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Investigation of relationship between sake-making parameters and sake metabolites using a newly developed sake metabolome analysis method

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We developed a sake metabolome analysis method using liquid chromatography–quadrupole/time-of-flight mass spectrometry to investigate the metabolome of various types of sakes and other alcohol beverages. Our method identified 198 compounds by comparison with standard metabolites. Using this method, we investigated the relationship between several sake-making parameters and sake metabolites by conducting combination experiments of these parameters using small-scale fermentation. The results indicated that all parameters significantly affected sake metabolites ($P < 0.005$) and most peaks were affected by multiple sake-making parameters. Interestingly, the effect of the rice cultivar on sake metabolites was higher for *koji* rice than for *kake*-rice. This result suggests that the rice cultivar used has a greater effect on the characteristics of *Aspergillus oryzae* compared to sake yeast and affects sake metabolites. In this study, we also evaluated the combined effect of several parameters. We demonstrated the different effects of each parameter on several amino acids. The results showed a new aspect of the science of sake making. For example, the amount of α-ethylglucoside, which can affect the taste of sake, was negatively correlated with α-glucosidase activity in *koji* ($r = -0.84$). In this study, various unidentified peaks were observed; detectable peaks can be increased by analyzing additional standard reagents. Investigating these unidentified peaks and accumulating datasets for sake-making parameters will give us insight into how to improve sake taste and quality.

[**Key words:** Japanese sake; Rice polishing ratio; Sake rice; Sake yeast; Metabolomics; Liquid chromatography–mass spectrometry]

Japanese sake is a traditional alcoholic beverage made from rice, *koji*, and water. Simultaneous saccharification of rice by *koji* (*Aspergillus oryzae*) and alcohol fermentation by yeast (*Saccharomyces cerevisiae*) lead to the formation of a variety of ingredients of Japanese sake that give it its characteristic taste. Sake contains more than 280 metabolites that affect its quality. The metabolite composition of sake depends on the combination of raw materials and sake-making parameters (e.g., rice species, rice polishing ratio, water quality, *koji* mold, yeast strains, sake mash fermentation methods) used during production. Thus, investigating the association between sake metabolites and sake-making parameters is important for controlling the taste and quality of sake.

Recent developments in mass spectrometry have enabled analysis of total compounds in foods and beverages (1). Many studies have examined the metabolomics of Japanese sake using capillary electrophoresis time-of-flight mass spectrometry (CE-TOF-MS) (2,3), gas chromatography–mass spectrometry (4), and two-dimensional gas chromatography with TOF-MS (5,6). Recently, the oligosaccharide composition of sake was analyzed by hydrophilic interaction chromatography TOF-MS (7), and the process of fermentation of *yamahai-ginjo-shikomi* was analyzed by nano–liquid chromatography–mass spectrometry (nano-LC-MS) (8). However, there have been no metabolomics studies of sake using liquid chromatography–quadrupole/time-of-flight mass spectrometry (LC-Q/TOF-MS), which is suitable for analyzing nonvolatile metabolites.

To investigate the association between sake-making parameters and sake metabolites, we developed a sake metabolome analysis method using ultraperformance LC with quadrupole/TOF-MS (UPLC-Q/TOF-MS). First, we focused on the effect of the rice polishing ratio on sake metabolites, which is one of the most important factors influencing sake quality. When this ratio is low, sake becomes richer in flavor and smooth in taste. According to the Japanese Liquor Tax Law for sake labeling, specially designed sake is categorized according to its rice polishing ratio. Studies have reported an association between the rice polishing ratio and sake metabolites. The protein content of rice grains linearly depends on the rice polishing ratio (9). Furthermore, the release of amino acids into sake mash and rice polishing ratio showed a positive correlation (10). In contrast, the uptake of amino acids increases with yeast growth (11). Yeast growth and amino acid uptake also increase in sake mash with a high rice polishing ratio. Therefore, the total amino acid content in sake has been reported to be highest in 75% polished rice sake mash (12). Mineral and lipid contents in rice grains also depend on the rice polishing ratio (13,14). Furthermore,

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the rice polishing ratio greatly affects the enzyme activity and metabolites of *koji* (15,16), thereby affecting sake metabolites.

In the sake industry, parameters such as rice cultivars, yeast species, and fermentation temperature are altered according to the rice polishing ratio. For example, when the rice polishing ratio is low, Yamadanishiki (sake rice) and sake yeast K1801 (suitable for *Ginjo* sake) are used. The combined effects of these parameters on the sake metabolome have not been examined. In this study, we analyzed several types of commercial sake using our sake metabolome analysis method and revealed that sake metabolites are correlated with the rice polishing ratio. As previously mentioned, some parameters tend to change with the rice polishing ratio. Thus, to examine the statistical correlation between each parameter, we performed combination experiments to evaluate sake-making parameters, such as rice polishing ratio, rice cultivars, and yeast strains used in small-scale fermentation. The results revealed that many metabolites are affected by various parameters and that these parameters have combined effects on sake metabolites.

