K. Takoi

Behaviour of hop-derived branched-chain fatty acids during fermentation and their sensory effect on hopped beer flavours

In this study, branched-chain fatty acids (isobutyric acid, isovaleric acid, and 2-methylbutyric acid) were focused on. These fatty acids were originated from malts, hops, and yeast fermentation. It was thought that the concentrations of isobutyric acid and isovaleric acid in both wort and beer were affected by hops used for brewing, and that the behaviour of 2-methylbutyric acid might be less affected without/with hops. When fresh hops were used, these fatty acids were at relatively narrow ranges in both worts and beers. From the analysis by scatter diagrams of the concentrations of three fatty acids and corresponding ethyl esters (ethyl isobutyrate, ethyl isovalerate, and ethyl 2-methylbutyrate), it was suggested that the concentration of ethyl isovalerate showed relatively good correlation with isovaleric acids derived from hops, that ethyl 2-methylbutyrate in beer could be mainly affected by 2-methylbutyric acid generated during fermentation, and that ethyl isobutyrate in beer could be affected by hop-derived isobutyric esters rather than by hop-derived isobutyric acid. In the viewpoint of sensory effect, it was suggested that branched-chain fatty acids could enhance the flavours of monoterpene alcohols, and that only threshold levels of fatty acids were enough for this effect. In addition, it was assumed that branched-chain fatty acids could change total flavour profile of the mixture of monoterpene alcohols at threshold levels.

Descriptors: beers, hops, flavour, branched-chain fatty acids, ethyl esters of branched-chain fatty acids, monoterpene alcohols, additive effect, synergy

1 Introduction

It is well-known that various beers contain many flavour compounds derived from barley malts, hops, yeast fermentation and other raw materials. Among these raw materials, hops (*Humulus lupulus* L.) especially contain many types of flavour compounds. The flavour compounds of hops are mainly derived from the hop oil included in the lupulin gland of hop cone. Various terpenoids, esters, aldehydes, ketones, and sulphur compounds were well known as the major flavour compounds in the hop oil. Until now, our group have focused on monoterpene alcohols, volatile thiols, and hop-derived esters having branched-chain structures and revealed their contribution to the hop-derived varietal aromas, the behaviour of these compounds during beer production, and the mechanism of varietal aroma formation based on the synergy among various flavour compounds [27-36].

https://doi.org/10.23763/BrSc19-24takoi

Authors

Kiyoshi Takoi, Product & Technology Innovation Department, Sapporo Breweries Ltd., Shizuoka, Japan; corresponding author: kiyoshi.takoi@sapporobeer.co.jp

Many researchers have reported various hop-derived esters, which have branched-chain structures (Figure 1). Isobutyric esters of branched-chain alcohols (isobutyl isobutyrate, isoamyl isobutyrate, and 2-methylbutyl isobutyrate) were widely found in various hops [4-8, 10, 12, 15-16, 24, 27, 29, 32, 36-37]. These isobutyric esters have a green apple, apricot-like flavour [27, 29]. Almost of hop varieties contained these compounds at relatively high levels, while Saaz and Lublin had few of these compounds [12, 27, 29]. *Seaton* et al. reported that isobutyric esters could be unstable during boiling and fermentation [24]. In fact, commercial beers brewed with kettle hopping contain only small amounts of these compounds [27]. These compounds are found in late-hopped beer and dry-hopped beer [15-16, 24]. Isobutyric esters are expected to contribute to some of the special flavours of late-hopped/dry-hopped beers [27, 29, 32].

Isobutyric esters consist of isobutyric acid and three branched-chain alcohols (isobutanol, isoamyl alcohol, and 2-methybutanol). These structures are corresponding to those of branched-chain fatty acids (isobutyric acid, isovaleric acid, and 2-methylbutyric acid) and amino acids (L-valine, L-leucine, and L-isoleucine) (Fig. 1). In the field of brewing science, these structures also have been well-known as side-chain structures of hop bitter acids (alpha acids, iso-alpha acids, and beta acids) (Fig. 2). The oxidative degradation of hop bitter acids are resulted in the formation of branched-chain fatty acids. In general, bitter hops/high alpha hops contain higher levels of such fatty acids. Several researchers have reported that a part of

hop-derived fatty acids was esterified to ethyl esters (ethyl isobutyrate, ethyl isovalerate, and ethyl 2-methylbutyrate (Fig. 1)) during fermentation [11, 19]. These esters are expected to contribute to a part of the flavours of hopped beers, because of their very low thresholds, 6.3 µg/L for ethyl isobutyrate, 2.0 µg/L for ethyl isovalerate, and 1.1 µg/L for ethyl 2-methylbutyrate (in beer) [11].

In previous study [36], we investigated hop-derived isobutyric esters (isobutyl isobutyrate, isoamyl isobutyrate, and 2-methylbutyl isobutyrate) and ethyl esters of branched-chain fatty acids (ethyl isobutyrate, ethyl isovalerate, and ethyl 2-methylbutyrate). The wort, green beer, and finished beer samples hopped with total 42 hop varieties were analyzed and compared. Of the three isobutyric esters, 2-methylbutyl isobutyrate is most dominant component. All isobutyric esters gradually decreased during fermentation. All ethyl esters of branched-chain fatty acids were almost absent in wort and gently increased during total fermentation period, except for the fermentation using Huell Melon hops. Surprisingly, the wort made by Huell Melon hops only contained ethyl isobutyrate and ethyl 2-methylbutyrate at relatively high levels. On the other hand, the concentrations of all three ethyl esters in the Ekuanot (HBC366) beer were at relatively high levels and ethyl isovalerate was most dominant component. In addition, it is suggested that a part of 2-methylbutyl isobutyrate could be transesterificated to ethyl isobutyrate.

In this study, the concentrations of branched-chain fatty acids (isobutyic acid, isovaleric acid, and 2-methylbutyric acid) in test-brewed worts and beers were analysed, and their sensory effects on hopped beer flavours were also investigated.

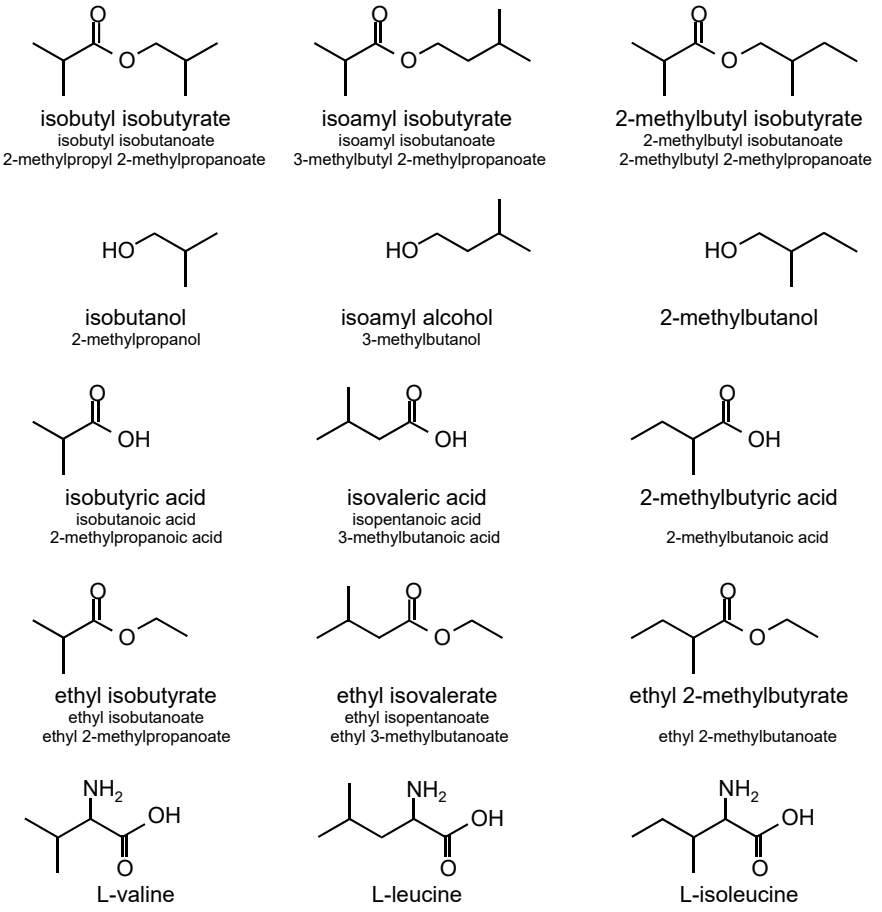

Fig. 1 **Chemical structures of isobutyric esters of branched-chain alcohols (isobutyl isobutyrate, isoamyl isobutyrate, and 2-methylbutyl isobutyrate), ethyl esters of branched-chain fatty acids (ethyl isobutyrate, ethyl isovalerate, and ethyl 2-methylbutyrate), and related alcohols, fatty acids, and amino acids**

