Original paper

Effect of Maturation Time on Koji-like Smell and Volatile Compounds of Barley Miso (Japanese Soybean Paste) during Fermentation

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Barley miso (Japanese soybean paste) is generally classified into either the light-colored type or the red type based on a short or long maturation time, respectively. In this study, we characterized barley miso with a focus on its koji-like smell. Sensory evaluation showed that koji-like smell was significantly reduced during the maturation process, especially after two months. However, comprehensive analysis of volatile compounds indicated that known constituents of koji-like smell, 1-octen-3-ol and phenylacetaldehyde, were present in similar and lower concentrations, respectively, in the light-colored barley miso compared to the red barley miso. In addition, most compounds, such as aldehydes and esters, were found in larger concentrations in the red barley miso than in the light-colored barley miso. These results suggest that maturation time has an important role in koji-like smell, and the contributions of decreases in 1-octen-3-ol and phenylacetaldehyde to koji-like smell during the maturation process remains unclear.

Keywords: light-colored barley miso, red barley miso, volatile compounds, koji-like smell, 1-octen-3-ol, phenylacetaldehyde

Introduction

Miso is a traditional Japanese soybean paste and can be divided into four types: rice miso, barley miso, soybean miso, and blended miso. According to a 2017 survey by the Japan Federation of Miso Manufacturers Cooperatives¹⁾, the market shares of different miso types are as follows: rice miso (81.2 %), barley miso (4.3 %), soybean miso (4.8 %), and blended miso (9.7 %). Different types of miso are based on different types of koji, which are cereals covered with the yellow koji fungus *Aspergillus oryzae*. Moreover, the taste and color of miso vary among Japanese regions, making miso a local specialty. According to its taste and color, rice miso can

be further classified into three categories: red salty rice miso, thin-colored salty rice miso, and weak salty rice miso, which are generally matured for about 12 months, 6 months, and 20 days, respectively (Ohata *et al.*, 2009). The maturation time of light-colored miso is generally shorter than that of the dark-colored one. Similarly, barley miso can also be classified into two categories: light-colored barley miso and red barley miso (Domoto, 1982). The former is matured for about 1 to 3 months, and the latter is matured for about 6 months to 1 year.

In this study, we attempted to characterize the koji-like smell of light-colored barley miso in comparison to red barley miso. Barley miso is mainly produced and consumed in the

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Yamaguchi and Ehime Prefectures as well as in the Kyushu–Okinawa region of Japan. In Kagoshima Prefecture, the southern part of Kyushu, barley miso is generally matured around 1 month. The short maturation time is thought to prevent the consumption of sugar by the yeast population and results in a sweet taste and fresh koji aroma, which is preferred by consumers in Kagoshima Prefecture.

The koji fungus *A. oryzae* has an important role in degrading polysaccharides and proteins in the product (Machida *et al.*, 2008). In addition, *A. oryzae* also produces odoriferous compounds, including 1-octen-3-ol, which is described as a main component of the mushroom-like smell of koji (Ito *et al.*, 1990; Kamiński *et al.*, 1972). It was previously reported that the mixture of five compounds, including 2-methyl-2-hepten-6-one, methional, 1-octen-3-one, 1-octen-3-ol, and phenylacetaldehyde, could synergistically represent koji-smell with a similarity level of 88.3% during the study course of the sake koji (Takahashi *et al.*, 2006). Among these five compounds, three compounds, methional, 1-octen-3-one, and phenylacetaldehyde, which have a rose-like odor, especially contributed to the koji-smell. We, therefore, investigated the effect of maturation time on volatile compounds associated with the koji-like smell of barley miso, using GC-MS equipped with a headspace automatic concentration device.