MATERIALS AND METHODS

Samples and reagents Various types of sake samples were commercially obtained and are described in Tables S1 and S2. A model sake sample contained a mixture of 68 compounds (Table S3) in 15% ethanol. Next, 1 mL of each type of sake was aliquoted into Eppendorf tubes and stored at −80°C. Two cultivars of rice, Yamadanishiki (sake rice) and Nipponbare (cooking rice), were used to make sake. The rice grains were polished to different ratios (40%, 50%, 60%, and 70%) using a milling machine (HS-08CNC, Chiyoda, Hiroshima, Japan). *A. oryzae* strain RIBOIS01 was used to make *koji* and *S. cerevisiae* strains K1801 and K701 were used for subsequent fermentation.

Small-scale *koji* making During small-scale *koji* making, the rice was washed for 30 s using tap water and drained for 15 s; this procedure was repeated once more. To achieve 130% water absorption (100% absorption indicates original raw rice), the rice was soaked in water and drained for 30 min at 15°C, followed by steaming for 50 min. The steamed rice was cooled to room temperature and left to dry until 130% water absorption. Next, the rice was inoculated with conidia of *A. oryzae* (5×10^5 conidia/g *koji*) and incubated in a thermo controlled and humidity-regulated incubator (KCL2000, EYRA, Tokyo, Japan) for 46 h to mimic the *koji*-making process. To make *Tsuki-haze koji*, the temperature was initially maintained at 32°C for 22 h and then gradually increased as follows: 34°C for 30 h→37°C for 33 h→40°C for 36 h→42°C for 38 h. The moisture level was initially maintained at 90% for 22 h and then gradually decreased as follows: 80% for 30 h→70% for 33 h→60% for 38 h. To cool the *koji*, the temperature of the incubator was decreased to 10°C and the moisture level was increased to 80% after 46 h. The *koji* was stored at −30°C until sake production.

Small-scale sake making Small-scale sake making was performed using a one-step addition of raw material (rice, *koji*, and yeast culture). Sake was made using a total of 200 g of rice (including *koji*) and yeast (K701 or K1801). First, the rice was washed for 30 s using tap water and drained for 15 s; this procedure was repeated once. The washed rice was soaked for 10–30 min, including washing time (depending on water absorption: ∼130% of the original rice weight), and then drained for 30 min at 15°C. Next, the rice was steamed for 50 min. The water absorption of the steamed rice was adjusted to 130%, assuming that the water content of the original rice was 13.5%. The steamed rice was added to 260 mL of water with 90 μL of 90% lactate, 2 mL of yeast (1×10^8 cells/mL), and 40 g of *koji*. Yeast cells were precultured overnight at 30°C in rice *koji* extract medium (Baume scale 10), and the culture was inoculated into fresh medium at a 100-fold dilution and then statically incubated for 2 days at 30°C. Each sake-making test was conducted in duplicate. The fermentation temperature was maintained at 15°C in a water bath for 20 days and fermentation was monitored by measuring the reduction in sake mash weight, which corresponds to CO_2 evolution. After fermentation, the mash was centrifuged (4000 ×*g*, 10 min) and the supernatant recovered as refined sake. To compare the effects of rice parameters of *kake*-rice and *koji*-rice, two sake-making tests were conducted (Fig. 1, Table S4). In the first test, only the parameters of *kake*-rice were changed, while those of *koji*-rice remained constant (Yamadanishiki, rice polishing ratio = 40%; upper part of Table S4); this process is referred to as kake-rice sake making in this study. In the second test, the parameters of both *koji*-rice and *kake*-rice were changed (lower part of Table S4); this process is referred to as +koji-rice sake making in this study. Combinations of several sake-making parameters, such as rice cultivars (Yamadanishiki and Nipponbare), rice polishing ratio (40%, 50%, 60%, and 70%), and yeast strains (K1801 and K701), were examined, as shown in Fig. 1 and Table S4.

Analysis of sake The alcohol concentration, sake meter value, acidity, and amino acidity were analyzed using the National Tax Administration method (17).

Analysis of *koji* enzyme activity The enzyme activity in *koji* was measured using the National Tax Administration method. The α-amylase, glucoamylase, α-glucosidase, and acid carboxypeptidase enzyme activities were measured using assay kits (Kikkoman, Noda, Japan).

