a splitter. The flow rate of the carrier gas, purified helium (≥ 99.99995%), was 3.8 mL/min at 70 °C. The inlet system was a split/splitless mode. The inlet pressure was 170 kPa. The oven temperature was programmed from 70 °C (maintained for 5 min) to 240 °C at 3°C/min. The injector and the interface temperatures were maintained at 250 °C. The mass spectra in the electron impact mode were generated at 70 eV. Data were collected in full scan mode. The mass scan range was *m/z* 27-300. Olfactory detection operated during the above chromatographic separation. The temperature of the olfactory port was maintained at

260 °C. Damp air (nitrogen/oxygen = 80/20) was constantly pulled at a rate of 30 mL/min via the head of the olfactory port during operation. Using the retention times of the hydrocarbons C6-C27, retention indices (RI) were calculated according to the literature (Kováts, 1958). The samples were injected under the conditions shown in Table 2. Data handling was performed using GCMS solution 1.01 Su3 (Shimadzu).

2.5.2 Aroma Extract Dilution Analysis (AEDA) (Ullrich & Grosch, 1987)

The hop volatile oil was diluted stepwise with ethanol (1:1). Aliquots were analyzed by GC-MS/O equipped with an InertCap WAX capillary column. The volume of the injection was 3.0 μL, and the split ratio was 1:20. The flavor dilution (FD) factors (2^n) of the target compounds were estimated rom the values of the dilution degree (n).

Table 2. GC-MS/O analysis conditions

Sample[a)]	Injection volume (μL)	Injection mode (split/splitless)
Hop volatile oil[b)]	3.0	1:10
Fraction I	3.0	1:20
Fraction II	1.0	1:20
Fraction III	2.0	1:20
Fraction IV	2.0	1:20
Fraction V	3.0	1:20

a) I: neutral/basic fraction; II: acidic fraction; III: silica gel column chromatographic fraction (95/5, v/v); IV: the concentrated fraction by preparative GC (9.0–11.0 min); V: the concentrated fraction by preparative GC (15.0–17.5 min)

b) Injection volume: 3.0 μL; injection mode: 1:20 for AEDA.

2.5.3 Gas Chromatography-Flame Photometric Detector (GC-FPD)

GC-FPD analysis was carried out using an Agilent 6890N GC gas chromatograph connected with a flame photometric detector (FPD). The chromatographer was fitted with an InertCap Pure WAX (polyethylene glycol) fused silica capillary column (60 m × 0.25 mm i.d.; film thickness 0.25μm) (GL Sciences Inc.). The flow rate of the carrier gas, purified helium (≥ 99.99995%), was 0.8 mL/min. The inlet pressure was 230 kPa. The injector temperature was 250 °C. The oven temperature was increased from 70 °C (maintained for 5 min) to 240 °C at a rate of 3 °C/min. The FPD temperature was 250 °C; H_2 flow, 75 mL/min; airflow, 100 mL/min; and the make-up flow (He), 10 mL/min. The fraction V (3.0 μL) was injected in a split ratio of 1:5. Data handling was performed via Chemstation software G1701DJ MSD, version C.00.01J (Agilent Technologies Japan). Using the retention times of the hydrocarbons C6-C27, retention indices (RI) were calculated according to the literature (Kováts, 1958).

