Analysis of Volatile Compounds in Shochu Koji, Sake Koji, and Steamed Rice by Gas Chromatography-Mass Spectrometry

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ABSTRACT

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The volatile compounds in three types of koji (yellow, white, and black) and steamed rice were investigated using gas chromatography-mass spectrometry. The objective of this study was to characterize each koji type based on its profile of volatile compounds. From the steamed rice and rice koji samples examined, a total of 29 volatile compounds were identified. Butanal, hexanal, dimethyl trisulfide and cyclohexyl isothiocyanate were detected only in steamed rice. The volatile profile of yellow koji (sake koji) was distinct from those of white and black kojis (shochu koji). On the basis of relative peak areas, alcohols and aldehydes were identified as being more abundant in yellow koji. White and black kojis had similar volatile profiles, and had a greater abundance of furans than yellow koji. We assume that the different suites of volatile compounds identified in shochu and sake kojis impart the respective characteristic flavours to shochu and sake.

Key words: *Aspergillus* sp., GC-MS, large volume static headspace (LVSH), rice koji, shochu, volatile compounds.

INTRODUCTION

Shochu is a traditional Japanese distilled liquor made from various raw materials, including rice, barley, sweet potato, and buckwheat. In Asian countries, including Japan, there has been a long tradition of using moulds for brewing and in the production of fermented foods. Shochu production also involves the use moulds in the manufacturing process. It is well known that in beer and whisky brewing, starch is saccharified by β-amylase in the malt. In Asian brewing, moulds are also used as a source of starch hydrolases[14,18]. In the manufacturing processes of sake and shochu, moulds are grown as solid cultures on cereal crops such as rice and barley. These solid fungal cultures are referred to as 'koji'. The koji is subsequently incorporated into a mash containing the raw materials and yeast. The saccharification of starch materials and alcohol fermentation by yeast proceed simultaneously in the mash. This style of fermentation is thought to have originated 2,000–3,000 years ago in China[5,13]. The use of koji in brewing contributes to the distinctive character of Asian liquors, and sets them apart from other liquors from around the world.

In Japan, the representative koji mould is *Aspergillus* sp., which is cultured exclusively on rice; this is described as a rice koji. Yellow koji mould, *Aspergillus oryzae,* has been used for more than 2,000 years in Japan for the production of fermented foods, i.e., *sake,* soy sauce, and *miso*. In contrast, white koji mould (*Aspergillus kawachii*) and black koji mould (*Aspergillus awamori*) have been used exclusively in the manufacture of shochu. White koji mould is considered to be a mutant strain of the black koji mould, and each type has the ability to synthesize citric acid. Shochu is manufactured mainly in the southern part of Japan, which experiences a warm climate throughout the year. It is thought that the citric acid produced by white and black koji moulds decreases the pH of the mash and thus prevents contamination. Consequently, the white and black koji moulds are historically and technologically important microorganisms in the manufacture of shochu.

The volatile compounds of shochu have been the subject of previous investigations[11,22]. Furthermore, it has been shown that extracellular β-glucosidase from rice koji plays an important role in determining the flavour of shochu[16,17]. β-Glucosidase is involved in the hydrolysis of odourless glycosides in plant materials to volatile forms. For example, when sweet potato is used as a substrate, pleasant floral odour compounds such as linalool, α-terpineol, nerol, and geraniol are released from glycoside forms[17]. However, the monoterpenoid content in shochu is below the odour detection threshold. Consequently, the contribution of the sensory properties of monoterpenoids to the character of shochu has remained inadequately documented.

Analysis of the odour from rice koji itself has been carried out for yellow koji using gas chromatography-mass spectrometry (GC-MS). Ito et al.[8] identified 16 types of alcohols, ketones, and aldehydes and an ester from rice koji, and it was demonstrated that the growth rate of koji mould affects the composition of these volatile compounds. Subsequently, other volatile compounds, namely, 2-methyl-3-hepten-6-one, 1-octene-3-one, and phenylacetaldehyde, were identified as key odour molecules in rice

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koji; these are generally associated with the descriptors of nutty, mushroom, and rosy odour, respectively[20]. However, the respective volatile components of black and white koji moulds, and how each of these contributes to the flavour of shochu, have yet to be analysed. To date, research on shochu koji, in particular white koji, has been pursued from the perspective of producing koji and has focused on koji mycelia production, enzyme activity, and the composition of amino acids[9,15].

