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Key volatile compounds in red *koji-shochu*, a *Monascus*-fermented product, and their formation steps during fermentation

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ABSTRACT

Red *koji*, which refers the solid culture grown *koji* mold on the steamed rice, is one of the ingredients of Asian fermented foods including the Japanese spirit *shochu*. This study was aimed at elucidating the characteristic flavor and key volatile compounds of red *koji-shochu* as well as the mechanism of their formation. Sensory evaluation showed that red *koji-shochu* has the distinctive flavors cheese, sour, milky, and oily. Fifteen key volatile compounds of red *koji-shochu* were identified by gas chromatography–mass spectrometry and high-performance liquid chromatography, and by comparison between red *koji-shochu* and white *koji-shochu*, as another typical *shochu*. The mash analysis revealed that ketone compounds and short-chain acids derive from red *koji*. Furthermore, although other key compounds were produced by yeast, it is highly likely that their concentrations were affected directly or indirectly by the high activities of protease and lipase in red *koji*.

1. Introduction

Red *koji*-mold (*Monascus* sp.) is well known as an industrially important mold that produces valuable metabolites including red pigments, γ-aminobutyric acid, and monacolin K (Endo, 1980; Kono & Himeno, 2000; Srianta et al., 2014). Therefore, the research on red *koji*-mold has been centered on therapeutic properties in relation to several chronic diseases including hyperlipemia, hypertension, and dengue virus infection, even prevention of cancer (Adnan, Suharto, & Triyono, 2012; Endo, 1980; Lee & Pan, 2012).

Nevertheless, red *koji* mold also has a long history of use for preparation of fermented foods in Asian countries before it was discovered that it could be used to produce the functional compounds. *Koji* is the ingredient used for production of fermented foods and beverages, and it means the steamed rice covered with the grown-*koji* mold. *Koji* plays an important role as a source of various enzymes for fermentation because *koji* mold produces and secretes many hydrolytic enzymes such as α-amylase, glucoamylase, protease, and lipase during its growth. *Aspergillus* sp., *Rhizopus* sp., and *Mucor* sp. are generally used for *koji* preparation. Red *koji*-mold is also used for preparation of *koji*. Red *koji* is used for preparation of several fermented foods: rice wine, vinegar, and fermented soybean curd (*tofuyo* in Japan and *furu* in China). Although red *koji*-mold and red *koji* are important ingredients of fermented foods in Asia, the relevant research on their food chemistry is scarce.

The odor of a food is one of the important factors affecting consumer preferences. Therefore, volatile compounds from foods have been a major topic of research. *Shochu* is a traditional Japanese distilled spirit. It is made from *koji*. It has become increasingly popular, in fact, despite *sake's* popularity outside Japan. Since 2003, *shochu* sales exceeded those of *sake* in Japan (National Tax Agency Japan. Status report of sales (consumption) volumes of alcohol beverages, 2003). Starchy substrates such as rice, barley, and sweet potato are used as the main materials for *shochu* making. Because *shochu* has a characteristic aroma from the main materials, the volatile compounds in *shochu* made from different main materials have been well researched by gas chromatography–mass spectrometry (GC–MS) and GC–MS/olfactometry and compared between them. For example, unlike *shochu* made from other materials, *shochu* made from sweet potato has citrus and floral flavors that are attributable to monoterpene alcohols such as linalool, geraniol, and nerol; it also has a sweet aroma, which is

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imparted by β-damascenone (Kamiwatari, Setoguchi, Kanda, Setoguchi, & Ogata, 2006).

Furthermore, some studies have been conducted with respect to the direct or indirect effects of *koji* on *shochu* flavor. *Koji* mold produces extracellular β-glucosidase and contributes to the release of monoterpene alcohols from a glycoside precursor during fermentation (Ohta et al., 1991). *Koji* itself also has characteristic mushroom or chestnut flavors distinct from those of rice as a material. Ito, Yoshida, Ishikawa, and Kobayashi (1990) identified 16 types of alcohols, ketones, and aldehydes and an ester in *koji*, and it was demonstrated that the growth stage of *koji* mold affects the composition of these volatile compounds (Takahashi et al., 2006, 2007). In addition, it has been shown that the type of species used for preparation of *koji* affects the odor of *koji*. The volatile compounds from white and black *koji* that are prepared by *Aspergillus luchuensis* mut. *kawachii* and *Aspergillus luchuensis*, respectively, are distinct from those of yellow *koji* prepared by *Aspergillus oryzae* (Yoshizaki, Yamato et al., 2010). These volatile compounds are also detectable in *shochu* products (Shiraishi et al., 2016). Thus far, a lot of attention has been given to the volatile compounds of *shochu* made with *koji* prepared by *Aspergillus* sp., whereas the studies on *shochu* made with red *koji* (red *koji-shochu*) have hardly attracted any attention.

The aim of this study was to characterize the aroma profile and to identify the key volatile compounds in red *koji-shochu*. Furthermore, we studied the formation steps of key volatile compounds in the fermentation process to reveal the effects of red *koji*-mold on the flavor formation.

2. Materials and methods

2.1. Strains and chemicals

A seed culture of a single strain of red *koji*-mold (*Monascus anka*) was purchased from Akita-Konno Co., Ltd. (Akita, Japan) to produce red *koji*, whereas white *koji*-mold (*Aspergillus luchuensis* mut. *kawachii*), a fungus used for preparation of white *koji*, was purchased from Kawachi Gen-ichiro Company (Kagoshima, Japan). The yeast strain was Kagoshima-5, supplied by the Kagoshima prefectural brewing association (Kagoshima, Japan). The chemicals used in this study were acquired from Sigma-Aldrich (Steinheim, Germany), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and Nacalai Tesque, Inc. (Kyoto, Japan).