2.5.4 Heart-Cut Multidimensional Gas Chromatography-Mass Spectrometry (MDGC-MS)

Heart-cut MDGC-MS analysis was carried out using an Agilent two-dimensional gas chromatography system, which consisted of Agilent 6890A gas chromatographs for the first (1D) and the second dimensions (2D). The 1D chromatograph was connected with a flame ionization detector (FID), while the 2D chromatograph was coupled to a mass spectrometer (Agilent 5973N MSD, Agilent Technologies Japan, Tokyo, Japan). The 1D chromatograph was connected with the 2D chromatograph via a Gerstel Cryo Trap system CTS1 (Gerstel K.K., Tokyo, Japan). The 1D chromatograph was equipped with a Gerstel multi column switching system MCS2 (Gerstel K. K.) for the heart-cutting operation. Moreover, the 1D chromatograph was equipped with a TC-WAX (polyethylene glycol) fused silica capillary column (30 m × 0.25 mm i.d.; film thickness, 0.25 μm) (GL Sciences Inc.), while the 2D chromatograph was fitted with an InertCap 1 capillary column (60 m × 0.25 mm i.d.; film thickness, 0.25 μm) (GL Sciences Inc.). The flow rate of the carrier gas, purified helium (≥ 99.99995%), was 3.8 mL/min at 70 °C. The inlet system was a split/splitless mode. The inlet pressure was 103 kPa. The injector and the transfer line temperatures were maintained at 250 °C. The FID temperature was 250 °C; H_2 flow, 60 mL/min; airflow, 100 mL/min; and the make-up flow (He), 20 mL/min. The mass spectra in the electron impact mode were generated at 70 eV with the ion source temperature at 230 °C. Data were collected in full scan mode. The mass scan range was *m/z* 27-300. The heart-cutting operations for the target compounds were performed under

the conditions shown in Table 3. Data handling was performed via Chemstation software G1701CA, version C.00.00 21-Dec-1999 (Agilent Technologies Japan). Private and commercially available databases (Wiley 275 and NIST 02) were used for identification.

2.5.5 Nuclear Magnetic Resonance

^{1}H NMR spectra were recorded on a FT-NMR JMN-ECS 400 (400 MHz) spectrometer (JEOL Ltd., Tokyo, Japan). Chemical shifts are reported in parts per million (δ) using solvent as an internal standard ($CDCl_3$ at 7.26 ppm). The coupling constants, *J*, are given in hertz. Chemical patterns are indicated as follows: s, singlet; d, doublet; t, triplet; q, quartet; dt, double triplet. ^{13}C NMR spectra were recorded on a FT-NMR JMN-ECS 400 (100 MHz) spectrometer (JEOL Ltd.). Chemical shifts are reported in parts per million (δ) using solvent as an internal standard ($CDCl_3$ at 77.00 ppm).

2.5.6 Infrared (IR) Spectroscopy

IR spectra were recorded using an FTIR-8200PC spectrometer (Shimadzu). The spectrum peaksare reported as wave numbers (cm^{-1}). The intensities are indicated as follows: w, weak; m, medium; s, strong.

Table 3. Heart-cut MDGC-MS analysis conditions

Unknown target compound	Heart-cutting time span (time)	Injection volume (μL)	Injection mode (split/splitless)	1D temperature program[a)]	2D temperature program[b)]	CTS temperature (°C)
A	39.80–40.80	1.0	1:5	70 °C (5 min) to 240 °C, at 3 °C/min	70 °C (45 min) to 240 °C, at 3 °C/min	250
B	43.20–43.80	2.0	Splitless (1 min)	70 °C (5 min) to 240 °C, at 3 °C/min	70 °C (45 min) to 240 °C, at 3 °C/min	250
C	19.90–21.50	1.0	Splitless (1 min)	70 °C (5 min) to 240 °C, at 3 °C/min	70 °C (25 min) to 240 °C, at 3 °C/min	250
D[c)]	23.75–25.30	3.0	1:5	70 °C (5 min) to 120 °C (1 min), at 3 °C/min, to 160 °C (1 min), at 1 °C/min, to 200 °C (40 min), at 3 °C/min	80 °C (10 min) to 100 °C (5 min), at 1 °C/min, to 240 °C (50 min), at 4 °C/min	−20[d)]

a) TC-WAX (30 m × 0.25 mm i.d.; film thickness, 0.25 μm).

b) InertCap 1 (60 m × 0.25 mm i.d.; film thickness, 0.25 μm).

c) Repeated 5 times.

d) Raised to 250 °C after the repetition of heart-cutting operation.

3. Results and Discussion

Hallertau Perle hop was used as a standard sample according to Steinhaus et al. (2007). The hop volatile oil was extracted from the hop pellets by simultaneous steam distillation–extraction and then analyzed by GC-MS/O fitted with a polar or an apolar capillary column. The perceived sweat-like-odor active compounds are

summarized in Table 4. The sniffing test revealed seven sweat-like-odor active compounds in Hallertau Perle hop volatile oil. Further, the AEDA result clarified the contribution of these compounds to the overall aroma at flavor dilution (FD) factors in the range of 1 to 32. Among these, 3-methylbutanoic acid and 2-methylbutanoic acid were easily identified due to their high FD factor values of 32. Interestingly, five unknown sweat-like-odor active compounds were detected. We pursued the identification of these unknown compounds.