Sake metabolome analysis Sake samples were filtered using Amicon Ultra 0.5 3K (Merck Millipore, Billerica, MA, USA) and then diluted by 10-fold using MS-grade water. UPLC-Q/TOF-MS analysis was performed using a UPLC Xevo Q/TOF-MS system (Waters, Milford, MA, USA). The metabolites were separated on an Acquity UPLC HSS T3 (2.1×150 mm^2, 1.8-μm column; Waters). Mobile phases A and B were 0.1% (v/v) formic acid in Milli-Q water and 0.1% (v/v) formic acid in acetonitrile, respectively. Gradients of phases A and B were as follows: $t = 0$ min,

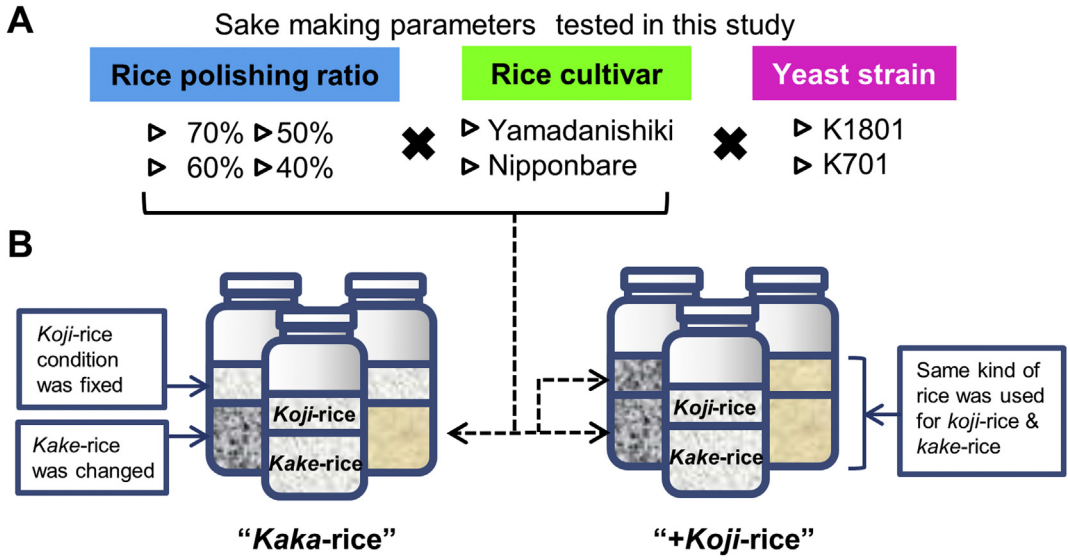

FIG. 1. Combination of sake-making parameters. (A) Sake-making parameters, such as rice cultivars (Yamadanishiki and Nipponbare), rice polishing ratio (40%, 50%, 60%, and 70%), and yeast strains (K1801 and K701), were combined and examined. (B) Two types of sake-making tests were conducted. First, common *koji* (Yamadanishiki, rice polishing ratio = 40%) was prepared and different types of rice cultivars and rice polishing ratios were used to make *kake*-rice, referred to as *kake*-rice sake making in this study (left). Second, *koji* was prepared using different types of rice cultivars and rice polishing ratios, but the sake-making parameters were the same as those in *kake*-rice sake making. This process was referred to as +koji-rice sake making (right). The precise combinations of the sake-making parameters are summarized in Table S4.

0% B; $t = 5$ min, 0% B; $t = 15$ min, 100% B; $t = 20$ min, 100% B; $t = 21$ min, 0% B; and $t = 30$ min, 0% B. The MS conditions were as follows: ionization, electrospray ionization positive; capillary voltage, 3 kV; sample cone voltage, 15 kV; source temperature, 140°C; desolvation temperature, 450°C; cone gas flow rate, 50 L/h; desolvation gas flow rate, 800 L/h; MS mode, MS (MS1 scan mode); measuring range of m/z, 50–1000; and data scanning, 5 scans/s. Each sample was analyzed at least three times.

Data processing Peak detection, peak data alignment, and peak area calculation were conducted using MassLynx XS Rev. 1.0 software (Waters). The mass range and retention time (RT) range for peak detection were determined from the error range of 14 selected peaks from the model sake samples (Table S3). The 14 peaks were selected depending on their variations in retention time. To evaluate the accuracy of the metabolome data, model sake samples were analyzed at least five times using 5–10 samples per interval. The error range of each mass spectrum was calculated by five-fold standard deviation of each of the 14 selected peaks, and the largest value was used as the error range for the peak-picking method of MassLynx. The error range of the retention time was also calculated as the mass spectrum error. Other settings used in the peak-picking method of metabolome data were as follows: initial and final retention times of monitoring, 0–15 min; peak width at 5% height, 4 s; intensity threshold of peak picking, 50; mass window, 0.05 Da; and retention time window, 0.3 min. Other settings were used according to the manufacturer's instructions. After preparing a peak table, preliminary data processing was performed. If the maximum intensity among the samples was smaller than the six-fold chromatogram noise or included 0, these markers were eliminated. Metabolites were identified based on the metabolite list (Table S3). The thresholds of mass and retention time error for identification were 0.05 Da and 0.3 min, respectively.