2.2. Preparation of red koji and white koji

Red *koji* and white *koji* were prepared in our laboratory. *Koji* preparation was performed as described in our previous studies (Yoshizaki, Susuki et al., 2010; Yoshizaki, Yamato et al., 2010). Polished rice (1 kg) was soaked in water for 1 h, and excess liquid was drained off for 1 h. The rice was then steamed for 1 h, and the steamed rice was cooled to 40 °C before inoculation. Red *koji*-mold starter (10 g) or white *koji*-mold starter (1 g) were inoculated into the steamed rice, mixed properly at 35 °C and relative humidity of 95%. During the preparation of *koji*, the temperature of the inoculated rice was monitored with a thermometer at the center of mass. The rice was mixed and cooled when its temperature was higher than 40 °C. The incubation was continued for 72 or 43 h for red *koji* or white *koji* preparation, respectively. Then, the *koji* was stored at −80 °C until use.

2.3. Preparation of shochu

Shochu was made according to the method used in our previous study (Shiraishi et al., 2016). Yeast was cultured in a static YPD medium at 30 °C for 48 h in advance. The mixing ratio was determined by the weight of water and rice used for *koji* making = 1.8:1. Approximately 658 g or 480 g of red or white *koji* (water content was 46.5% and 25.6%, respectively) equivalent to 400 g of polished rice was used. Water was added (462 g or 640 g) containing 5 ml of the seed culture of yeast to red or white *koji*, respectively, in such a way that the total weight of water was 720 g. The mash was incubated at 30 °C in a water bath for 10–12 days. *Shochu* was obtained from single-batch distillation in a glass distillation apparatus (a glass pot still coupled with a glass column). After fermentation for 10 days, approximately 1 kg of the mash was distilled using the steam generated from water in a round flask heated by a mantle heater. The distillate was then water-cooled. The end of distillation was the point when the alcohol content in the bundled distillate reached approximately 40%. The distillate was filtered and diluted with deionized water until 25% alcohol. *Shochu* samples were stored at room temperature in a dark place prior to GC–MS analysis. The mash samples for determination of amino acids and organic acids were prepared separately in the same manner and 10 g of mash was used for analysis.

2.4. Sensory evaluation

Red *koji-shochu* and white *koji-shochu* were evaluated in terms of odor. Sensory-profile analysis constituted blind testing and was conducted by 14 panelists from Kagoshima University (eight females and six males, aged between 20 and 50 years). Most of the panelists were previously trained on sensory evaluation techniques. The panelists evaluated the intensity of each aroma descriptions on a six-point scale (0 = not detected, 1 = slightly detected, 2 = weak intensity, 3 = moderate intensity; 4 = strong intensity, 5 = very strong intensity). The results of the sensory-profile analysis were averaged for each aroma description and plotted on a spider web diagram.

2.5. Protease activity in koji

Koji (10 g) was added to 50 ml of 50 mM acetate buffer (pH 5.5) containing 0.5% NaCl. The supernatant (10 ml) was dialyzed against the same buffer at 4 °C overnight to remove the peptide and free amino acids and was used as a crude extract. Protease activity was determined by glutelin as a substrate. A mixture of 160 μl of a glutelin solution (2% [w/v] glutelin in 50 mM acetate buffer, pH 5.5) and 160 μl of the sample were added to a tube. The reactions were carried out at 37 °C for 10 min and terminated by the addition of 320 μl of 0.4 M trichloroacetic acid. After incubation for 5 min at 37 °C, the reaction mixture was centrifuged at 13,700*g* for 5 min. The supernatant (160 μl) was mixed with 0.4 M Na_2CO_3 (800 μl) and 0.5 M Folin-phenol reagent (160 μl). After incubation for 20 min at 37 °C, the absorbance at 760 nm was measured. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μmol of tyrosine in 20 min at 37 °C.

2.6. Lipase activity in koji

The crude extract containing lipase was prepared from *koji* according to previous research with a minor modification (Kudo, Mizutani, Honbu, & Ohta, 2001). *Koji* (20 g) was added to 60 ml of 50 mM acetate buffer (pH 5.0) and homogenized at 13,700*g* for 1 min. Then, 40 ml of ethanol was added and mixed well (final ethanol concentration 40%). The homogenate was centrifuged at 13,700*g* for 5 min, and the supernatant was used as a crude extract for the lipase assay. Lipase activity was determined by 2,3′-dimercaptopropan-1-ol tributyrate (BALB) as a substrate (Furukawa, Kurooka, Arisue, Kohda, & Hayashi, 1982). A mixture

of 400 μl of 0.3 mM 2,2′-dithiodipyridine in 50 mM acetate buffer (pH 5.5) and 40 μl of 20 mM BALB dissolved in ethanol was preincubated at 30 °C for 5 min. The sample (20 μl) was added to the tube and allowed to react for 15 h at 30 °C. The enzymatic reaction was stopped by adding 800 μl of acetone. Absorbance at 343 nm was measured. One unit of lipase activity was defined as the amount of enzyme forming 1 μmol of 2-mercaptopyridine per hour at 30 °C.

2.7. Analysis of amino acids by high performance liquid chromatography (HPLC)

Koji (10 g) was added to 100 ml of prechilled deionized water, and the mixture was centrifuged at 3400*g* for 10 min. The supernatant was passed through a microfilter (0.45-μm-pore size). Mash samples were prepared as the supernatant by the centrifugation at 3400*g* for 10 min. The concentrations of amino acids of *koji* and mash samples were determined by HPLC (Prominence HPLC system, Shimadzu Corp., Kyoto, Japan) using a fluorescence detector (RF-10AXL, Shimadzu Corp.) by the post column fluorescence derivatization method. Separation of amino acids was achieved by Shimadzu Shim-pack Amino-Na column (100 × 6.0 mm I.D., Shimadzu Corp.) at 60 °C at a flow rate of 0.6 ml/min using the amino acid mobile phase kit, Na type (Shimadzu Corp.). The fluorescence detector was set to the excitation/emission wavelength pair of 350/450 nm. The reaction reagents were taken from an amino acid reaction kit (Shimadzu Corp.) and maintained at the flow rate of 0.2 ml/min.

2.8. Analysis of short-chain acids by HPLC

Koji and mash samples for analysis were prepared in the same way for amino acid analysis. Short-chain acids were identified using HPLC (Prominence HPLC system, Shimadzu Corp.) using a conductivity detector (CDD-10A VP, Shimadzu Corp.). The separation of organic acids was achieved by Shimadzu Shim-pack SCR-102H HPLC column (300 × 8 mm I.D., Shimadzu Corp.) at 50 °C using 4 mM *p*-toluenesulfonic acid monohydrate as a mobile phase at the flow rate of 0.8 ml/min. The mixture solution of 4 mM *p*-toluenesulfonic acid monohydrate, 16 mM bis-Tris, and 80 μM EDTA served as a post column reaction solution at the flow rate of 0.8 ml/min. Standard curves were constructed by linear regression of analyte peak areas versus known concentrations of each compound.