The unknown compounds **A** and **B** could be perceived by GC-MS/O in the acidic fraction II, while **C** and **D** could be sniffed by GC-MS/O in both neutral/basic fraction I and the silica gel column chromatographic fraction III (95/5, v/v) of fraction I; however, the corresponding chromatographic peaks could not be detected (Table 4). Based on this result, **A** and **B** were predicted to be volatile acids according to the literature (Miyazato et al., 2013), while **C** and **D** were empirically predicted to be volatile ketones or esters according to the literature (Miyazato, Hashimoto, & Hayashi, 2007).

Table 4. Sweat-like-odor active compounds identified in hop volatile oil by GC-MS/O and AEDA

Compound	RI (polar)[a)]	RI (apolar)[b)]	Fraction[c)]	Representation of odor[d)]	FD factor[e)]
Acid					
3-Methylbutanoic acid	1671	813	II	Sweaty, rancid, cheese-like	32
2-Methylbutanoic acid	1671	824	II	Sweaty, rancid, cheese-like	32
Unknown **A**	1953	974	II	Sweaty, urine-like, malodor in laundry	4
Unknown **B**	2052	1070	II	Sweaty, urine-like, malodor in laundry	32
Others					
Unknown **C**	1367	1012	I, III, IV	Sweaty, malodor in laundry, slightly fruity	8
Unknown **D**	1577	1178	I, III, V	Sweaty, rubber	1
Unknown	1339	—	I, III	Sweaty	1

a) InertCap WAX (60 m × 0.32 mm i.d.; film thickness, 0.25 μm).

b) InertCap 1 (60 m × 0.25 mm i.d.; film thickness, 0.25 μm).

c) I: neutral/basic fraction; II: acidic fraction; III: silica gel column chromatographic fraction (95/5, v/v); IV: the concentrated fraction by preparative GC (9.0–11.0 min); V: the concentrated fraction by preparative GC (15.0–17.5 min).

d) Perceived by GC-MS/O.

e) Estimated by AEDA: 2^n (n, value of dilution degree).

In addition, **C** and **D** could also be detected by GC-MS/O in the concentrated fractions IV and V obtained from hop volatile oil by preparative GC; however, the corresponding chromatographic peaks could not be detected. The fraction V was also analyzed by gas chromatography-flame photometric detector (GC-FPD) to give the corresponding chromatographic peak (Figure 2). The result indicates that **D** has a sulfur atom.

Next, the acidic fraction II and the concentrated fractions IV and V were analyzed by heart-cut MDGC-MS. The analyses provided the corresponding chromatographic peaks and mass spectra (Figure 3–6). The identification procedure was carried out by comparing the mass spectrum, the retention indices (RIs) by a polar and an apolar column, and the sniffing odor quality by GC-MS/O with the synthesize dreference substances (Table 5). As a result, **A** and **B** were identified as 4-methyl-3-pentenoic acid and (*E*)-4-methyl-3-hexenoic acid, respectively, while **C** and **D** were determined to be methyl (*E*)-4-methyl-3-hexenoate and *S*-methyl (*E*)-4-methyl-3-hexenethioate, respectively (Figure 7).

4-Methyl-3-pentenoic acid has been described in hop essential oil (Sandra & Verzele, 1975; Tressl, Friese, Fendesack, & Köppler, 1978a; Tressl & Friese, 1978b). In our study, we determined that 4-methyl-3-pentenoic acid could be responsible for the sweat-like odor in hops. 4-Methyl-3-pentenoic acid, along with

2-methylpropanoic acid, 3-methylbutanoic acid, and 2-methylbutanoic acid, is generated via the oxidative degradation of certain hop acids (Sandra et al., 1975). We also observed the generation of 4-methyl-3-pentenoic acid from isohumulone via heat-promoted oxidative degradation (data not shown).