In this study, the volatile compounds from koji were collected using a large volume static headspace (LVSH) vessel, then concentrated, and finally analysed by GC-MS. LVSH is a simple, rapid, solventless, and low-cost sampling method for collecting volatile compounds, and it has previously been applied to the analysis of trace amounts of volatile compounds in drinking water and orange waste[23]. The present study focussed mainly on identifying and compiling profiles of the volatile compounds in the different types of rice koji.

MATERIALS AND METHODS

Chemicals

Authentic reference compounds for all of the volatile compounds identified in this study were purchased from Sigma-Aldrich (Steinheim, Germany), Wako (Osaka, Japan), and Tokyo Chemical Industry Co. (Tokyo, Japan).

Strains

The *Aspergillus* spp. used to investigate the volatile compounds of steamed rice and rice kojis were purchased from Kawachi Gen-ichiro Company (Kagoshima, Japan). Yellow koji mould (*Aspergillus oryzae*) is used for saccharification. White koji mould (*Aspergillus kawachii*) and black koji mould (*Aspergillus awamori* NK) are used in the manufacture of shochu. The characteristics of these moulds are described in Table I.

Preparation of rice koji

The process of manufacturing rice koji is typically carried out under conditions of controlled temperature and humidity for 2 days. Rice (1.6 kg) was soaked in water for 1 h, and then excess liquid was drained off for 1 h. The rice was then steamed for 1 h to produce a final moisture content of 37–38% (w/w). The steamed rice was cooled to 30°C and divided into four equal parts. Three of the separated portions of steamed rice were inoculated with 0.8 g conidia of one of the above-mentioned *Aspergillus* fungi. The remaining portion was left uninoculated. The fungi were grown at approximately 38°C. It is not necessary to heat the inoculated rice using an external source since the koji mould generates heat by respiration; however, cooling using an automatic blower is necessary in order to prevent overheating. Further, it was necessary to mix the rice grain in order to achieve effective temperature homogenization, having initially confirmed that the fungal mycelium had spread over the rice surface. In the final stages of the process, the temperature used for each koji mould type must correspond to that used in their commercial production. For the manufacture of yellow koji, the mould was then incubated continuously at approximately 38°C for 43 h. Growth of white and black kojis was controlled at 38°C for 16 h after inoculation, after which they were cultivated at 35°C for 27 h. This lower temperature is required for the production of citric acid by these moulds. The preparation of koji was repeated at least three times and GC-MS analysis was carried out on individual samples.

LVSH sampling

Headspace volatile components released from rice koji were collected in an LVSH system (Entech 7100A series; Entech Instruments Inc., Simi Valley, CA). Owing to differences in the water content of each sample, the following different weights of material were used to obtain the same solid content (70 g) for GC-MS analysis: steamed rice, 95.5–98.9 g; yellow koji, 85.3–86.1 g; white koji, 83.7–84.9 g; black koji, 81.1–83.7 g. The rice kojis were transferred to a 450-mL sample bottle and then incubated at 30°C for 30 min. Following incubation, the 100 mL headspace gas was vacuum extracted. The headspace 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 using an Entech 7100A preconcentrator and applied to the GC-MS system.

Analysis of volatiles by gas chromatography/mass spectroscopy

The identification and quantification of the volatile compounds released from steamed rice and rice kojis were performed using an Agilent 6890 series gas chromatograph (Agilent Technologies Inc., Palo Alto, CA), equipped with an Agilent 5975B mass-selective detector. All mass spectra were acquired in the 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.). The GC operation conditions were as follows: injector temperature, 220°C; transfer line, 250°C; quadruple ion trap temperature, 150°C; ion source temperature, 250°C. Analyses were carried out using helium as a carrier gas at a flow rate of 1.0 mL/min and with the following temperature programme: 40°C for 5 min, 3°C per min to 58°C, 0.2°C per min to 62°C, and 10°C per min to 240°C. The injector and detector were held at 250°C and 300°C, respectively. Peak identification was performed using Agilent ChemStation software and the NIST05a mass spectral database.