2.9. Headspace GC–MS

The headspace gas was collected by Entech 7100A preconcentrator (Entech 7100A series; Entech Instruments Inc., Simi Valley, CA). A 10 ml sample was transferred to a 200-ml sample bottle and then incubated at 30 °C for 30 min. Subsequently, the 100 ml headspace gas was vacuum extracted. The volatile compounds were adsorbed onto two tandemly arranged commercial traps (Entech Instruments Inc.), which were packed with different stationary phases: Trap 1, glass beads/tenax mixture resin; Trap 2, tenax resin. The volatile compounds were then desorbed by thermodesorption and applied to the GC–MS system. Identification and quantification of the volatile compounds was performed using an Agilent 6890 series gas chromatograph (Agilent Technologies Inc., Palo Alto, CA) equipped with an Agilent 5975B mass spectrometer. All mass spectra were acquired in electron impact (EI) mode. The GC–MS system was equipped with a DB-WAX column (60 m × 0.25 mm I.D., 0.25-μm film thickness; Agilent Technologies Inc.). Analyses were carried out with helium as a carrier gas at the flow rate of 1.0 ml/min using the following temperature program: 40 °C for 5 min, 3 °C per min to 240 °C. Quantitative data were obtained using 1-pentanol (*m*/*z* 55) as an internal standard. Identification of volatile compounds was confirmed by comparison of their mass spectra with those of the NIST05a mass-spectral database and the retention index (RI) in the AromaOffice database (Nishikawa Keisoku Co., Ltd., Tokyo, Japan). Standard curves were constructed by linear regression of the ratio of the peak area for the analyte to that for 1-pentanol (internal standard) versus known concentrations of each compound.

2.10. GC–MS with stir bar sorptive extraction

Mash samples were prepared as the supernatant by the centrifugation at 3400*g* for 10 min. *Shochu* or mash sample (10 ml) were transferred to a sample vial and a 15-mm stir bar coated with 0.5 mm polydimethylsiloxane was added (Twister, Gerstel K.K., Japan). The sample was stirred at 1200 rpm on magnetic stirrer for 1 h at room temperature. Then, the stir bar was removed, washed with deionized water, and dried off with a tissue. The stir bar was placed into a glass insert. The volatile compounds were desorbed from the stir bar using the temperature program of thermal desorption system (Gerstel TDS3 and Gerstel CIS4, Gerstel K.K.): 20 °C for 1 min, 60 °C/min to 260 °C (hold for 1 min). In the meantime, cold trap was set to −150 °C to cryofocus (Gerstel CIS4, Gerstel K.K.). After the desorptive program was completed, the cryotrap was heated to inject the compounds into the GC analytical column: −150 °C for 1 min, 12 °C/s to 270 °C (hold for 2 min). The GC–MS system was equipped with an Inner Pure-WAX column (60 m × 0.25 mm I.D., 0.25-μm film thickness; GL Sciences Inc., Tokyo, Japan). The analytical conditions of GC–MS and data analysis were the same as those shown in the section about headspace GC–MS. Standard curves were constructed by linear regression of analyte peak areas versus known concentrations of each compound.

2.11. HPLC with tandem mass spectrometry (LC–MS/MS)

Total lipids containing long chain fatty acids (LCFAs) in *koji* and mash were extracted by the method of Bligh and Dyer (Bligh & Dyer, 1959). LCFAs in the samples were analyzed using an HPLC system (Prominence HPLC system, Shimadzu Corp.) equipped with triple quadrupole/linear ion trap mass spectrometer (3200 QTRAP, AB Sciex, MA, USA). The separation was accomplished on an Acuity UPLC CSH C18 column (particle size 1.7 μm, 30 × 2.1 mm I.D., Waters Corp., MA, USA). LCFAs were analyzed under the following conditions: mobile phase A (acetonitrile:H_2O = 60:40) and mobile phase B (isopropanol) at a flow rate of 0.2 ml/min; 10% B linearly changed to 80% B within 15 min. All mobile phases were contained 0.0028% ammonia and 0.1% formic acid. The sample (10 μl) was then injected. The mass spectrometer was set up as follows. The ion source temperature was maintained at 600 C. Ion intensity of fatty acids was set to negative mode, based on the full-scan electrospray ionization (ESI) sources. The declustering potential was set to −72 V. The precursor/product ion transitions in multiple reaction monitoring mode were used for mass analysis and quantitation [Q1/Q3 were set to 255/255, 279/279, 281/281, and 283/283, which correspond to palmitic acid (16:0), linoleic acid (18:2), oleic acid (18:1), and stearic acid (18:0), respectively]. The procedure was controlled by Analyst 1.5.1 data acquisition and processing software (AB SCIEX, CA, USA). Standard curves were constructed by linear regression of analyte peak areas versus known concentrations of each compound.

3. Results and discussion

3.1. The characteristic flavor and key volatile compounds of red koji-shochu

To identify the characteristic flavor and key volatile compounds of red *koji-shochu*, we used white *koji-shochu* as a control, which is one of popular *shochu* types. Red *koji-shochu* and white *koji-shochu* commonly contain volatile compounds from rice as the main material and the metabolites of yeast. Therefore, it is worthwhile to determine the characteristic flavor of red *koji-shochu* and the key volatile compounds that contribute to its flavor. Red *koji-shochu* and white *koji-shochu* were prepared in our laboratory and compared by sensory evaluation of 11 odor descriptors. Red *koji-shochu* had a higher intensity of cheese, sour, oily, and milky flavors than white *koji-shochu* (Fig. 1). Moreover, red *koji-shochu* had a slightly higher intensity of fruity odor than white *koji-shochu*. On the other hand, white *koji-shochu* had a slightly higher intensity of cereal and *koji* odor than red *koji-shochu*. These results revealed that both *shochu* types have a distinctive characteristic flavor. Next, we attempted to analyze the volatile compounds that contribute to these distinctive flavors of *shochu*.

