C. Tanigawa, S. Otori, K. Takoi, A. Tanigawa and T. Kiyosaki

Effects of branched-chain fatty acids derived from aged hops on the aroma of beer brewed with flavour hop varieties

Hops stored at room temperature for an extended period are commonly referred to as 'aged hops'. Degradation of alpha and beta acids occurs during ageing, so the ageing process is accompanied by a significant increase in the concentration of branched-chain fatty acids (BCFAs): isobutyric acid (IBA), isovaleric acid (IVA), and 2-methylbutyric acid (2MBA). These three compounds, having rancid, cheesy, and/or sweaty flavours, are regarded as "off-flavours". It was previously reported that BCFAs contained at their threshold levels could enhance the flavour intensity of monoterpene alcohols (linalool, geraniol, and β-citronellol; LGC) and change their aroma profiles. In this study, the behaviours of BCFAs during a 12-months ageing process were compared using five Japanese hop varieties. All BCFAs in the hops increased in all varieties during the total ageing period and a drastic increase was especially observed from 9 to 12 months. The content of linalool gradually decreased but plateaued at relatively high levels. In thiol-containing hops, 3-sulfanyl-4-methylpentan-1-ol (3S4MP) decreased drastically from 6 to 12 months, whereas 4-methyl-4-sulfanylpentan-2-one (4MSP) gradually decreased but was plateaued at relatively high levels. Next, to investigate the sensory effect of aged hops on aroma and taste profile of beer, model beers spiked with several combinations of monoterpene alcohols (LGC), 4MSP, and each BCFA were prepared. As a result, BCFAs commonly enhanced the characteristics of fruity, fullness, and maturity in the model beer spiking LGC. BCFAs also more enhanced the characteristics of tropical in coexistence with 4MSP. The sensory effect of BCFAs was expected to enhance hop-derived flavour of beer brewed with both geraniol-rich hops and 4MSP-rich hops. However, the sensory tests also showed an increase in rancid characteristics due to the presence of BCFAs. Therefore, test beers were brewed using fresh and aged 4MSP-rich hops. As a result, although the negative characteristics of rancid gradually increased depending on the dosage of aged hops, the positive characteristics of fruity, tropical, fullness, and maturity increased by mixing only 0.1 g/L of aged hops.

Descriptors: beers; hops; flavour; branched-chain fatty acids; monoterpene alcohols; volatile thiols; 4-methyl-4-sulfanylpentan-2-one; synergy

1 Introduction

Hops (*Humulus lupulus* L.) contain many types of compounds, such as resins, polyphenols, and oils. The flavour compounds of hops are mainly derived from the hop oil found in the lupulin glands of the hop cone. Hop oil contains various terpenoids, esters, aldehydes, ketones, and sulphur compounds. Among these compounds, monoterpene alcohols, volatile thiols, and hop-derived esters having branched-chain structures have been studied as contributors to characteristic hop varietal aromas [1–10]. However, resins could also induce flavour compounds, such as fatty acids.

In general, harvested hop cones are very instable because of their high moisture contents and instability to oxidation. Therefore, hop cones are quickly dried using kilning apparatus around 60 °C. After that, dried whole hop cones are crushed, pelletized, and packaged into vacuum-sealed bags. To prevent the oxidation after unsealing of bags, brewers use hop pellets as soon as possible. However, hops are sometimes used as oxidized form. Hops stored at room temperature for an extended period are commonly referred to as 'aged hops' [11–14]. Aged hops have been traditionally used in Lambic style beer, and usually impart an unpleasant cheesy aroma [15]. In aged hops, a large reduction of oils is observed mainly due to the loss of terpene hydrocarbons, such as myrcene. Degradation of alpha and beta acids occurs during ageing. The three side chains of the bitter acids are degraded into three fatty acids (Fig. 1); thus, the ageing process has been reported to be accompanied by a significant increase in the concentration of branched-chain fatty acids (BCFAs): isobutyric acid (IBA), isovaleric acid (IVA), and 2-methylbutyric acid (2MBA) [11–14]. These three compounds, having rancid, cheesy, and/or sweaty flavours, are regarded as

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"off-flavours" [14]. However, they can also act as precursors to fruity pineapple-like esters, which have lower perception thresholds, during fermentation [10, 13]. Most research on hops ageing has focused on traditional aroma hop varieties [11–14, 16]; only a few studies investigated "flavour hop" varieties, which have gained popularity in recent years [17–19]. Some studies reported oxidative deterioration of hop bitter acids during the process of hops ageing, as well as the generation of BCFAs as degradation products and/or a decrease of hop oil components [11–14, 17, 19].

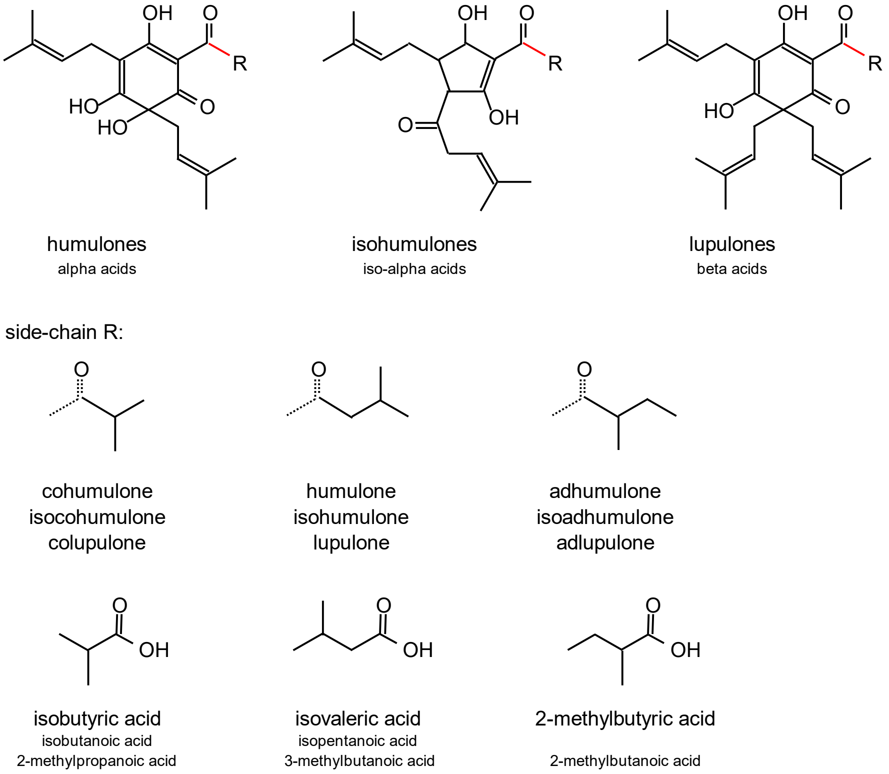

Fig. 1 **Chemical structures of humulones (alpha acids), isohumulones (iso-alpha acids), lupulones (beta acids), and related branched-chain fatty acids (isobutyric acid, isovaleric acid, and 2-methylbutyric acid): dotted line in the structures of side-chain R is indicated the link points between bitter acids and side chain**

It was recently reported that BCFAs, whose concentrations increased with hops ageing, could enhance the flavour intensity of monoterpene alcohols (linalool, geraniol, and β-citronellol; LGC) and change their aroma profiles [20]. However, to date, only a few studies have investigated the effect of ageing on characteristic aroma components in flavour hop varieties and the impact on finished beers. In this study, various hop varieties including traditional aroma and high alpha hops were aged for 12 months and compared the behaviour of both BCFAs and characteristic hop-derived aroma components, such as monoterpene alcohols, geranic acid, esters, and volatile thiols. Based on the analytical profiles of a test beer made of flavour hop variety, model beers spiked with several combinations of LGC, 4-methyl-4-sulfanylpentan-2-one (4MSP), and each BCFA were prepared to investigate the sensory effects of using aged hops on the aroma and taste profiles of beers. In addition, it was also confirmed the sensory effect of BCFAs derived from aged hops by brewing test beers using fresh and aged hops at a pilot plant.

2 Materials and methods

2.1 Hop plant materials and ageing (storage) conditions

Seven varieties of hops, Little Star [21], Sorachi Ace [22], Furano Special [23], Furano Magical [24], Furano Queen [25], Saaz, and Columbus were used for the ageing trials. The hops were grown in an experimental field in Hokkaido, Japan. After harvest, the hop cones were dried with hot air at 60 °C until the moisture content reached about 10 %. Each 100 g of whole hop cones was collected in a mesh bag for the ageing trial. From September 2021 to September 2022, all hop samples were stored at room temperature in an un-air-conditioned warehouse of hops processing plant, Iwate, Japan. During storage period, the temperature in this district ranged between – 7 °C and 28 °C (https://weather.time-j.net/Stations/Jp/Ninohe/). Hop samples in mesh bags were collected at intervals of three months (3, 6, 9, and 12M) except for Saaz and Columbus. These two varieties were collected only at 12 months. For each hop variety, hop samples kept frozen (not stored in the warehouse) were used as the controls (0M). The ageing conditions are summarized in table 1. All hop cones were ground by a cutter mill (ZM200, Verder Scientific Co. Ltd., Germany) at 15,000 rpm with 0.5 mm mesh, vacuum-sealed, and stored at − 20 °C until chemical analysis and brewing trials. In addition, Hallertauer Tradition (HHT) hops grown in Germany in 2021 were used for test-brewing as described below.