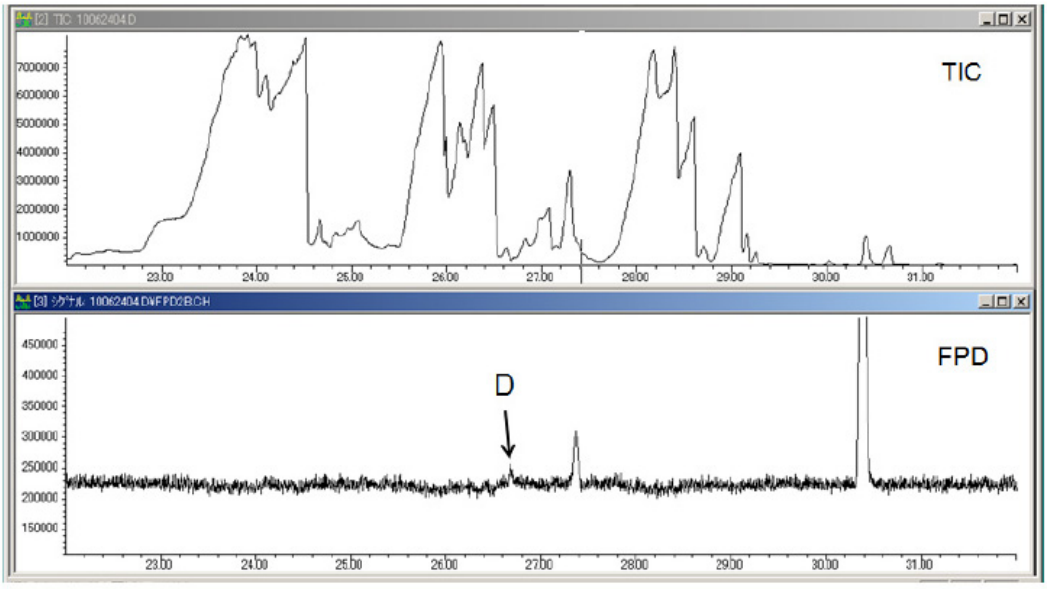

Figure 2. Detection of unknown compound **D** in fraction Vby GC-FPD

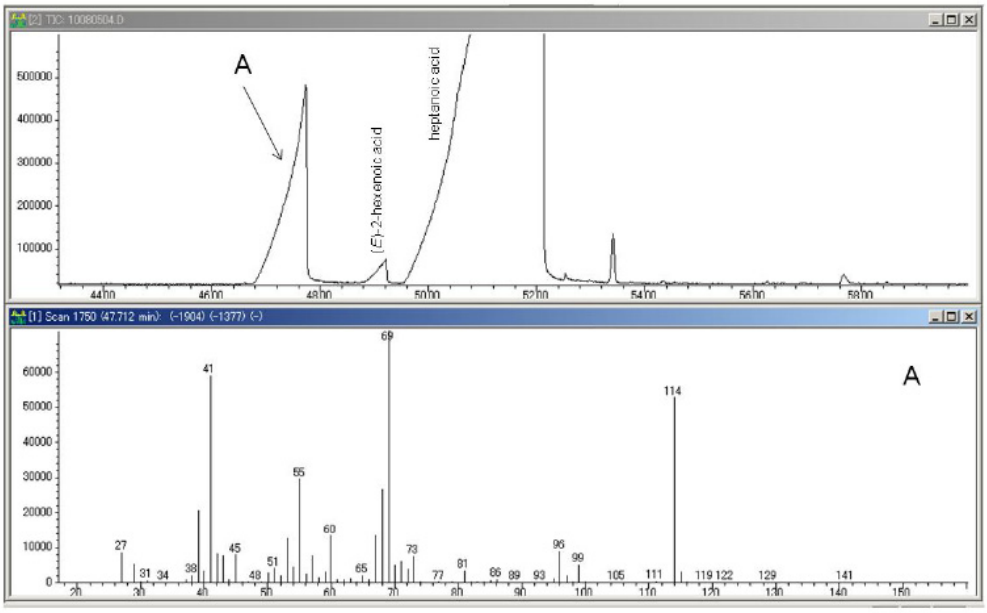

Figure 3. Enlarged images of the total ion chromatogram (2D) (upper panel) and the mass spectrum (lower panel) of unknown compound **A** as analyzed by heart-cut MDGC-MS