3.2. The comparison of volatile compounds in red koji-shochu and white koji-shochu

To identify key volatile compounds of red *koji-shochu*, we analyzed volatile compounds by GC–MS and HPLC. A total of 52 volatile compounds were identified in both *shochu* types (Table 1). These volatile compounds were classified into seven chemical classes including 11 alcohols, 25 esters, five ketones, four aldehydes, two furans, three acids, and two other classes. Forty-four volatile compounds were typically detected in both types of *shochu*.

Most alcohol compounds were present at higher concentrations in red *koji-shochu* than in white *koji-shochu* except for 1-octen-3-ol. Isobutyl alcohol, isoamyl alcohol, and β-phenylethyl alcohol were present at the level above their odor thresholds in both types of *shochu*. These compounds have an alcohol-like odor. However, sensory evaluation detected a slightly stronger odor in white *koji-shochu* than that in red *koji-shochu* (Fig. 1). It was suggested that the alcohol-like odor in red *koji-shochu* was masked by other detectable volatile compounds that were present at a higher concentration in red *koji-shochu* than in white *koji-shochu*.

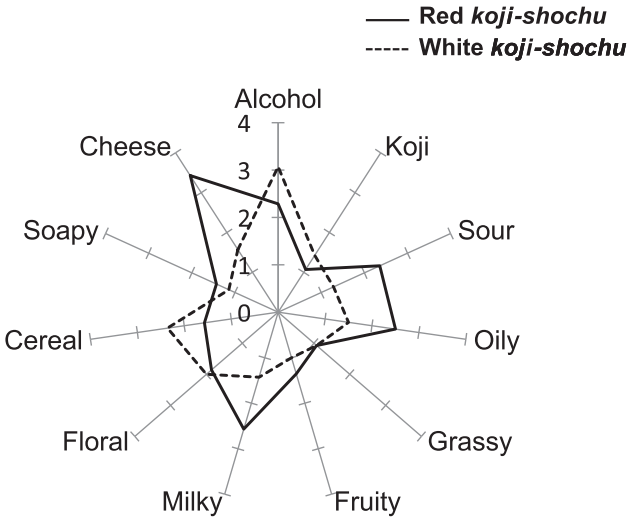

Fig. 1. Comparison of the aroma profile of red *koji-shochu* (solid line) and white *koji-shochu* (dotted line). The sensory evaluation was carried out by 14 panelists. The panelists evaluated the intensity of odor in the following manner: 0 = not detected, 1 = slightly detected, 2 = weakly detected, 3 = detected, 4 = strongly detected, 5 = very strongly detected. The values are shown as the means.

Ester compounds were the largest group detected in both types of *shochu*. Esters are the most important group of volatile compounds and affect the quality of fermented beverages due to their low threshold concentrations and desirable fruity aromas (Chen & Xu, 2010). Although the total ester concentrations were almost equal between red *koji-shochu* (22.3 mg/l) and white *koji-shochu* (24.8 mg/l), the compositions were different. Typically, esters of short-chain acids and ethyl esters of LCFAs (C14–C18) were predominant esters in red *koji-shochu*, whereas ethyl esters of MCFAs (C6–C12) were found to be prevalent in white *koji-shochu*. Most ethyl esters of LCFAs impart an oily odor to foods (Burdock, 2004). Ethyl palmitate, which belongs to the group of ethyl esters of LCFAs exclusively showed odor activity value (OAV) $\geqslant 1$ in red *koji-shochu*. Therefore, it was shown here that this compound contributes to the oily odor of red *koji-shochu* (Fig. 1). Among esters of short-chain acids, acetate ester, ethyl isovalerate, ethyl butyrate, and isoamyl acetate showed high OAV in red *koji-shochu*. In particular, ethyl butyrate that was identified as one of the key aroma components of strawberry (Ulrich, Hoberg, Rapp, & Kecke, 1997) was found to be approximately 15-fold more abundant in red *koji-shochu* than in white *koji-shochu*. Among ethyl esters of MCFAs, ethyl hexanoate, ethyl octanoate, and ethyl decanoate were detected above their odor thresholds in both *shochu* types.

Ketone compounds and short-chain acids were found to be the important compounds in red *koji-shochu* because most of them were exclusively found in red *koji-shochu*, in addition, 2-pentanone, diacetyl, isobutyric acid, and isovaleric acid showed OAVs $\geqslant 1$. Diacetyl generally contributed cheesy-to-butter scotch odor (Giri, Osako, & Ohshima, 2010). A similar flavor is imparted by other ketones detected, which belong to the methyl ketone group (2-pentanone, 2-heptanone, and 2-nonanone) and short-chain acids (isobutyric acid and isovaleric acid). They are major volatile compounds in cheese, and were reported to be fungal metabolites (Gehrig & Knight, 1961). Therefore, it is highly likely that ketone compounds and short-chain acids contributed to the cheese, sour, and milky odors in red *koji-shochu* because they correspond to its distinctive flavor characteristics (Fig. 1). The flavor characteristics of these compounds detected at higher concentrations in red *koji-shochu* were consistent with the flavor characteristics of red *koji-shochu*. Therefore, we assumed that our GC–MS results could explain the flavor characteristics of red *koji-shochu* well.

Most aldehydes and furan compounds were present at higher concentrations in white *koji-shochu* than in red *koji-shochu*. Among aldehydes, isovaleraldehyde is known as a key volatile compound of *koji* made from *Aspergillus* sp. including white *koji*-mold (Yoshizaki, Yamato et al., 2010). It was reported that *shochu* made with white *koji* also contains isovaleraldehyde (Shiraishi et al., 2016). Furfural of furan compounds is a volatile compound commonly detected in distilled spirits because it is produced during distillation via degradation of monosaccharides under high-temperature acidic conditions (Pino, 2014). White *koji* showed a high concentration of citric acid because white *koji* mold produces and secrets a lot of this acid. It was confirmed that mash pH of white *koji* (pH 3.6) is lower than that of red *koji* (pH 4.9). Therefore, it is likely that the mash conditions of white *koji* promoted the production of furfural. In fact, when we distilled red *koji* mash, whose pH was adjusted to the same level as in white *koji* mash, the furfural level in red *koji-shochu* became almost the same as that in white *koji-shochu* (data not shown).