Materials and Methods

Materials Miso samples used in this study are listed in Table 1. The production of five types of barley miso with different maturation times was carried out at a miso factory (Hotaru Jyozo, Kagoshima, Japan). Approximately 240 kg of steamed barley was mixed with *A. oryzae* (Kawachi Genichiro Shoten, Kagoshima, Japan) and incubated in the koji manufacturing room for 40 h. The temperature was gradually increased from 32 °C to 36–38 °C during the initial 10 h, maintained at 36–38 °C for 18 h, and reduced to 33–35 °C for 12 h. Then, the barley koji was mixed with approximately 72 kg of steamed soybeans and 50 kg of sodium chloride, and incubated at 28 °C during the maturation period. The sampling of barley miso was performed after incubation for 20, 30, 60, 90, or 365 days, and named as H20, H30, H60, H90, and H365, respectively (Table 1). H20 and H365 were used as the representative light-colored barley miso sample and red barley miso sample, respectively, because H20 and H365 are from the commercial line-up.

In addition, four commercial light-colored barley miso samples (A, B, C, and D) and four commercial red barley miso samples (E, F, G, and I) were randomly selected. No addition of lactic acid bacteria and yeast was confirmed by interviews with manufacturers. All light-colored barley misos were produced in different factories in the Kagoshima Prefecture. The miso samples were preserved at –20 °C until further use for analysis.

Sensory evaluation of miso samples Barley and rice miso

odors were evaluated by 13 assessors (six males and seven females) from Kagoshima University. Koji is an ingredient of both miso and shochu (a traditional distilled spirit). Assessors were therefore trained by smelling miso and shochu. We suspended 10 g of miso in 20 mL of boiled water in a 100 mL Erlenmeyer flask to represent the drinking condition of miso soup. Assessors evaluated the level of koji-like smell of barley miso samples matured for different times (H20, H30, H60, H90, and H365) based on a blind sniff test. The sensory score was conducted on a scale of 0, 1, 2, 3, 4, and 5 points, where 5 represented a strong koji-like smell and 0 represented the absence of a koji-like smell.

GC-MS analysis Large-volume static-headspace sampling and subsequent GC-MS analysis were performed as previously described with some modifications (Shiraishi *et al.*, 2016; Rahayu *et al.*, 2017). Briefly, 10 g of miso was dissolved in 10 mL of water in a 500 mL bottle, and 1 mL of 10 mg/L 1-pentanol was spiked as an internal standard. In order to compare the aromas in miso soup, the bottle containing the sample was kept in a water bath at 70 °C for 30 min. Then, 200 mL of headspace gas was aspirated and subsequently concentrated by the headspace automatic concentration device (ENTECH 7100 A; Entech Instruments Inc., CA, USA).

Volatile components of miso were analyzed with GC-MS (GC, Agilent 6890; MS, Agilent 5979B; Agilent Technologies, CA, USA) equipped with a DB-WAX column (60 m × 0.25 mm, i.e., 0.25-μm film; Agilent Technologies). The procedure was repeated three times, and the average value was computed. Volatile components were identified using the Agilent ChemStation software with the NIST 05a mass spectral library, and by the retention index retrieved from the database in the Aroma Offices software (Nishikawa Meter Co., Ltd., Tokyo, Japan). Standard solution containing *n*-Paraffin Mix C₅, C₆, C₇, C₈ (Supelco, PA, USA) and *n*-Paraffin Mix C₁₀, C₁₂, C₁₄, C₁₆ (Supelco) was used to calculate the retention index.

Statistical analysis Differences in volatile compounds detected by GC-MS in light-colored barley miso and red barley miso were statistically evaluated with the Wilcoxon rank-sum test and principal component analysis (PCA), using the FactoMineR package in R version 3.4.1 (Lê *et al.*, 2008).

Results and Discussion

Sensory evaluation of barley miso with different maturation periods To verify the decrease in the koji-like smell of barley miso during maturation, we performed a sensory evaluation of the barley miso samples H20, H30, H60, H90, and H365 with different maturation periods (20, 30, 60, 90, and 365 days, respectively) prepared at the same miso factory (Fig. 1). Although the scores were similar for barley miso samples H20, H30, and H60, the score was significantly reduced in H90 and further reduced in H365 (a typical red barley miso). This result indicates that the fresh aroma of koji could be maintained for a period of 20 to 60 days of maturation time. In addition, this

Table 1. Volatile compounds detected by GC-MS analysis in barley miso samples.