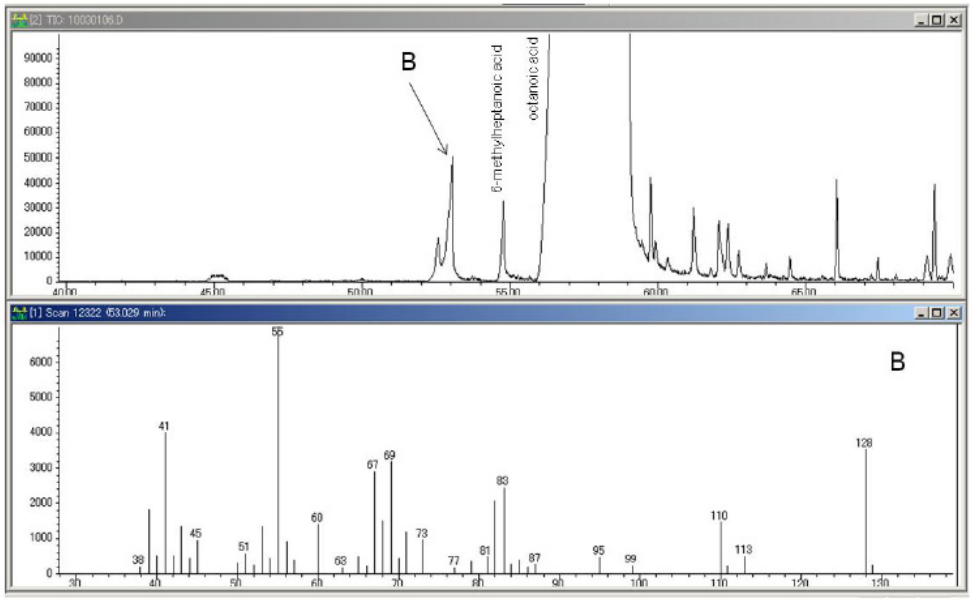

Figure 4. Enlarged images of the total ion chromatogram (2D) (upper panel) and the mass spectrum (lower panel) of unknown compound **B** as analyzed by heart-cut MDGC-MS

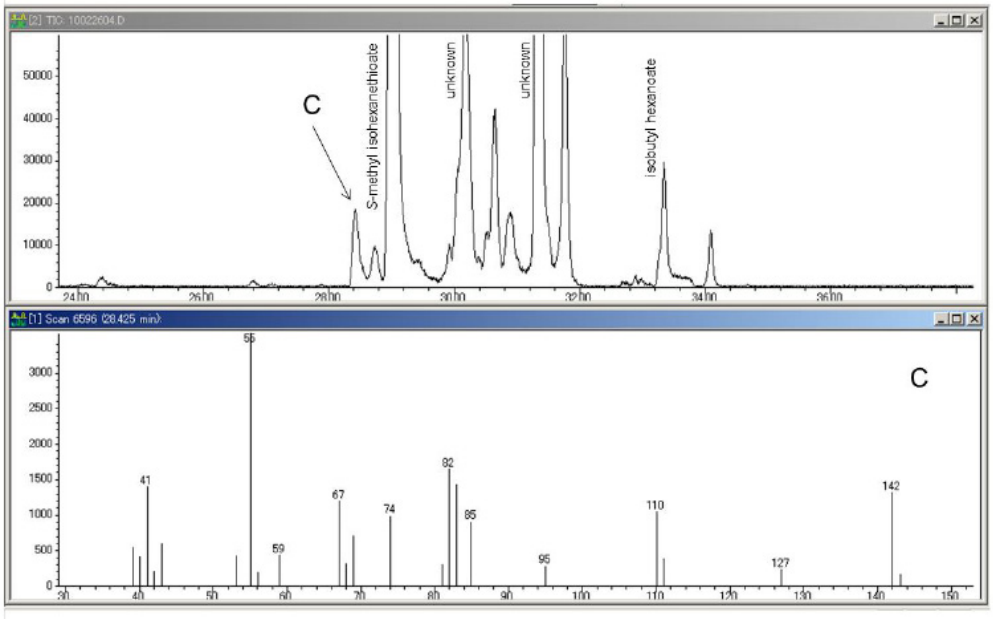

Figure 5. Enlarged images of the total ion chromatogram (2D) (upper panel) and the mass spectrum (lower panel) of unknown compound **C** as analyzed by heart-cut MDGC-MS