Moreover, if we review the compounds detected predominantly in white *koji-shochu*, 1-octen-3-ol was exclusively detected in

Table 1
Volatile compounds identified in red and white *koji-shochu*.

Compounds	Concentration (μg/L)		Identification	RI[¶]	Odor Threshold[§](μg/L)		OAV	
	Red	White					Red	White
GC–MS analysis								
(Alcohols)								
Isobutyl alcohol	1,45,000 ± 16,000*	76,000 ± 2000	MS, std	1196	40,000	[1]	3.6	1.9
Isoamyl alcohol	1,69,000 ± 10,000*	1,34,000 ± 8000	MS, std	1322	30,000	[1]	5.6	4.5
β-Phenethyl alcohol	65,000 ± 2000*	16,000 ± 100	MS, std	2109	14,000	[1]	4.6	1.1
3-Methyl-3-buten-1-ol	1000 ± 40*	160 ± 30	MS, std	1355	600	[2]	1.7	<1
2-Ethyl-1-butanol	50 ± 7*	n.d.	MS, std	1418	n.f.		–	–
4-Methyl-1-pentanol	24.4 ± 0.4*	n.d.	MS, std	1425	50,000	[2]	<1	<1
2-Heptanol	0.07 ± 0.03**	n.d.	MS, std	1431	200	[2]	<1	<1
1-Hexanol	70 ± 9*	n.d.	MS, std	1465	8000	[1]	<1	<
2-Nonanol	0.035 ± 0.005*	n.d.	MS, std	1654	58	[2]	<1	<1
1-Octen-3-ol	n.d.	17.3 ± 3.2*	MS, std	1572	1	[2]	<1	17
2-Ethyl-1-hexanol	167 ± 33**	29.4 ± 11.7	MS, std	1619	8000	[2]	<1	<1
(Esters)								
Short-chain acid ethyl ester								
Ethyl acetate	14,000 ± 1000	20,000 ± 1000	MS, std, RI	887	12,000	[2]	1.2	1.7
Ethyl propionate	109 ± 13*	75 ± 4	MS, std, RI	957	5500	[2]	<1	<1
Ethyl isobutyrate	1.0 ± 0.1*	n.d.	MS, std, RI	968	0.1	[5]	10	<1
Ethyl butyrate	573 ± 71*	39 ± 12	MS, std, RI	1037	20	[1]	29	2.0
Ethyl 2-methyl-butyrate	92 ± 8*	14 ± 2	MS, std, RI	1052	18	[1]	5.1	<1
Ethyl isovalerate	105 ± 24*	16 ± 1	MS, std, RI	1068	3	[1]	35	5.3
MCFA ethyl ester								
Ethyl hexanoate	93 ± 2*	155 ± 10	MS, std, RI	1241	14	[1]	6.6	11.1
Ethyl heptanoate	1.2 ± 0.2	0.6 ± 0.1	MS, std, RI	1337	2	[2]	<1	<1
Ethyl octanoate	187 ± 23*	487 ± 18	MS, std, RI	1440	5	[1]	37	97
Ethyl decanoate	193 ± 29*	372 ± 40	MS, std, RI	1643	200	[1]	1.0	1.9
Ethyl laurate	150 ± 4*	191 ± 12	MS, std, RI	1830	3500	[2]	<1	<1
LCFA ethyl ester								
Ethyl myristate$^{\Psi}$	471 ± 32*	322 ± 33	MS, std, RI	2040	800	[2]	<1	<1
Ethyl palmitate$^{\Psi}$	2126 ± 252*	954 ± 38	MS, std, RI	2246	1500	[2]	1.4	<1
Ethyl oleate$^{\Psi}$	61 ± 5*	15.1 ± 0.4	MS, std, RI	2467	n.f.		–	–
Ethyl linoleate$^{\Psi}$	248 ± 20*	102 ± 1	MS, std, RI	2513	n.f.		–	–
Acetate ester								
n-Propyl acetate	13 ± 1*	30 ± 2	MS, std, RI	974	54	[3]	<1	<1
Isobutyl acetate	131 ± 8*	86 ± 5	MS, std, RI	1014	66	[3]	2.0	1.3
Butyl acetate	7.7 ± 1.0*	2.6 ± 0.3	MS, std, RI	1071	1800	[2]	<1	<1
Isoamyl acetate	827 ± 63*	463 ± 44	MS, std, RI	1126	30	[1]	28	15
β-Phenethyl acetate	2301 ± 76**	594 ± 44	MS, std, RI	1807	250	[1]	9.2	2.4
2-Ethylhexyl acetate	9.3 ± 1.4*	n.d.	MS, std	1387	n.f.		–	–
Other ester								
Isobutyl isobutyrate	16 ± 3*	n.d.	MS, std, RI	1091	n.f.		–	–
Isobutyl octanoate	0.7 ± 0.2*	0.9 ± 0.1	MS, std, RI	1558	1	[2]	<1	1.1
Isoamyl 2-methylbutanoate	0.91 ± 0.02*	n.d.	MS, std	1282	n.f.		–	–
Diethyl succinate	629 ± 36*	897 ± 49	MS, std, RI	1677	1200	[2]	0.5	<1
(Ketones)								
2-Pentanone	197 ± 7*	10.7 ± 2.3	MS, std	1044	50	[4]	3.9	<1
2-Heptanone	20 ± 2*	n.d.	MS, std	1278	140	[3]	<1	<1
2-Nonanone	4.6 ± 0.7*	n.d.	MS, std	1501	200	[2]	<1	<1
Diacetyl	84,158 ± 3891**	n.d.	MS, std	1042	100	[1]	842	<1
Acetoin	18,574 ± 1300*	7000 ± 100	MS, std	1390	1,50,000	[2]	<1	<1
(Aldehydes)								
Acetaldehyde	1700 ± 70*	4614 ± 131	MS, std	950	500	[2]	3.4	9.2
2-Methylbutanal	78 ± 28**	164 ± 19	MS, std	966	4.4	[5]	18	37
Isovaleraldehyde	184 ± 98**	306 ± 75	MS, std	972	0.35	[2]	526	874
Hexanal	32 ± 2*	42 ± 1	MS, std	1168	5	[2]	6.4	1.0
(Furans)								
2-Penthylfuran	0.4 ± 0.1*	n.d.	MS, std	1331	6	[2]	<1	<1
Furfural	3336 ± 21*	4580 ± 67	MS, std	1580	14,100	[1]	<1	<1
(Others)								
Dimethyl trisulfide	n.d.	1.24 ± 0.12**	MS, std	1487	0.1	[5]	<1	12
Isobutanal diethyl acetal	10.7 ± 0.8	8.3 ± 0.3	MS, std	1047	n.f.		<1	<1
HPLC analysis								
(Short-chain acids)								
Acetic acid†	92,000 ± 4000	95,000 ± 1000	std	–	2,00,000	[1]	<1	<1
Isobutyric acid†	65,000 ± 2000*	n.d.	std	–	2300	[1]	28	–
Isovaleric acid†	52,000 ± 6000*	n.d.	std	–	33	[1]	1576	–