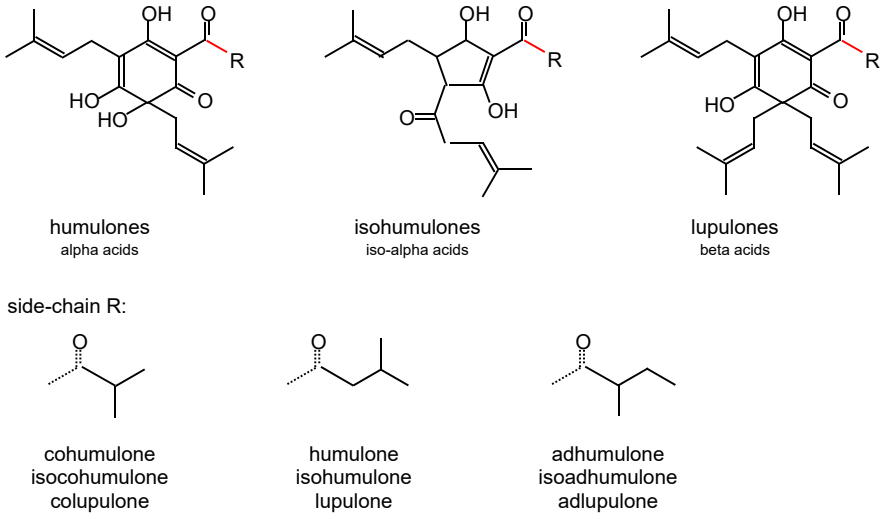

Fig. 2 **Chemical structures of humulones (alpha acids), isohumulones (iso-alpha acids), and lupulones (beta acids): dotted line in the structures of side-chain R is indicated the link points between bitter acids and side chain**

2 Materials and methods

2.1 Hop raw materials

Saaz from Czech Republic in 2007 and 2008 (type 90 pellet). Hallertauer Magnum (Magnum) and Hallertauer Tradition (HHT) were grown and pelletized in Germany in 2006 and 2008, respectively (type 90 pellet). Nelson Sauvin from New Zealand in 2007 (type 90 pellet). Amarillo, Apollo, Bravo, Cascade, Chinook, Glacier, Mosaic (formerly named as HBC369), Mt. Hood, and Simcoe were harvested in the U.S. in 2008 (hop powder). Citra was grown and pelletized in the U.S. in 2008 (type 90 pellet).

2.2 Pilot-scale brewing

For the comparison between unhopped and hopped conditions, two sets of brewing trial were carried out. Beers were made with

the same recipe according to the standard method of the Production & Technology Development Centre, Sapporo Breweries, Ltd.. Briefly, the wort was prepared using commercially available 67% malts, 33% adjuncts (starch, corn, and rice), and hops in a 400-L pilot-scale apparatus. Boiling period was 90 min. For unhopped trials, no hops were added. For hopped trials, all hops were added at the beginning of boiling. The Hallertauer Magnum or the Saaz hops were used. The hop dosage was adjusted in each trial, so that the bitterness unit (BU) in the finished beer was at 22. Each cooled wort was collected to fermentation tank (400 L/tank). Subsequently, the fermentation was started by adding 15.0 x 10^6 cells/ml lager yeast (brewery collected; *Saccharomyces pastorianus*) to the wort. The temperature of the fermentation was maintained at 10–12 °C (primary fermentation). After transferring the fermented wort to another storage tank under a CO_2 atmosphere, the maturation was carried out at 13 °C for 8 days, then at 0 °C for 2-3 weeks. Kieselguhr filtration and bottling were done using the pilot-scale equipment under anti-oxidative conditions. The alcohol contents of all test-brewed beers were at approximately 5.0 %.

Late-hopped beers were made with the same recipe according to the arranged method of the Production & Technology Development Centre, Sapporo Breweries, Ltd.. Briefly, the wort was prepared using commercially available malts and hops in 400-L scale pilot apparatus. Boiling period was 90 min. For prevention of over boiling, HHT hops were added at the beginning of boiling (0.2 g of hop/L). Cooled wort was collected to fermentation tanks (30 L/tank) and medium bottles (900 ml/bottle). For hop-flavouring, 24.8 g of hop was added to each bottle and was autoclaved at 105 °C for 5min. After cooling, the hop-flavoured wort was mixed with 30 L of wort in each fermentation tank. This condition was corresponding to that of the late-hopping with 0.8 g of hop/L. Subsequently, the fermentation, maturation, filtration, and bottling were done using the same equipment under the conditions, as described above. The alcohol contents of all test-brewed beers were at approximately 5.5 %.

2.3 Standard products

Linalool (> 98 %, racemic mixture) and β-citronellol (> 92 %, racemic mixture) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Geraniol (98 %) was purchased from Aldrich Chemical Company Inc.. Ethyl isobutyrate (> 98 %), ethyl isovalerate (> 97 %), isobutyric acid (> 98 %), isovaleric acid (> 98 %), and 2-methylbutyric acid (> 97 %, racemic mixture), were purchased from FUJIFILM Wako Pure Chemical Co., Ltd. (Osaka, Japan). Ethyl 2-methylbutyrate (> 98 %, racemic mixture) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

2.4 Quantification of hop-derived flavour compounds

2.4.1 (Semi-) Quantification of branched-chain fatty acids by solid phase microextraction-gas chromatography-mass spectroscopy (SPME-GC-MS)

For analysis of branched-chain fatty acids (isobutyric acid, isovaleric acid, and 2-methylbutyric acid), GC-MS analyses were carried out using a 6890N gas chromatograph (Agilent Technologies) and a MS 5973 mass spectrometer (Agilent Technologies) according to the following method. The carrier gas was helium, with a column-head pressure of 7.6 psi and flow rate of 1.0 mL/min. The detector was functioned in the EI mode (70 eV) and was connected to the GC by a transfer line heated to 250 °C. For analysis of wort and finished beer, 8 mL of each sample was put into a 20-mL glass vial including 3 g of sodium chloride at 0 °C on ice water. The vial, including a sample, was sealed with a magnet cap. The vial was preincubated with stirring at 40 °C for 15 min using a Combi-PAL autosampler (CTC Analytics). After preincubation, an SPME fibre [PDMS/DVB (polydimethylsiloxane/divinylbenzene), 65-µm film thickness; Supelco] was inserted into the head space of the vial and adsorption was carried out for 15 min. After the adsorption, the SPME fibre was injected into a splitless injector (260 °C; purge time = 3 min, purge flow = 30 mL/min) at oven temperature (50 °C) onto a type HP-INNOwax capillary column (30 m, 0.25-mm i.d., 0.25-µm film thickness; Agilent Technologies). For all the analyses, the temperature program was as follows: 50 °C for 1 min, raised at 10 °C/min to 250 °C, followed by a 5-min isotherm. The branched-chain fatty acids (isobutyric acid, isovaleric acid, and 2-methylbutyric acid) were (semi-) quantified in the SIM mode, selecting *m/z* 73, 60, and 74, respectively. Calibration curves were determined using test-worts/test-beers containing these compounds at final concentrations ranging from 0 to 1.0 mg/L. All calibration produced a linear response with an R^2 value > 0.98. The analysis was performed in duplicate.

2.4.2 (Semi-) Quantification of monoterpene alcohols and ethyl esters of branched-chain fatty acids by solid phase microextraction-gas chromatography-mass spectroscopy (SPME-GC-MS)

For analysis of monoterpene alcohols (linalool, β-citronellol, and geraniol), GC-MS analyses were carried out using a 6890N gas chromatograph (Agilent Technologies) and a MS 5973 mass spectrometer (Agilent Technologies) according to the method described in previous papers [32]. The carrier gas was helium, with a column-head pressure of 15 psi and flow rate of 1.8 mL/min. The detector was functioned in the EI mode (70 eV) and was connected to the GC by a transfer line heated to 280 °C. For analysis of wort and finished beer, 8 mL of each sample was put into a 20-mL glass vial including 3 g of sodium chloride at 0 °C on ice water. The vial, including a sample, was sealed with a magnet cap. The vial was preincubated with stirring at 40 °C for 15 min using a Combi-PAL autosampler (CTC Analytics). After preincubation, an SPME fibre [PDMS (polydimethylsiloxane), 100-µm film thickness; Supelco] was inserted into the head space of the vial and adsorption was carried out for 15 min. After the adsorption, the SPME fibre was injected into a splitless injector (260 °C; purge time = 3 min, purge flow = 20 mL/min) at oven temperature (50 °C) onto a type HP-1MS capillary column (30 m, 0.25-mm i.d., 1.0-µm film thickness; Agilent Technologies). For all the analyses, the temperature program was as follows: 50 °C for 1 min, raised at 5 °C/min to 250 °C, followed by a 1-min isotherm. The monoterpene alcohols (linalool, α-terpineol, nerol, β-citronellol, and geraniol) were (semi-) quantified in the SIM mode, selecting the following ions: *m/z* 93 (for geraniol), and 109 (for linalool and β-citronellol). Ethyl isobutyrate, ethyl isovalerate, and ethyl 2- methylbutyrate were (semi-) quantified in the SIM mode, selecting *m/z* 116, 88, and 102, respectively.Calibration