Table 1 **Ageing (storage) conditions of hops in a warehouse of processing plant, Iwate, Japan**

hop variety	storage period (months)				
	0	3	6	9	12
Little Star	LS-0M	LS-3M	LS-6M	LS-9M	LS-12M
Sorachi Ace	SA-0M	SA-3M	SA-6M	SA-9M	SA-12M
Furano Special	FS-0M	FS-3M	FS-6M	FS-9M	FS-12M
Furano Magical	FM-0M	FM-3M	FM-6M	FM-9M	FM-12M
Furano Queen	FQ-0M	FQ-3M	FQ-6M	FQ-9M	FQ-12M
Saaz	SZ-0M	–	–	–	SZ-12M
Columbus	CO-0M	–	–	–	CO-12M

2.2 Chemicals

2.2.1 Reference Compounds

The chemicals used for quantification were as follows: Ethyl isobu-

Table 2 **Late-hopping conditions of test-brewed beers**

test beer	LS-F	LS-A	SA-F	SA-A	FS-F	FS-A	FM-F	FM-A	FQ-F	FQ-A	SZ-F	SZ-A	CO-F	CO-A
hop variety (late-hopping dosage)	Little Star (g of hops/L)		Sorachi Ace (g of hops/L)		Furano Special (g of hops/L)		Furano Magical (g of hops/L)		Furano Queen (g of hops/L)		Saaz (g of hops/L)		Columbus (g of hops/L)	
0M hops	1,5	1,5	1,5	1,5	1,5	1,5	1,5	1,5	1,5	1,5	1,5	1,5	1,5	1,5
12M hops	–	0,3	–	0,3	–	0,3	–	0,3	–	0,3	–	0,3	–	0,3

tyrate (> 98 %), ethyl isovalerate (> 97 %), isobutyl isobutyrate (> 98 %), isoamyl isobutyrate (> 98 %), isobutyric acid (IBA, > 98 %), isovaleric acid (IVA, > 98 %), and 2-methylbutyric acid (2MBA, > 97 %, racemic mixture), were purchased from FUJIFILM Wako Pure Chemical Co., Ltd. (Osaka, Japan). Geranic acid (> 85 %, racemic mixture) and myrcene (> 90 %) were purchased from Sigma-Aldrich Japan Co., Ltd. (Tokyo, Japan). Ethyl 2-methylbutyrate (> 98 %, racemic mixture), 2-methylbutyl isobutyrate (> 98 %), linalool (> 96 %), β-citronellol (> 92 %), geraniol (> 97 %), citral (> 98 %, racemic mixture), benzyl acetate (> 99 %), and o-cymene (> 99 %) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Methyl geranate (> 94 %, mixture of isomers) was purchased from Thermo Fisher Scientific Inc. (NYSE: TMO). 2-Methylbutyl isobutyrate-d7 was purchased from Alfa Chemistry (Holbrook, NY, USA). Ethyl 2-methylbutyrate-d3 was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Linalool-d5 was purchased from HPC Standards GmbH (Borsdorf, Germany).

The chemicals used to quantitate volatile thiols were as follows: 4-Methyl-4-sulfanylpentane-2-one (4MSP) was purchased from Combi-Blocks (San Diego, CA, USA). 3-Sulfanylhexan-1-ol (3SH) was purchased from Alfa Aesar (Lancashire, United Kingdom). 3-Sulfanylhexyl acetate (3SHA) was purchased from Matrix Scientific (Columbia, SC, USA). 3-Sulfanylpropyl hexanoate (3SPH) was purchased from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan). 3-Sulfanyl-4-methylpentan-1-ol (3S4MP) and 3-sulfanyl-4-methylpentyl acetate (3S4MPA) were synthesized as described in a previous study [2].

2.2.2 Miscellaneous Chemicals.

Acetonitrile, anhydrous sodium sulfate, dichloromethane, ethyl acetate, phosphoric acid, sodium chloride, sodium hydrogen carbonate, and thioglycerol were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). 5,5'-Dithiobis(2-nitrobenzoic acid) was purchased from Tokyo Chemical Industry (Tokyo, Japan).

2.3 Hop Extraction with Hot Water

A citrate buffer (0.05 M, pH 5.5) was prepared from citric acid monohydrate (0.05 M) and trisodium citrate (0.05 M). Each hop sample was crushed in a mortar and three grams of the hop sample was placed in a 200 mL Erlenmeyer flask, and 150 mL of the citrate buffer was added. Then, the flask was covered with aluminium foil and autoclaved at 105 °C for 30 minutes. After adjusting for the amount of water that evaporated, the extract was filtered through filter paper using suction with a Buchner funnel. The filtrate was used to quantify hop-derived flavour compounds.

2.4 Pilot-scale Brewing

In the brewing trial, a pilot-scale brewing was conducted with and without aged hops. The wort was prepared using commercially available malts in a 100 L pilot scale apparatus. The same series of test brewing was performed using same lots of malts. To adjust the BU in the finished beer to approximately 30, the HHT hops (1.0 – 2.1 g of hop/L) were added at the beginning of the boiling process and the wort was boiled for 90 min. For late-hopping, fresh hops (0M, 1.5 g of hop/L) with and without aged hops of the same variety (12M, 0.3 g of hop/L) were added 5 min before the end of the boiling period. The late-hopping conditions are summarized in table 2. After cooling, each wort was transferred to a fermentation tank (100 L/tank) and fermentation was initiated by adding 15.0 × 10^6 cells/mL of lager yeast (*Saccharomyces pastorianus*) to the wort. The fermentation temperature was maintained at 12 °C. After transferring the green beer to another storage tank (30 L/tank) under a CO_2 atmosphere, maturation was allowed to occur at 12 °C for 6 days and at 0 °C for 2 – 3 weeks. Filtration and bottling were performed using pilot-scale equipment under antioxidative conditions.

2.5 Quantitation of Hop-derived Flavour Compounds

2.5.1 Determination of the Ratio of Isomers in the Reference Compounds by Gas Chromatography−Flame Ionization Detection (GC-FID)

The reference compounds citral, geranic acid, and methyl geranate contained isomers. Citral is a mixture of geranial and neral. Commercial geranic acid and methyl geranate contained small amounts of neric acid and methyl nerolate, respectively. All the reference compounds contained small amounts of contaminants and/or degradation products. For isomer analysis, GC-FID measurements were conducted on a 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) under the same conditions described in our previous paper [26]. The carrier gas was helium with a flow rate of 1.7 mL/min in the constant-flow mode. The detector used was an FID at 250 °C. Hydrogen gas at a flow rate of 40 mL/min and air at 450 mL/min were used for the FID. Aliquots (1 µL) of each chemical (500 mg/L) were injected into a split injector (250 °C; split rate, 100:1; purge flow, 168.5 mL/min) at an oven temperature of 50 °C onto a type HP-INNOwax capillary column (30 m × 0.25 mm internal diameter (i.d.); 0.25 µm film thickness; Agilent Technologies). For all measurements, the temperature program was as follows: 50 °C for 2.5 min, heated at 10 °C/min to 240 °C, and a 5 min isotherm. The ratio of isomers and contaminants was calculated on the basis of the areas of all the peaks obtained using the FID detector. The calculated isomer ratios were used for the subsequent quantification of each isomer.

2.5.2 Quantitation of Branched-chain Fatty Acids (BCFAs) by Solid Phase Microextraction-Gas Chromatography-Mass Spectrometry (SPME-GC-MS)

For analysis of BCFAs (IBA, IVA, and 2MBA), GC-MS analyses were carried out using a 7890B GC with a 5977A MS (Agilent Technologies) according to the following method. Eight millilitres of each sample were placed in a 20 mL glass vial with 3 g of sodium chloride, followed by spiking with 40 µL of 10 mg/L benzyl acetate as the internal standard. In addition, 100 µL of phosphoric acid was added to evaporate fatty acids into the headspace. The vial was then sealed with a magnetic cap and agitated at 80 °C for 15 min using the Combi-PAL system (CTC Analytics, Zwingen, Switzerland). The SPME fibre (PDMS/DVB [polydimethylsiloxane/divinylbenzene], 65 µm film thickness, Supelco, Bellefonte, PA) was inserted into the vial's headspace and held at 50 °C for 30 min for adsorption. After adsorption, the SPME fibre was immediately inserted into a GC injector for thermal desorption for 3 min at 270 °C. Volatiles were injected in the splitless mode. The separation of volatiles was conducted using an InertCap Pure-WAX column (30 m × 0.25 mm i.d., 0.25 µm film thickness; GL Science Inc., Tokyo, Japan) with a helium carrier gas at a constant flow rate of 1.0 mL/min. The oven temperature was set to 40 °C for 3 min and raised to 250 °C at a rate of 5 °C/min. The MS was operated under the following conditions: ionization voltage of 70 eV (EI), ion source temperature of 230 °C, a quadrupole temperature of 150 °C, and a transfer line temperature of 250 °C. The MS detector in the selected ion monitoring (SIM) mode was used for quantitation.

Calibration curves were constructed using beer samples spiked with reference compounds. An appropriate calibration curve range was determined to calculate the concentrations of the compounds in the hot water extracts and beers. Each hot water extract was diluted with cold water to one-fifth of its original concentration before the analysis. The monitored ions in the reference compounds are listed in table S1. All calibrations produced a linear correlation with an R^2 value > 0.99, across the entire concentration range analysed. All the tests were performed twice to reduce errors.