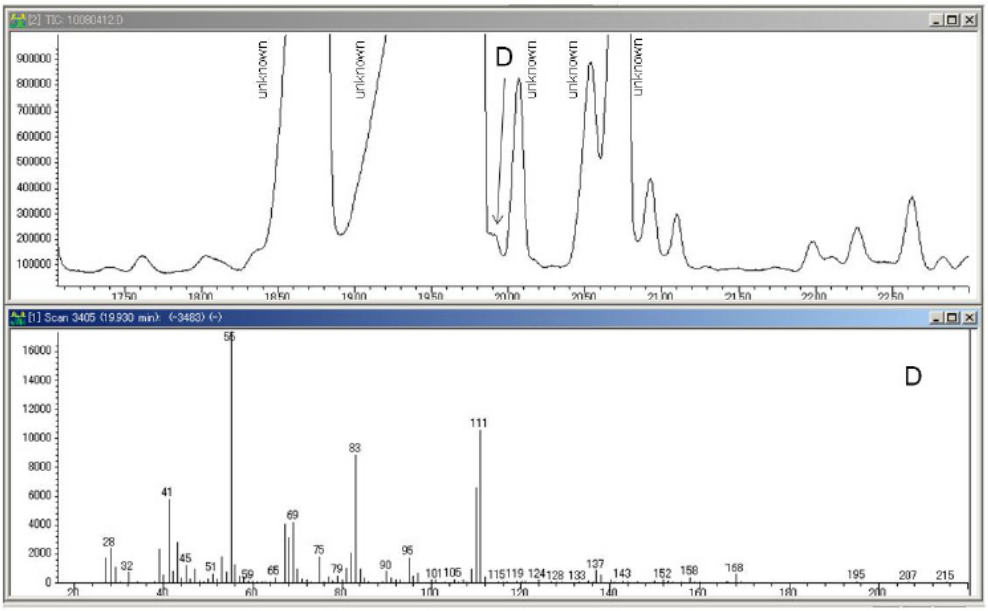

Figure 6. Enlarged images of the total ion chromatogram (2D) (upper panel) and the mass spectrum (lower panel) of unknown compound **D** as analyzed by heart-cut MDGC-MS

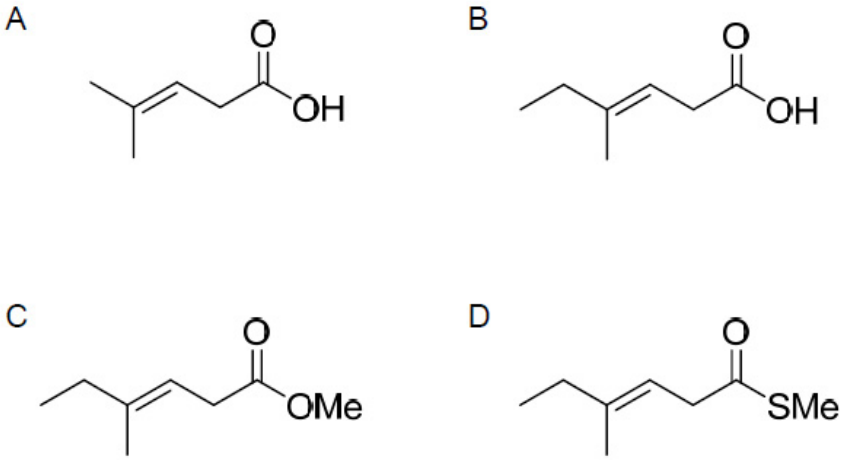

Figure 7. Thechemical structures of the sweat-like-odor active compounds identified in HallertauPerle hop volatile oil: **A**, 4-methyl-3-pentenoic acid; **B**, (*E*)-4-methyl-3-hexenoic acid; **C**, methyl (*E*)-4-methyl-3-hexenoate; **D**, *S*-methyl (*E*)-4-methyl-3-hexenethioate

