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Behaviour of Hop-derived Branched-chain Esters During Fermentation and Unique Characteristics of Huell Melon and Ekuanot (HBC366) Hops

A preliminary report of some of this work was given at the 35th Congress of the European Brewery Convention, Porto, Portugal, 24-28 May, 2015.

In this study, 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) have been focused on. 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 (threshold in beer, 78 µg/L) could be transesterificated to ethyl isobutyrate (threshold in beer, 6.3 µg/L). The transesterification between these esters might be an important reaction for hop aromas in beer. From this study, it is concluded that hop-derived various esters including branched-chain structures might be very important contributors to hop varietal aromas, having two roles, their own fruity flavours and precursors of ethyl esters of branched-chain fatty acids.

Descriptors: beer, hop, varietal aroma, flavour compounds, branched-chain esters

1 Introduction

In the 2000s, new types of hops have been bred and widely used for craft beers all around the world [1, 7, 10, 13, 17–18, 22]. These hops, so-called 'Flavour hops' [9, 13, 20], impart very characteristic 'Varietal Aroma', for example citrus-like and/or exotic fruit-like (tropical) flavours, to finished beer. Therefore, beer/hop researchers have been focused on key flavour compounds contributing to such varietal aromas. Certain 'flavour hop' varieties, for example Bravo, Cascade, Citra, Mosaic and Nelson Sauvin, have been investigated. Until now, our group have revealed the contribution of monoterpene alcohols and volatile thiols 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 [23–32].

In the previous paper [32], hop-derived monoterpene alcohols (linalool, geraniol, β-citronellol, nerol, and α-terpineol) and their behaviour during fermentation have been focused on. Total 42 hop varieties were compared. As a result, it was suggested that a group of geraniol-rich 'Flavour hop' varieties, which could generate a large amount of β-citronellol to finished beer, could be furthermore classified into two types, 'free geraniol dominant hops' and 'geraniol precursor dominant hops'. 'Free geraniol dominant hops' mainly contain free geraniol at high levels and subsidiary geraniol precursors. Most of 'geraniol-rich hops' was classified into this type, for example Motueka, Bravo, Cascade, Citra, Mosaic, Sorachi Ace, 0612B, and so on. 'Geraniol precursor dominant hops' might mainly contain geraniol precursors at high levels and subsidiary

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free geraniol, for example, Vic Secret, Comet, Hallertau Blanc, Polaris, Amarillo, Ekuanot (HBC366), and Summit.

It is well-known that hops contains not only terpenoids but also various esters, which have branched-chain structures. For example, isobutyric esters of branched-chain alcohols, including isobutyl isobutyrate, isoamyl isobutyrate, and 2-methylbutyl isobutyrate (Fig. 1), were widely found in various hops [2–6, 8, 12, 14, 15, 21, 23, 25, 28, 33]. These isobutyric esters have a green apple, apricot-like flavour [23, 25].Traditional bitter hops and modern high alpha hops, for example Northern Brewer, Nugget, and Magnum, contain relatively high amount of these compounds. When looking at traditional aroma hops, German aroma hops, for example Hallertauer Tradition, contained these compounds while Saaz and Lublin had few of these compounds [12, 23, 25]. *Seaton* et al. reported that isobutyric esters could be unstable during boiling and fermentation [21]. In fact, commercial beers brewed with kettle hopping contain only small amounts of these compounds [23]. These compounds are found in late-hopped beer and dry-hopped beer [14, 15, 21]. Isobutyric esters are expected to contribute to some of the special flavours of late-hopped/dry-hopped beers [23, 25, 28].

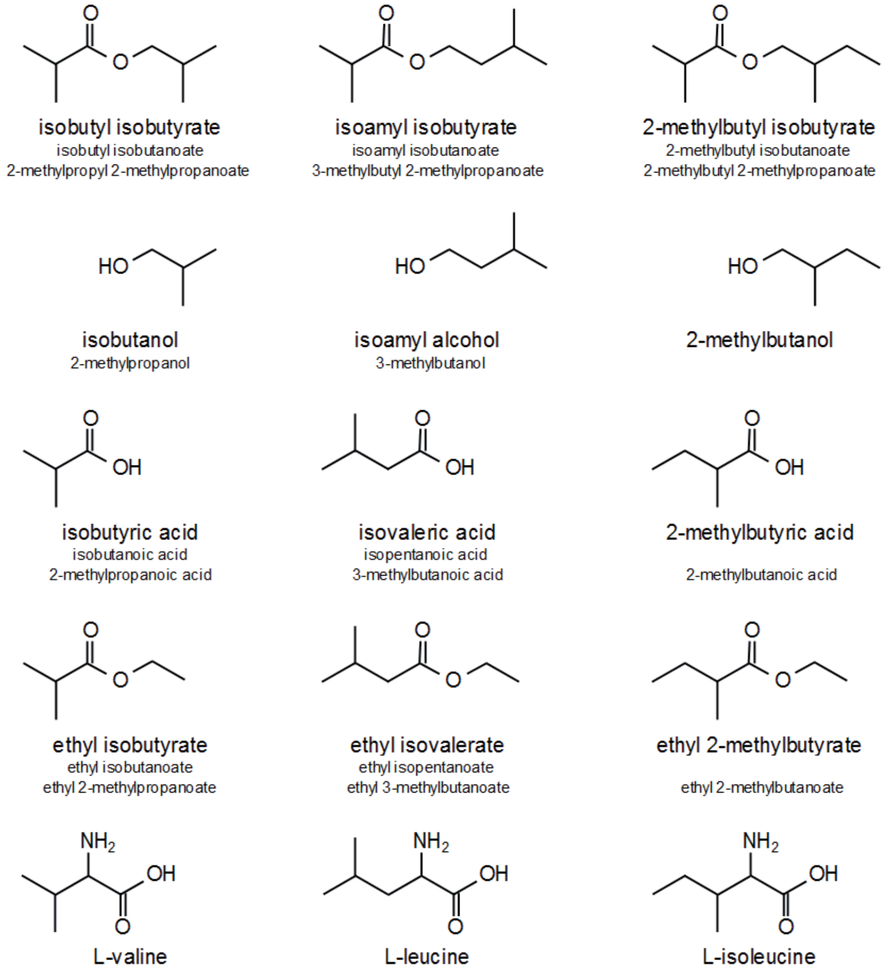

Fig. 1 **Chemical structures of isobutyric esters of branched-chain alcohols (isobutyl isobutyrate, isoamyl isobutyrate, and 2-methylbutyl isobutyrate), ethyl esters of branched-chain fatty acids (ethyl isobutyrate, ethyl isovalerate, and ethyl 2-methylbutyrate), and related alcohols, fatty acids, and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

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 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]. The odor thresholds of these esters have been estimated at very low levels, 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]. Therfore, these esters are also expected to contribute to a part of the special flavours of beers made with various hops.

In this study, 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) during total fermentation period were analysed, and behaviours of hop-derived esters among total 42 hop varieties were compared.