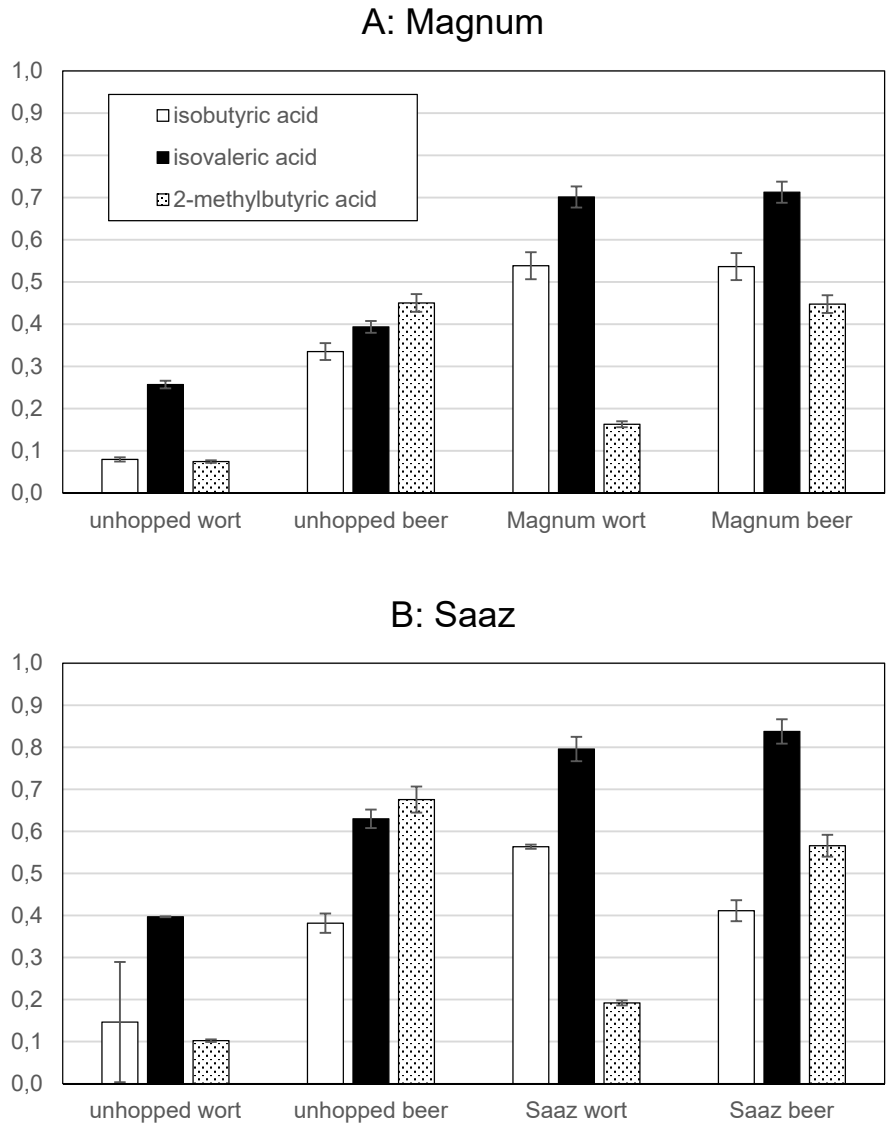

Fig. 3 Behaviour of branched-chain fatty acids (isobutyric acid, isovaleric acid, and 2-methlbutyric acid) (mg/L) in test-brewed worts and beers: A, brewed without/with Magnum; B, brewed without/with Saaz

curves were determined using water (including 5 % ethanol) containing these compounds at final concentrations ranging from 0 to 10 µg/L. All calibration produced a linear response with an R^2 value > 0.98 over the concentration range analysed. The analysis was performed in duplicate.

2.5 Sensory evaluation

2.5.1 Determining flavour thresholds of branched chain fatty acids and ethyl esters of branched chain fatty acids

Each sensory evaluation was performed by 10 – 14 well-trained panellists. Perception threshold of branched chain fatty acids (isobutyric acid, isovaleric acid, and 2-methylbutyric acid) and ethyl esters of branched chain fatty acids (ethyl isobutyrate and ethyl isovalerate) was assessed by a forced-choice ascending concentration series method of limits [1]. Briefly, the directional triangular tests of six increasing concentrations in model carbonated dilute alcohol solution (5 %v/v ethanol). The 50 mL of each sample solution was presented in plastic cups. The best estimate threshold was calculated for each panellist as the geometric mean of the highest concentration missed and the next highest concentration. The group threshold was calculated as the geometric mean of the best estimate thresholds of the panellists.

2.5.2 Study of additive effect between branched-chain fatty acids and other flavour compounds (monoterpene alcohols and ethyl esters of branched chain fatty acids)

Each sensory evaluation was performed by 10 – 14 well-trained panellists. Evaluation of an additive effect was performed according to the methods previously described [28-30, 34]. Namely, in order to assess an additive effect among branched-chain fatty acids and other flavour compounds (monoterpene alcohols and ethyl esters of branched chain fatty acids), triangular tests were carried out in model carbonated dilute alcohol solution (5 %v/v ethanol), as follows. A control solution containing the estimated threshold concentrations of branched-chain fatty acids (isobutyric acid, 5000 µg/L; isovaleric acid, 40 µg/L; 2-methylbutyric acid, 800 µg/L) was compared with test solutions containing the same concentration of branched-chain fatty acids together with 3 µg/L of linalool, 5 µg/L of geraniol, 5 µg/L of β-citronellol, 0.6 µg/L of ethyl isobutyrate, or 0.4 µg/L of ethyl isovalerate. The 50 mL of each sample solution was presented in plastic cups. The significance of the results was determined according to the binominal law.

2.5.3 Sensory evaluation of synergy between branched-chain fatty acids and monoterpene alcohols

Each sensory evaluation was performed by 10 well-trained panellists. The change of flavour characters by synergy between branched-chain fatty acids and monoterpene alcohols was assessed in a model solution (5 % v/v ethanol, carbonated), as follows. A control solution 'LGC mix' (simulating the test-brewed Citra beer; linalool, 100 µg/L; geraniol, 25 µg/L; β-citronellol, 30 µg/L) was compared with test solutions containing the same concentration of monoterpene alcohols together with 5000 µg/L of isobutyric acid (LGC mix + IBA), 40 µg/L of isovaleric acid (LGC mix + IVA), or 800 µg/L of 2-methylbutyric acid (LGC mix + 2MBA). The concentrations of branched-chain fatty acids were adjusted to the estimated thresholds. A 50 mL aliquot of each sample solution was presented in a plastic cup and the six flavour characters (flowery, fruity, citrus, tropical, green, and rancid) were scored from 0 (no flavour) to 3 (strong flavour) in intervals of 1.0.

Table 1 **Concentrations of monoterpene alcohols (linalool, β-citronellol, and geraniol) (µg/L), branched-chain fatty acids (isobutyric acid, isovaleric acid, and 2-methlbutyric acid) (mg/L), and ethyl esters of branched-chain fatty acids (ethyl isobutyrate, ethyl isovalerate acid, and ethyl 2-methlbutyrate) (µg/L) in test-brewed beers late-hopped with various hop varieties**