2.5.3 Quantitation of Monoterpenes and Esters.

To quantitate monoterpenes and esters, HS-SPME-GC-MS analysis was conducted on a 7890 B GC instrument with a 5977A MS (Agilent Technologies). The SPME fibre (PDMS, 100 µm film thickness, Supelco) was used in this analysis. Absorption and desorption were performed under the conditions described above. Volatiles were injected in the splitless mode. The separation of volatiles was performed on an HP-1MS column (30 m × 0.25 mm i.d., 1 µm film thickness; Agilent Technologies) with a helium carrier gas at a constant flow rate of 1.0 mL/min. The oven temperature was set as follows: 40 °C for 3 min, increased to 200 °C at a rate of 5 °C/min, and raised to 320 °C at a rate of 10 °C/min. The MS was operated under the following conditions: ionization voltage of 70 eV (EI), ion source temperature of 230 °C, quadrupole temperature of 150 °C, and transfer line temperature of 320 °C. The MS detector in the SIM mode was used for quantitation. Calibration curves were constructed according to the method described in 2.5.2. The monitored ions in the reference compounds are listed in table S1.

2.5.4 Quantitation of Aldehydes.

To analyse aldehydes, selectable one-dimensional or two-dimensional gas chromatography/mass spectrometry (^{1}D or ^{2}D GC/MS was performed using a dual low thermal mass gas chromatograph on a 7890A GC × LTM System with a 5975C inert XL MSD with Triple-Axis Detector (Agilent Technologies). The GC was equipped with a capillary flow technology (CFT) Deans Switch and a 3-way splitter with a makeup gas line, which were controlled by a pressure control module (PCM) (Agilent Technologies). The conditions for ^{1}D or ^{2}D GC/MS operation were sourced from the protocol described by *Tokita* et al. [27]. The SPME fibre (PDMS, 100 µm film thickness, Supelco) was used in this analysis. Absorption and desorption were performed under the conditions described above. Volatiles were injected in the splitless mode. The separation of volatiles was performed on a DB-WAX LTM (30m × 0.25 mm i.d., 0.25 µm film thickness; Agilent Technologies) as the ^{1}D column and a DB-5 LTM (10 m × 0.18 mm i.d., 0.40 µm film thickness; Agilent Technologies) as the ^{2}D column with a helium carrier gas at a constant pressure of 410 psi. The oven temperature for ^{1}D column was set to 40 °C for 3 min and raised to 250 °C at a rate of 5 °C/min. That for ^{2}D column was set to 35 °C for 28 min, raised to 120 °C at a rate of 5 °C/min, continued to raise to 240 °C at a rate of 25 °C/min, and followed by an isotherm for 1 min. The MS was operated under the following conditions: ionization voltage of 70 eV (EI), ion source temperature of 230 °C, quadrupole temperature of 150 °C, and transfer line temperature of 250 °C. The MS detector in the SIM mode was used for quantitation. Calibration curves were constructed according to the method described in 2.5.2. The monitored ions in the reference compounds are listed in table S1.

2.5.5 Quantitation of Geranic Acid and Neric Acid by Gas Chromatography-Tandem Mass Spectrometry (GC-MS/MS)

Geranic acid and neric acid were quantitated via GC-MS/MS analysis using a 7890A GC coupled to a 7000B triple quadrupole MS (Agilent Technologies) under the improved analysis conditions described in our previous paper [26]. Four millilitres of each test-brewed beer sample and 4 mL of 0.1 % (v/v) aqueous phosphoric acid were added to a 20 mL glass containing 3 g of sodium chloride, followed by spiking with 20 µL of 10 mg/L benzyl acetate as the internal standard. The vial was hermetically sealed using a magnetic cap and agitated at 60 °C for 15 min on a Combi-PAL autosampler (CTC Analytics). The SPME fibre (PDMS/ DVB, 65 µm film thickness, Supelco) was inserted into the head space of the vial and held for 15 min for adsorption. After adsorption, the SPME fibre was immediately inserted into a GC injector for thermal desorption for 3 min at 270 °C. The volatiles were injected in the splitless mode. The separation of volatiles was performed on a DB-FFAP (30 m × 0.25 mm i.d., 0.25 µm thickness; Agilent Technologies) with a helium carrier gas at a constant flow rate of 1.0 mL/min. The oven temperature was increased from 40 °C (held for 3 min) to 210 °C at a rate of 5 °C/min, and reised to 250 °C at a rate of 15 °C/min, followed by a 4 min isotherm. The triple quadrupole mass spectrometer was operated in the selected reaction monitoring (SRM) mode. The conditions for the SRM mode are listed in table S1. Calibration curves were constructed using test-brewed beers containing standard substances at concentrations of 10, 25, 50, 100, 250, 500, and 1000 µg/L. All calibrations indicated a linear correlation with an R2 value of > 0.99 over the entire concentration range analysed. All tests were run twice to reduce error.

2.5.6 Quantitation of Volatile Thiols by GC-MS/MS

2.5.6.1 Specific Extraction of Volatile Thiols from Beer.

The specific extraction method of volatile thiols from hops and beers was performed according to the method reported by *Takazumi* et al. [28], as follows: In a 50 mL glass centrifuge tube, 6 g of sodium chloride, 20 mL of beer, 20 mL of dichloromethane, and 20 µL of internal standard solution (10 mg/L 3SPH in ethanol) were added and shaken for 15 min. After centrifugation at 1800 g for 15 min, the organic phase was obtained. The organic phase was dried on anhydrous sodium sulfate and concentrated to 8 mL under nitrogen flow. Solid phase extraction using silver ion was carried out with an ASPEC GX-274 (Gilson, Middleton, WI, USA). First, a solid phase extraction cartridge (Meta-Sep IC-Ag; GL Sciences, Tokyo, Japan) was conditioned with 6 mL of dichloromethane. Then, 10 mL of each beer extract was loaded onto the cartridge at a flow rate of 2 mL/min. The cartridge was then rinsed with 10 mL of dichloromethane and 20 mL of acetonitrile in succession. The cartridge was reversed and washed with 10 mL of dichloromethane. Volatile thiols were eluted with 6 mL of 10 g/L thioglycerol in dichloromethane at a flow rate of 0.66 mL/min. The eluate and 30 mL of saturated salt solution were added to a 50 mL glass centrifuge tube and shaken for 15 min. After centrifugation at 1800 g for 15 min, the organic phase was obtained and dried on anhydrous sodium sulfate. Finally, 1 mL of ethyl acetate was added to the eluate, and the mixture was concentrated to 20 µL under nitrogen flow.

2.5.6.2 Quantitation of Volatile Thiols by GC-MS/MS.

The method of GC−MS/MS analysis for volatile thiols was performed according to the method reported by Takazumi et al. [28]. The analysis was performed with a 7890A gas chromatograph coupled to a 7000B triple quadrupole mass spectrometer (Agilent Technologies). An InertCap PureWAX capillary column (30 m × 0.25 mm internal diameter and 0.25 µm film thickness; GL Sciences) was used for separation. Here, 3 µL of extract was injected using a CombiPAL system (CTC Analytics). The inlet was operated in a pulsed splitless mode (30 psi, 1 min) at 250 °C. The flow rate of the helium carrier gas was 1 mL/min. The oven temperature was increased from 70 °C (held for 1 min) to 250 °C at a rate of 5 °C/min and was held at 250 °C for 10 min. The triple quadrupole mass spectrometer was operated in the SRM mode. Target and qualification transitions and the corresponding collision energy values are listed in table S2. Calibration curves were obtained by analyzing standard mixture solutions. The concentration of each volatile thiol stock solution was determined according to Ellmans's method using 5,5-dithiobis (2-nitrobenzoic acid) before mixing. Standard mixture solutions were prepared by mixing five volatile thiol stock solutions and diluting them with ethyl acetate. To all standard mixture solutions, 3SPH (final concentration, 10 mg/L) and thioglycerol (final concentration, 10 g/L) were added as an internal standard and an analyte protectant, respectively.

2.6 Sensory Tests

2.6.1 Sensory Evaluation of Synergy between BCFAs, Monoterpene Alcohols, and 4MSP

Each sensory evaluation was performed by eight to nine panellists well-trained using GMP Beer Flavour Standards & Kits (FlavorActiV, Oxfordshire, UK). The change in flavour characteristics due to the synergy between BCFAs, monoterpene alcohols, and 4MSP was assessed in model beers spiked with four solutions, as follows. A Japanese commercial kettle-hopped beer was used as the base beer. First, a control beer spiked only with 'LGC mix (mixture of linalool, geraniol, and β-citronellol)' solution (simulating a test beer brewed with Furano Magical; linalool, 84 µg/L; geraniol, 2.5 µg/L; β-citronellol, 14 µg/L) was compared with test beers containing the same 'LGC mix' together with 5000 µg/L of IBA (LGC mix + IBA), 40 µg/L of IVA (LGC mix + IVA), or 800 µg/L of 2MBA (LGC mix + 2MBA) according to a previous study [20]. The concentrations of BCFAs were adjusted to the estimated thresholds. Second, a beer spiked with 'LGC mix' and 4MSP solution (simulating a test beer brewed with Furano Magical; 4MSP, 57 ng/L) was compared with test beer containing the same 'LGC mix' and 4MSP solutions together with each BCFA solution (LGC mix + 4MSP + IBA, LGC mix + 4MSP + IVA, or LGC mix + 4MSP + 2MBA). The spiking compositions of all model beers are listed in table 3. Fifty millilitres of each model beer