2 Materials and methods

2.1 Hop raw materials

Saaz from Czech Republic (type 90 pellet). Hallertauer Tradition, Comet, Hallertau Blanc, Huell Melon, Mandarina Bavaria, and Polaris were grown and pelletized in Germany (type 90 pellet). Aramis, Barbe Rouge, Mistral, and Triskel were bred and grown in France (type 90 pellet). Furano Beauty, Furano No. 18, 9702A, 9803A, and 0612B were bred and grown in Japan (Bioresources Research & Development Department, Sapporo Breweries, Ltd.) (hop powder). Galaxy and Vic Secret were produced in Australia (type 90 pellet). Motueka, Nelson Sauvin, Pacific Jade, Rakau, Riwaka, Southern Cross, Wai-iti, and Waimea were harvested and pelletized in New Zealand (type 90 pellet). Apollo and Bravo were harvested in the U.S. in 2008 (hop powder) and 2010 (type 90 pellet). Cascade was grown in the U.S. in 2007 (type 90 pellet) and 2008 (hop powder).Citra was grown in the U.S. in 2007 and 2008 (type 90 pellet). Amarillo, Chinook, Glacier, Ekuanot (formerly named as HBC366), Mosaic (formerly named as HBC369), Mt. Hood, Simcoe, Sorachi Ace and Summit were harvested in the U.S. (hop powder). Nugget was grown in the U.S. (type 90 pellet). Crop years of all hops are summarized in table 1 and 2 (see page 103–104).

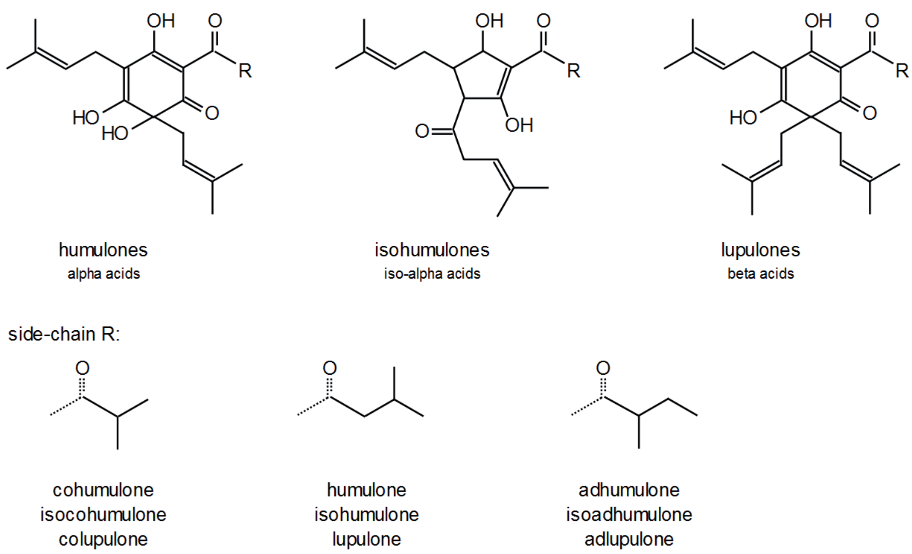

Fig. 2 **Chemical structures of humulones (alpha acids), isohumulones (iso-alpha acids), and lupulones (beta acids): dotted line in the structures of side-chain R is indicated the link points between bitter acids and side chain**

2.2 Pilot-scale brewing

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 malts (or malts and 33 % adjuncts (starch, corn and rice)) and hops in 100-L or 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 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 5.5 %.

2.3 Standard products

Isobutyl isobutyrate (> 98 %), isoamyl isobutyrate (> 98 %), isobutyric acid (> 98 %), and 2-methylbutan-1-ol (> 97 %) were purchased from Fujifilm Wako Pure Chemical Co., Ltd. (Osaka, Japan). 2-Methylbutyl isobutyrate (> 97 %) was synthesized by esterification of isobutyric acid and 2-methylbutan-1-ol, as previously described [24]. Ethyl isobutyrate (> 98 %) and ethyl isovalerate (> 97 %) 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 (Semi-) Quantification of hop-derived esters including branched-chain structures by solid phase microextraction-gas chromatography-mass spectrosco-py (SPME-GC-MS)

For analysis of esters including branched-chain structures (isobutyl isobutyrate, isoamyl isobutyrate, 2-methylbutyl isobutyrate, ethyl isobutyrate, ethyl isovalerate, and ethyl 2-methylbutyrate), 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 [26–28]. 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, fermenting beer, 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. 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 fiber [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 fiber was injected into a splitless injector (260 °C; purge time = 3 min, purge flow = 20 mL/min) at oven temperature (50 °C) onto a type HP-1MS capillary column (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 isobutyric esters (isobutyl isobutyrate, isoamyl isobutyrate, and 2-methylbutyl isobutyrate) were (semi-) quantified in the SIM mode, selecting the following ions: *m/z* 71 and 87. Ethyl isobutyrate, ethyl isovalerate, and ethyl 2- methylbutyrate were (semi-) quantified in the SIM mode, selecting *m/z* 116, 88, and 102, respectively. Calibration 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 analyzed. The analysis was performed in duplicate.

3 Results and discussions

3.1 Comparison of behaviours of hop-derived isobutyric esters of branched-chain alcohols among total 42 hop varieties

In previous paper [32], we have compared the behaviours of all monoterpene alcohols (linalool, geraniol, β-citronellol, nerol, and α-terpineol) among 42 hop varieties, including not only newly bred ‘Flavour hops’ but also traditional aroma hops and high alpha hops all around the world. All brewing trials were conducted with late-hopping using same flavouring hop dosage (0.8 g/L). In this study, we tried to compare the behaviours of hop-derived esters

Table 1 Comparison of isobutyric esters (isobutyl isobutyrate, isoamyl isobutyrate, and 2-methylbutyl isobutyrate) (µg/L) during fermentation of late-hopped beer: area, grown area; rp, repeating trial using same crop sample; ref, reference number (previously reported data); F., primary fermentation; 3d, 3 days; S., storage; 1w, 1 week