		Amarillo		Apollo		Bravo		Cascade		Chinook		Citra	
concentrations		**wort**	**beer**	**wort**	**beer**	**wort**	**beer**	**wort**	**beer**	**wort**	**beer**	**wort**	**beer**
linalool	(µg/L)	191[a]	120[a]	60.0[a]	51.7[a]	111[a]	78.0[a]	63.7[a]	35.7[a]	72.1[a]	44.3[a]	195[a]	108[a]
β-citronellol	(µg/L)	0.2[a]	31.7[a]	0.4[a]	16.2[a]	0.7[a]	35.6[a]	0.2[a]	29.2[a]	0.6[a]	24.3[a]	0.5[a]	27.4[a]
geraniol	(µg/L)	64.3[a]	73.0[a]	81.6[a]	26.8[a]	252[a]	69.0[a]	137[a]	22.1[a]	146[a]	41.4[a]	87.5[a]	22.6[a]
isobutyric acid	(mg/L)	0,1	0,4	0,1	0,5	0,1	0,6	0,2	0,5	0,1	0,4	0,2	0,5
isovaleric acid	(mg/L)	0,2	0,4	0,2	0,8	0,2	0,6	0,3	0,5	0,2	0,4	0,4	0,6
2-methylbutyric acid	(mg/L)	0,1	0,3	0,1	0,5	0,1	0,4	0,1	0,4	0,1	0,3	0,1	0,4
ethyl isobutyrate	(µg/L)	tr[b]	3.5[b]	tr[b]	4.5[b]	tr[b]	5.4[b]	0.1[b]	2.0[b]	0.3[b]	11.7[b]	0.1[b]	3.1[b]
ethyl isovalerate	(µg/L)	tr[b]	0.4[b]	tr[b]	1.0[b]	n.d.[b]	0.5[b]	tr[b]	0.4[b]	tr[b]	0.5[b]	tr[b]	0.9[b]
ethyl 2-methylbutyrate	(µg/L)	n.d.[b]	0.5[b]	tr[b]	1.6[b]	tr[b]	0.8[b]	tr[b]	0.5[b]	tr[b]	1.0[b]	tr[b]	0.6[b]
		Galcier		**Mosaic**		**Mt. Hood**		**Nelson Sauvin**		**Saaz**		**Simcoe**	
concentrations		**wort**	**beer**	**wort**	**beer**	**wort**	**beer**	**wort**	**beer**	**wort**	**beer**	**wort**	**beer**
linalool	(µg/L)	83.8[a]	49.6[a]	110[a]	70.1[a]	149[a]	78.6[a]	66.2[a]	40.4[a]	39.1[a]	19.4[a]	41.2[a]	27.0[a]
β-citronellol	(µg/L)	0.7[a]	7.8[a]	0.6[a]	27.8[a]	0.5[a]	16.3[a]	0.2[a]	14.5[a]	0.2[a]	6.1[a]	0.2[a]	8.9[a]
geraniol	(µg/L)	13.0[a]	5.9[a]	171[a]	47.0[a]	54.2[a]	12.2[a]	19.2[a]	13.2[a]	4.6[a]	3.8[a]	38.0[a]	10.9[a]
isobutyric acid	(mg/L)	0,1	0,4	0,2	0,5	0,1	0,5	0,2	0,6	0,2	0,4	0,1	0,4
isovaleric acid	(mg/L)	0,3	0,4	0,2	0,5	0,3	0,5	0,4	0,6	0,4	0,5	0,3	0,5
2-methylbutyric acid	(mg/L)	0,1	0,4	0,1	0,3	0,1	0,4	0,1	0,4	0,1	0,4	0,1	0,4
ethyl isobutyrate	(µg/L)	tr[b]	1.7[b]	0.1[b]	6.6[b]	tr[b]	2.2[b]	0.1[b]	4.4[b]	tr[b]	1.6[b]	0.1[b]	2.7[b]
ethyl isovalerate	(µg/L)	n.d.[b]	0.5[b]	n.d.[b]	0.5[b]	n.d.[b]	0.7[b]	tr[b]	0.9[b]	n.d.[b]	0.6[b]	tr[b]	0.6[b]
ethyl 2-methylbutyrate	(µg/L)	n.d.[b]	0.5[b]	tr[b]	0.7[b]	n.d.[b]	0.6[b]	tr[b]	0.8[b]	n.d.[b]	0.6[b]	tr[b]	0.5[b]

[a] Previously reported in ref 35. [b] Previously reported in ref 36.

3 Results and discussions

3.1 Comparison of behaviours of branched-chain fatty acids before and after fermentation

3.1.1 Comparison between unhopped and hopped conditions

In general, branched-chain fatty acids (isobutyric acid, isovaleric acid, and 2-methylbutyric acid (Fig. 1)) are major metabolite, which are derived from various metabolic pathways. Therefore, these compounds are contained in various foods and beverages. The branched-chain fatty acids in beer are derived from malts, hops, and also yeast metabolism. In order to investigate origins of these fatty acids in beer, we brewed test-beers without/with hops. The concentrations of branched-chain fatty acids in worts and beers were compared. We carried out two sets of trials, each set included unhopped and hopped trials, and one set used Magnum hops (Fig. 3A), the other Saaz hops (Fig. 3B).

In both unhopped worts, all three branched-chain fatty acids were detected. Therefore, these compounds were firstly derived from malts. After fermentation, all compounds increased in both unhopped beers. It is thought that brewing yeasts generated these compounds as fermentation by-products. Of all compounds, 2-methylbutyric acid drastically increased after fermentation. In comparison with both sets of unhopped and hopped worts, all compounds increased by adding hops. Especially, isobutyric acid and isovaleric acid clearly increased in both hopped worts. On the other hand, apparent concentrations of isobutyric acid and isovaleric acid in both hopped beers were not almost changed after fermentation. It is assumed that brewing yeasts could not only generate but also consume these two fatty acids during fermentation. Only 2-methylbutyric acid increased after fermentation. It is thought that the behaviour of 2-methylbutyric acid might be less affected without/with hops.

It is well-known that branched-chain fatty acids in general increase during storage of hops [19]. Therefore, it is expected that the concentrations of branched-chain fatty acids in wort made with aged hops could increase more than those in wort with fresh hops. In the case of Magnum and Saaz hops, branched-chain fatty acids significantly increase by using aged hops. In such case, isobutyric acid and isovaleric acid were at very high levels in wots, and these compounds decreased after fermentation. However, 2-methylbutyric acid increased after fermentation in these cases (data not shown). Therefore, it is thought that the concentrations of isobutyric acid and isovaleric acid in both wort and beer are sensitive to hops used for brewing.

3.1.2 Comparison between various hopped varieties

In order to compare the concentrations of branched-chain fatty acids in worts and beers between various hop varieties, we analysed 12 test-brewed worts and beers, which were late-hopped with fresh hops (hop powders or pellets not treated with any aging methods). Table 1 showed the concentrations of these compounds together with those of monoterpene alcohols and ethyl esters of fatty acids.

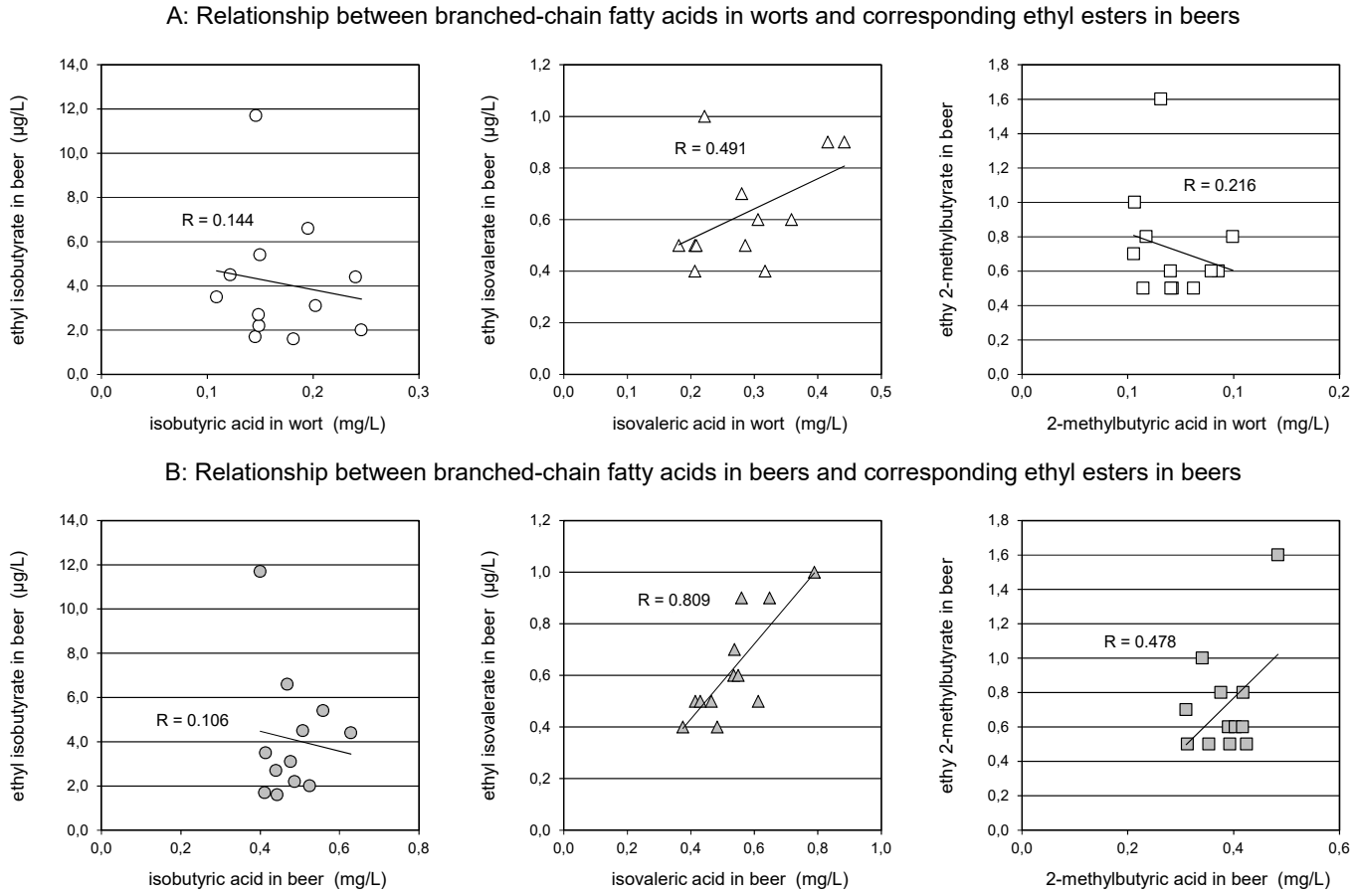

Fig. 4 Relationship between branched-chain fatty acids (isobutyric acid, isovaleric acid, and 2-methylbutyric acid) (mg/L) and ethyl esters of branched-chain fatty acids (ethyl isobutyrate, ethyl isovaleracte, and ethyl 2-methylbutyracte) (µg/L): A, relationship between branched-chain fatty acids in worts and corresponding ethyl esters in beers; B, relationship between branched-chain fatty acids in beers and corresponding ethyl esters in beers

As a result, the concentration of each fatty acid was ranged, as follows: isobutyric acid, 0.1–0.2 mg/L in worts, 0.4–0.6 mg/L in beers; isovaleric acid, 0.2–0.4 mg/L in worts, 0.4–0.8 mg/L in beers; 2-methylbutyric acid, 0.1 mg/L in worts, 0.3–0.5 mg/L in beers. When fresh hops were used, these fatty acids were at relatively narrow ranges in both worts and beers.