Evaluation of metabolome data Correlation analysis of the commercial and small-scale sakes was performed using the peak table. The Pearson correlation coefficient was calculated using the rice polishing ratio and intensity of each peak. P values were calculated using Student's t-test, and a P value of <0.005 was considered to indicate a correlation with the rice polishing ratio. In the case of yeast strains and rice cultivars, significant differences were calculated using Student's t-test. P values were calculated using the average of the intensity of each group, and a P value of <0.005 was considered to indicate a significant difference between the two groups (Yamadanishiki and Nipponbare, or K1801 and K701). The results are presented in Table S5.

RESULTS AND DISCUSSION

Development of sake metabolome analysis method To develop the sake metabolome analysis method using LC-Q/TOF-MS, which can detect a wide range of compounds in sake, several analytical conditions were investigated. First, a model sake sample and five different commercial sake types were examined using seven columns: Acquity UPLC HSS T3, Acquity UPLC BEH130 C18, Acquity UPLC CSH C18 (Waters), SeQuant ZIC-pHILIC, Hypercarb (Merck Millipore), Scherzo SM-C18 (Intakt, Kyoto, Japan), and Ascentis RP-Amide (Sigma–Aldrich, St. Louis, MO, USA). Of these, the Acquity UPLC HSS T3 column was selected based on its peak number and resolution (data not shown). The gradient program for UPLC was also used to obtain the maximum number of peaks and high resolution in a minimum run time (see the Materials and methods section). Using this method, we examined an additional 233 standard reagents and detected 198 compounds (Table S3). We used this method and Table S3 for metabolome analysis and identification. Metabolite data was obtained by MS scanning rather than by MS/MS. Thus, we cannot distinguish co-eluted isobaric metabolites in this method. There were 28 such compounds among the 198 compounds (Table S3). Thirteen compounds among the 28 may be distinguishable using their specific in-source fragment peaks, but it is difficult to distinguish the remaining 15 compounds because they do not have specific in-source fragment peaks. To distinguish these compounds, MS/MS analysis is required.

Metabolite profiles of 40 commercial sakes To investigate sake metabolite profiles, various types of commercial sake samples were analyzed using the sake metabolome analysis method. The metabolite profiles of 40 commercial sakes (Table S1) were analyzed by principal component analysis (PCA) (Fig. 2A). Specially designed sakes and ordinary sakes, categorized by the Japanese Liquor Tax Law, were clearly separated in principal component 1 (PC1). This difference was predicted to be related to the large differences between these sakes in terms of the permitted alcohol amount added to the sake mash. Sake samples were also separated into PC1 according to the rice polishing ratio. This tendency suggested that the rice polishing ratio is one of the major factors affecting the sake metabolome. Loading plot analysis of the PCA in Fig. 2B showed that arginine made the highest contribution to the positive axis of PC1, whereas agmatine made the highest contribution to the negative axis. The sakes made from highly polished rice (<35%) were clearly separated into principal component 2 (PC2) in the positive direction (Fig. 2A). Unknown-1 and unknown-2 peaks contributed to the positive axis of PC2 (Fig. 2B). Further investigations are needed to evaluate low rice polishing ratios.

Combined sake fermentation experiment Metabolome analysis of the commercial sakes suggested that rice polishing ratio is one of the most important parameters in sake making. However, other parameters, such as rice cultivars, yeast strains, and