					isobutyl isobtyrate (µg/L)					isoamyl isobtyrate (µg/L)					2-methylbutyl isobtyrate (µg/L)				
variety	area	crop	rp	ref	wort	F. 3d	F. end	S. 1w	beer	wort	F. 3d	F. end	S. 1w	beer	wort	F. 3d	F. end	S. 1w	beer
Saaz	CZ	2008			0.9	0.7	0.5	0.4	1.0	0.3	0.3	0.3	0.3	1.1	0.2	0.1	0.1	0.1	0.2
Hallertauer Tradition	GER	2007			10.1	5.6	3.7	3.5	4.8	1.5	0.9	0.5	0.5	0.8	18.5	10.1	6.3	6.7	10.5
Hallertauer Tradition	GER	2009	#1		132	114	91.4	72.6	76.4	21.5	22.7	20.2	12.9	17.7	14.9	16.3	14.6	9.8	13.2
Hallertauer Tradition	GER	2009	#2		13.7	10.0	8.9	7.2	7.4	2.3	1.8	1.8	1.3	1.8	15.0	11.9	11.8	8.0	9.3
Comet	GER	2014			14.7	10.2	8.6	7.2	6.8	4.3	3.3	2.6	1.9	2.1	25.9	19.8	16.9	13.0	13.1
Hallertau Blanc	GER	2012		30	20.0	13.8	12.4	9.1	9.5	10.6	8.3	8.0	5.2	5.9	31.4	25.8	25.1	16.2	18.9
Huell Melon	GER	2012		30	54.0	38.1	30.7	26.0	25.3	13.8	10.9	8.6	6.8	7.3	69.6	56.6	48.3	38.3	41.4
Mandarina Bavaria	GER	2012		30	42.0	31.9	25.1	21.1	21.8	5.6	5.0	4.1	3.0	3.4	42.8	40.1	33.8	24.7	27.6
Polaris	GER	2012		30	71.9	69.9	57.5	47.4	46.7	23.7	25.5	20.0	15.3	16.5	82.1	85.0	72.8	59.0	63.6
Aramis	FR	2013			11.2	5.7	4.6	4.7	4.4	5.8	2.9	2.3	1.9	1.7	18.1	9.8	8.3	7.0	6.0
Barbe Rouge	FR	2013			49.8	40.0	32.7	29.8	25.7	19.2	19.3	15.0	11.5	10.0	80.3	80.8	66.2	52.6	48.4
Mistral	FR	2015			25.9	15.7	14.2	11.7	12.1	6.9	4.5	4.5	3.4	3.7	63.4	45.4	42.7	35.2	37.8
Triskel	FR	2013			7.2	4.3	3.7	3.6	3.4	5.7	3.4	2.7	2.3	2.0	22.0	13.0	11.0	9.4	8.6
Furano Beauty	JP	2009			41.7	32.7	28.2	21.9	22.1	19.8	19.0	17.9	11.6	13.3	130	127	124	95.8	104
Furano No. 18	JP	2009			0.9	0.9	0.7	0.7	1.1	0.5	0.5	0.4	0.4	0.6	2.7	3.0	2.5	2.0	4.5
9702A	JP	2007			7.8	4.7	3.2	2.7	4.0	6.0	3.2	2.3	1.9	3.2	26.5	14.1	10.2	8.7	14.6
9803A	JP	2007			5.7	3.2	2.2	1.8	2.7	2.2	1.1	0.7	0.6	1.1	11.7	5.6	4.0	3.3	5.8
0612B	JP	2009			18.8	15.3	11.6	9.1	9.3	24.1	23.0	18.2	12.5	13.9	78.6	76.9	64.4	45.7	52.1
Galaxy	AUS	2013			8.0	6.4	5.2	4.3	4.4	10.1	9.0	7.9	5.7	6.6	53.2	51.8	44.7	33.3	36.6
Vic Secret	AUS	2014			33.9	29.3	22.8	20.9	19.5	14.7	12.2	9.5	8.9	9.1	93.2	81.0	67.8	66.1	68.2
Motueka	NZ	2010		30	9.9	8.3	6.2	5.3	4.6	2.6	2.5	1.7	1.4	1.2	34.4	31.9	23.3	19.7	18.1
Nelson Sauvin	NZ	2007			191	121	98.3	81.0	86.3	49.9	37.2	38.3	28.4	29.7	50.2	39.2	39.4	31.5	34.7
Pacific Jade	NZ	2010		30	40.5	35.4	22.1	22.0	20.6	5.7	5.8	3.8	3.4	3.1	120	127	87.6	80.4	78.1
Rakau	NZ	2012		30	38.7	25.1	17.2	9.9	8.5	8.5	6.6	4.4	2.0	1.8	95.6	79.5	55.8	29.4	26.3
Riwaka	NZ	2010		30	17.9	16.7	13.1	12.7	10.8	6.8	7.0	5.0	4.6	4.0	56.1	58.1	46.1	42.3	38.6
Riwaka	NZ	2012			10.5	5.8	4.1	2.5	2.3	7.4	5.0	3.3	1.6	1.5	37.6	26.4	18.2	9.1	8.5
Southern Cross	NZ	2010		30	39.9	31.1	28.5	22.6	21.2	9.2	8.7	7.7	5.6	5.3	149	143	141	106	104
Wai-iti	NZ	2012		30	12.2	8.2	5.6	3.2	3.1	5.7	4.6	2.9	1.2	1.4	56.6	46.8	32.2	14.8	16.4
Waimea	NZ	2012		30	37.8	29.8	21.6	12.8	12.8	5.0	4.8	3.4	1.5	1.7	85.0	84.2	64.5	35.0	35.2
Amarillo	US	2008	#1		14.0	10.6	8.2	7.0	5.5	12.8	11.7	9.7	6.9	6.5	29.1	26.2	21.8	16.5	14.2
Amarillo	US	2008	#2		122	93.9	85.8	62.5	60.1	109	94.8	101	56.9	69.9	23.0	21.6	23.0	13.5	16.5
Apollo	US	2008	#1		5.8	6.5	5.9	4.8	4.1	9.5	13.4	13.5	9.3	10.3	49.4	65.5	67.8	49.6	51.6
Apollo	US	2008	#2		6.0	7.4	5.7	4.7	4.5	9.9	13.9	11.9	8.9	9.9	48.8	65.9	59.1	45.5	49.6
Apollo	US	2010		30	1.7	1.7	1.4	1.1	1.1	2.7	2.7	2.0	1.4	1.5	17.3	17.1	13.4	9.5	9.6
Bravo	US	2008	#1		16.9	15.4	12.7	10.2	8.8	14.3	16.1	15.0	10.1	10.1	81.2	90.7	87.8	63.3	62.3
Bravo	US	2008	#2	28	19.1	18.4	13.4	10.4	9.7	15.5	19.1	15.5	11.5	10.9	87.3	105	88.7	68.4	65.7
Bravo	US	2010		30	2.4	1.8	1.5	1.1	1.0	4.1	3.3	2.7	1.8	1.9	20.5	16.8	13.7	9.5	10.3
Cascade	US	2007			6.2	3.5	2.9	–	1.9	6.0	3.2	3.0	–	2.0	18.7	10.3	9.5	–	6.2
Cascade	US	2008	#1	28	7.3	5.0	3.7	2.4	3.2	6.8	5.4	4.2	2.2	3.4	19.8	16.1	12.7	7.0	11.1
Cascade	US	2008	#2		35.9	28.3	19.5	15.8	16.9	34.5	32.4	23.0	19.1	18.8	10.7	10.0	7.3	6.4	6.5
Cascade	US	2008	#3		30.8	28.5	22.6	16.2	17.5	29.9	33.7	28.9	16.8	21.8	10.0	11.2	9.9	5.9	7.9
Chinook	US	2008			10.8	8.2	6.3	5.3	4.1	11.8	10.9	8.9	6.1	5.7	62.7	58.3	49.0	36.0	30.3
Citra	US	2007			7.4	5.0	3.4	–	1.9	6.4	4.1	3.0	–	1.7	21.9	14.3	10.4	–	5.8
Citra	US	2008	#1		133	96.2	72.9	57.4	62.7	81.1	72.8	62.9	46.7	48.7	27.5	24.5	22.2	17.2	18.9
Citra	US	2008	#2		9.5	9.0	6.8	5.5	5.7	6.4	6.9	5.4	3.8	4.6	22.3	24.3	19.1	12.7	15.5
Citra	US	2008	#3	30	1.4	1.2	1.0	0.8	0.4	1.7	1.2	1.0	0.8	0.4	6.6	4.6	3.9	3.1	1.6
Galcier	US	2008			14.7	14.9	9.4	7.6	8.2	15.7	19.3	12.4	10.0	10.3	4.9	6.0	3.9	3.3	3.6
Ekuanot (HBC366)	US	2012			42.4	35.2	30.3	25.1	26.1	28.1	23.8	20.0	15.1	13.6	78.6	65.6	60.0	47.5	42.2
Millenium	US	2007			31.1	20.9	15.2	–	8.6	31.7	20.1	15.4	–	9.1	78.7	51.8	39.9	–	24.3
Mosaic	US	2008	#1		36.5	32.7	22.5	18.0	14.0	37.8	39.8	29.3	20.3	18.5	81.1	87.2	68.4	50.0	43.5
Mosaic	US	2008	#2	28	29.9	31.3	26.3	17.0	16.9	33.1	38.0	35.6	21.9	22.1	73.5	82.4	79.7	48.0	52.8
Mosaic	US	2012		30	18.6	16.0	13.2	10.4	10.3	17.2	17.3	14.4	9.9	10.8	36.7	39.0	33.8	23.5	26.0
Mt.Hood	US	2008			60.0	47.0	32.7	25.6	26.9	74.1	70.3	52.5	40.4	38.9	13.3	12.9	9.7	7.8	8.0
Nugget	US	2007			18.8	12.4	8.7	–	4.9	26.6	16.6	12.1	–	6.9	51.1	32.7	23.5	–	13.8
Simcoe	US	2008	#1		2.2	1.9	1.5	1.1	1.0	14.8	15.3	12.9	8.3	8.9	12.2	12.8	11.0	7.1	7.5
Simcoe	US	2008	#2		2.1	2.0	1.4	1.3	1.3	13.3	15.0	10.4	8.2	8.6	10.1	11.7	8.2	6.1	7.5
Sorachi Ace	US	2009			3.0	3.1	2.2	2.1	2.1	2.2	2.7	2.1	1.7	2.0	10.2	12.7	9.9	8.1	9.2
Summit	US	2012		30	4.3	4.0	3.2	2.6	3.0	3.9	4.5	3.6	2.6	3.1	8.1	9.9	8.0	5.7	7.3