Table 3 Concentrations of spiked compounds in model beers for the sensory test

		beer FM-A (late-hopped with Furano Magical)	concentraions of spiked compounds												percep-tion threshold
			model beer 1-1	model beer 1-2	model beer 1-3	model beer 1-4	model beer 2-1	model beer 2-2	model beer 2-3	model beer 2-4	model beer 3-1	model beer 3-2	model beer 3-3	model beer 3-4	
linalool	(µg/L)	84	84	84	84	84	84	84	84	84	84	84	84	84	3[a]
geraniol	(µg/L)	2,5	2,5	2,5	2,5	2,5	2,5	2,5	2,5	2,5	2,5	2,5	2,5	2,5	7[a]
β-citronellol	(µg/L)	14	14	14	14	14	14	14	14	14	14	14	14	14	9[a]
4MSP	(ng/L)	57	–	–	57	57	–	–	57	57	–	–	57	57	1.2[b]
IBA	(µg/L)	–	–	5000	–	5000	–	–	–	–	–	–	–	–	5000[c]
IVA	(µg/L)	–	–	–	–	–	–	40	–	40	–	–	–	–	40[c]
2MBA	(µg/L)	–	–	–	–	–	–	–	–	–	–	800	–	800	800[c]

[a]aperception threshold reported in ref 4; [b]perception threshold reported in ref 8; [c]perception threshold reported in ref 20

4MSP, 4-methyl-4-sulfanylpentane-2-on; IBA, isobutyric acid; IVA, isovaleric acid; 2MBA, 2-methylbutyric acid

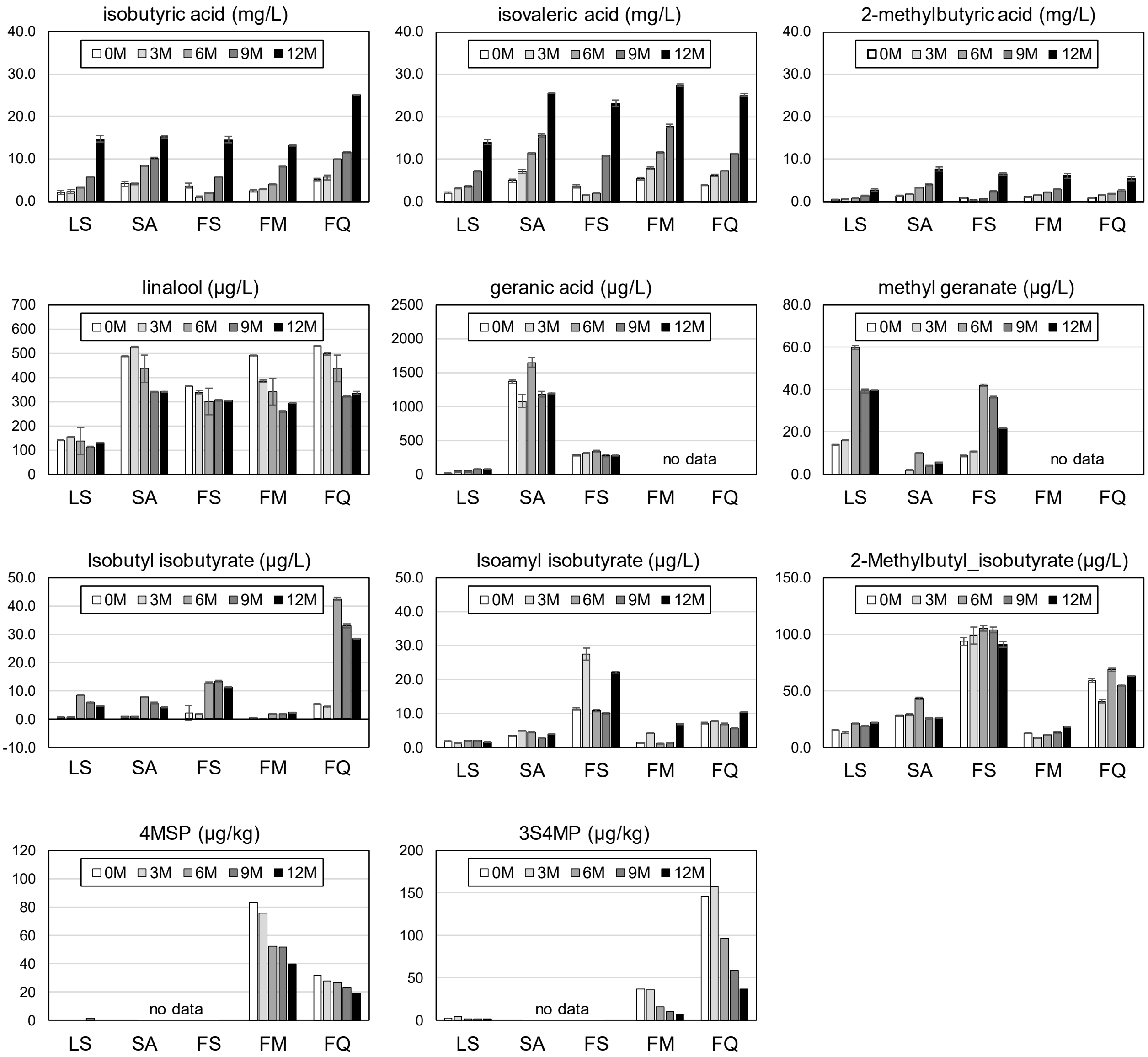

Fig. 2 **Changes in concentrations of flavour compounds during ageing of hops; LS, Little Star; SA, Sorachi Ace; FS, Furano Special; FM, Furano Magical; FQ, Furano Queen; 4MSP, 4-methyl-4-sulfanylpentan-2-one; 3S4MP, 3-sulfanyl-4-methylpentan-1-ol**

were presented in a plastic cup and the six flavour characteristics (“flowery”, “fruity”, “citrus”, “tropical”, “green”, and “rancid”) and two taste characteristics (“fullness” and “maturity”) of each sample were scored from 0 (no flavour) to 3 (strong flavour) in intervals of 1.0. “Rancid” was scored based on the cheesy and/or sweaty odour of the sample beer. “Fullness” was evaluated based on the body and fullness of the taste of the sample beer. “Maturity” was scored based on the mature fruit-like impression of the overall flavour/taste. The mean intensity value of each characteristic was calculated, and paired t-tests were conducted using Microsoft Excel for Microsoft 365 MSO.

2.6.2 Sensory Evaluation of Test Beer Brewed with and without Aged Hops

To evaluate the result obtained in the model beers, a sensory evaluation was performed by eight well-trained panellists. The Furano Magical beers with and without aged hops (beer FM-F and FM-A) were used for this test. In addition, another test beer (FM-a) was also prepared by mixing FM-F and FM-A in a 2:1 ratio. The FM-A beer was made with 0.3 g of hops/L of aged Furano Magical (12 M), so the FM-a beer was corresponding to the beer made with 0.1 g of hops/L of the same aged hops. Fifty millilitres of each beer were presented in a plastic cup and the six flavour characteristics and two taste characteristics of each sample were scored from 0 (no flavour) to 3 (strong flavour) in intervals of 1.0, as in 2.6.1. The mean intensity value of each characteristic was calculated, and paired t-tests were conducted using Microsoft Excel for Microsoft 365 MSO.

3 Results and Discussions

3.1 Changes in Hop-derived Flavour Compounds during Ageing Process

First, the aroma properties of each hop variety are explained. Five varieties, except for Saaz and Columbus, were bred by Sapporo Breweries Ltd. in Hokkaido and registered in Japan [21–25]. Little Star is a daughter of Tettnanger, and its aroma is similar to that of Saaz and Tettnanger. This hop is used as one of the major Japanese