Compounds	Retention index ^a	Peak area of analyte / Peak area of internal standard											
		Test products				Commercial samples (light-colored barley miso)				Commercial samples (red barley miso)			
		H20 (20 days)	30 days (30 days)	H60 (60 days)	H90 (90 days)	H365 (365 days)	A (2 months)	B (2 months)	C (1 to 2 months)	D (1 to 2 months)	E (more than 1 year)	F (3 to 5 months)	G (more than 3 years)
acetaldehyde	739	0.04490	0.02723	0.03919	0.04305	0.33322	0.08971	0.12821	0.29175	0.39436	0.30232	0.33614	0.19648
isobutyraldehyde	795	0.01883	0.02160	0.08233	0.20125	0.23095	0.02779	0.02344	0.00539	0.10312	0.07465	0.06399	0.43388
ethyl acetate	872	1.62954	1.40704	1.61404	1.63528	8.33188	9.02154	2.81788	21.38140	19.80140	8.67186	9.54263	19.74710
2-methylbutyraldehyde	900	0.13628	0.16971	0.74915	1.59792	1.57822	0.15361	0.11202	0.04103	0.65435	0.59149	0.60693	4.89599
isovaleraldehyde	904	0.39838	0.29517	0.61579	0.85532	4.81779	0.24239	0.21755	0.07489	0.74137	1.82107	1.64696	5.36092
ethyl propionate	944	0.02332	0.01829	0.02141	0.02087	0.51729	0.42621	0.08367	1.92014	0.93435	0.24476	1.14953	2.17117
ethyl isobutyrate	952	0.06212	0.05772	0.08341	0.08093	1.61981	0.19092	0.60833	0.52769	3.58015	0.36970	3.52566	4.16055
1-propanol	1031	0.09393	0.06228	0.00765	0.05603	0.10812	0.32022	0.14196	0.66933	0.35774	0.04320	0.27259	0.11992
ethyl 2-methylbutyrate	1043	0.03171	0.03014	0.05238	0.05124	0.90106	0.28961	0.20944	0.14985	1.03385	0.13480	1.35149	1.91673
hexanal	1071	0.03424	0.02589	0.04739	0.04135	0.14945	0.00462	0.04983	0.05026	0.06015	0.09539	0.07717	0.10357
2-methyl-1-propanol	1091	0.00200	0.20855	0.17885	0.20684	2.19546	4.80718	1.63794	6.33698	3.72374	1.07925	6.53551	1.78327
isoamyl acetate	1116	0.02183	0.00838	0.01100	0.00998	1.35562	0.33031	0.06195	1.35652	1.84207	0.77550	1.26429	1.15901
1-butanol	1139	0.40480	0.00852	0.00857	0.00901	0.10289	1.35896	0.42531	1.37297	1.78944	0.17501	3.96440	0.62421
isoamyl alcohol	1207	0.00052	0.58943	0.52593	0.50137	12.01510	0.00169	3.21534	31.45190	0.01632	9.05055	16.62072	7.54624
ethyl hexanoate	1225	0.01199	0.00884	0.01566	0.01283	0.16877	0.23228	0.12200	0.38972	0.65542	0.16138	0.18267	0.21717
ethyl <i>n</i> -octanoate	1424	0.01817	0.00680	0.00811	0.00636	0.06008	0.03438	0.05628	0.21317	0.19645	0.05529	0.12107	0.09880
1-octen-3-ol	1442	0.19243	0.14992	0.21646	0.19045	0.48590	0.42464	1.20333	0.17996	0.70442	0.20288	0.22906	0.21262
furfural	1451	0.03121	0.03327	0.07802	0.10129	0.19011	0.04221	0.05564	0.06184	0.06487	0.19983	0.36455	0.12963
2-ethyl-1-hexanol	1481	0.01621	0.01695	0.01881	0.02003	0.03359	0.02896	0.01312	0.01939	0.01989	0.02429	0.02671	0.03262
benzaldehyde	1513	0.21680	0.18705	0.41226	0.39901	0.26248	0.19395	0.11313	0.06692	0.10323	0.39907	0.20789	0.27629
ethyl decanoate	1631	0.00822	0.00508	0.00458	0.00414	0.01286	0.00643	0.04696	0.04162	0.02889	0.00716	0.01435	0.01149
phenylacetaldehyde	1634	0.11235	0.10779	0.17889	0.19591	0.61896	0.08088	0.16368	0.18918	0.20683	0.77720	0.45836	0.50286
ethyl benzoate	1662	0.03719	0.03465	0.04941	0.04222	0.31739	0.07685	0.21977	0.33155	0.13793	0.10698	0.72735	1.28703
ethyl phenylacetate	1779	0.03446	0.03761	0.05079	0.04519	0.17061	0.00006	0.09412	0.10778	0.18226	0.09923	0.54994	0.59364