ref 28; concentrations of isobutyric esters in ‚beer' were previouely reported
ref 30; concentrations of isoamyl isobutyrate and 2-methylbutyl isobutyrate in ‚beer' were previouely reported

Table 2 **Comparison of ethyl esters of branched-chain fatty acids (ethyl isobutyrate, ethyl isovalerate, and ethyl 2-methylbutyrate) (µg/L) during fermentation of late-hopped beer: area, grown area; rp, repeating trial using same crop sample; ref, reference number (previously reported data); F., primary fermentation; 3d, 3 days; S., storage; 1w, 1 week; tr, trace; n.d., not detected**

					ethyl isobtyrate (µg/L)					ethyl isovalerate (µg/L)					ethyl 2-methylbtyrate (µg/L)				
variety	**area**	**crop**	**rp**	**ref**	**wort**	**F. 3d**	**F. end**	**S. 1w**	**beer**	**wort**	**F. 3d**	**F. end**	**S. 1w**	**beer**	**wort**	**F. 3d**	**F. end**	**S. 1w**	**beer**
Saaz	CZ	2008			tr	0.4	0.6	1.4	1.6	n.d.	0.1	0.1	0.5	0.6	n.d.	0.1	0.1	0.5	0.6
Hallertauer Tradition	GER	2007			–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Hallertauer Tradition	GER	2009	#1		tr	0.4	1.1	2.1	2.2	n.d.	0.1	0.3	0.7	0.7	n.d.	0.1	0.2	0.5	0.6
Hallertauer Tradition	GER	2009	#2		tr	0.7	1.4	2.6	2.6	n.d.	0.1	0.3	0.7	0.7	n.d.	0.1	0.2	0.5	0.6
Comet	GER	2014			tr	1.8	2.5	3.9	4.4	tr	0.6	0.9	1.4	1.7	n.d.	0.3	0.4	0.7	0.7
Hallertau Blanc	GER	2012			0.1	1.1	2.0	3.1	3.7	n.d.	0.1	0.2	0.4	0.5	tr	0.1	0.3	0.5	0.7
Huell Melon	GER	2012			5.1	4.8	5.7	8.7	9.2	0.1	0.3	0.5	0.8	0.9	2.3	1.7	1.6	2.0	2.2
Mandarina Bavaria	GER	2012			0.4	1.3	2.0	3.4	4.2	tr	0.1	0.2	0.4	0.5	0.1	0.3	0.4	0.7	0.9
Polaris	GER	2012			0.1	2.5	4.4	8.4	10.0	n.d.	0.1	0.2	0.5	0.6	tr	0.3	0.6	1.0	1.2
Aramis	FR	2013			tr	1.5	1.9	2.5	2.6	n.d.	0.3	0.5	0.7	1.1	n.d.	0.1	0.2	0.4	0.5
Barbe Rouge	FR	2013			0.1	4.4	5.2	6.8	9.2	tr	0.1	0.3	0.6	0.9	tr	0.2	0.3	0.5	0.8
Mistral	FR	2015			tr	1.3	1.9	3.0	3.4	n.d.	0.2	0.4	0.7	0.8	n.d.	0.2	0.2	0.4	0.5
Triskel	FR	2013			tr	2.0	2.6	3.2	4.0	n.d.	0.4	0.7	0.8	1.3	n.d.	0.2	0.3	0.5	0.7
Furano Beauty	JP	2009			tr	2.1	4.9	8.1	8.8	n.d.	0.2	0.4	0.9	0.9	n.d.	0.3	0.7	1.2	1.3
Furano No. 18	JP	2009			tr	0.4	0.7	1.4	1.6	n.d.	0.1	0.2	0.4	0.5	n.d.	0.1	0.1	0.4	0.5
9702A	JP	2007			–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9803A	JP	2007			–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
0612B	JP	2009			tr	1.3	2.5	4.7	5.1	n.d.	0.1	0.2	0.4	0.4	n.d.	0.2	0.4	0.7	0.8
Galaxy	AUS	2013			tr	2.5	3.6	5.9	5.9	tr	0.7	1.0	1.6	1.8	n.d.	0.2	0.3	0.5	0.5
Vic Secret	AUS	2014			0.1	5.0	7.5	12.5	12.4	tr	1.6	2.2	3.4	3.5	n.d.	0.4	0.5	0.9	1.0
Motueka	NZ	2010			0.1	2.2	2.2	2.5	1.9	n.d.	0.1	0.2	0.3	0.3	tr	0.2	0.3	0.5	0.4
Nelson Sauvin	NZ	2007			0.1	0.7	1.6	3.7	4.4	tr	0.1	0.2	0.6	0.9	tr	0.1	0.3	0.7	0.8
Pacific Jade	NZ	2010			0.1	4.4	3.8	5.2	4.2	n.d.	0.1	0.2	0.3	0.4	tr	0.3	0.4	0.6	0.6
Rakau	NZ	2012			0.4	2.6	3.2	3.5	3.9	tr	0.1	0.2	0.3	0.3	tr	0.3	0.4	0.5	0.5