In the literatures [11, 19], the biotransformation pathway from branched-chain fatty acids to corresponding ethyl esters has been proposed. In order to verify this pathway, the concentrations of three fatty acids and ethyl esters shown in Table 1 were plotted in scatter diagrams (Fig. 4); figure 4A shows fatty acids in worts and esters in beers, and figure 4B fatty acids in beers and esters in beers. As a result, only the concentrations of isovaleric acid in worts had weak positive correlation with those of ethyl isovalerate in beers (Fig. 4A). The concentrations of isovaleric acid in beers showed relatively high correlation with those of ethyl isovalerate in beers (Fig. 4B).

In general, the structure of isovaleric acid is related to humulone and lupulone (Fig. 2), which are major isomers of alpha/beta acids, and is dominant component among all branched-chain fatty acids derived from hops. As described above, it is thought that the concentrations of this compound in both worts and beers were affected by hops used for brewing (Fig. 3). Therefore, the concentration of ethyl isovalerate showed relatively good correlation with isovaleric acids derived from hops. From the comparison between figure 4A and 4B, it was suggested that isovaleric acid generated during fermentation could additively affect to ethyl isovalerate in beer.

As described above, it is thought that 2-methylbutyric acid mainly increased by yeast fermentation, and that the behaviour of 2-methylbutyric acid might be less affected without/with hops. Therefore, it was suggested that 2-methylbutyric acid generated during fermentation could mainly affect to ethyl 2-methylbutyrate in beer.

As well as isovaleric acid, it is also thought that the concentrations of isobutyric acid in both worts and beers were affected by hops (Fig. 3). However, there were no correlation between isobutyric acid and ethyl isobutyrate, interestingly (Fig. 4). In previous study [36], it was observed that there was relatively good correlation between ethyl isobutyrate and 2-methybutyl isobutyrate, which is dominant component among hop-derived isobutyric esters. Therefore, it was suggested that ethyl isobutyrate in beer could affect by hop-derived isobutyric esters rather than by hop-derived isobutyric acid. Based on these results we hypothesize the following biotransformation pathway of branched-chain esters by brewing yeast (Fig. 5). The enzymes involved in the biotransformation pathway are not fully characterized so far. It is expected that the quality of hops and the selection of different yeast strains might strongly affect the fate of short chain fatty acids and esters during beer production. Therefore, the revealing of the mechanism of this pathway could

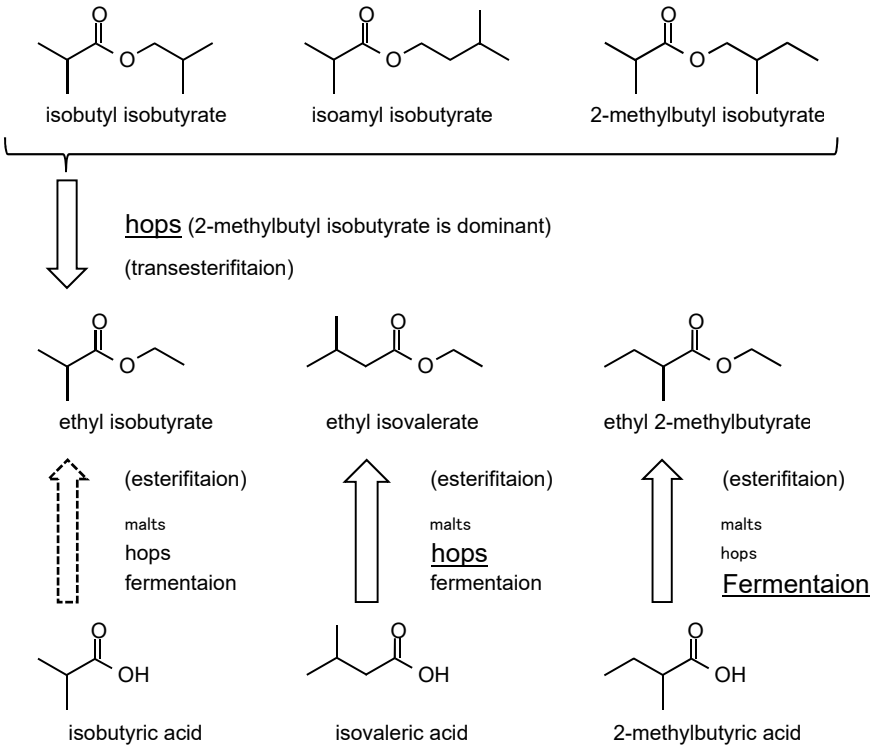

Fig. 5 Biotransformation pathway of branched-chain esters by brewing yeast

be useful for hop breeders and/or brewers

3.2 Sensrory effect between branched-chain fatty acids and other flavour compounds (monoterpene alcohols and ethyl esters of branched-chain fatty acids)

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 [9, 23]. For example, 3-sulfanylhexan-1-ol (C6) can enhance the odour intensities of 3-sulfanypentan-1-ol (C5) and 3-sulfanylheptan-1-ol (C7) [23]. On the other hand, we have already observed that a certain volatile thiols, 3-sulfanyl-4-methylpentan-1-ol (3S4MP) and 4-methyl-4-sulfanylpentan-2-one (4MSP), could enhance intensities of other flavour compounds, for example 3-sulfanyl-4-methylpentyl acetate (3S4MPA), 2-methylbutyl isobutyrate, linalool, and geraniol [28-29, 34]. In addition, the intensities of several coffee aroma compounds could be enhanced by certain carboxylic acids, such as acetic acid (C2) and butyric acid (C4), at sub-threshold levels [13-14]. From these studies [9, 13-14, 23, 28-29, 34], we assumed that branched-chain fatty acids can enhance the intensities of other flavour compounds at sub-threshold levels. In this study, we focused on branched-chain fatty acids, monoterpene alcohols, and ethyl esters of branched-chain fatty acids. We assumed branched-chain fatty acids as possible enhancers.

Firstly, we determined group threshold of all candidate compounds in model solution (5 % v/v ethanol, carbonated) by using our own panellists. The group thresholds of three monoterpene alcohols (linalool, geraniol, and β-citronellol) have been determined by same panellists [30]. Table 2 lists the flavour descriptions and newly estimated group thresholds of monoterpene alcohols, branched-chain fatty acids, and ethyl esters of branched-chain fatty acids, together with reference thresholds of these compounds reported in previous papers [2-3, 11, 17-18,

Table 2 Olfactory descriptions and perception thresholds in model solution (5 % v/v ethanol, carbonated) of monoterpene alcohols (linalool, β-citronellol, and geraniol) (μg/L), branched-chain fatty acids (isobutyric acid, isovaleric acid, and 2-methlbutyric acid) (μg/L), and ethyl esters of branched-chain fatty acids (ethyl isobutyrate, ethyl isovalerate acid, and ethyl 2-methlbutyrate) (μg/L)

ref compd		flavour descriptions	newly estimated threshold[a] in model solution[b]	reference threshold in previous papers
linalool	(μg/L)	lavender	3[c,d]	1–2[e]
β-citronellol	(μg/L)	lemon, lime	9[c,d]	40[f]
geraniol	(μg/L)	rose	7[d]	40[g]
isobutyric acid	(μg/L)	rancid, buttery	5000	8100[h]
isovaleric acid	(μg/L)	cheesy	40	120–700[i]
2-methylbutyric acid	(μg/L)	cheesy	800[c]	100, 50[j]
ethyl isobutyrate	(μg/L)	grape	0,6	6.3[k]
ethyl isovalerate	(μg/L)	melon	0,4	2.0[k]
ethyl 2-methylbutyrate	(μg/L)	melon	–	1.1[k]

[a] Flavor threshold determined by 10-13 panelists

[b] Model carbonated dilute ethanol solution (5% v/v Ethanol, Carbonated)

[c] Determined using racemic mixture

[d] Previously reported in ref 30

[e] Previously reported in ref 11 (estimated in beer), 18, and 25

[f] Previously reported in ref 17

[g] Previously reported in ref 26

[h] Previously reported in ref 21

[i] Previously reported in ref 2, 3, 17, and 21

[j] Previously reported in ref 20; 100 for *R* isomer and 50 for *S* isomer

[k] Previously reported in ref 11 (estimated in beer)

20-21, 25-26]. The odour of isobutyric acid is somewhat reminiscent of rancid and/or buttery, and its threshold was estimated at 5000 µg/L, the ones of isovaleric acid and 2-methylbutyric acid are of cheesy, and their thresholds were at 40 and 800 µg/L, respectively. In the literature [11], the thresholds of ethyl esters of branched-chain fatty acids were reported as follows; 6.3 µg/L for ethyl isobutyrate, 2.0 µg/L for ethyl isovalerate, and 1.1 µg/L for ethyl 2-methylbutyrate (in beer). Our panellists newly estimated the thresholds of ethyl isobutyrate and ethyl isovalerate at 0.6 and 0.4 µg/L (in model solution), respectively. These thresholds were much lower than those in the literature.