^aRetention index was analyzed by comparison with the retention indexes in the AromaOffice database (Nishikawa Meter Co., Ltd., Tokyo, Japan).

Fig. 1. Effect of maturation time of barley miso on the koji-like smell. Sensory evaluation of barley miso with different maturation periods (20, 30, 60, 90, and 365 days) was scored on a scale of 0–5 points, where 5 was the score given to a strong koji-like smell.

result corresponded to the fact that light-colored barley miso is generally shipped out at maturation times of less than 2 months.

Comparison of volatile compounds of barley miso with different maturation periods To investigate the effect of maturation time on the volatile compounds impacting the aroma of koji, we analyzed the barley miso samples H20, H30, H60, H90, and H365 of different maturation periods, which could be classified by the sensory evaluation of koji-like smell (Fig. 1). GC-MS analysis identified a total of 24 compounds commonly detected in all barley miso samples (Table 1). We focused on the concentrations of 1-octen-3-ol and phenylacetaldehyde, as these compounds are known to contribute to koji-like smell (Ito *et al.*, 1990; Shiraishi *et al.* 2016; Takahashi *et al.*, 2006; Takahashi *et al.*, 2007).

The concentration of 1-octen-3-ol did not change from 20 days (H20) to 90 days (H90) and then increased from 90 days (H90) to 365 days (H365), whereas the concentration of phenylacetaldehyde increased gradually from 20 days (H20) to 365 days (H365) (Table 1). The fresh koji aroma was significantly reduced from 60 days (H60) to 90 days (H90) (Fig. 1); thus, this result might imply that the reduction of koji-like smell might not be caused by the decrease of 1-octen-3-ol and phenylacetaldehyde. The results of GC-MS analysis also indicated that most compounds, except for 1-propanol and 1-butanol, increased during the maturation periods (Table 1). When the concentrations of 1-octen-3-ol and phenylacetaldehyde were evaluated by the proportion of area value to all area values, the area value of 1-octen-3-ol was reduced from 60 days (H60) to 365 days (H365), whereas that of phenylacetaldehyde was reduced from 90 days (H90) to 365 days (H365) (Fig. 2). Although the concentrations of both 1-octen-3-ol and phenylacetaldehyde increased depending on the length of the maturation period, the relative amount of 1-octen-3-ol and phenylacetaldehyde compared to the volatile compounds

Fig. 2. Proportion of area values of 1-octen-3-ol and phenylacetaldehyde to all the area values. Calculation was performed from the dataset in Table 1.

as a whole decreased.