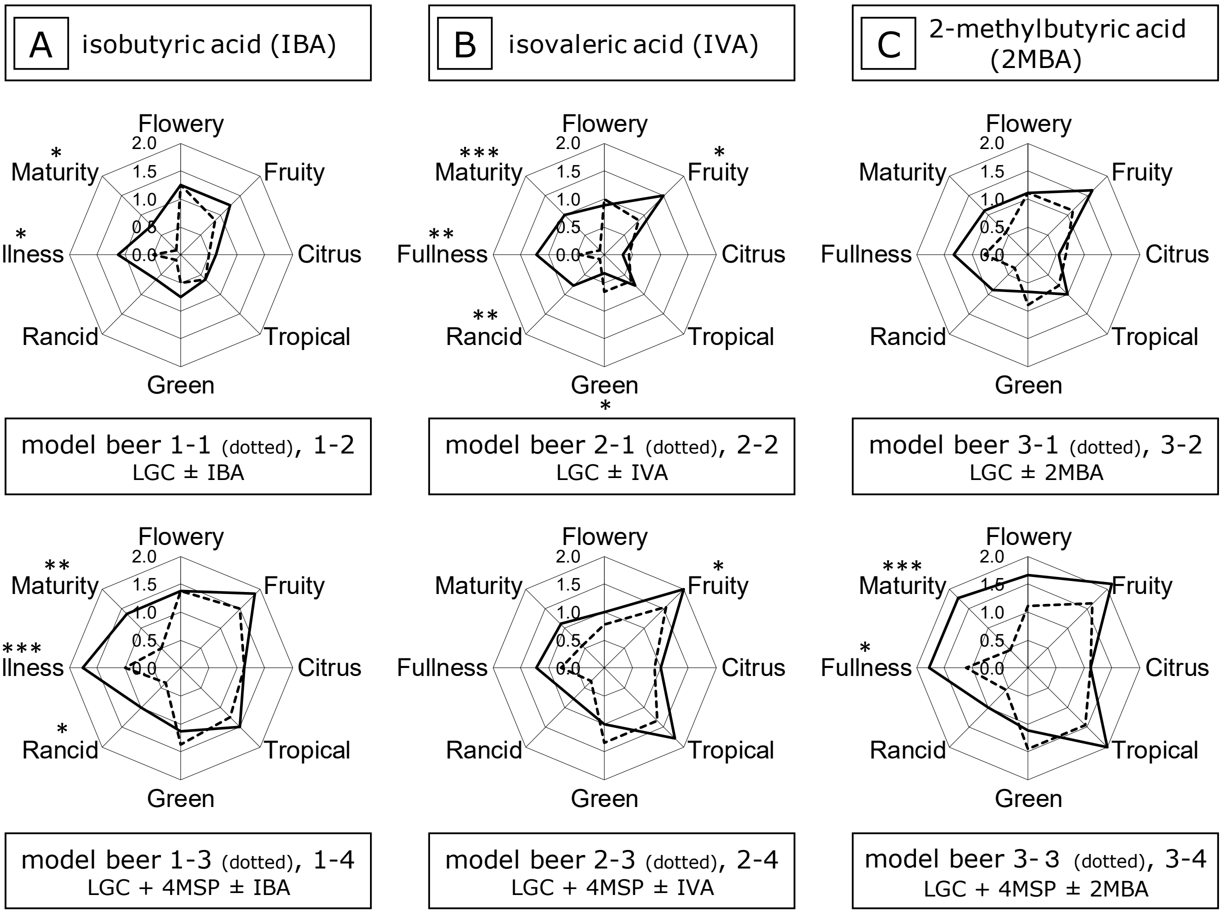

Fig. 3 **Flavour profiles of model beers containing three monoterpene alcohols, 4-methyl-4-sulfanylpentan-2-one (4MSP), and/or branched-chain fatty acids (BCFAs; isobutyric acid (IBA), isovaleric acid (IVA), and 2-methylbutyric acid (2MBA)): concentrations of spiked compounds in model beers are as follows; LGC mix, containing 84 µg/L of linalool, 2.5 µg/L of geraniol, and 14 µg/L of β-citronellol; 4MSP, containing 57 ng/L of 4MSP; IBA, containing 5000 µg/L of IBA; IVA, containing 40 µg/L of IVA; 2MBA, containing 800 µg/L of 2MBA; dotted line, profile of model beer not using BCFAs; *, significant difference with a risk of 5 % in paired t-test; **, significant difference with a risk of 1 % in paired t-test; ***, significant difference with a risk of 0.1 % in paired t-test**

varieties, along with Shinshu Wase. Sorachi Ace is used for craft beer as a flavour hop variety worldwide and its variety-specific compound is geranic acid [26]. Furano Special is a daughter of Sorachi Ace and is used in Japan. Furano Magical imparts mango-like, tropical aroma to beer owing to the volatile thiol, 4MSP [29], and its aroma is similar to that of Mosaic and Citra. Furano Queen is most recently bred variety and its specific compound is another thiol, 3S4MP, which is a specific compound of Nelson Sauvin [2].

In previous studies, various hop-derived flavour compounds have been reported as contributors to hop varietal aromas. Therefore, to investigate the changes in hop-derived flavour compounds during the ageing process, monoterpene alcohols, geranic acid, and volatile thiols were focused on in this study. Monoterpene alcohols have lavender-, rose-, or lime-like aromas; geranic acid contributes a lemongrass-like aroma; and volatile thiols, 4MSP and 3S4MP, impart black currant-like and grapefruit-like aromas, respectively [1–9]. These thiols are specific to certain varieties, such as Mosaic, Nelson Sauvin, and others. Among tested varieties, only Furano Magical and Furano Queen contained both thiols. As described above, each hop sample was stored at room temperature in the warehouse of a hops processing plant in Japan. The changes in hop-derived flavour compounds during the ageing process in five Japanese hops are summarized in table S3.

Among all measured compounds, the BCFAs, linalool, geranic acid, methyl geranate, isobutyric esters (isobutyl isobutyrate, isoamyl isobutyrate, and 2-methylbutyl isobutyrate), 4MSP, and 3S4MP are shown in figure 2. All BCFAs in the hops increased in all varieties during the entire ageing period and a drastic increase was especially observed from 9 to 12 months (July to September 2022). Although the temperature in this district was over 25 °C during this period, the temperature was also over 25 °C during the initial month (September 2021). Therefore, the increase of BCFAs in hops during the last 3 months couldn't be sufficiently explained by only storage temperature. It was assumed that the oxidative reaction in hops could be repressed for 0 – 9 months perhaps due to the existence of antioxidant substances such as polyphenols. A similar delay of oxidation was also observed in beer [30]. The delay of oxidative reaction during hop ageing is an unknown phenomenon. This is one of the important themes in future work. The content of linalool gradually decreased but plateaued at relatively high levels. The content of geranic acid was mostly stable or increased only slightly. On the other hand, the levels of methyl geranate and isobutyric esters increased mainly during the first 0 – 6 months but decreased in several varieties from 9 to 12 months. From these results, it was assumed that certain carboxylic acids in hop cones could be esterified by remaining ester biosynthetic activities. For example, methyl geranate increased as hop harvest time was delayed [31]. In addition, it was thought that these esters might gradually decrease from 9 to 12 months due to escalating evaporation from prolonged ageing period, during which the storage temperature increased over 25 °C. Among all hops tested, Furano Magical and Furano Queen were thiol-rich hops. The concentration of 3S4MP decreased drastically from 6 to 12 months. In contrast, the concentration of 4MSP gradually decreased but plateaued at relatively high levels. Therefore, in comparison with 3S4MP, 4MSP was thought to be a more resistant aroma compound to the ageing process.

3.2 Sensory Effect among BCFAs, Monoterpene Alcohols, and 4MSP

In the 2000s, new types of hops have been bred and widely used for craft beers all around the world [32–38]. These hops, so-called 'Flavour hops' [35–36], impart very characteristic 'Varietal Aroma', for example citrus-like and/or exotic fruit-like (tropical) flavours, to finished beer. In the last two decades, several mechanisms of hop aroma formation have been proposed [2–5, 8]; most of such research have focused on monoterpene alcohols and volatile thiols. Among these mechanisms, synergy between monoterpene alcohols can contribute to the varietal aroma of geraniol-rich hops, such as Bravo [6, 9], and that between monoterpene alcohols and 4MSP can form the varietal aromas of 4MSP-rich hops such as Citra, Mosaic, and others [8, 28–29, 39]. It is thought that these mechanisms could contribute to the varietal aromas of recently bred flavour hop varieties.

In the field of flavour science, it is well known that there is an additive effect between classes of compounds with similar structures at sub-threshold levels [40–41]. For example, 3-sulfanylhexan-1-ol (C6) can enhance the odour intensities of 3-sulfanypentan-1-ol (C5) and 3-sulfanylheptan-1-ol (C7) [41]. Whereas certain volatile thiols, 3S4MP and 4MSP, could enhance the intensities of other flavour compounds, for example 3S4MPA, 2-methylbutyl isobutyrate, linalool, and geraniol [2–3, 8]. In addition, certain carboxylic acids, such as acetic acid (C2) and butyric acid (C4), could enhance the intensities of several coffee aroma compounds at sub-threshold levels [42–43]. As well as in the case of straight-chain fatty acids, synergy between BCFAs and monoterpene alcohols was also revealed [20]. However, the sensory effect of BCFAs and monoterpene alcohols against volatile thiols, important contributors to flavour hops, has not been sufficiently examined yet. As described in the previous section, the remaining concentrations of 4MSP in certain hops after 12 months were relatively high. Therefore, 4MSP was the focus of this study.

To assess a possible synergy among BCFAs, monoterpene alcohols, and 4MSP, a sensory test was conducted according to table 3. Figure 3 shows the six flavour characteristics (flowery, fruity, citrus, tropical, green, and rancid) and two taste characteristics (fullness and maturity) of model beers, evaluated by the panellists. The model solution 'LGC mix' (containing 84 µg/L of linalool, 2.5 µg/L of geraniol, and 14 µg/L of β-citronellol) and '4MSP' (containing 57 ng/L of 4MSP) were designed to simulate the composition of these compounds in previously brewed Furano Magical beer (data not shown). The results are shown in figure 3 and table S4.

In the model beers 1-1 to 1-4, all the model beers contained linalool geraniol, and β-citronellol (LGC mix). In addition, beers 1-3 and 1-4 contained 4MSP. The sensory effect of IBA was evaluated under these conditions. Comparing beer 1-2 with 1-1, the characteristics of fruity, fullness, and maturity were enhanced in the presence of IBA (Fig. 3A). Especially, the scores of fullness and maturity were significantly higher with a risk of 5 % (Table S4). In the presence of 4MSP (beer 1-3 and 1-4), the characteristics of fruity, tropical, rancid, fullness, and maturity were more enhanced together with IBA (Fig. 3A). The scores of rancid, fullness, and maturity were significantly higher with a risk of 5 %, 0.1 %, and 1 %, respectively (Table S4).

In the model beers 2-1 to 2-4, all the model beers contained the LGC mix, and beers 2-3 and 2-4 additionally contained 4MSP. The effect of IVA was evaluated. In beer 2-2, the characteristics of fruity, rancid, fullness, and maturity were higher than those in 2-1, and the characteristic of green was lower than that in 2-1 (Fig. 3B). The significant differences are shown in table S4. In the presence of 4MSP (in beers 2-3 and 2-4), the characteristics of fruity, tropical, rancid, fullness, and maturity were enhanced together with IVA (Fig. 3B). The score of fruity was significantly higher with a risk of 5 % (Table S4).