Secondly, in order to assess a possible additive effect between branched-chain fatty acids and other flavour compounds, triangular tests were designed according to the method used in previous studies [28-30, 34] and carried out, as shown in Table 3 and 4. A control model solution (5 % v/v ethanol, carbonated), which contains each fatty acid at its perception threshold level in model solution, was compared with test solutions containing each fatty acid together with 3 µg/L of linalool, 5 µg/L of geraniol, 5 µg/L of β-citronellol, 0.6 µg/L of ethyl isobutyrate, or 0.4 µg/L of ethyl isovalerate. The concentrations of linalool, ethyl isobutyrate, and ethyl isovalerate corresponded to their threshold levels. Those of geraniol and β-citronellol were lower than their thresholds. The concentrations of these monoterpene alcohols in model solutions were adjusted to same concentrations used in previous studies [28-30, 34]. In general, in a triangular test corresponding to the threshold of a certain compound, about half of panellists judge correctly. It is not at significant level. In a triangular test under the threshold of a certain compound, panellists couldn't judge at significant level, as well. Such triangular test could mainly evaluate a flavour intensity. Therefore, in our previous studies [28-30, 34], the 'additive effect' has been defined as a sensory effect of candidate enhancer compound by which a flavour intensity of reference compound could increase.

Table 3 **Additive effects between branched-chain fatty acids (isobutyric acid, isovaleric acid, and 2-methlbutyric acid) and monoterpene alcohols (linalool, β-citronellol, and geraniol): Triangular test involving 12 – 14 panelists (in model solution (5 % v/v ethanol, carbonated))**

test solution	control solution	correct answers/ total answers	*p*
5000 µg/L isobutyric acid + 3 µg/L linalool	5000 µg/L isobutyric acid	8/12	0.05
5000 µg/L isobutyric acid + 5 µg/L β-citronellol	5000 µg/L isobutyric acid	6/12	–
5000 µg/L isobutyric acid + 5 µg/L geraniol	5000 µg/L isobutyric acid	10/12	0.001
40 µg/L isovaleric acid + 3 µg/L linalool	40 µg/L isovaleric acid	11/14	0.001
40 µg/L isovaleric acid + 5 µg/L β-citronellol	40 µg/L isovaleric acid	8/14	–
40 µg/L isovaleric acid + 5 µg/L geraniol	40 µg/L isovaleric acid	12/14	< 0.001
800 µg/L 2-methlbutyric acid + 3 µg/L linalool	800 µg/L 2-methlbutyric acid	9/13	0.01
800 µg/L 2-methlbutyric acid + 5 µg/L β-citronellol	800 µg/L 2-methlbutyric acid	9/13	0.01
800 µg/L 2-methlbutyric acid + 5 µg/L geraniol	800 µg/L 2-methlbutyric acid	12/13	< 0.001

Table 4 **Additive effects between branched-chain fatty acids (isobutyric acid, isovaleric acid, and 2-methlbutyric acid) and ethyl esters of branched-chain fatty acids (ethyl isobutyrate and ethyl isovalerate acid): Triangular test involving 12 – 14 panelists (in model solution (5 % v/v ethanol, carbonated)).**

test solution	control solution	correct answers/ total answers	*p*
5000 µg/L isobutyric acid + 0.6 µg/L ethyl isobytyrate	5000 µg/L isobutyric acid	6/12	-
5000 µg/L isobutyric acid + 0.4 µg/L ethyl isovalerate	5000 µg/L isobutyric acid	6/12	-
40 µg/L isovaleric acid + 0.6 µg/L ethyl isobytyrate	40 µg/L isovaleric acid	11/14	0,001
40 µg/L isovaleric acid + 0.4 µg/L ethyl isovalerate	40 µg/L isovaleric acid	10/14	0,01
800 µg/L 2-methlbutyric acid + 0.6 µg/L ethyl isobytyrate	800 µg/L 2-methlbutyric acid	9/13	0,01
800 µg/L 2-methlbutyric acid + 0.4 µg/L ethyl isovalerate	800 µg/L 2-methlbutyric acid	7/13	-

3.2.1 Additive effect between branched-chain fatty acids and monoterpene alcohols

We investigated whether branched-chain fatty acids could enhance the flavour intensities of monoterpene alcohols. Table 3 presents the results of total 9 sets of sensory triangular tests. In the sets containing isobutyric acid, 8 of the 12 panellists could recognize the test sample containing linalool at a significant level with a risk of 5 %, 10 of the 12 panellists could recognize the one containing geraniol with a risk of 0.1 %. There was no significant difference between the control solution and the test solution containing β-citronellol. In the sets containing isovaleric acid, 11 of the 14 panellists could recognize the test sample containing linalool at a significant level with a risk of 0.1 %, 12 of the 14 panellists could recognize the one containing geraniol with a risk of below 0.1 %. In the sets containing 2-methylbutyric acid, 9 of the 13 panellists could recognize the test sample containing linalool at a significant level with a risk of 1 %, 9 of the 13 panellists could recognize the one containing β-citronellol with a risk of 1 %, 12 of the 13 panellists could recognize that containing geraniol with a risk of below 0.1%. These results suggested that odours of linalool and geraniol could be enhanced by the occurrence of branched-chain fatty acids at a threshold level. Interestingly, only 2-methylbutyric acid could enhance odours of all three monoterpene alcohols. Therefore, we concluded that there might be an additive effect between branched-

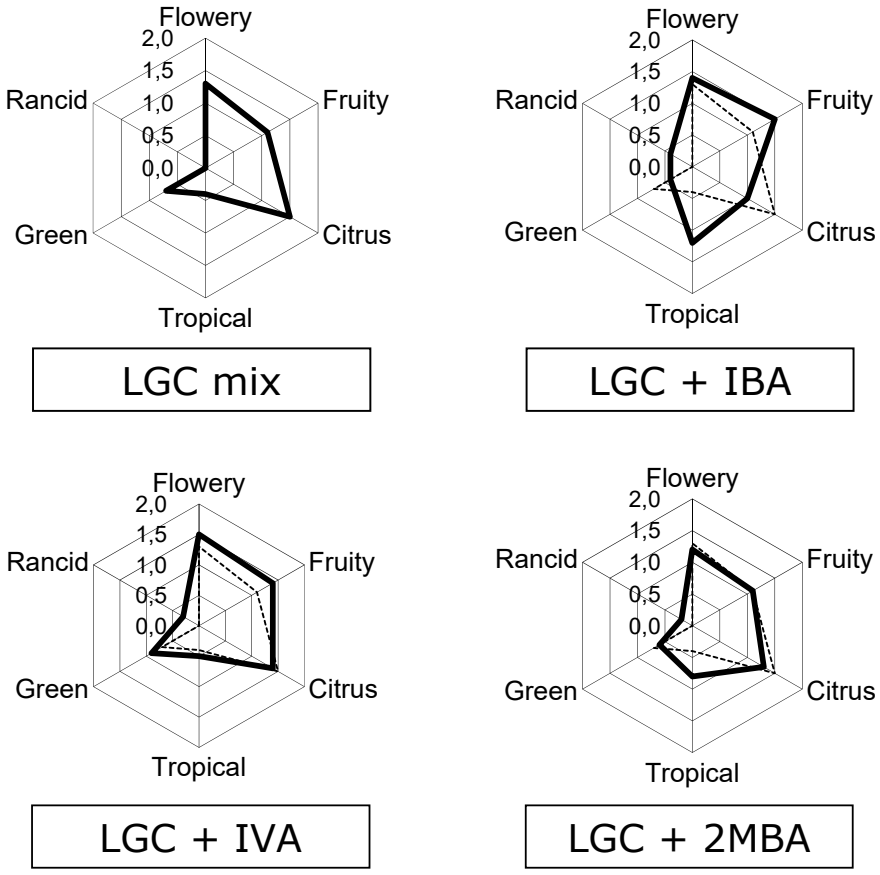

Fig. 6 **Flavor profiles of model-solutions (5 % v/v Ethanol, Carbonated) containing three monoterpene alcohols and/or branched-chain fatty acids (isobutyric acid, isovaleric acid, and 2-methlbutyric acid): LGC mix, containing 100 µg/L of linalool, 25 µg/L of geraniol, and 30 µg/L of β-citronellol; IBA, containing 5000 µg/L of isobutyric acid; IVA, containing 40 µg/L of isovaleric acid; 2MBA, containing 800 µg/L of 2-methlbutyric acid; dotted line, profile of LGC mix model solution**

chain fatty acids and monoterpene alcohols and that only threshold levels of fatty acids were enough for this effect.