n.d., not detected; n.f., not found. $^{\Psi}$Compound analyzed by stir bar sorptive extraction GC–MS. †Compound analyzed by HPLC. Asterisks show a significant difference (** $p < 0.05$, * $p < 0.01$) between red and white *koji-shochu* according to Student's *t* test. MS, identification by comparison with the NIST05a mass spectral database; std, identification by comparison of their mass spectra with those of the authentic compounds. [¶]RI, analyzed by comparison with the retention indexes from the literature. [§]Odor threshold from the literature: [1] Ferreira, López, & Cacho, 2000; [2] Welke, Zanus, Lazzarotto, & Zini, 2014; [3] Pino & Quijano, 2012; [4] Ulrich et al., 1997; [5] Feng et al., 2015.

white *koji-shochu* with OAV ⩾ 1 (Table 1). 1-Octen-3-ol has been reported as a predominant compound produced by molds of the *Aspergillus* genus (Kaminski, Stawicki, & Wasowicz, 1974), and was detected in white *koji-shochu* (Yoshizaki, Yamato et al., 2010). Thus, it appears that 1-octen-3-ol detected in white *koji-shochu* derives from white *koji*. In this experiment, we also found the key volatile compounds of white *koji-shochu*.

3.3. The changes of key volatile compounds of red koji-shochu during fermentation

Among all the detected compounds, 15 were selected as key volatile compounds of red *koji-shochu* based on the ⩾1.5-fold difference in concentration between the two types of *shochu* and the concentration above the odor thresholds (OAV ⩾ 1). They are three higher alcohols (isobutyl alcohol, phenethyl alcohol, and 3-methyl-3-buten-1-ol), seven esters of short-chain acids (ethyl isobutyrate, ethyl butyrate, ethyl 2-methyl-butyrate, ethyl isovalerate, isobutyl acetate, isoamyl acetate, and β-phenethyl acetate), one ester of LCFA (ethyl palmitate), two ketones (2-pentanone, diacetyl), and two short-chain acids (isobutyric acid and isovaleric acid). To find the reason why these compounds were present at high concentrations in red *koji-shochu*, we studied the formation steps of key volatile compounds by analyzing the changes in concentrations during fermentation. 2-Heptanone and 2-nonanone were also added to the list of research targets to understand the tendencies of the methyl ketone compounds containing 2-pentanone in more detail. The measurement was started on day 1 because the mash just after preparation (day 0) could not be sampled homogeneously from the mash. Unfortunately, diacetyl in the mash could not be measured well by GC–MS. This problem is probably the effect of the non-volatile compounds in the mash.

We found that concentrations of alcohols and esters increased during fermentation (Fig. 2). Levels of all alcohols, acetate ester, and ethyl palmitate increased dramatically from day 1 to day 3, and then their concentrations reached a steady state. In contrast, ethyl isobutyrate, ethyl butyrate, ethyl-2-methyl-butyrate, and ethyl isovalerate concentrations increased gradually during fermentation, and reached a maximum on day 7. The higher alcohols and esters during alcohol fermentation have long been thought to be produced by yeast during brewing (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008). Therefore, it seems that red *koji* has some effects on yeast metabolism.

The concentrations of ketone compounds showed two tendencies during fermentation (Fig. 2). 2-Pentanone concentration increased gradually during fermentation, whereas that of 2-heptanone and 2-nonanone decreased gradually throughout fermentation. These results indicated that ketone compounds except