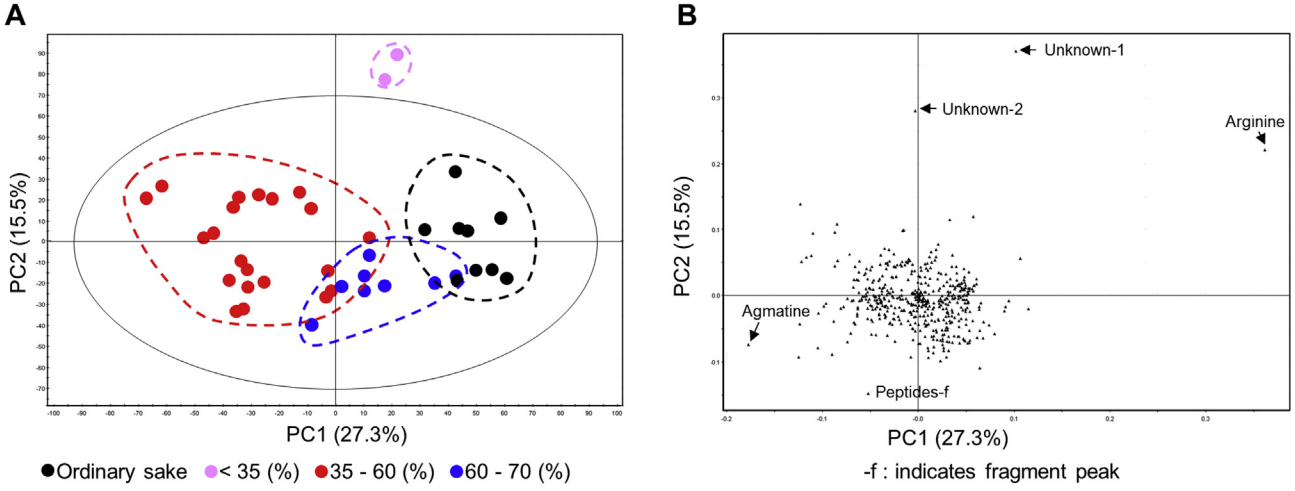

FIG. 2. PCA analysis of 40 commercial sakes. (A) Score plot of metabolome data. Pink, red, and blue dots indicate sakes in which the rice polishing ratios were <35%, 35–60%, and 60–70%, respectively. Pink, red, and blue dots indicate specially designed sake, and black dots indicate ordinary sake. Pink, red, and blue dotted lines include sake in which the rice polishing ratios were <35%, 35–60%, and 60–70%, respectively. The black dotted line includes ordinary sake. (B) Corresponding loading plots.

fermentation temperature, often change with the rice polishing ratio. Furthermore, the effects of a combination of these parameters on the sake metabolome have not been evaluated. To reveal the association between sake-making parameters and sake metabolites, combination experiments using the rice polishing ratio (40–70%), rice cultivars (Yamadanishiki and Nipponbare), and yeast strains (K1801 and K701) were performed. First, a combined fermentation test was conducted using *koji* made of 40% polished Yamadanishiki, which was designated as *kake*-rice. Another fermentation test was performed using *koji* made from the same ingredients as *kake*-rice, which was designated as +koji-rice (Fig. 1, Table S4). The enzyme activities of each *koji* sample are summarized in Tables S6 and S7. The enzyme activities of α-amylase and glucoamylase were correlated with the rice polishing ratio and rice cultivars. Acid carboxypeptidase activity showed an inverse correlation with the rice polishing ratio; this was higher for *koji* made from Yamadanishiki than for *koji* made from Nipponbare. These results were mostly in accordance with those of previous reports (15,16). The fermentation rates of all small-scale sakes increased with the rice polishing ratio (Figs. S1 and S2). Among the general analytical characteristics of sake samples, the sake meter value and amino acidity were correlated with the rice polishing ratio. In contrast, acidity and amino acidity were higher in Nipponbare sake than in Yamadanishiki sake (Table S8). These results are consistent with those reported by previous studies and the general experiential knowledge in the industry.

PCA analysis of sake samples Because the fermentation process and general characteristics of sake agreed with those of previous reports, we performed metabolome analysis of our small-scale sake samples. The PCA score plots of the *kake*-rice sake samples showed a rather high (43.3%) contribution ratio of PC1 (Fig. 3A). On this axis, the *kake*-rice sake samples were clearly separated for the yeast strain K701 or K1801. In contrast, sake samples were plotted on the PC2 axis according to the rice polishing ratio. These results suggest that the rice polishing ratio and yeast strains used have greater effects on sake metabolites compared to the rice cultivar of *kake*-rice. In the loading plot analysis of PCA, among the identified peaks, phenylalanine, choline, and glutamic acid showed a higher contribution to PC1 in the positive direction. In PC2, proline, glutamic acid, arginine, and α-ethylglucoside were in the positive direction, whereas phenylalanine and a fragment of saccharide were in the negative direction (Fig. 3B). Interestingly, the PCA results of the +*koji*-rice sake samples differed from those of the *kake*-rice sake samples. The +*koji*-rice sake samples were separated on the PC1 axis by a difference in the rice cultivars Yamadanishiki and Nipponbare and on the PC2 axis by a difference in yeast strains (Fig. 3C). These results suggest that compared to the rice cultivar of *kake*-rice that of *koji* had a greater effect on sake metabolites. In loading plot analysis of PCA, among the identified peaks, phenylalanine, proline, glutamic acid, and a fragment of α-ethylglucoside showed a high contribution to the positive axis of PC1, whereas arginine, phenylalanine, valine, tyrosine, and a fragment of succinic acid contributed to the positive axis of PC2 (Fig. 3D). To compare the metabolome profiles of small-scale sakes with those of commercial sakes, we analyzed 16 newly prepared commercial sakes (Table S2) and small-scale sakes. PCA analysis of the commercial sakes showed that the sake samples were separated according to the rice polishing ratio (Fig. S3). This result was similar to those of the 40 commercial sakes (Fig. 1).