Comparison of volatile compounds between commercial light-colored barley miso and red barley miso To further clarify differences in koji-like smell between light-colored barley miso and red barley miso, we additionally analyzed the volatile compounds of commercial light-colored barley miso (A, B, C, and D) and red barley miso (E, F, G, and I) using GC-MS. A total of 24 compounds were also detected in the commercial barley miso samples (Table 1). The H20 and H365 samples were from the commercial line-up of the miso company (Hotaru Jyozo). Therefore, these two samples were included in the comparative analysis.

Fig. 3A and B shows PCA scores of the first principal component and second principal component (PC1 and PC2) and a biplot of their factor loadings, respectively, for all the commercial barley miso samples, consisting of five light-colored barley misos (A, B, C, D, and H20) and red barley misos (E, F, G, I, and H365). The cumulative contribution ratio of PC1 and PC2 showed that they accounted for 64.98 % of variation in the dataset. Along the first component, most of the miso samples were grouped into two clusters according to the maturation time. In particular, four of five red barley misos (F, G, I, and H365) were clustered on the right of the plot, whereas four of five light-colored barley misos (A, B, C, and H20) were clustered on the left of the plot (Fig. 3A). The first principal component (PC1) correlated positively with ethyl acetate, isovaleraldehyde, 2-methylbutyraldehyde, ethyl isobutyrate, ethyl propionate, ethyl 2-methylbutyrate, ethyl benzoate, isoamyl acetate, ethyl phenylacetate, isobutyraldehyde, acetaldehyde, hexanal, ethyl *n*-octanoate, and 2-ethyl-1-hexanol (Fig. 3B, Table 2). This result is consistent with the result in which the concentrations of most volatile compounds

Fig. 3. PCA plot based on volatile compounds of commercial light-colored barley miso (A, B, C, D, and H20) and red barley miso (E, F, G, I, and H365). The analysis was performed on the dataset in Table 1. The individuals factor map (A) and variables factor map (B) for the two first principal components.

increased during the maturation time (Table 1).

In addition, the average concentrations of all the compounds that showed a statistically significant difference between the two barley miso types (Table 3; p -value < 0.05; furfural, hexanal, isovaleraldehyde, phenylacetaldehyde, benzaldehyde, 2-ethyl-1-hexanol, 2-methylbutyraldehyde, and isobutyraldehyde) were higher in the red barley miso than in the light-colored barley miso. The concentration of phenylacetaldehyde was 3.6-fold higher in the red barley miso than that in the light-colored barley miso (Table 3; p -value < 0.05). This was contrary to our expectations because phenylacetaldehyde is one of the known important aromas of

Table 2. Loadings of the principal components.

Variables	PC1	PC2
ethyl acetate	0.711	0.523
isoamyl alcohol	0.451	0.433
isovaleraldehyde	0.780	-0.535
2-methylbutyraldehyde	0.802	-0.338
ethyl isobutyrate	0.866	0.062
ethyl propionate	0.781	0.371
ethyl 2-methylbutyrate	0.877	-0.131
2-methyl-1-propanol	0.328	0.742
ethyl benzoate	0.847	-0.140
isoamyl acetate	0.835	0.364
1-butanol	0.440	0.492
ethyl phenylacetate	0.756	-0.155
phenylacetaldehyde	0.493	-0.589
isobutyraldehyde	0.804	-0.400
benzaldehyde	0.218	-0.851
ethyl hexanoate	0.365	0.705
1-octen-3-ol	-0.356	0.149
acetaldehyde	0.683	0.293
furfural	0.543	-0.253
1-propanol	0.020	0.916
hexanal	0.724	-0.395
ethyl n-octanoate	0.621	0.598
2-ethyl-1-hexanol	0.752	-0.296
ethyl decanoate	0.020	0.674
% of Variance	40.88	24.10
Cumulative % of variance	40.88	64.98

koji (Takahashi *et al.*, 2006). On the other hand, the PCA (Fig. 3B) showed that only the 1-octen-3-ol tended to present at a higher concentration in the light-colored barley miso than in the red barley miso. The concentration of 1-octen-3-ol was 0.5-fold lower in red barley miso than that in light-colored barley miso; however, this was not statistically significant (Table 3). Based on this result, it was difficult to conclude that the larger amount of 1-octen-3-ol contributes to the characteristic koji-like smell of light-colored barley miso.