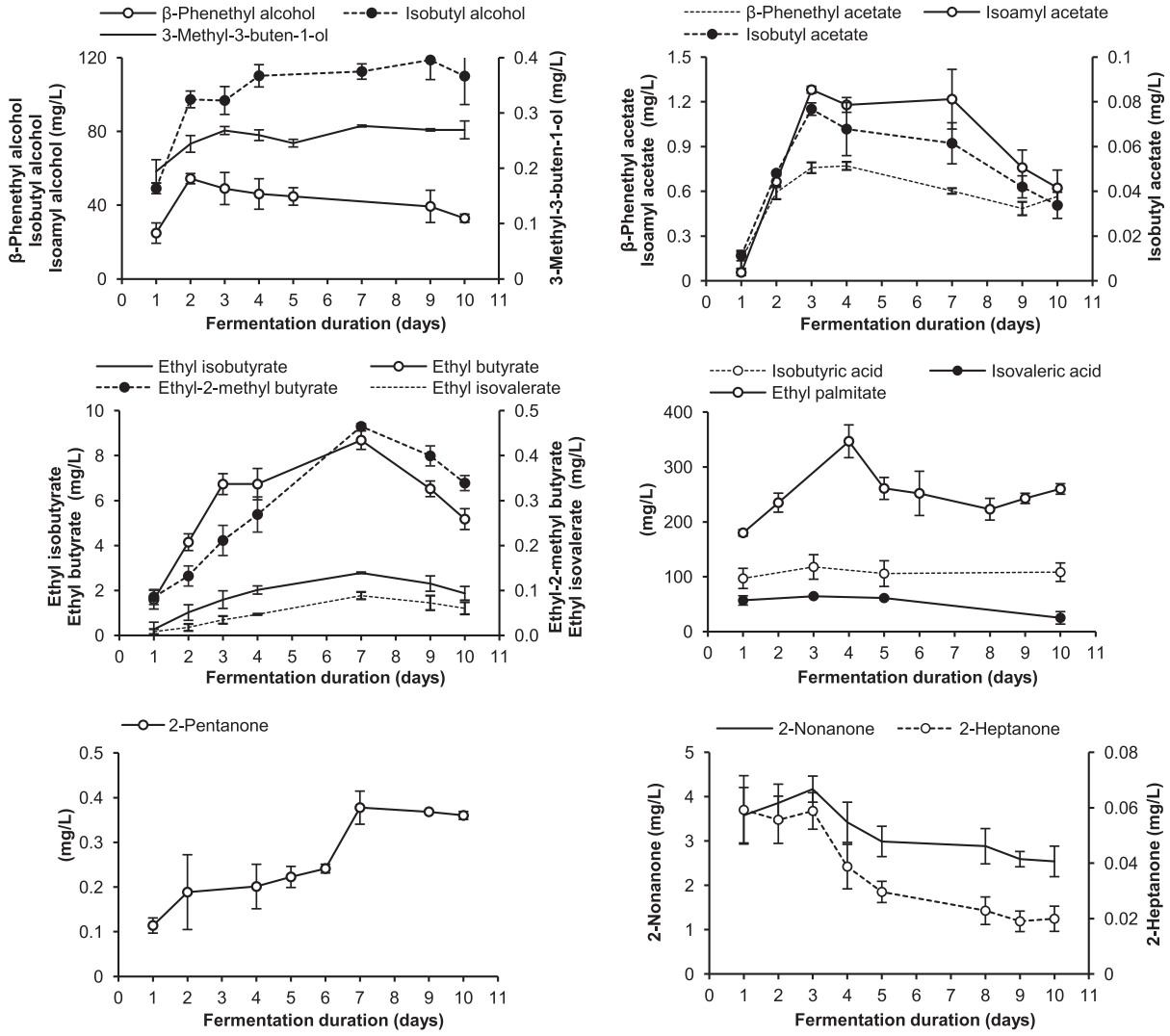

Fig. 2. Time course of the concentration of the volatile compounds in red *koji*-mash. The compounds except ethyl palmitate, isobutyric acid, and isovaleric acid were analyzed by GC–MS with stir bar sorptive extraction. Ethyl palmitate was measured by stir bar sorptive extraction-GC–MS. Isobutyric acid and isovaleric acid were analyzed by HPLC.

2-pentanone were not produced during fermentation by yeast metabolism but rather derived from red *koji*. The ability of *Monascus purpureus* to oxidize fatty acids to methyl ketones via the β-oxidation pathway has already been reported (Kranz, Panitz, & Kunz, 1992). This observation is in agreement with our results.

Isobutyric acid and isovaleric acid levels did not change significantly throughout the fermentation (Fig. 2). Therefore, it appears that these compounds are also contained in red *koji*, not produced exclusively by yeast. These branched short-chain acids are formed in valine and leucine degradation pathways (Kuzdzal-Savoie, 1980). Although these short-chain acids are known to be produced by lactic acid bacteria in cheese, these bacteria were not detected in our red *koji* (data not shown). It is possible that isobutyric acid and isovaleric acid are common characteristic compounds of red *koji*-fermented products. It has not been reported elsewhere that *Monascus* sp. produces branched short-chain acids. This finding is reported here for the first time.

3.4. Factors affecting the formation of key volatile compounds of red koji-shochu

There are a lot of data showing that higher alcohols are produced by amino metabolism and the Ehrlich pathway of yeast (Hazelwood et al., 2008). Valine, leucine, isoleucine, and phenylalanine are metabolized to isobutyl alcohol, isoamyl alcohol, 2-methylbutyl alcohol (active amyl alcohol), and β-phenylethyl alcohol, respectively. It has been reported that the addition of amino acids to a yeast fermentation mixture upregulates the production of the corresponding alcohols (Hazelwood et al., 2008). Therefore, we analyzed the concentrations of amino acids in mash and protease activity of *koji*. Red *koji* and its mash contained more amino acids than white *koji* did. Correspondingly, protease activity of red *koji* was higher than that of white *koji* (Table 2). Therefore, it is likely that the high protease activity of red *koji* results in high amino acids content in the mash and eventually leads to a high concentration of higher alcohols in red *koji-shochu*. In addition, it was reported that the production of acetate ester such as isoamyl acetate depends strongly on the isoamyl alcohol amount (Ashida, Ichikawa, Suginami, & Imayasu, 1987). Therefore, it seems that the production of isobutyl acetate, isoamyl acetate, and β-phenethyl acetate, which were found to be the key compounds, was promoted by the high concentration of higher alcohols in the mash.

It is likely that ethyl esters of LCFAs are produced in a reaction during fermentation (as other esters are) between alcohol and acyl-CoA by yeast (Nykänen, 1986). Furthermore, *Monascus* sp. is reported to have an esterification-activity that could produce ethyl hexanoate from ethanol and hexanoic acid (Fang, 2011). However, these results do not explain the difference between ester levels in red and white *koji-shochu*. It has been reported that ethyl esters of LCFAs in the alcoholic beverage made from *koji* are also synthesized by lipase activity of *koji* (Kudo et al., 2001). It appears that the LCFA level in the mash affects the levels of the corresponding esters. Thus, we measured the LCFA concentration in the mash and lipase activity in *koji*. Red *koji* mash contained a higher LCFA concentration than that in white *koji* (Fig. 3), and red *koji* showed a higher lipase activity than white *koji* did (Table 2). These results suggested that lipase from molds plays an important role in formation of ethyl esters of LCFAs during fermentation of *shochu* mash.