Riwaka	NZ	2010			0.1	3.6	3.7	4.8	4.4	n.d.	0.1	0.2	0.3	0.3	tr	0.3	0.4	0.6	0.6
Riwaka	NZ	2012			0.4	1.9	2.5	3.0	3.6	0.1	0.1	0.2	0.3	0.3	tr	0.2	0.3	0.3	0.4
Southern Cross	NZ	2010			0.1	4.5	4.5	5.0	4.5	n.d.	0.1	0.2	0.3	0.4	tr	0.4	0.6	0.7	0.7
Wai-iti	NZ	2012			0.4	1.7	2.2	2.6	3.2	0.1	0.2	0.3	0.4	0.5	tr	0.2	0.3	0.3	0.4
Waimea	NZ	2012			0.4	2.3	3.3	3.8	5.2	0.1	0.1	0.2	0.3	0.4	0.1	0.3	0.5	0.6	0.7
Amarillo	US	2008	#1		tr	0.7	1.7	2.7	3.5	tr	0.1	0.2	0.4	0.4	n.d.	0.1	0.3	0.5	0.5
Amarillo	US	2008	#2		0.1	0.8	2.1	3.4	3.3	tr	0.1	0.2	0.5	0.5	n.d.	0.2	0.4	0.7	0.7
Apollo	US	2008	#1		tr	0.6	1.5	3.2	4.5	tr	0.2	0.4	0.9	1.0	tr	0.4	0.9	1.6	1.6
Apollo	US	2008	#2		tr	0.9	2.0	3.7	3.9	tr	0.2	0.5	0.9	0.9	tr	0.3	0.8	1.3	1.2
Apollo	US	2010			tr	0.6	1.1	1.2	1.2	n.d.	0.0	0.2	0.2	0.3	n.d.	0.1	0.5	0.6	0.6
Bravo	US	2008	#1		tr	0.8	2.1	3.9	5.4	n.d.	0.1	0.2	0.5	0.5	tr	0.2	0.4	0.7	0.8
Bravo	US	2008	#2	28	0.3	1.2	2.3	5.2	5.7	0.2	0.2	0.3	0.6	0.7	0.3	0.5	0.7	1.0	1.0
Bravo	US	2010			tr	0.8	1.2	1.4	1.4	n.d.	0.0	0.1	0.2	0.2	n.d.	0.1	0.3	0.3	0.4
Cascade	US	2007			0.1	0.4	0.9	–	1.1	n.d.	0.0	0.2	–	0.3	n.d.	0.1	0.1	–	0.2
Cascade	US	2008	#1	28	0.1	0.5	0.8	1.4	2.1	n.d.	0.0	0.1	0.2	0.5	n.d.	0.1	0.1	0.2	0.3
Cascade	US	2008	#2		0.1	0.5	0.7	1.6	2.0	tr	0.0	0.1	0.3	0.4	tr	0.1	0.1	0.4	0.5
Cascade	US	2008	#3		0.1	0.4	1.0	1.7	1.8	tr	0.1	0.2	0.3	0.4	n.d.	0.1	0.2	0.4	0.4
Chinook	US	2008			0.3	4.5	7.2	9.0	11.7	tr	0.1	0.3	0.5	0.5	tr	0.4	0.7	1.0	1.0
Citra	US	2007			0.1	0.4	0.7	–	0.9	tr	0.0	0.1	–	0.2	n.d.	0.0	0.1	–	0.2
Citra	US	2008	#1		0.1	0.6	1.2	2.6	3.1	tr	0.1	0.2	0.7	0.9	tr	0.1	0.2	0.5	0.6
Citra	US	2008	#2		0.3	1.3	2.1	3.6	4.0	0.1	0.2	0.5	1.0	1.2	tr	0.1	0.3	0.6	0.7
Citra	US	2008	#3		tr	0.8	0.9	1.1	1.1	n.d.	0.1	0.1	0.2	0.3	n.d.	0.1	0.2	0.2	0.3
Galcier	US	2008			tr	0.4	0.6	1.4	1.7	n.d.	0.1	0.1	0.4	0.5	n.d.	0.1	0.1	0.4	0.5
Ekuanot (HBC366)	US	2012			1.4	7.9	8.2	11.8	11.3	0.6	12.8	12.1	12.3	14.5	tr	1.0	1.2	1.4	1.7
Millenium	US	2007			0.1	0.6	1.6	–	1.6	n.d.	0.0	0.1	–	0.2	n.d.	0.1	0.2	–	0.2
Mosaic	US	2008	#1		0.1	1.0	2.7	4.8	6.6	n.d.	0.1	0.2	0.4	0.5	tr	0.2	0.3	0.6	0.7
Mosaic	US	2008	#2	28	0.2	1.2	2.4	5.0	5.7	0.2	0.3	0.4	0.5	0.6	0.2	0.4	0.5	0.7	0.7
Mosaic	US	2012			tr	1.6	2.8	4.9	5.8	n.d.	0.1	0.2	0.5	0.6	n.d.	0.2	0.3	0.7	0.8
Mt.Hood	US	2008			tr	0.4	0.8	1.8	2.2	n.d.	0.1	0.2	0.5	0.7	n.d.	0.1	0.1	0.5	0.6
Nugget	US	2007			0.1	0.5	1.3	–	1.3	n.d.	0.0	0.1	–	0.2	n.d.	0.0	0.1	–	0.2
Simcoe	US	2008	#1		0.1	0.6	1.1	1.8	2.7	tr	0.1	0.2	0.5	0.6	tr	0.1	0.2	0.4	0.5
Simcoe	US	2008	#2		0.2	1.0	1.7	2.8	2.9	0.1	0.2	0.4	0.9	1.0	tr	0.1	0.3	0.5	0.6
Sorachi Ace	US	2009			tr	0.5	0.8	1.8	2.0	n.d.	0.0	0.1	0.3	0.4	n.d.	0.1	0.1	0.4	0.4
Summit	US	2012			tr	0.8	1.5	2.4	3.1	tr	0.1	0.4	0.7	0.8	tr	0.3	0.7	1.1	1.3

ref 28; concentrations of ethyl esters of branched-chain fatty acids in ‚beer' were previouely reported