Table I. Characterization of koji moulds and kojis.

Product	Microorganism	Source	Citric acid synthesis
Yellow koji	*Aspergillus oryzae* (for sake)	Commercial	–
White koji	*Aspergillus kawachii* (for shochu)	Commercial	+
Black koji	*Aspergillus awamori* NK (for shochu)	Commercial	+

Identification of volatile components

The identity of the volatile components was confirmed by comparing their retention times and mass spectra with those of the authentic compounds. Otherwise, tentative identifications were made based on comparisons with the mass spectra data of the NIST05a mass spectral database.

Data analysis

Principal component analysis (PCA) was performed using Ekuseru-Toukei 2008 statistical software (Social Survey Research Information Co., Ltd., Tokyo, Japan) to assess differences in the volatile compounds of steamed rice and rice koji samples.

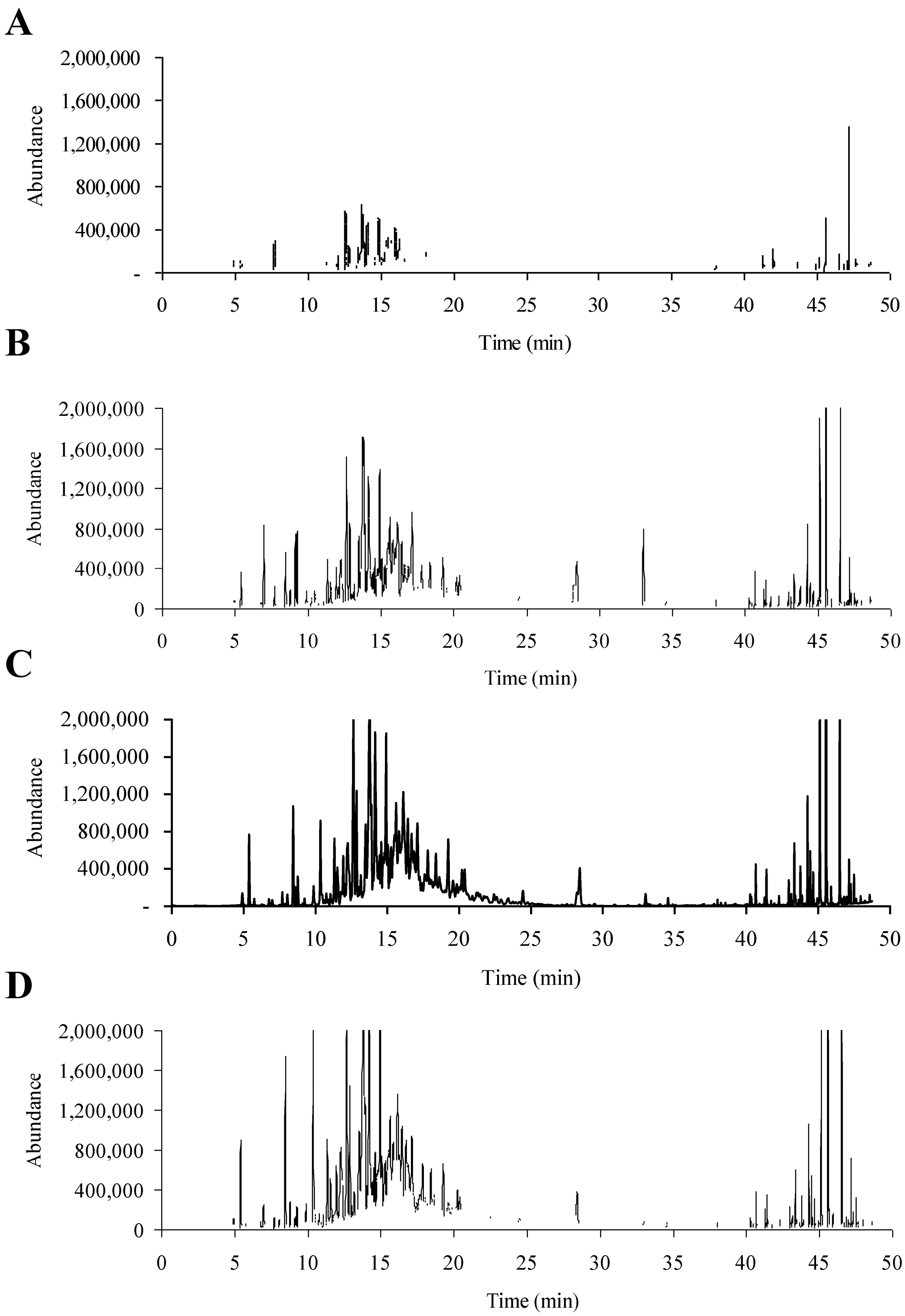

Fig. 1. Typical LSVH-HS-GC-MS chromatograms for steamed rice (A), yellow koji (B), white koji (C), and black koji (D).

RESULTS AND DISCUSSION

GC-MS results

Rice koji and steamed rice samples, ranging in weight from 81.1 to 98.9 g according to the water content of the sample, were placed in screw-capped bottles directly connected to a large volume static headspace system and analysed by GC-MS. It was observed that the total peak area for each of the rice koji samples was clearly greater than that for steamed rice (Fig. 1). In contrast, the total peak areas for the different rice kojis were all relatively similar. The total number of countable peaks (volatile compounds) for steamed rice, yellow koji, white