Furthermore, we can hypothesize that MCFA and short-chain acid levels in the mash also affect those corresponding esters because it is known that the addition of fatty acids to the growth

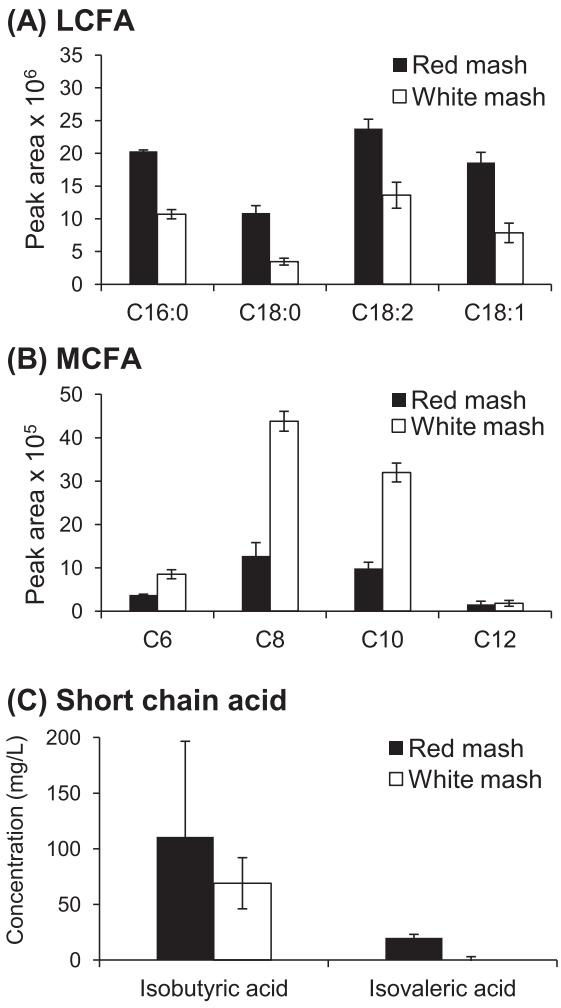

Fig. 3. LCFAs, MCFAs, and short-chain acids contents in red and white *koji* mash. (A) LCFAs were analyzed by HPLC–MS/MS. (B) MCFAs were analyzed by GC–MS with stir bar sorptive extraction. (C) Isobutyric acid and isovaleric acid were analyzed by HPLC. C16:0, C18:0, C18:2, C18:1, C6, C8, C10, and C12 were palmitic acid, stearic acid, linoleic acid, oleic acid, hexanoic acid, octanoic acid, decanoic acid, and dodecanoic acid, respectively.

Table 2
Amino acid content of *koji* and its mash and enzyme activities in *koji*.

	Koji (μg/g dry *koji*)		Mash (mg/L)	
	Red	White	Red mash	White mash
Valine	127.5 ± 0.3*	2887 ± 63	3.72 ± 0.03*	2.89 ± 0.06
Isoleucine	92.1 ± 2.0*	1365 ± 27	2.18 ± 0.01*	1.36 ± 0.03
Leucine	241.1 ± 2.8*	6027 ± 126	6.78 ± 0.05*	6.03 ± 0.13
Phenylalanine	198.1 ± 5.5*	4072 ± 202	4.61 ± 0.03**	4.07 ± 0.20
Protease (U/g dry koji)	0.49 ± 0.01*	0.33 ± 0.0	–	–
Lipase (U/g dry koji)	38 ± 4*	20 ± 4	–	–

Values are means of three independent measurements ± S.D., and asterisks show a significant difference (**$p < 0.05$, *$p < 0.01$) between red and white *koji-shochu* according to Student's *t* test.

medium of yeast results in an increase in ethyl ester levels, and several fungal lipases can synthesize ethyl esters of MCFAs and short-chain acids (Saerens et al., 2006; Xu, Wang, Mu, Zhao, & Zhang, 2002). Ethyl esters of MCFAs were detected at higher concentrations in white *koji-shochu*. Ethyl esters of short-chain acids which are ethyl isobutyrate and ethyl isovalerate, were detected at higher concentrations in red *koji-shochu*. Therefore, we expected that MCFA and short-chain acid levels would be higher in white *koji*-mash and red *koji*-mash, respectively. Accordingly, we compared MCFA and short-chain acid levels between white and red *koji*-mash. MCFAs were detected at higher concentrations in white *koji* mash. In contrast, isobutyric acid and isovaleric acid were detected at higher concentrations in red *koji*-mash than in white *koji*-mash. Therefore, it is likely that the concentration of a precursor in the mash affects the ester compounds in red and white *koji-shochu*. It is well known that MCFAs and LCFAs are formed via the fatty acid synthesis pathway from acetyl-CoA during yeast fermentation (Styger, Prior, & Bauer, 2011). Although red *koji-shochu* and white *koji-shochu* were prepared with the same yeast strain, MCFA and LCFA levels were different between the two types of *koji* mash. Thus, it is possible that ester compounds and fatty acids are also affected by the type of *koji*.

Our results can advance the understanding of the flavor characteristics of red *koji shochu* and the steps of formation of volatile compounds during red *koji-shochu* production. These results may help to improve its aroma quality, and we expect that red *koji shochu* could be an alternative to the regular type of *shochu*.

4. Conclusion

It was demonstrated that red *koji-shochu* has the characteristic flavors cheese, sour, oily, and milky. Fifteen key volatile compounds were identified based on OAV and concentration ratio by comparison with white *koji-shochu*: isobutyl alcohol, phenethyl alcohol, 3-methyl-3-buten-1-ol, ethyl isobutyrate, ethyl butyrate, ethyl 2-methyl-butyrate, ethyl isovalerate, isobutyl acetate, isoamyl acetate, β-phenethyl acetate, ethyl palmitate, 2-pentanone, diacetyl, isobutyric acid, and isovaleric acid. The mash analysis revealed that ketones, isobutyric acid, and isovaleric acid were not produced by yeast, but rather derived from red *koji*. Furthermore, high protease and lipase activities in red *koji* produced high concentrations of amino acids and LCFAs and upregulated the productions of higher alcohols and ethyl esters of LCFAs. At the same time, it appears that the high lipase activity promoted the esterification of short-chain acids. Furthermore, it is likely that the high concentration of higher alcohols is due to the corresponding acetate esters. As a result, red *koji* has direct and indirect effects on red *koji-shochu*.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.12.005.

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