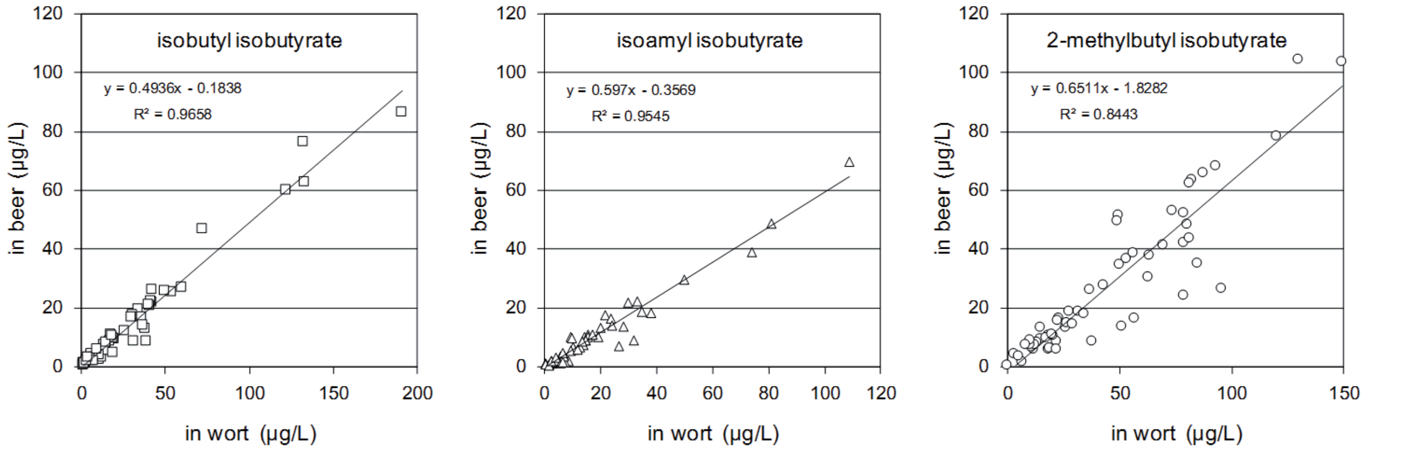

Fig. 3 **Relationship between isobutyric esters (isobutyl isobutyrate, isoamyl isobutyrate, and 2-methylbutyl isobutyrate) (µg/L) in worts and those in beers**

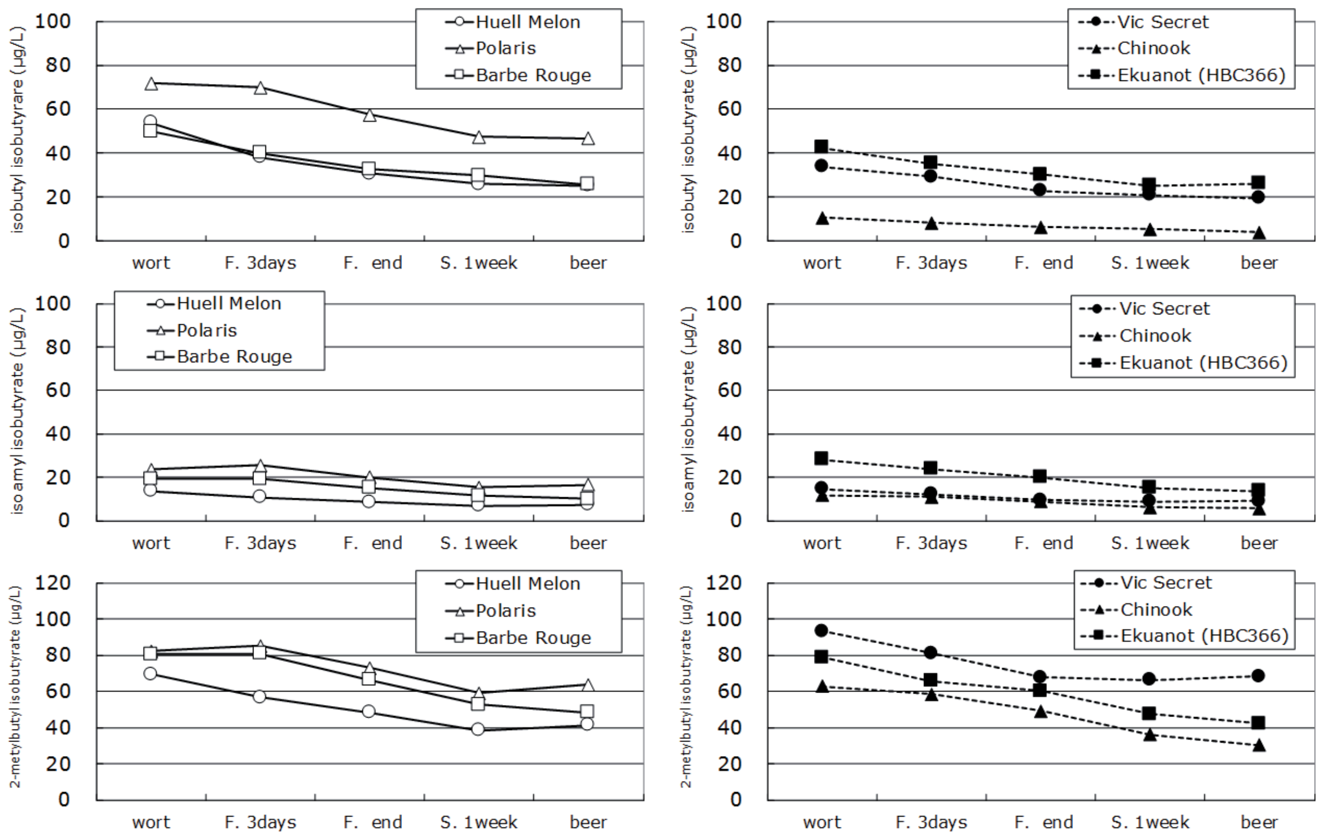

Fig. 4 **Comparison of isobutyric esters (isobutyl isobutyrate, isoamyl isobutyrate, and 2-methylbutyl isobutyrate) (µg/L) during fermentation of beers late-hopped with Huell Melon, Polaris, Barbe Rouge, Vic Secret, Chinook, or Ekuanot (HBC366): F., primary fermentation; S., srorage**

including branched-chain structures (Fig. 1) among 42 hop varieties. For simultaneous (semi-) quantification of many samples, we used relatively simple procedure as described above. All results are summarized in table 1 and 2.

Table 1 shows the behaviours of isobutyric esters of branched-chain alcohols. Of the three isobutyric esters, 2-methylbutyl isobutyrate is most dominant component. As well as reported in previous studies [12, 23, 25], these compounds were at very low levels during total fermentation period using Saaz hops. It has been also reported that isobutyric esters could be unstable during boiling and fermentation [21]. In this study, we investigated the behaviours of isobutyric esters during fermentation, from wort to beer. The concentrations of three isobutyric esters in worts and finished beers shown in table 1 were plotted in scatter diagrams (Fig. 3). As a result, the concentrations of isobutyric esters in the worts were highly related to those in the beers, and each slope of regression equation could be regarded as an average transfer rate of each ester from wort to beer; 0.49 for isobutyl isobutyrate, 0.60 for isoamyl isobutyrate, and 0.65 for 2-methylbutyl isobutyrate.