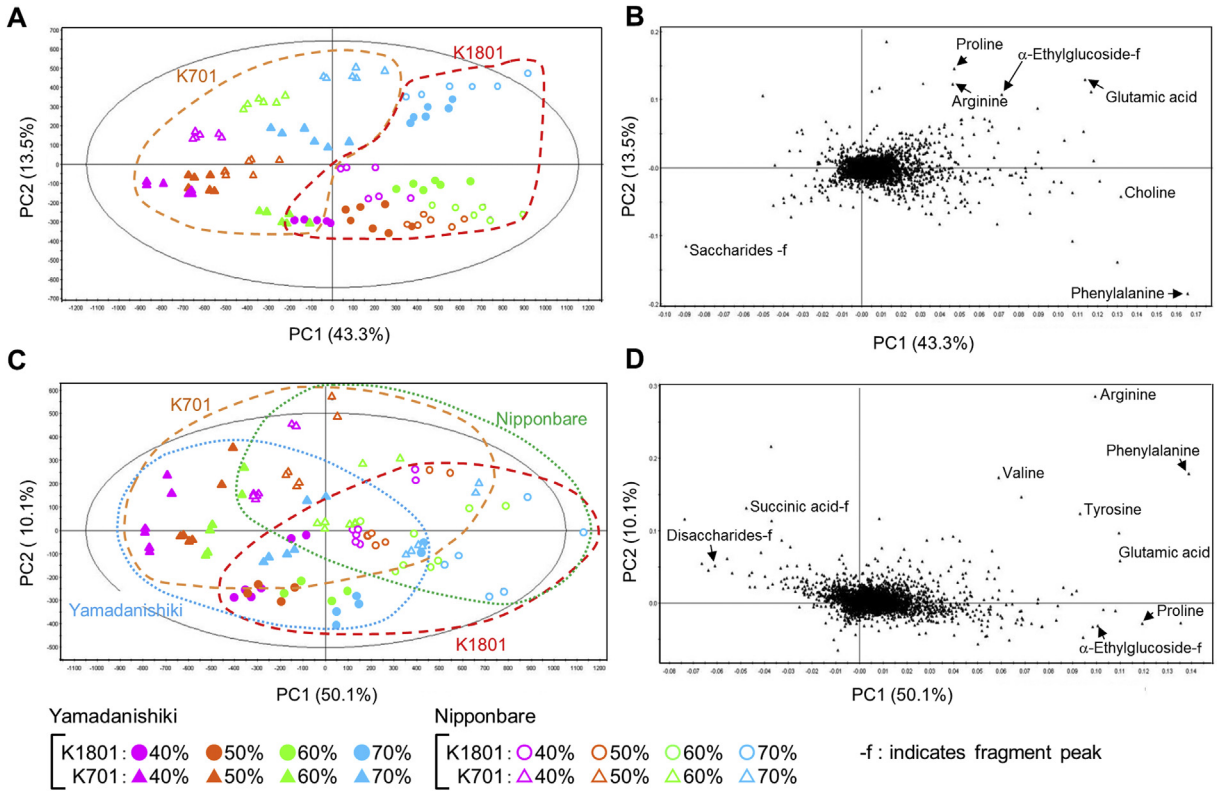

FIG. 3. PCA analysis of small-scale sake making. (A) Score plot of *kake*-rice sake making. (C) Score plot of +*koji*-rice sake making. Solid dots indicate sake made from Yamadanishiki, while open dots indicate sake made from Nipponbare. Circles and triangles indicate sake made using K1801 and K701, respectively. Pink, orange, green, and light blue dots indicate sake in which the rice polishing ratios were 40%, 50%, 60%, and 70%, respectively. Red, orange, blue, and green dotted lines include sake made using K1801, K701, Yamadanishiki, and Nipponbare, respectively. (B) Loading plot of *kake*-rice sake making. (D) Loading plot of +*koji*-rice sake making.