koji, and black koji were 90, 138, 123, and 132, respectively. Volatile compounds were identified from mass spectra and retention times using authentic standards, or tentatively identified using the NIST05a MS database. In the steamed rice samples, the peaks of unidentified compounds mainly appeared at retention times between 10 and 20 min (Fig. 1A). Almost all of the unidentified peaks were assumed to be alkanes based on reference to the MS database. Since alkanes have a variety of molecular structures, it was difficult to accurately identify these compounds. The same tendency was observed in rice kojis, although the total content of these compounds in the kojis was greater than that in steamed rice. It has been suggested that the release of a large number of alkanes from steamed rice is attributable to the activity of enzymes such as lipases produced by the koji moulds (Fig. 1B, 1C, 1D)[20,21].

Comparison of volatile compounds in steamed rice and rice kojis

Four alkanes, four alcohols, five aldehydes, one ketone, four furans, one ester, six phenols, and four other compounds were identified and quantified in the steamed rice and rice koji samples. The largest peak areas for the steamed rice and rice koji samples were designated as 100, and all other peak areas were scored relative to these maximum values. The relative peak areas of these compounds are listed in Table II. Eleven volatile compounds were detected in all samples. Of these compounds, cyclopentane, heptane, n-octane, 2-pentylfuran, α-methyl-

Table II. Relative percentage (%) of volatile compounds identified by LVSH-GC/MS analysis.