In this study, we showed that maturation time has a significant role in the decrease of the koji-like smell of barley miso. However, interestingly, known components of koji-like smell, i.e., 1-octen-3-ol and phenylacetaldehyde, were present at similar and lower concentrations, respectively, in light-colored barley miso compared to red miso. In addition, we showed that most compounds, such as aldehydes and esters, were increased during the maturation process. One hypothesis is that there are other compounds that contribute to the koji-like odor in light-colored barley miso. Previous studies have investigated the odoriferous compounds of miso, and more than 200 compounds have been identified in rice miso (Honnma, 1987). For example, alkylpyrazines have been

Table 3. Evaluation of volatile compounds between light-colored barley miso and red barley miso by Wilcoxon rank sum test.

Compounds	Ratio of average ^a (red/light)	<i>p</i> -value ^b
furfural	4.0	0.008
hexanal	2.8	0.008
isovaleraldehyde	10.6	0.008
phenylacetaldehyde	3.6	0.008
benzaldehyde	2.0	0.016
2-ethyl-1-hexanol	1.6	0.032
2-methylbutyraldehyde	10.3	0.032
isobutyraldehyde	6.2	0.032
ethyl phenylacetate	3.8	0.056
ethyl benzoate	4.0	0.095
ethyl 2-methylbutyrate	3.2	0.151
isoamyl alcohol	1.8	0.151
1-propanol	0.5	0.222
acetaldehyde	1.6	0.222
ethyl isobutyrate	2.6	0.222
ethyl propionate	1.6	0.310
isoamyl acetate	1.8	0.421
ethyl <i>n</i> -octanoate	1.3	0.548
ethyl decanoate	0.6	0.690
1-octen-3-ol	0.5	0.841
ethyl hexanoate	0.8	0.841
1-butanol	1.4	1.000
2-methyl-1-propanol	0.9	1.000
ethyl acetate	1.2	1.000

^aAverage of area values of red barley miso (E, F, G, I, and H365) was divided by that of light-colored barley miso (A, B, C, D, and H20).

^bAnalysis was performed for area values between the light-colored barley miso and red barley miso.

identified as basic flavor compounds and are known to contribute to miso flavor (Mori *et al.*, 1983). In addition, 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone (HEMF) has also been identified as a key odoriferous compound in rice and barley misos (Sugawara, 1991; Hayashida *et al.*, 1998). HEMF is produced by the yeast *Zygosaccharomyces rouxii* and is characterized by a sweet smell resembling that of caramel and shortcake (Kobayashi and Sugawara, 1999). In this study, we could detect 1-octen-3-ol and phenylacetaldehyde, but not other key compounds such as HEMF. Our findings provided insight into the characteristic koji-like smell of light-colored barley miso; however, further studies are required for a more comprehensive analysis.

Additionally, the possibility exists that the high levels of other odoriferous compounds may mask the koji-like smell in red barley miso, as the relative amounts of 1-octen-3-ol and phenylacetaldehyde in the volatile compounds might be decreased during the maturation process (Fig. 2). For example,

when wine is exposed to oxygen, furfural and hexanal are correlated with the appearance of cooked vegetable and pungent odors, respectively, thus, furfural and hexanal are known to mask the wine's aroma (Jackson, 2011). In addition, benzaldehyde is also known to enhance the odor-masking effect of trimethylamine (fishy odor) (Shiraishi *et al.*, 1982). To obtain a better understanding of the importance of maturation time on barley miso, it is clear that quantitative analysis of volatile compounds is needed to reconstruct the aroma of different types of barley miso. In addition, the effects of aldehyde and ester compounds should also be investigated to verify our latter hypothesis, that aldehyde and ester compounds mask the koji-like smell of barley miso aged for an extended duration. We are currently focusing on these studies.

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