In the model beers 3-1 to 3-4, all the model beers contained the LGC mix, and beers 3-3 and 3-4 additionally contained 4MSP as well. The effect of 2MBA was evaluated. In beer 3-2, the characteristics of fruity, rancid, fullness, and maturity slightly increased compared to 3-1 (Fig. 3C). However, there is no significant difference in any of the increased scores. In the presence of 4MSP (in beers 3-3 and 3-4), the characteristics of flowery, fruity, tropical, rancid, fullness, and maturity were enhanced together with 2MBA (Fig. 3C). The scores of fullness and maturity were significantly higher with a risk of 5 % and 1 %, respectively (Table S4). From these results, BCFAs commonly enhanced the characteristics of fruity, fullness, and maturity in the model beer spiking LGC. BCFAs also more enhanced the characteristics of tropical in coexistence with 4MSP.

3.3 Sensory Effect of Aged Hops on Hop-derived Aroma and Taste in Beer

3.3.1 Hop-derived Flavour Compounds in Test Beers with and without Aged Hops

As described in 3.2, the sensory effect of BCFAs was expected to enhance the hop-derived flavour of beer brewed with both geraniol-rich hops and 4MSP-rich hops. In other words, aged hops rich in BCFAs could be used as an enhancing agent for hop-derived beer flavour. However, the sensory tests also showed an increase in rancid characteristics due to the presence of BCFAs. Therefore, test beers using fresh and aged hops of seven varieties including Saaz and Columbus were brewed (Table 2). Each 'F' test beer was brewed with 1.5 g/L of fresh hop (0M) and 'A' beer with 1.5 g/L of fresh hop (0M) and 0.3 g/L of aged hop of the same variety (12M). The comparison of hop-derived flavour compounds in test beers is summarized in table S5. Each amount of aged hops (0.3 g/L) is smaller than that of fresh hops, so that most of the hop-derived flavour compounds in each beer 'A' were at similar levels in comparison with those in beer 'F' of the same variety (Table S5). BCFAs in test beers with aged hops significantly increased, whereas ethyl esters of BCFAs (ethyl

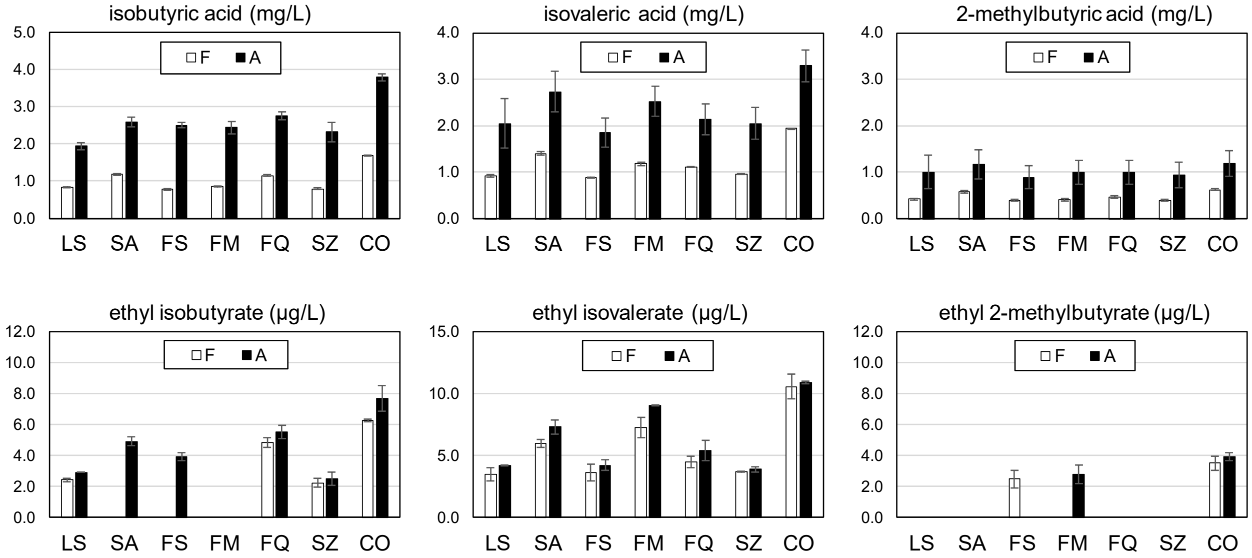

Fig. 4 **Changes in concentrations of flavour compounds with and without aged hops: LS, Little Star; SA, Sorachi Ace; FS, Furano Special; FM, Furano Magical; FQ, Furano Queen; SZ, Saaz; CO, Columbus; F, brewed with 1.5 g/L of each fresh hop (0M); A, 1.5 g/L of each fresh hop (0M) and 0.3 g/L of aged hop of same variety (12M)**

isobutyrate, ethyl isovalerate, and ethyl 2-methylbutyrate) were slightly increased (Fig. 4). However, the concentrations of these esters were small and/or at similar levels between ‘F’ and ‘A’ beers, as well as other flavour compounds. Therefore, it was decided that these test beers were suitable for evaluating the sensory effect of BCFAs on hop-derived aroma and taste in beer under the condition in which various flavour compounds, except for BCFAs, have almost no difference.

3.3.2 Sensory Effect of Aged Hops on Hop-derived Aroma and Taste in Beer

In this sensory test, three test beers were prepared: beer FM-F was brewed with 1.5 g/L of fresh FM hops, and beer FM-A with 1.5 g/L of fresh FM and 0.3 g/L of aged FM hops (Table 2). In addition, beer FM-a was prepared by mixing FM-F and FM-A in a 2:1 ratio, so that FM-a corresponded to a test beer brewed with 1.5 g/L of fresh FM and 0.1 g/L of aged FM hops (Table S5).

Comparing beer FM-a with FM-F, the characteristics of fruity, tropical, rancid, fullness, and maturity were enhanced (Fig. 5A). Especially, the scores of fruity, tropical, fullness, and maturity were significantly higher with a risk of 5 % (Table S6). In beer FM-A, the characteristics of fruity, tropical, rancid, fullness, and maturity were higher than those in FM-F (Fig. 5B). The score of fruity and maturity were significantly higher with a risk of 5 % (Table S6). Indeed, while the negative characteristics of rancid gradually increased depending on the dosage of aged hops, the positive characteristics of fruity, tropical, fullness, and maturity increased by using only 0.1 g/L of aged hops.

4. Conclusions

This study focused on BCFAs in aged hops. The behaviours of BCFAs during a 12-months ageing process were compared using five Japanese hop varieties. All BCFAs in the hops increased in all varieties during the entire ageing period, with a drastic increase observed especially from 9 to 12 months. The increase of BCFAs in hops during this period couldn’t be sufficiently explained by only storage temperature. It was assumed that the oxidative reaction in hops could be repressed for 0 – 9 months perhaps due to the existence of antioxidant substances. The content of linalool gradually decreased but plateaued at relatively high levels. The content of geranic acid was mostly stable or increased only slightly. The levels of esters (methyl geranate and isobutyric esters) increased from 0 to 6 months but decreased from 9 to 12 months. It was assumed that certain carboxylic acids in hop cones could be esteri-

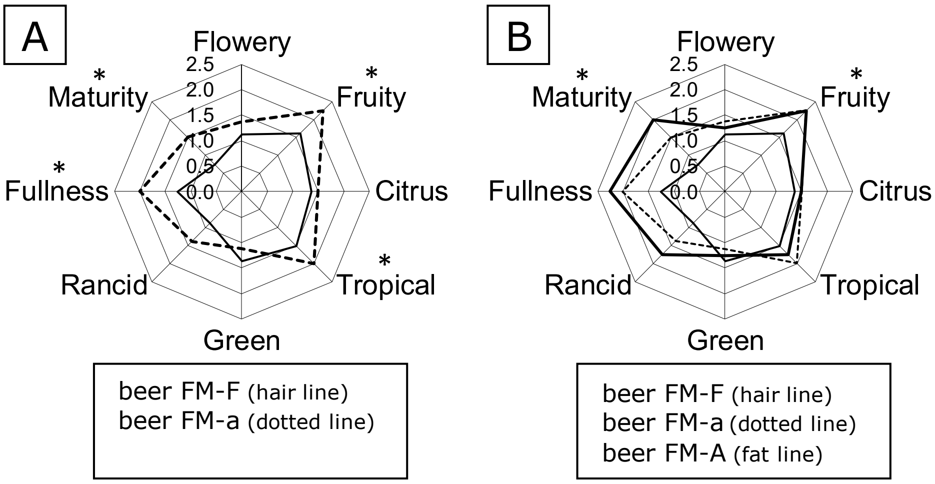

Fig. 5 **Flavour profiles of test beers made of Furano Magical (FM) with and without aged hops: beer FM-F (hair line), 1.5 g/L of fresh FM; FM-a (dotted line), 1.5 g/L of fresh FM and 0.1 g/L of aged FM; beer FM-A (fat line), 1.5 g/L of fresh FM and 0.3 g/L of aged FM; *, significant difference with a risk of 5 % in paired t-test comparing beer FM-a or FM-A against beer FM-F**

fied by remaining ester biosynthetic activities and that these esters might gradually decrease from 9 to 12 months due to escalating evaporation during long ageing period, during which the storage temperature increased over 25 °C. In thiol-containing hops, the concentration of 3S4MP decreased drastically from 6 to 12 months, whereas the concentration of 4MSP gradually decreased but plateaued at relatively high levels.