No.	Compounds	Retention time (min)	Quantification ions (m/z)	Identification	Samples[a]			
					Yellow koji	White koji	Black koji	Steamed rice
	alkanes (4)							
(1)	cyclopentane	5.40	55	MS[b], Std[c]	50.9	91.9	100	51.7
(2)	heptane	5.76	71	MS, Std	64.6	87.6	80.3	100
(3)	n-octane	6.78	85	MS, Std	52.7	55.1	100	46.0
(4)	trans 2-octene	7.82	55,112	MS, Std	100	N.D.[d]	93.7	N.D.
	alcohols (4)							
(5)	isobutyl alcohol	17.11	43,31	MS, Std	100	62.4	N.D.	7.43
(6)	2-methyl-1-butanol	28.36	57	MS, Std	100	42.3	14.4	N.D.
(7)	3-methyl-1-butanol	28.63	55	MS, Std	100	67.3	28.2	N.D.
(8)	1-octen-3-ol	40.45	57,72	MS, Std	100	44.1	89.6	N.D.
	aldehydes (5)							
(9)	isobutyraldehyde	7.00	72	MS, Std	100	17.6	48.1	1.76
(10)	butanal	8.19	72,43	MS, Std	N.D.	N.D.	N.D.	100
(11)	2-methylbutyraldehyde	9.13	57	MS, Std	100	10.4	15.7	N.D.
(12)	isovaleraldehyde	9.25	44	MS, Std	100	10.8	19.1	N.D.
(13)	hexanal	16.15	56,44	MS, Std	N.D.	N.D.	N.D.	100
	ketones (1)							
(14)	2-butanone	8.77	43,72	MS, Std	56.2	100	67.9	6.63
	furans (4)							
(15)	2-methylfuran	8.05	82,53	MS, Std	18.2	100	64.5	7.37
(16)	3-methylfuran	8.62	82,53	MS, Std	43.8	100	44.3	N.D.
(17)	2,5-dimethylfuran	10.36	96,53	MS, Std	N.D.	57.3	100	N.D.
(18)	2-pentylfuran	30.83	81,138	MS, Std	42.3	41.8	30.9	100
	ester (1)							
(19)	isopropyl formate	7.64	73	MS, Std	N.D.	100	N.D.	N.D.
	phenols (6)							
(20)	ethylbenzene	19.26	91,106	MS, Std	69.0	93.8	100	16.0
(21)	propylbenzene	27.33	91,120	MS, Std	N.D.	94.3	100	N.D.
(22)	α-methylstyrene	36.95	118,103	MS, Std	100	95.0	85.0	68.0
(23)	benzaldehyde	41.95	106,77	MS, Std	50.2	N.D.	N.D.	100
(24)	phenylacetaldehyde	43.93	91,120	MS, Std	100	N.D.	N.D.	N.D.
(25)	acetophenone	44.10	105,77	MS, Std	81.0	57.9	71.2	100
	others (4)							
(26)	limonene	26.15	68,93	MS, Std	75.8	90.0	98.8	100
(27)	1,8-cineole	27.50	81	MS, Std	N.D.	92.0	100	N.D.
(28)	dimethyl trisulfide	38.55	126	MS	N.D.	N.D.	N.D.	100
(29)	cyclohexyl isothiocyanate	44.48	55,141	MS, Std	N.D.	N.D.	N.D.	100

[a] 100, largest peak area obtained. Results are expressed to three significant figures.
[b] Compounds identified by comparison with the NIST05a mass spectral database.
[c] Components identified by comparing their retention times and mass spectra with those of the authentic compounds.
[d] Not detected.

styrene, acetophenone, and limonene had similar peak areas despite the differences in sample type. The compound 2-pentylfuran was previously reported as an odorant in cooked rice of California long-grain and brown rice cultivars[2,10]. It is considered that 2-pentylfuran contributes to the distinctive rice flavour of rice koji.

Seventeen volatile compounds were identified in steamed rice samples. Butanal, hexanal, dimethyl trisulfide, and cyclohexyl isothiocyanate were characterized as unique compounds in steamed rice. It is known that hexanal is produced nonenzymatically or by an unknown pathway from linoleic acid in rice[12,19,25]. Moreover, it was previously shown that aldehydes represent the highest percentage of total volatiles in cooked rice[24], and dimethyl trisulfide has been identified as one of the compounds that make a significant contribution to the odour of rice cakes[3]. Accordingly, it is assumed that these aliphatic aldehydes and sulfide compounds were produced by steam heating, and were then reduced during the koji production process. Dimethyl trisulfide is responsible for the 'off-flavour' associated with sake, and its prominence increases as the sake ages[7]. Thus, during the production of rice koji, the off-flavour derived from rice decreases.