Table 5. Characteristics of reference substances used for unknown target compound identification

Unknown target compound	Reference substance	Mass spectrum[a)]	RI (polar)[b)]	RI (apolar)[c)]	Odor quality[d)]
A	4-Methyl-3-pentenoic acid	39 (28), 41 (100), 42 (10), 43 (12), 45 (15), 53 (14), 55 (12), 56 (14), 67 (12), 68 (16), 69 (90), 114 (M^+, 38).	1951	974	Sweaty, urine-like, malodor in laundry
B	(*E*)-4-Methyl-3-hexenoic acid	27 (38), 29 (21), 39 (36), 41 (69), 43 (19), 45 (31), 51 (10), 53 (19), 55 (100), 56 (11), 67 (31), 68 (13), 69 (33), 71 (11), 82 (20), 83 (22), 110 (9), 128 (M^+, 29).	2052	1070	Sweaty, urine-like, malodor in laundry
C	Methyl (*E*)-4-methyl-3-hexenoate	39 (14), 41 (32), 43 (10), 53 (10), 55 (100), 59 (11), 67 (31), 68 (10), 69 (17), 74 (28), 82 (51), 83 (50), 85 (7), 95 (7), 110 (35), 127 (7), 142 (M^+, 45).	1368	1012	Sweaty, malodor in laundry, fruity
D	*S*-Methyl (*E*)-4-methyl-3-hexenethioate	39 (11), 41 (28), 55 (100), 67 (11), 75 (11), 82 (12), 83 (64), 110 (41), 111 (44), 158 (M^+, 2).	1578	1176	Sweaty, rubber

a) EI, 70 eV, *m*/*z* (relative intensity).

b) InertCap WAX (60 m × 0.32 mm i.d.;film thickness, 0.25 μm).

c) InertCap 1 (60 m × 0.25 mm i.d.;film thickness, 0.25 μm).

d) Perceived by GC-MS/O.

(*E*)-4-Methyl-3-hexenoic acid was identified for the first time in hops. Very recently, (*E*)-4-methyl-3-hexenoic acid has been determined to be an odor-active compound in some natural sources, such as yuzu (Miyazato et al., 2013) and kabosu (Tomiyama, Aoki, Oikawa, Sakurai, Kasahara, & Kawakami, 2012). Further, Takeuchi, Hasegawa, Ishida, and Kashiwagi (2012) have reported (*E*)-4-methyl-3-hexenoic acid as the key odorous component responsible for the malodor in laundry. In our study, among the seven sweat-like-odor active compounds, (*E*)-4-methyl-3-hexenoic acid, along with 3-methylbutanoic acid and 2-methylbutanoic acid, has a high FD factor value of 32. Therefore, (*E*)-4-methyl-3-hexenoic acid was found to be the key odorous component responsible for the sweat-like odor in hops.

The generation pathway of (*E*)-4-methyl-3-hexenoic acid found in hops remains unclear. (*E*)-4-Methyl-3-hexenoic acid is formed from myrcene (Narushima, Omori, & Minoda, 1982; Mikami, 1988) or linalool (Mizutani, Hayashi, Ueda, & Tatsumi, 1971) via enzyme-catalyzed oxidative degradation. In yuzu (Miyazato et al., 2013), the (*E*)-isomer is predominant and, in contrast, the (*Z*)-isomer was not detected. Additionally, in hops, the (*Z*)-isomer was not detected, which is similar to yuzu. Therefore, similar to yuzu, the (*E*)-4-methyl-3-hexenoic acid present in hops, may originate from myrcene and/or linalool because hop volatile oil contains myrcene and linalool as major volatile components (Nijssen et al., 2013). Further investigation is needed to clarify the formation of (*E*)-4-methyl-3-hexenoic acid in hops.