In previous studies [9, 23], it has been often reported that there was an additive effect among same class of compounds, having similar chemical structures and similar odours, for example 3-sulfanypentan-1-ol, 3-sulfanylhexan-1-ol, and 3-sulfanylheptan-1-ol [23]. We have also reported similar additive effect, for example among monoterpene alcohols (linalool, geraniol, and β-citronellol) [30], among geranic acid and monoterpene alcohols [22], having similar chemical structures. On the other hand, it was thought that certain volatile thiols (3S4MP and 4MSP) could enhance flavour intensities of various flavour compounds, having different chemical structures and different odours. It is thought that branched-chain fatty acids could enhance various flavour compounds, having different chemical structures and different odours, as well.

3.2.2 Additive effect between branched-chain fatty acids and ethyl esters of branched-chain fatty acids

In addition, we investigated whether branched-chain fatty acids could enhance the flavour intensities of ethyl esters of branched-chain fatty acids. Table 4 presents the results of total 6 sets of sensory triangular tests. In the sets containing isobutyric acid, there was no significant difference between the control solution and the test solution containing ethyl isobutyrate or ethyl isovalerate. In the sets containing isovaleric acid, 11 of the 14 panellists could recognize the test sample containing ethyl isobutyrate at a significant level with a risk of 0.1 %, 10 of the 14 panellists could recognize the one containing ethyl isovalerate with a risk of 1 %. There was no significant difference between the control solution and the test solution containing ethyl isovalerate. These results suggested that odours of ethyl isobutyrate and ethyl isovalerate could be enhanced by the occurrence of isovaleric acid at a threshold level, only 40 µg/L. However, an enhancing effect of 2-methylbutyric acid on ethyl esters of branched-chain fatty acids were much weaker than that of isovaleric acid. Surprisingly, isobutyric acid had no enhancing effect on esters having similar branched structures. From the results in 3.2.1 and 3.2.2, branched-chain fatty acids could strongly enhance the intensities of monoterpene alcohols, rather than those of ethyl esters of branched-chain fatty acids.

3.3 Synergy of branched-chain fatty acids and monoterpene alcohols

In addition, we tried to confirm an effect of branched-chain fatty acids on flavour profile, for further understanding of characteristics of branched-chain fatty acids. Such flavour profile could evaluate not only flavour intensity but also change in flavour characters. Therefore, in our previous studies [28-31, 34], the 'synergy' has been defined as a sensory effect of candidate enhancer compound by which flavour characters of reference compounds could change.

A test was conducted to assess changes of flavour character by synergy of branched-chain fatty acids and monoterpene alcohols, which were selected because of the result as described above. Figure 6 shows the six flavour characters (flowery, fruity, citrus, tropical, green, and rancid) of model solutions, evaluated by the panelists. The model solution 'LGC mix' (containing 100 µg/L of linalool, 25 µg/L of geraniol, and 30 µg/L of β-citronellol) was designed to simulate the composition of the three monoterpene alcohols in test-brewed Citra beer (Table 1). The model solutions 'LGC mix + IBA', 'LGC mix + IVA', and 'LGC mix + 2MBA' contained same concentrations of three monoterpene alcohols together with 5000 µg/L of isobutyric acid (IBA), 40 µg/L of isovaleric acid (IVA), or 800 µg/L of 2-methylbutyric acid (2MBA).

As a result, in the flavour profile of the model solution 'LGC mix', the average scores of 'Flowery', 'Fruity', 'Citrus', and 'Green' were relatively high, that of 'Tropical' was relatively low, and that of 'Rancid' was 0 point. This result is similar to those shown in our previous studies [31, 34]. With the addition of isobutyric acid (LGC mix + IBA), the average score of the 'Citrus' character decreased

and 'Fruity' and 'Tropical' increased in comparison with the control solution (LGC mix). With the addition of isovaleric acid (LGC mix + IVA), the average scores of 'Flowery' and 'Fruity' characters increased slightly in comparison with the control solution. The spider chart of the test solution was almost similar shape to that of the control solution. With the addition of 2-methylbutyric acid (LGC mix + 2MBA), the average score of 'Tropical' character increased in comparison with the control solution. The spider chart of the test solution was relatively similar shape to that of the control solution.

In the viewpoint of flavour impression, isobutyric acid could change the total flavour profile most drastically, especially the 'Tropical' score characteristically increased. The shapes of spider charts of 'LGC mix + IVA' and 'LGC mix + 2MBA' were less changed than that of 'LGC mix + IBA'. As described above, isovaleric acid could affect to the 'fruity' character, and 2-Methylbutyric acid to the 'Tropical' character. Therefore, it was assumed that a part of the 'Tropical' character of hopped beer could formed by synergy among branched-chain fatty acids and other flavour compounds.

4. Conclusions

In this study, branched-chain fatty acids (isobutyric acid, isovaleric acid, and 2-methylbutyric acid) were focused on. Firstly, the behaviours of branched-chain fatty acids before and after fermentation were compared between unhopped and hopped conditions. In general, these fatty acids were originated from malts, hops, and yeast fermentation. It was thought that the concentrations of isobutyric acid and isovaleric acid in both wort and beer were affected by hops used for brewing, and that the behaviour of 2-methylbutyric acid might be less affected without/with hops. Next, the behaviours of these fatty acids were compared among 12 hop varieties. When fresh hops were used, these fatty acids were at relatively narrow ranges in both worts and beers. From the analysis by scatter diagrams of the concentrations of three fatty acids and corresponding ethyl esters, it was suggested that the concentration of ethyl isovalerate showed relatively good correlation with isovaleric acids derived from hops, that ethyl 2-methylbutyrate in beer could be mainly affected by 2-methylbutyric acid generated during fermentation, and that ethyl isobutyrate in beer could be affected by hop-derived isobutyric esters, for example 2-methylbutyl isoutyrate, rather than by hop-derived isobutyric acid.

In addition, the sensory effect of branched-chain fatty acids on hopped beer flavours were also investigated. As a result, it was suggested that branched-chain fatty acids could enhance the flavours of monoterpene alcohols, and that only threshold levels of fatty acids were enough for this effect. In the viewpoint of flavour impression, it is assumed that branched-chain fatty acids could change total flavour profile of the mixture of monoterpene alcohols at threshold levels, and that a part of the 'Tropical' character of hopped beer could formed by synergy among branched-chain fatty acids and monoterpene alcohols.

Acknowledgment

The author thanks Masanori Shirai and Sawako Mochizuki at the Product and Technology Development Centre for test-brewing; all panellists at Sapporo Breweries Ltd. for their sensory work; Junji Takayanagi and Reika Miyamoto at the Frontier Laboratories of Value Creation for technical assistance; Yutaka Itoga and Narushi Suda at the Bioresources Research & Development Department, Yasuyuki Nakayama and Junji Watari at the Value Creation Department, and Ichiro Matsumoto and Masahiro Nomura at the Product and Technology Innovation Department for their kind help.

A preliminary report of some of this work was given at the 44th Annual Meeting of the Japanese Association for the Study of Taste and Smell, Kitakyushu, Fukuoka, Japan, September 8-10, 2010.

5. References

1. American Society for Testing and Materials. Subcomittee E-18. Standard Practice E-679 for Determination of Odor and Taste Thresholds by a Forced-Choice Ascending Concentration Series Method of Limits, ASTM: Philadelphia, PA, 1979.
2. Buttery, R. G.; Teranishi, R.; Ling, L. C.; and Turnbaugh, J. G.: Quantitative and sensory studies on tomato paste volatiles, J. Agric. Food Chem., **38** (1990), no. 1, pp. 336-340.
3. Amoore, J. E.: Specific anosmia and the concept of primary odors, Chem. Senses, **2** (1977), no. 3, pp. 267-281.
4. Dresel, M.; Van Opstaele, F.; Praet, T.; Jaskula-Goiris, B.; Van Holle, A.; Naudts, D.; De Keukeleire, D.; De Cooman, L. and Aerts, G.: Investigation of the impact of the hop variety and the hopping technology on the analytical volatile profile of single-hopped worts and beers, BrewingScience – Monatsschrift für Brauwissenschaft, **66** (2013), no. 11/12, pp. 162-175.
5. Dresel, M.; Praet, T.; Van Opstaele, F.; Van Holle, A.; Naudts, D.; De Keukeleire, D.; De Cooman, L. and Aerts, G.: Comparison of the analytical profiles of volatiles in single-hopped worts and beers as a function of the hop variety, BrewingScience – Monatsschrift für Brauwissenschaft, **68** (2015), no. 1/2, pp. 8-28.
6. Forster, A.; Schmidt, R.: The characterization and classification of hop varieties. EBC Monograph, XXII (1994), pp. 251-269.
7. Forster, A. and Gahr, A.: On the fate of certain hop substances during dry hopping, BrewingScience – Monatsschrift für Brauwissenschaft, **66** (2013), no. 7/8, pp. 93-103.
8. Gahr, A.; Forster, A. and Van Opstaele, F.: Reproducibility Trials in a Research Brewery and Effects on the Evaluation of Hop Substances in Beer – Part 1: Reproducibility in fresh beers, BrewingScience, **69** (2016), no. 11/12, pp. 103-111.
9. Guadagni, D. G.; Buttery, R. G.; Okano, S. and Burr, H. K.: Additive effect of sub-threshold concentrations of some organic compounds associated with food aromas, Nature, **200** (1963), pp. 1288-1289.
10. Haley, J. and Peppard, T. L.: Difference in utilization of the essential oil of hops during the production of dry-hopped and late-hopped beers, J. Inst. Brew., **89** (1983), no. 2, pp. 87-91.
11. Kishimoto, T.; Wanikawa, A.; Kono, K. and Aoki, K.: Odorants comprising hop aroma of beer: hop-derived odorants increased in the beer hopped with aged hops, Proc. 31st EBC Congr., (2007), pp. 226-235 (CD-ROM).
12. Lermusieau, G.; Bulens, M. and Collin, S.: Use of GC-olfactometry to identify the hop aromatic compounds in beer, J. Agric. Food Chem., **49** (2001), no. 8, pp. 3867-3874.
13. Miyazawa, T.; Gallagher, M.; Preti, G. and Wise, P. M.: Synergistic