Seventeen compounds were detected in all the koji samples analysed. Previously, isobutyraldehyde, isovaleraldehyde, 1-octen-3-ol, and phenylacetaldehyde were identified as characteristic compounds in yellow koji[8,20]. In this study, isobutyraldehyde, isovaleraldehyde, and 1-octen-3-ol were also found in all rice koji samples; however, phenylacetaldehyde was detected only in yellow koji samples. Yellow rice koji was found to contain the largest amounts of isobutyraldehyde, isovaleraldehyde, and 1-octen-3-ol, suggesting that these compounds are important for the aroma of yellow koji. Isopropyl formate was only detected in white koji and no unique compounds were identified in black koji. White and black kojis have only three volatile compounds in common, namely, 2,5-dimethylfuran, propylbenzene, and 1,8-cineole, that were not found in yellow koji and steamed rice. 1,8-Cineole is the compound often used in the fragrance industry to impart a fresh odour, and the term *eucalyptus* is used to describe the spicy, mint-like aroma that 1,8-cineole imparts to red wines[6]. It has been reported that 1,8-cineole is produced by the chemical transformation of limonene and α-terpineol[4]. Amounts of 1,8-cineol and limonene were small in all samples analysed in this study. However, it is expected that these compounds contribute to the respective characteristic odours because both compounds typically have low odour thresholds of approximately 1–64 μg/L and 4–229 μg/L, respectively[1].

Assessment of the relative importance of different volatile compounds to each koji type

In order to assess the importance of individual volatile compounds to each of the different types of rice koji, we compared steamed rice and rice koji samples in terms of the total peak area of volatile compounds divided into five classes according to functional group and molecular structure: alkanes, alcohols, aldehydes, furans, and phenols (Fig. 2). Alkanes were not significantly changed among all samples, whereas the proportions of alcohols and aldehydes were highest in yellow koji. It is accordingly assumed that alcohols and aldehydes are responsible for the distinctiveness of this koji type. Among the three rice koji types analysed, the total contents of phenols were almost equivalent. Further, the compound profiles of white and black kojis were almost identical, and the content of furan compounds was considerably higher in the white and black kojis than in yellow koji (Fig. 2).

In order to identify which volatile compounds in steamed rice and rice kojis are likely to contribute most to the different characters of these products (Fig. 3), principal component analysis, (PCA) using the relative per-

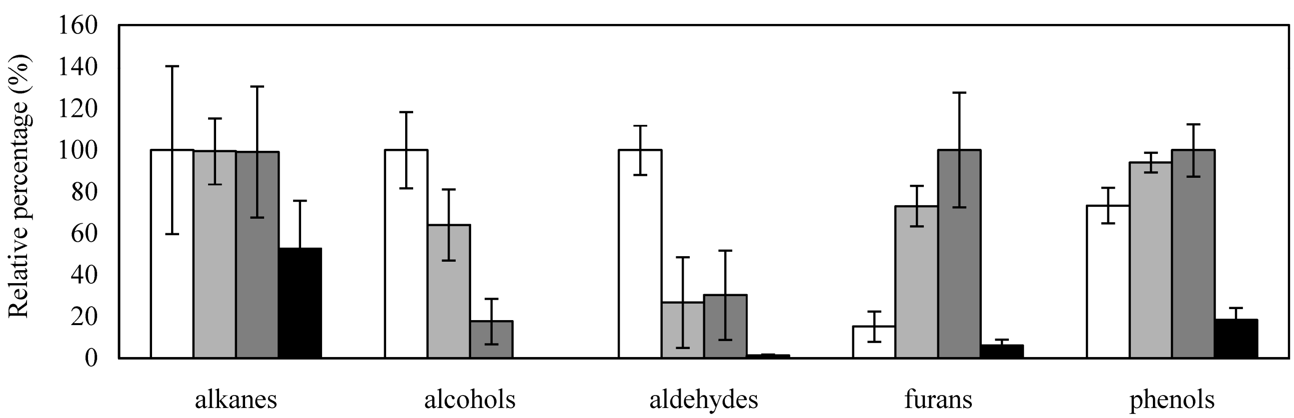

Fig. 2. Relative percentage of total peak areas of the compound classes in steamed rice and the different rice koji samples. The largest peak areas for steamed rice and rice kojis samples were designated as 100%. 'Alkanes' is the sum of the alkane compounds cyclopentane, heptanes, n-octane, and trans-2-octene. 'Alcohols' is the sum of isobutyl alcohol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 1-octen-3-ol. 'Aldehydes' is the sum of isobutyraldehyde, butanal, 2-methylbutyraldehyde, isovaleraldehyde, and hexanal. 'Furans' is the sum of the furan compounds 2-methylfuran, 3-methylfuran, 2,5-dimethylfuran, and 2-pentylfuran. 'Phenols' is the sum of the phenol compounds ethylbenzene, propylbenzene, benzaldehyde, and phenylacetaldehyde. Yellow rice koji (white bars), white rice koji (light grey bars), black rice koji (grey bars), and steamed rice (black bars). Three independent experiments were performed for yellow koji and white koji. Five independent experiments were performed for steamed rice and black koji. The error bars show the standard deviations.