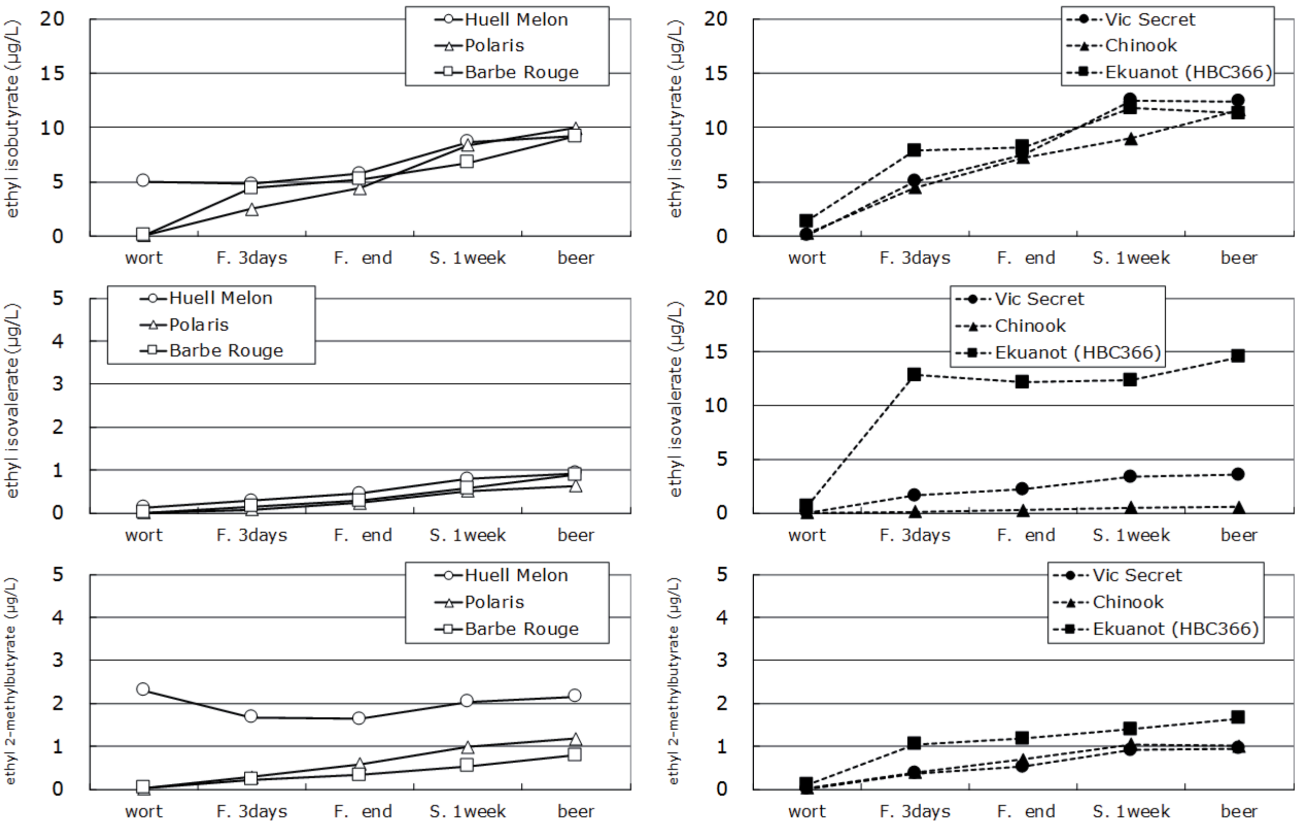

Fig. 5 **Comparison of ethyl esters of branched-chain fatty acids (ethyl isobutyrate, ethyl isovalerate, and ethyl 2-methylbutyrate) (µg/L) during fermentation of beers late-hopped with Huell Melon, Polaris, Barbe Rouge, Vic Secret, Chinook, or Ekuanot (HBC366): F., primary fermentation; S., srorage**

The behaviours of isobutyric esters during total fermentation period in the characteristic six varieties (Huell Melon, Polaris, Barbe Rouge, Vic Secret, Chinook, and Ekuanot (HBC366)) were shown in figure 4. All isobutyric esters gradually decreased during fermentation. In previous report, the contents of isobutyric esters present in the finished beers could increase by delaying hop-addition timing [28]. As described above, these esters were perceived as having fruity, green apple-like, and apricot-like flavour, and an odour threshold of 2-methylbutyl isobutyrate was 78 µg/L in beer [25]. Seaton et al. also suggested that the hop fraction containing myrcene and 2-methylbutyl isobutyrate had a citrus-like flavour [21]. Therefore, these isobutyric esters are expected to contribute to a part of the specific flavour in dry-hopped craft beers.

3.2 Comparison of behaviours of ethyl esters of branched-chain fatty acids among total 40 hop varieties

Table 2 shows the behaviours of ethyl esters of branched-chain fatty acids among 40 hop varieties (There is no data of these compounds in 9702A and 9803A beers). Of the three ethyl esters, ethyl isobutyrate is most dominant component. The behaviours of ethyl esters of branched-chain fatty acids during total fermentation period in the characteristic six varieties (Huell Melon, Polaris, Barbe Rouge, Vic Secret, Chinook, and Ekuanot (HBC366)) were shown in figure 5.

All ethyl esters 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. Ethyl isobutyrate and ethyl 2-methylbutyrate have grape-like and melon-like odours, respectively. In fact, Huell Melon hops (cone and/or pellet) has melon-like odour. Therefore, it is thought that Huell Melon hops might contain these two esters. This unique behaviour of these esters in the fermentation using Huell Melon hops has been reported first time in the 35th Congress of European Brewery Convention (2015) [29]. In recent times, *Neiens* et al. also reported the occurrence of ethyl isobutyrate and ethyl 2-methylbutyrate in this hop [16]. These two ethyl esters were at relatively high levels during total fermentation period. As described above, the thresholds of these esters in beer are as follows: 6.3 µg/L for ethyl isobutyrate, and 1.1 µg/L for ethyl 2-methylbutyrate. The concentrations of these esters in the Huell Melon beer were over their thresholds (Table 2). Therefore, it is thought that ethyl isobutyrate and ethyl 2-methylbutyrate could contribute to the specific flavour of the Huell Melon beer.

In addition, the behaviour of ethyl esters of branched-chain fatty acids in the Ekuanot (HBC366) beer was also unique. Indeed all ethyl esters were almost absent in the wort made with this hop, but the concentrations of all three ethyl esters were over their thresholds and ethyl isovalerate was most dominant component (Fig. 5 and Table 2). Therefore, it is thought that all three ethyl esters, especially ethyl isovalerate, could contribute to the specific flavour of the Ekuanot (HBC366) beer.