Based on the analytical profiles of a test beer made from a flavour hop variety, model beers spiked with several combinations of monoterpene alcohols (LGC), 4MSP, and each BCFA were prepared to investigate the sensory effects of using aged hops on aroma and taste profiles of beers. As a result, BCFAs commonly enhanced the characteristics of fruity, fullness, and maturity in the model beer spiking LGC. BCFAs also more enhanced the characteristics of tropical in coexistence with 4MSP.

The sensory effect of BCFAs was expected to enhance the hop-derived flavour of beer brewed with both geraniol-rich hops and 4MSP-rich hops. However, the sensory tests also showed an increase in rancid characteristics due to the presence of BCFAs. Therefore, test beers using fresh and aged hops were brewed. These test beers were thought to be suitable for evaluating the sensory effect of BCFAs on hop-derived aroma and taste in beer under the condition in which various flavour compounds, except for BCFAs, have almost no difference. As a result, although the negative characteristics of rancid gradually increased depending on the dosage of aged hops, the positive characteristics of fruity, tropical, fullness, and maturity increased by using only 0.1 g/L of aged hops. In addition, several researchers have reported that ageing of hops could increase the fruity flavour of beer [12, 32]. It is assumed that the sensory enhancing effect of ageing-derived BCFAs could explain such flavour change in beer brewed with aged hops.

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Supplementary Information

2.5 Quantitation of Hop-derived Flavour Compounds

Table S1 **GC-MS parameters for quantitation of selected flavor compounds**

group	reagent	isomers	ion 1 *(m/z)*	ion 2 *(m/z)*	column	internal standard (ISTD)
alcohols	linalool	–	136[a]	121[a]	HP-1MS	linalool-d$_5$
	β-citronellol	–	138[a]	109[a]	HP-1MS	*o*-cymene
	geraniol	–	123[a]	93[a]	HP-1MS	*o*-cymene
aldehydes	citral	geranial	152[a]	137[a]	DB-WAX LTM[c] DB-5 LTM[c]	*o*-cymene
		neral	137[a]	119[a]	DB-WAX LTM[c] DB-5 LTM[c]	*o*-cymene
carboxylic acids	geranic acid	geranic acid	100[b]	82[b]	DB-FFAP	nonanoic acid
		neric acid	168[b]	123[b]	DB-FFAP	nonanoic acid
	isobutyric acid	–	88[a]	73[a]	InterCap-WAX	benzyl acetate
	isovaleric acid	–	60[a]	87[a]	InterCap-WAX	benzyl acetate
	2-methylbutyric acid	–	74[a]	57[a]	InterCap-WAX	benzyl acetate
esters	methyl geranate	methyl geranate	114[a]	182[a]	HP-1MS	*o*-cymene
		methyl nerolate	114[a]	182[a]	HP-1MS	*o*-cymene
	isobutyl isobutyrate	–	89[a]	101[a]	HP-1MS	2-methylbutyl isobutyrate-d$_7$
	isoamyl isobutyrate	–	89[a]	115[a]	HP-1MS	2-methylbutyl isobutyrate-d$_7$
	2-methylbutyl isobutyrate	–	89[a]	101[a]	HP-1MS	2-methylbutyl isobutyrate-d$_7$
	ethyl isobutyrate	–	71[a]	43[a]	HP-1MS	ethyl 2-methyl-butyrate-d$_3$
	ethyl isovalerate	–	88[a]	85[a]	HP-1MS	ethyl 2-methyl-butyrate-d$_3$
	ethyl 2-methylbutyrate	–	102[a]	85[a]	HP-1MS	ethyl 2-methyl-butyrate-d$_3$
hydrocarbons	myrcene	–	93[a]	69[a]	HP-1MS	*o*-cymene
internal standard	*o*-cymene	–	134[a]	119[a]	–	–
	linalool-d$_5$	–	141[a]	126[a]	–	–
	nonanoic acid	–	129[b]	87[b]	–	–
	benzyl acetate	–	108[a]	91[a]	–	–
	2-methylbutyl isobutyrate-d$_7$	–	108[a]	96[a]	–	–
	ethyl 2-methylbutyrate-d$_3$	–	105[a]	118[a]	–	–

[a] GC-MS SIM mode: ion 1, quantifier ion; ion 2, qualifier ion

[b] GC-MS/MS MRM mode: ion 1, precursor ion; ion 2, product ion

[c] analysed via GC×GC-MS: ^{1}D, DB-WAX LTM; ^{2}D, DB-5 LTM

Table S2 **SRM conditions for volatile thiols**

compound	target			qualification		
	precursor ion	production	collision energy (V)	precursor ion	production	collision energy (V)
4MSP	132	89	6	132	75	2
3S4MPA	116	88	4	116	101	4
3SHA	116	88	4	116	101	4
3S4MP	134	100	0	134	57	8
3SH	134	82	2	100	82	0
3SPH	106	60	4	106	88	0

4MSP, 4-methyl-4-sulfanylpentane-2-on; 3S4MPA, 3-sulfanyl-4-methylpentyl acetate; 3SHA, 3-sulfanylhexyl acetate; 3S4MP, 3-sulfanyl-4-methylpentan-1-ol; 3SH, 3-sulfanylhexan-1-ol; 3SPH, 3-Sulfanylpropyl hexanoate

3 Results and Discussions

3.1 Changes in Hop-derived Flavour Compounds during Ageing Process

Table S3 Changes in concentrations of flavour compounds during ageing of hops

			Little Star (LS)					Sorachi Ace (SA)					Furano Special (FS)					Furano Magical (FM)					Furano Queen (FQ)				
group	**compound**		**0M**	**3M**	**6M**	**9M**	**12M**	**0M**	**3M**	**6M**	**9M**	**12M**	**0M**	**3M**	**6M**	**9M**	**12M**	**0M**	**3M**	**6M**	**9M**	**12M**	**0M**	**3M**	**6M**	**9M**	**12M**
alcohols	linalool	(µg/L)*	142	155	137	112	132	489	525	436	342	341	364	339	300	306	304	491	384	341	261	295	531	498	437	322	335
	β-citronellol	(µg/L)*	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
	geraniol	(µg/L)*	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	n.d.	n.d.	tr	tr	tr	n.d.	n.d.	tr	tr	tr
aldehydes	geranial	(µg/L)*	tr	tr	tr	tr	4,7	tr	14,6	15,7	18,3	25,5	tr	tr	tr	tr	12,1	n.d.	n.d.	tr	tr	n.d.	n.d.	n.d.	tr	tr	n.d.
carboxylic acids	geranic acid	(µg/L)*	24,1	48,7	52,0	80,5	79,0	1370	1080	1649	1184	1200	287	315	341	278	280	–	–	–	–	–	–	–	–	–	–
	neric acid	(µg/L)*	tr	tr	tr	tr	tr	11,7	21,9	20,1	20,9	18,9	4,5	4,7	4,7	7,3	5,4	–	–	–	–	–	–	–	–	–	–
	IBA	(mg/L)*	2,1	2,3	3,4	5,6	14,7	4,1	4,1	8,4	10,1	15,3	3,7	1,0	2,1	5,8	14,5	2,5	2,9	4,0	8,2	13,2	5,1	5,6	9,9	11,5	25,2
	IVA	(mg/L)*	2,1	3,2	3,6	7,2	14,1	5,0	7,1	11,5	15,6	25,6	3,6	1,6	2,0	10,8	23,2	5,5	7,9	11,6	17,8	27,4	3,9	6,2	7,4	11,4	25,0
	2MBA	(mg/L)*	0,5	0,8	0,9	1,5	2,8	1,4	1,8	3,4	4,0	7,7	0,9	0,4	0,6	2,5	6,5	1,1	1,6	2,2	3,0	6,1	0,9	1,5	2,0	2,7	5,5
esters	methyl geranate	(µg/L)*	14,2	16,2	60,0	39,4	39,7	tr	2,2	10,2	4,3	5,8	8,8	10,7	42,1	36,5	21,9	–	–	6,6	5,1	–	–	–	4,6	2,8	–
	methyl nerolate	(µg/L)*	tr	tr	1,1	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	–	–	tr	tr	–	–	–	tr	tr	–
	isobutyl isobutyrate	(µg/L)*	0,6	0,7	8,5	5,9	4,8	0,9	0,9	7,8	5,7	4,2	2,2	1,9	12,8	13,3	11,4	0,3	tr	1,9	1,7	2,3	5,3	4,3	42,4	32,9	28,3
	isoamyl isobutyrate	(µg/L)*	1,9	1,3	2,0	1,9	1,5	3,4	4,9	4,4	2,7	4,0	11,3	27,4	10,8	10,1	22,2	1,5	4,2	1,1	1,3	6,9	7,1	7,8	6,9	5,7	10,3
	2-methyl-butyl isobutyrate	(µg/L)*	15,5	12,9	21,2	18,6	21,6	27,9	28,7	42,9	25,9	26,1	93,7	98,7	105	104	91,0	12,4	8,8	11,2	13,0	18,4	58,7	40,6	68,7	54,8	62,9
hydrocar-bons	myrcene	(µg/L)*	6,9	4,7	20,7	14,6	4,2	18,7	56,5	70,5	40,1	22,0	66,5	93,3	68,3	54,7	19,0	8,8	3,2	23,5	19,9	11,3	13,6	3,6	22,7	18,0	9,9
volatile thiols	4MSP	(µg/kg)	tr	tr	tr	1,2	tr	–	–	–	–	–	–	–	–	–	–	82,9	75,8	52,1	51,8	39,5	31,4	27,6	26,7	23,1	19,2
	3S4MPA	(µg/kg)	tr	tr	tr	tr	tr	–	–	–	–	–	–	–	–	–	–	1,7	1,7	tr	tr	1,9	3,2	3,4	1,6	tr	2,9
	3SHA	(µg/kg)	tr	tr	tr	tr	tr	–	–	–	–	–	–	–	–	–	–	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
	3S4MP	(µg/kg)	2,6	4,5	1,1	1,3	1,3	–	–	–	–	–	–	–	–	–	–	36,8	36,1	15,7	10,0	6,9	146	157	96,3	58,9	36,3
	3SH	(µg/kg)	2,3	tr	tr	tr	tr	–	–	–	–	–	–	–	–	–	–	2,4	2,3	1,4	1,3	tr	3,4	3,7	2,9	1,9	1,3

tr, trace; n.d., not detected; *, concentration in hot water extract of each hop sample