Profiles of peaks affected by sake-making parameters To investigate the effect of sake-making parameters on each peak, a correlation profile was generated (Table S5). All metabolome data (commercial sake and small-scale sake samples) were analyzed and combined into one peak table of 5362 peaks. The data include false positive peaks, such as isotopes, different adduct forms, and in-source fragment ions (18). For example, fragment peaks observed in the analysis of standard reagents (Table S3) would be in-source fragment peaks in the samples. Therefore, numerous peaks occur for in-source fragment ions. The actual metabolite number in sake in the present analysis may have been much lower than 5362. The original peak table included non-detected (intensity = 0) data and non-reliable data because of its low intensity. To eliminate such peaks, data processing was performed as described in the Materials and Methods. After processing the peak table data, the total number of peaks was reduced to 634 (Table S9). Using this peak table, peaks highly correlated with the rice polishing ratio ($P < 0.005$) were selected. Peaks with significantly different levels in sake in terms of K1801 and K701 and in terms of Yamadanishiki and Nipponbare were selected using Student's *t*-test ($P < 0.005$). Using these correlation profiles, a Venn diagram of the peaks affected by all parameters tested in this analysis was generated (Fig. 4A,B). Considering the high number of parameters for comparison, the Venn diagram was divided into *kake*-rice sake and +*koji*-rice sake diagrams (Fig. 4A,B). In the *kake*-rice samples, the parameter affecting the highest number of peaks was the yeast strain (496 peaks). The number of peaks affected by the rice cultivars was 220, which was less than half of those affected by yeast strains (Fig. 4B). In contrast, the number of peaks affected by rice cultivars in +*koji*-rice samples was 508, which was over two-fold the number in *kake*-rice, although the number of peaks affected by the yeast strains and rice polishing ratio changed similarly compared to those in *kake*-rice (Fig. 4A,B). These results suggest that differences in rice cultivars affect the quality and enzyme activity of *koji*, greatly affecting the sake metabolites. All enzyme activities except for acidic carboxypeptidase were changed with the rice cultivars (Table S6). There were 354 peaks correlated with the rice polishing ratio in commercial sakes, whereas 378 peaks in *kake*-rice experiments and 435 peaks in +*koji*-rice experiments were correlated with the rice polishing ratio. Approximately 84% (299/354) of the peaks affected by the rice polishing ratio in commercial sake were also affected by the rice polishing ratio in small-scale sake (Fig. 4C). The Venn diagram focusing on rice cultivars showed that 80% (176/220) of the peaks affected by rice cultivars in *kake*-rice were also affected by rice cultivars in +*koji*-rice (Fig. 4D). Regarding commercial sake, although the rice polishing ratio appeared to have a great effect on sake metabolites (Figs. 2 and S3), other parameters, such as yeast strain and rice cultivar, also had a large effects on the metabolites, and many peaks were affected by several sake-making parameters (Fig. 4A,B).

Effect of combined parameters on identified peaks Among the 634 peaks obtained, we identified 97 which were classified as follows: 41 carbohydrate, 34 nitrogen compound, 11 alcohol, 7 organic acid, 2 nucleic acid, and 2 vitamin peaks (Table S9). The association between some of these peaks and sake-making parameters has been reported previously, but the effects of a combination of these parameters remain unclear. Therefore, we focused on a few of the identified peaks to examine the effects of a combination of sake-making parameters.

In this analysis, 18 amino acids were detected, of which 13 in *kake*-rice, 10 in +*koji*-rice, and 13 in commercial sake samples were positively correlated ($P < 0.005$) with the rice polishing ratio (Table S5). These results agreed with those of previous reports (9–12). The amino acid content was also affected by other parameters. Compared to those in *kake*-rice, the levels of several amino