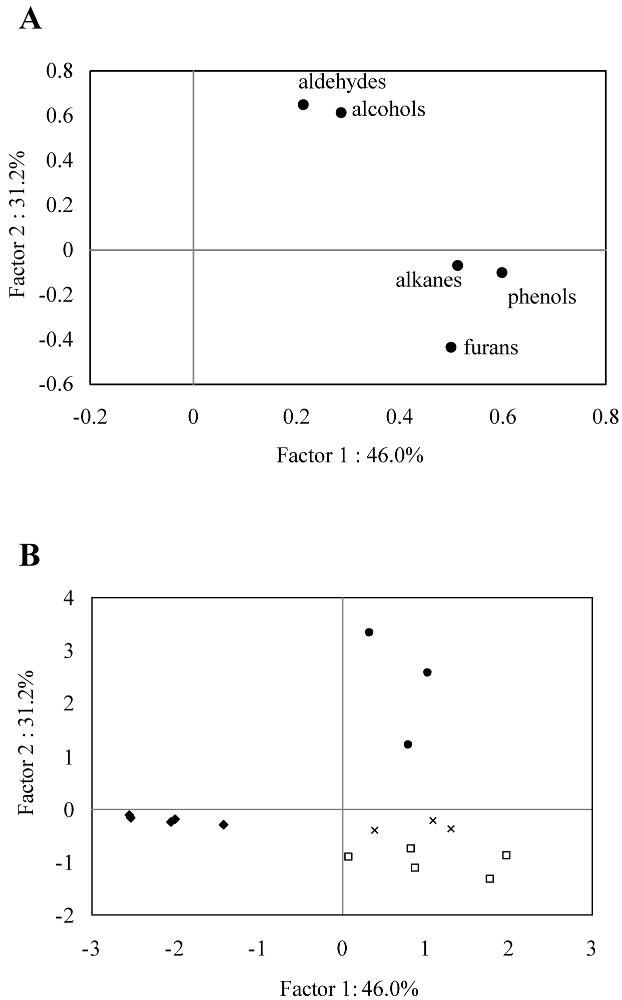

Fig. 3. PCA biplot of the volatile compounds in different rice koji samples and steamed rice. (A) The separation of five volatile compound classes based on the sum of the peak area of the volatile compounds. (B) The distribution of each sample in relation to the sum of the peak area of volatile compounds. Diamonds, circles, cross marks, and open squares indicate the individual steamed rice samples, and yellow, white and black koji samples, respectively.

centage values for the five classes of volatile compounds shown in Fig. 2, was performed. The five volatile compound classes segregated with 46.0 and 31.2% of total variance, accounted for by the first and second principal components, respectively (Fig. 3A). Alcohols and aldehydes were segregated from alkanes, phenols, and furans in factor 2. Furans were also segregated from alkanes and phenols in factor 2. Among the furans identified, 2,5-dimethylfuran was found to be unique to white and black kojis, and it was considered that the furan compounds in these kojis may contribute to their characteristic aroma. This analysis indicated that yellow koji is richer in total alcohols and aldehydes when compared with steamed rice and the other rice koji samples, regardless of the unique aldehyde compounds in steamed rice. PCA using all five volatile compound classes indicated that yellow koji could be readily separated from white and black kojis, and that the volatile compound profiles of white and black kojis are essentially similar (Fig. 3B).

Although the relative contributions of the different classes of volatile compounds to the flavour of each type of rice koji is unclear, the LVSH/GC-MS analysis enabled us to detect the uniqueness of the volatile compounds that may contribute to the characteristic aromas of the respective white and black koji cultivars. This analysis thus indicates that the koji moulds used for shochu manufacture contain characteristic compounds that are distinct from those of the yellow koji used for producing sake.

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