IBA, isobutyric acid; IVA, isovaleric acid; 2MBA, 2-methylbutyric acid

4MSP, 4-methyl-4-sulfanylpentane-2-on; 3S4MPA, 3-sulfanyl-4-methylpentyl acetate;

3SHA, 3-sulfanylhexyl acetate; 3S4MP, 3-sulfanyl-4-methylpentan-1-ol; 3SH, 3-sulfanylhexan-1-ol

3.2 Sensory Effect among BCFAs, Monoterpene Alcohols, and 4MSP

Table S4 Paired t-test in sensory tests of model beers

model beer	**1-1**	**1-2**		**1-3**	**1-4**		**2-1**	**2-2**		**2-3**	**2-4**		**3-1**	**3-2**		**3-3**	**3-4**	
spiking solution	**LGC**	**LGC**		**LGC**	**LGC**		**LGC**	**LGC**		**LGC**	**LGC**		**LGC**	**LGC**		**LGC**	**LGC**	
	–	–		4MSP	4MSP		–	–		4MSP	4MSP		–	–		4MSP	4MSP	
	–	IBA		–	IBA		–	IVA		–	IVA		–	2MBA		–	2MBA	
	intensity[a]		*p*[c]	intensity[a]		*p*[c]	intensity[b]		*p*[c]	intensity[b]		*p*[c]	intensity[b]		*p*[c]	intensity[b]		*p*[c]
flowery	1,25	1,25	–	1,38	1,38	–	1,00	0,89	–	0,78	1,00	–	1,11	1,11	–	1,11	1,67	–
fruity	0,88	1,25	–	1,50	1,88	–	0,88	1,50	0,022*	1,56	2,00	0,035*	1,13	1,63	–	1,63	2,13	–
citrus	0,50	0,63	–	1,13	1,13	–	0,44	0,33	–	0,89	1,00	–	0,67	0,56	–	1,11	1,11	–
tropical	0,63	0,63	–	1,25	1,50	–	0,67	0,78	–	1,33	1,78	–	0,78	1,00	–	1,44	2,00	–
green	0,50	0,75	–	1,38	1,13	–	0,67	0,33	0,035*	1,33	1,00	–	0,89	0,67	–	1,44	1,11	–
rancid	0,13	0,63	–	1,25	1,50	0,020*	0,11	0,78	0,0007**	0,33	0,78	–	0,33	0,89	–	0,56	1,00	–
fullness	0,50	1,13	0,011*	1,38	1,13	0,0002***	0,44	1,22	0,0022**	0,78	1,22	–	0,78	1,33	–	1,11	1,78	0,050*
maturity	0,13	0,75	0,049*	0,38	1,00	0,0062***	0,11	1,00	0,0003***	0,56	1,11	–	0,56	1,11	–	0,44	1,78	0,00004***

[a] mean intensity value of the scores from eight well-trained panellist

[b] mean intensity value of the scores from nine well-trained panellists

[c] paired t-test comparing model beer with and without BCFA

*, significant difference with a risk of 5 %; **, significant difference with a risk of 1 %; ***, significant difference with a risk of 0.1 %

3.3 Sensory Effect of Aged Hops on Hop-derived Aroma and Taste in Beer

3.3.1 Hop-derived Flavour Compounds in Test Beers with and without Aged Hops

Table S5 **Concentrations of flavour compounds in test beers brewed with and without aged hops**

			Little Star		Sorachi Ace		Furano Special		Furano Magical			Furano Queen		Saaz		Columbus	
group	**compound**		**LS-F**	**LS-A**	**SA-F**	**SA-A**	**FS-F**	**FS-A**	**FM-F**	**FM-a***	**FM-A**	**FQ-F**	**FQ-A**	**SZ-F**	**SZ-A**	**CO-F**	**CO-A**
alcohols	linalool	(µg/L)	32,0	35,8	67,0	61,5	61,6	65,8	57,8	56,6	54,3	68,7	74,9	45,4	44,7	48,9	49,4
	β-citronellol	(µg/L)	6,9	7,3	21,9	25,9	25,6	20,8	12,1	11,3	9,6	7,5	7,5	5,5	5,9	14,1	14,7
	geraniol	(µg/L)	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
carboxylic acids	geranic acid	(µg/L)	tr	tr	100	124	36,0	42,9	10,6	7,1	tr	tr	tr	tr	tr	tr	tr
	IBA	(mg/L)	0,8	1,9	1,2	2,6	0,8	2,5	0,9	1,4	2,4	1,1	2,8	0,8	2,3	1,7	3,8
	IVA	(mg/L)	0,9	2,0	1,4	2,7	0,9	1,9	1,2	1,6	2,5	1,1	2,1	1,0	2,0	1,9	3,3
	2MBA	(mg/L)	0,4	1,0	0,6	1,2	0,4	0,9	0,4	0,6	1,0	0,5	1,0	0,4	0,9	0,6	1,2
esters	methyl geranate	(µg/L)	3,4	3,7	0,8	0,8	3,0	2,1	1,0	1,0	1,0	0,9	1,0	1,2	1,5	2,8	2,2
	isobutyl isobutyrate	(µg/L)	1,9	2,4	1,8	2,5	3,5	3,9	tr	0,4	1,1	8,6	10,1	0,3	0,3	1,3	1,4
	isoamyl isobutyrate	(µg/L)	4,7	4,7	5,6	5,0	8,3	7,9	7,2	6,8	6,1	6,3	6,6	5,1	5,0	8,1	8,3
	2-methylbutyl isobutyrate	(µg/L)	7,6	7,9	10,8	11,1	19,4	18,1	10,7	9,8	8,1	13,7	15,0	6,1	5,9	9,8	9,9
	ethyl isobutyrate	(µg/L)	2,4	2,9	tr	4,9	tr	3,9	tr	tr	tr	4,8	5,5	2,2	2,5	6,3	7,7
	ethyl isovalerate	(µg/L)	3,5	4,2	5,9	7,3	3,6	4,2	7,2	7,8	9,1	4,4	5,4	3,7	3,9	10,5	10,9
	ethyl 2-methylbutyrate	(µg/L)	tr	tr	tr	tr	2,5	tr	tr	0,9	2,8	tr	tr	tr	tr	3,5	3,9
hydrocarbons	myrcene	(µg/L)	0,6	0,6	1,2	1,3	1,4	1,3	1,2	1,1	1,0	1,0	1,1	0,7	0,8	1,2	1,2
volatile thiols	4MSP	(ng/L)	tr	tr	–	–	–	–	33,0	34,1	36,3	15,4	15,9	–	–	12,1	12,6
	3S4MPA	(ng/L)	tr	tr	–	–	–	–	tr	tr	tr	tr	tr	–	–	tr	tr
	3SHA	(ng/L)	tr	tr	–	–	–	–	tr	tr	tr	tr	tr	–	–	tr	tr
	3S4MP	(ng/L)	8,1	11,0	–	–	–	–	25,9	27,1	29,6	78,7	78,8	–	–	14,6	18,9
	3SH	(ng/L)	150	150	–	–	–	–	121	126	136	128	128	–	–	122	126

tr, trace; n.d., not detected; *caluculated based on FM-F and FM-A, FM-a was mixed FM-A and FM-A in a 2:1 ratio

IBA, isobutyric acid; IVA, isovaleric acid; 2MBA, 2-methylbutyric acid

4MSP, 4-methyl-4-sulfanylpentane-2-on; 3S4MPA, 3-sulfanyl-4-methylpentyl acetate;

3SHA, 3-sulfanylhexyl acetate; 3S4MP, 3-sulfanyl-4-methylpentan-1-ol; 3SH, 3-sulfanylhexan-1-ol

3.3.2 Sensory Effect of Aged Hops on Hop-derived Aroma and Taste in Beer

Table S6 Paired t-test in sensory tests of test beers mede of FM

test beer	FM-F	FM-a		FM-A	
	intensity[a]	intensity[a]	*p*[b]	intensity[a]	*p*[b]
flowery	1,13	1,38	–	1,25	–
fruity	1,63	2,25	0,049*	2,25	0,049*
citrus	1,38	1,50	-	1,50	–
tropical	1,50	2,00	0,033*	1,75	–
green	1,38	1,13	–	1,25	–
rancid	0,88	1,38	–	1,75	–
fullness	1,25	2,00	0,020*	2,25	–
maturity	0,75	1,50	0,020*	2,00	0,038*

[a] mean intensity value of the scores from nine well-trained panellists

[b] paired t-test comparing beer FM-a or FM-A against beer FM-F

*, significant difference with a risk of 5 %