Although a great number of esters and thioesters have been described in hop volatile oil (Nijssen et al., 2013), in this study, methyl (*E*)-4-methyl-3-hexenoate and *S*-methyl (*E*)-4-methyl-3-hexenethioate were identified for the first time. Interestingly, these compounds have a sweat-like odor. This is the first study that indicates there are odorous components beyond volatile carboxylic acids responsible for the sweat-like odor of hops.

The biological synthesis of a great number of methyl esters present in hops remains unclear. Supriyadi, Suzuki, Wu, Tomita, Fujita, and Watanabe (2003) first reported the biogenesis of methyl esters in snake fruit. The authors demonstrated that a methyl ester is generated via the enzymatic esterification of an acid (acetyl-CoA) with methanol that is stemmed from methyl pectin via enzymatic degradation. Methyl (*E*)-4-methyl-3-hexenoate present in hops could be generated in the same way.

In addition, a majority of the methyl thioesters present in hops are biosynthesized via thioesterification of the

corresponding acids with methyl mercaptan originating from L-methionine (Lermusieau & Collin, 2003). Based on this finding, *S*-methyl (*E*)-4-methyl-3-hexenethioate may be generated in the same way.

S-Methylthiomethyl ester is present uniquely in hop volatile oil (Moir, Gallacher, Hobkirk, Seaton, & Suggett, 1980; Lermusieau et al., 2003). However, *S*-methylthiomethyl (*E*)-4-methyl-3-hexenethioate, which has a rubber odor, was not present in hops.

The odorous compound detected at RI (InertCap WAX) = 1339 remains unknown (Table 4). This compound could be detected in the same fraction I and III as methyl (*E*)-4-methyl-3-hexenoate and *S*-methyl (*E*)-4-methyl-3-hexenethioate. Subsequently, we determined that this compound is not a volatile carboxylic acid. Lermusieau et al. (2001) have stressed that 3-methylthiopropanal or *S*-methyl butanethioate, along with one unidentified compound, are the sweat-like odorous compounds in Challenger hop pellet volatile oil. In addition, Steinhaus et al. (2000, 2007) have not reported the sweat-like-odor active components in hop volatile oil except for butanoic acid, 3-methylbutanoic acid, and pentanoic acid, which in general have a sweat-like odor. This is the first study that demonstrably elucidated seven odorous components responsible for the sweat-like odor in hop volatile oil.

We did not examine whether the novel sweat-like-odor active compounds contribute to the aroma of hop-containing beer. The volatile compounds found in hops are transferred to beer, and these derived-volatile components have an influence on the aroma of beer (Sandra et al., 1975; Tressl, Friese, Fendesack, & Köppler, 1978c; Kishimoto, Wanikawa, Kono, & Shibata, 2006; Takoi et al., 2009). Sandra et al. (1975) have stressed the contribution of 3-methylbutanoic acid, 2-methylbutanoic acid, and 4-methyl-3-pentenoic acid to the flavor of beer because these compounds in hop-containing beer are present in higher concentration than those in hop-free beer. In addition, Steinhaus et al. (2007) have emphasized that the hop-derived esters do not contribute to the aroma of beer, most likely because these esters are decreased via hydrolysis during the kettle hop boiling in the brewing process, according to Tressl et al. (1978a). Thioesters also tend to be hydrolyzed. Based on these literature findings, we expect that 4-methyl-3-pentenoic acid and (*E*)-4-methyl-3-hexenoic acid, which were identified in this study, proceed with beer in the production and contribute to the beer overall aroma.

4. Conclusion

In this study, it was demonstrated that the seven odorants, such as 3-methylbutanoic acid, 2-methylbutanoic acid, 4-methyl-3-pentenoic acid, (*E*)-4-methyl-3-hexenoic acid, methyl (*E*)-4-methyl-3-hexenoate, *S*-methyl (*E*)-4-methyl-3-hexenethioate, and an unknown compound, are responsible for sweat-like odor in Hallertau Perle hop volatile oil. Among these, (*E*)-4-methyl-3-hexenoic acid, methyl (*E*)-4-methyl-3-hexenoate, and *S*-methyl (*E*)-4-methyl-3-hexenethioate were first identified.

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