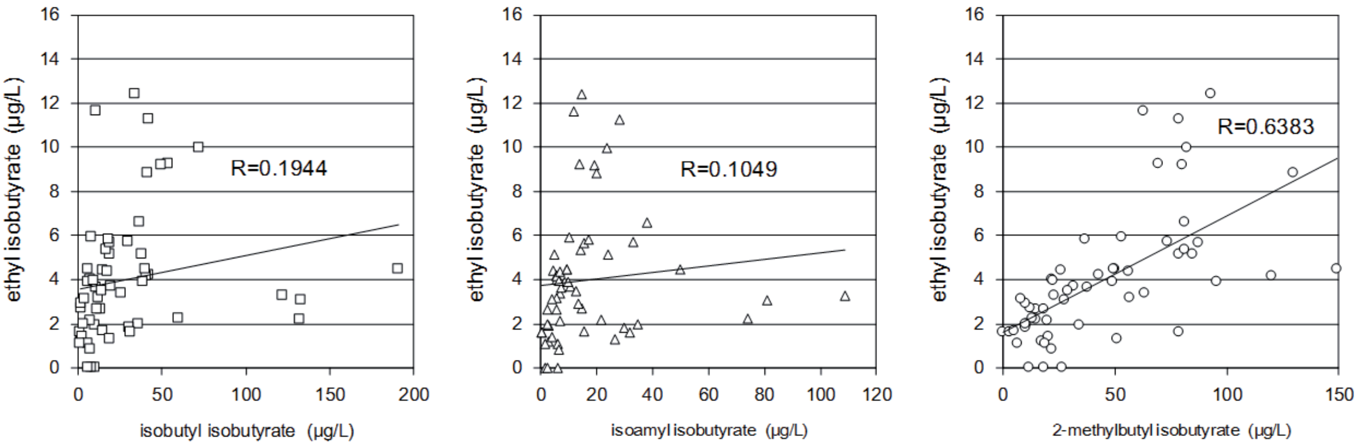

Fig. 6 Relationship between isobutyric esters (isobutyl isobutyrate, isoamyl isobutyrate, and 2-methylbutyl isobutyrate) (µg/L) in worts and ethyl isobutyrate in beers

3.3 Isobutyric esters as possible precursors of ethyl isobutyrate

In table 2, ethyl esters of branched-chain fatty acids in finished beers were widely varied depending on hop varieties and ethyl isobutyrate is most dominant component. If branched-chain fatty acids derived from hop bitter acids (alpha acids and/or beta acids), for example generated by aging of hops (oxidative degradation of hop bitter acids) were functioned as precursors of these ethyl esters, it is expected that ethyl isovalerate might become most dominant component, because humulone and lupulone, which have side-chains including isovaleric structure (Fig. 2), is most dominant components among their homologues. Therefore, it is assumed that a part of isobutyric esters derived from hops could function as possible precursors of ethyl isobutyrate, because isobutyric acid was a common structure of isobutyric esters and ethyl isobutyrate.

The concentrations of three isobutyric esters in the worts (Table 1) and ethyl isobutyrate in the finished beers (Table 2) were plotted in scatter diagrams (Fig. 6). As a result, the concentrations of ethyl isobutyrate in the beers were highly related to those of 2-methylbutyl isobutyrate in the worts (R = 0.64). As described above, the transfer rate of hop-derived 2-methylbutyl isobutyrate from wort to beer was approximately 0.65. These results suggested that a part of 2-methylbutyl isobutyrate could be transesterificated to ethyl isobutyrate.

In previous studies [8, 30], it has been assumed that ethyl heptanoate could be formed from methyl heptanoate derived from hops. Several researchers have reported an occurrence of methyl heptanoate in raw hop [4, 33]. Heptanoic acid is a minor metabolite induced during yeast fermentation. Therefore, methyl heptanoate in hop might behave as a precursor of ethyl heptanoate. As well as in the case of ethyl heptanoate, it is thought that isobutyric esters might function as precursors of ethyl isobutyrate. *Gahr* et al. have pointed out the occurrence of 2-methylbutyl 3-methylbutanoate (2-methylbutyl isovalerate) and 2-methylbutyl 2-methylbutanoate (2-methylbutyl 2-methylbutyrate) in the test-brewed beers late-hopped with Huell Melon [6]. These esters might also behave as precursors of ethyl isovalerate and ethyl 2-methylbutyrate. As described above, the thresholds of ethyl esters of branched-chain fatty acids were estimated at very low levels, 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]. The threshold of 2-methylbutyl isobutyrate (73 µg/L in beer) was at much higher level than that of ethyl isobutyrate (6.3 µg/L in beer). Therefore, it is thought that the transesterification between 2-methylbutyl isobutyrate and ethyl isobutyrate might be an important reaction for hop aromas in beer.

4. Conclusions

In this study, 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) and their behaviour during fermentation have been focused on. Total 42 hop varieties were compared.

Of the three isobutyric esters, 2-methylbutyl isobutyrate is most dominant component. These compounds were at very low levels during fermentation using Saaz hops. All isobutyric esters gradually decreased during fermentation. An average transfer rate of each isobutyric ester from wort to beer was as follows; 0.49 for isobutyl isobutyrate, 0.60 for isoamyl isobutyrate, and 0.65 for 2-methylbutyl isobutyrate.

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. The Huell Melon beer also contained these compounds. It is thought that Huell Melon hops might contain these two esters, and that ethyl isobutyrate and ethyl 2-methylbutyrate could contribute to the specific flavour of the Huell Melon beer. On the other hand, the behaviour of ethyl esters of branched-chain fatty acids in the Ekuanot (HBC366) beer was also unique. The concentrations of all three ethyl esters in the Ekuanot beer were at relatively high levels and ethyl isovalerate was most dominant component. Therefore, it is thought that all three ethyl esters, especially ethyl isovalerate, could contribute to the specific flavour of the Ekuanot beer.

In addition, it is suggested that a part of 2-methylbutyl isobutyrate could be transesterificated to ethyl isobutyrate. The threshold of 2-methylbutyl isobutyrate (73 µg/L in beer) was at much higher level than that of ethyl isobutyrate (6.3 µg/L in beer). Therefore, it is thought that the transesterification between 2-methylbutyl isobutyrate and ethyl isobutyrate might be an important reaction for hop aromas in beer.

From this study, it is concluded that hop-derived various esters including branched-chain structures might be very important contributors to hop varietal aromas, having two roles, their own fruity flavours and precursors of ethyl esters of branched-chain fatty acids.

It is assumed that esterification might be influenced several factors, for example yeast-derived esterase activity, chemical esterification, and so on. The formation/ degradation of esters could occur by such factors during fermentation and/or storage of packaged beer. In general, acids and esters undergo considerable changes during fermentation and storage in contrast to many other hop derived metabolites. In case yeast with high esterase activity is used, transesterification of hop derived isobutyic esters into ethyl esters and/or degradation of various esters by esterase might change in varietal aromas. Also, chemical esterification upon storage might influence beer flavour profile and could therefore explain flavour changes in hopped beer.

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