mixture inter-actions in detection of perithreshold odors by humans, Chem. Senses., **33** (2008), no. 3, pp. 363-369.

14. Miyazawa, T.; Gallagher, M.; Preti, G. and Wise, P. M.: Odor detection of mix-tures of homologous carboxylic acids and coffee aroma compounds by Humans, J. Agric. Food Chem., **57** (2009), no. 21, pp. 9895-9901.
15. Murakami, A.; Chicoye, E. and Goldstein, H.: Hop flavor constituents in beer by headspace analysis, J. Am. Soc. Brew. Chem., **45** (1987), no. 1, pp. 19-23.
16. Murakami., A.; Rader, S.; Chicoye, E. and Goldstein, H.: Effect of hopping on the headspace volatile composition of beer, J. Am. Soc. Brew. Chem., **47** (1989), no. 2, pp. 35-42.
17. Ohloff, G.: Importance of minor components in flavors and fragrances, Perfum, Flavor., **1** (1978), pp. 11-22.
18. Ong, P. K. C. and Acree, T. E.: Similarities in the aroma chemistry of Gewürztraminer variety wines and Lychee (*Litchi chinesis* Sonn.) fruit, J. Agric. Food Chem., **47** (1999), no. 2, pp. 665-670.
19. Rettberg, N.; Thörner, S.; Labus, A. B. and Garbe, L.-A.: Aroma Active Monocarboxylic Acids - Origin and Analytical Characterization in Fresh and Aged Hops, BrewingScience – Monatsschrift für Brauwissenschaft, **67** (2014), no. 3/4, pp. 33-47.
20. Rettinger, K.; Burschka, C.; Scheeben, p.; Fuchs, H. and Mosandl, A.: Chiral 2-alkylbranched acids, esters and alcohols. Preparation and stereospecific flavour evaluation, Tetrahedron: Asymmetry, **2** (1991), no. 10, pp. 965-968.
21. Salo, P.: Variability of odour thresholds for some compounds in alcoholic beverages, J. Sci. Food Agric., **21** (1970), no. 11, pp. 597-600.
22. Sanekata, A.; Tanigawa, A.; Takoi, K.; Nakayama, Y. and Tsuchiya, Y.: Identification and characterization of geranic acid as unique flavor compound of hops (*Humulus lupulus* L.) variety Sorachi Ace, J. Agric. Food Chem., **66** (2018), no. 46, pp. 12285–12295.
23. Sarrazin, E.; Shinkaruk, S.; Tominaga, T.; Bennetau, B.; Frérot, E. and Dubour-dieu, D.: Odorous impact of volatile thiols on the aroma of young botrytized sweet wines: Identification and quantification of new sulfanyl alcohols, J. Agric. Food Chem., **55** (2007), no. 4, pp. 1437-1444.
24. Seaton, J. C.; Moir, M. and Sugget, A.: The refinement of hop flavour by yeast action, Proceedings of the 17th Convention of the Institute of Brewing, Australia and New Zealand Section, Perth, Australia, (1982), pp. 117-124.
25. Steinhaus, M.; Fritsch, H. T. and Schieberle, P.: Quantitation of (*R*)- and (*S*)-linalool in beer using solid phase microextraction (SPME) in combination with a stable isotope dilution assay (SIDA), J. Agric. Food Chem., **51** (2003), no. 24, pp. 7100-7105.
26. Takeoka, G. R.; Flath, R. A.; Mon, T. R.; Teranishi, R. and Guentert, M.: Volatile constituents of apricot (*Prunus armeniaca*), J. Agric. Food Chem., **38** (1990), no. 2, pp. 471-477.
27. Takoi, K.; Tominaga, T.; Degueil, M.; Sakata, D.; Kurihara, T.; Shinkaruk, S.; Nakamura, T.; Maeda, K.; Akiyama, H.; Watari, J.; Bennetau, B. and Dubourdieu, D.: Identification of novel unique flavor compounds derived from Nelson Sauvin hop and development of new product using this hop, Proc. 31st EBC Congr., (2007), pp. 241-251 (CD-ROM).
28. Takoi, K.; Degueil, M.; Shinkaruk, S.; Thibon, C.; Maeda, K.; Ito, K.; Bennetau, B.; Dubourdieu, D. and Tominaga, T.: Identification and characteristics of new volatile thiols derived from the hop (*Humulus lupulus* L.) cultivar Nelson Sauvin, J. Agric. Food Chem., **57** (2009), no. 6, pp. 2493-2502.
29. Takoi, K.; Degueil, M.; Shinkaruk, S.; Thibon, C.; Kurihara, T.; Toyoshima, K.; Ito, K.; Bennetau, B.; Dubourdieu, D. and Tominaga, T.: Specific flavor compounds derived from Nelson Sauvin hop and synergy of these compounds, BrewingScience – Monatsschrift für Brauwissenschaft, **62** (2009), no. 7/8, pp. 108-118.
30. Takoi, K.; Koie, K.; Itoga, Y.; Katayama, K.; Shimase, M.; Nakayama, Y. and Watari, J.: Biotransformation of hop-derived monoterpene alcohols by lager yeast and their contribution to the flavor of hopped beer, J. Agric. Food Chem., **58** (2010), no. 8, pp. 5050-5058.
31. Takoi, K.; Itoga, Y.; Koie, K.; Kosugi, T.; Shimase, M.; Katayama, K.; Nakayama, Y. and Watari, J.: Contribution of geraniol metabolism to citrus flavour of beer: Synergy of geraniol and β-citronellol under coexistence with excess linalool, J. Inst. Brew., **116** (2010), no. 3, pp. 251-260.
32. Takoi, K.; Itoga, Y.; Takayanagi, J.; Kosugi, T.; Shioi, T.; Nakamura, T. and Watari, J.: Screening of geraniol-rich flavor hop and interesting behavior of β-citronellol during fermentation under various hop-addition timings, J. Am. Soc. Brew. Chem., **72** (2014), no. 1, pp. 22-29.
33. Takoi, K.; Tokita, K.; Sanekata, A.; Usami, Y.; Itoga, Y.; Koie, K.; Matsumoto, I. and Nakayama, Y.: Varietal difference of hop-derived flavour compounds in late-hopped/dry-hopped beers, BrewingScience, **69** (2016), no 1/2, pp. 1-7.
34. Takoi, K.; Itoga, Y.; Takayanagi, J.; Matsumoto, I. and Nakayama, Y.: Control of hop aroma impression of beer with blend-hopping using geraniol-rich hop and new hypothesis of synergy among hop-derived flavour compounds, BrewingScience, **69** (2016), no. 11/12, pp. 85-93.
35. Takoi, K.; Itoga, Y.; Koie, K.; Takayanagi, J.; Kaneko, T.; Watanabe, T.; Matsumoto, I. and Nomura, M.: Systematic analysis of behaviour of hop-derived monoterpene alcohols during fermentation and new classification of geraniol-rich flavour hops, BrewingScience, **70** (2017), no. 11/12, pp. 177-186.
36. Takoi, K.; Itoga, Y.; Koie, K.; Takayanagi, J.; Kaneko, T.; Watanabe, T.; Matsumoto, I. and Nomura, M.: Behaviour of hop-derived branched-chain esters during fermentation and unique characteristics of Huell Melon and Ekuanot (HBC366) hops, BrewingScience, **71** (2018), no. 11/12, pp. 100-109.
37. Tressel, R.; Kossa, M. and Koeppler, H.: Changes in aroma components during processing of hops. EBC Monograph, XIII (1987), pp. 116-129

Received 12 September 2019, accepted 25 November 2019