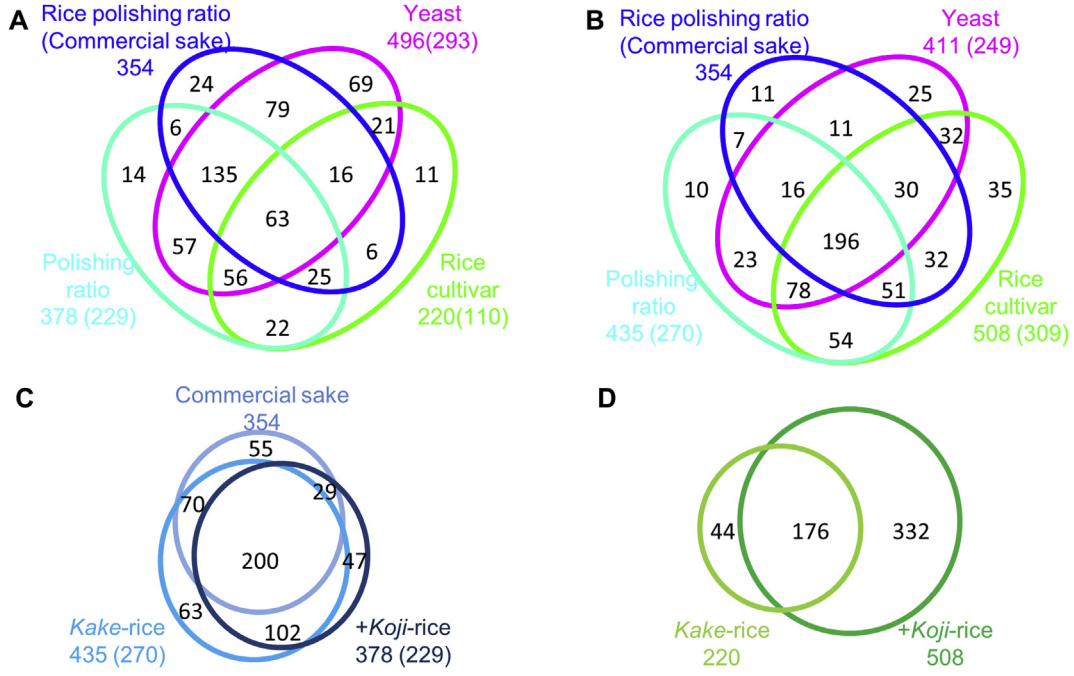

FIG. 4. Venn diagrams of peaks affected by each parameter. (A) Comparison of peaks affected by the rice polishing ratio in commercial sake and those affected in the *kake*-rice sake-making test. (B) Comparison of peaks affected by the rice polishing ratio in commercial sake and those affected in the +*koji*-rice sake-making test. Blue circles indicate the number of peaks affected by the rice polishing ratio in commercial sake. Light blue, pink, and green circles indicate the number of peaks affected by the rice polishing ratio, yeast strains, and rice cultivars, respectively, in both sake-making tests. Parenthetical numbers represent common peaks affected by the rice polishing ratio in commercial sake. (C) Comparison of peaks affected by the rice polishing ratio in commercial sake and the *kake*-rice and +*koji*-rice sake-making tests. (D) Comparison of peaks affected by rice cultivars in *kake*-rice and +*koji*-rice sake-making tests.

acid peaks in +*koji*-rice changed depending on the rice cultivars. In such peaks, the proteinogenic amino acid content (16 amino acids) in sake made from Nipponbare was greater than that in sake made from Yamadanishiki ($P < 0.005$). For *kake*-rice cultivars, only five amino acids increased in sake made from Nipponbare compared to in sake made from Yamadanishiki. This rice cultivar dependence on amino acid content in sake is expected because the protein content in a rice grain of Nipponbare is higher than that in Yamadanishiki (11). In our study, amino acid contents were more dependent on the difference in rice cultivars in +*koji*-rice samples than in *kake*-rice samples. However, the acidic carboxypeptidase activity of *koji* made from Nipponbare was similar to that of *koji* made from Yamadanishiki. The acidic protease activity of *koji* made from Nipponbare was slightly lower than that of *koji* made from Yamadanishiki (Table S6). Many genes have been predicted as acidic carboxypeptidase in *A. oryzae* (19). Some of these acidic carboxypeptidases show different substrate specificities in *koji*-rice (20–26). The gene expression of these enzymes in *A. oryzae* likely depends on the rice cultivars.

We found that many of these amino acids were affected by both the rice polishing ratio and rice cultivar of *koji*-rice. Moreover, some amino acids were affected by the differences in yeast strains. For example, tryptophan, serine, and methionine were affected by the rice cultivar of *koji*, rice polishing ratio, and yeast strains (Fig. 5A–C). The combination of Yamadanishiki, low rice polishing ratio, and K701 decreased these amino acids content, with the greatest effects on methionine (Fig. 5C). In the case of some amino acids, we found that the effect of various parameters can also be influenced by the combination of sake-making parameters. Some of these amino acids were affected by several parameters in a synergistic manner. For example, glutamine was heavily affected by the rice polishing ratio when Yamadanishiki was used to make *koji* ($r = 0.83$) but not when Nipponbare was used ($r = 0.55$; Fig. 5D). Histidine was affected more by the rice polishing ratio when Nipponbare was used to make *koji* ($r = 0.88$) than when Yamadanishiki was used ($r = -0.37$; Fig. 5E).

For carbohydrates, the production of α-ethylglucoside, which tastes sweet with late bitterness (27), was associated with the rice polishing ratio, *koji*-rice, and yeast strains (Fig. 6A, Table S5). The average concentration of α-ethylglucoside in commercial sake was higher than its bitterness threshold (27). Therefore, sake-making parameters, concerned with the amount of α-ethylglucoside, influenced the taste of sake. In sake mash, α-ethylglucoside is considered as enzymatically synthesized by transglucosylation from oligosaccharide to ethanol by α-glucosidase derived from *A. oryzae* in *koji* (28). Thus, α-ethylglucoside production is thought to be correlated with α-glucosidase activity. However, in this study, the amount of α-ethylglucoside was negatively correlated with α-glucosidase activity ($r = -0.85$; Fig. 6A, Tables S6 and S7). α-Glucosidase is known as a bidirectional enzyme that can degrade α-

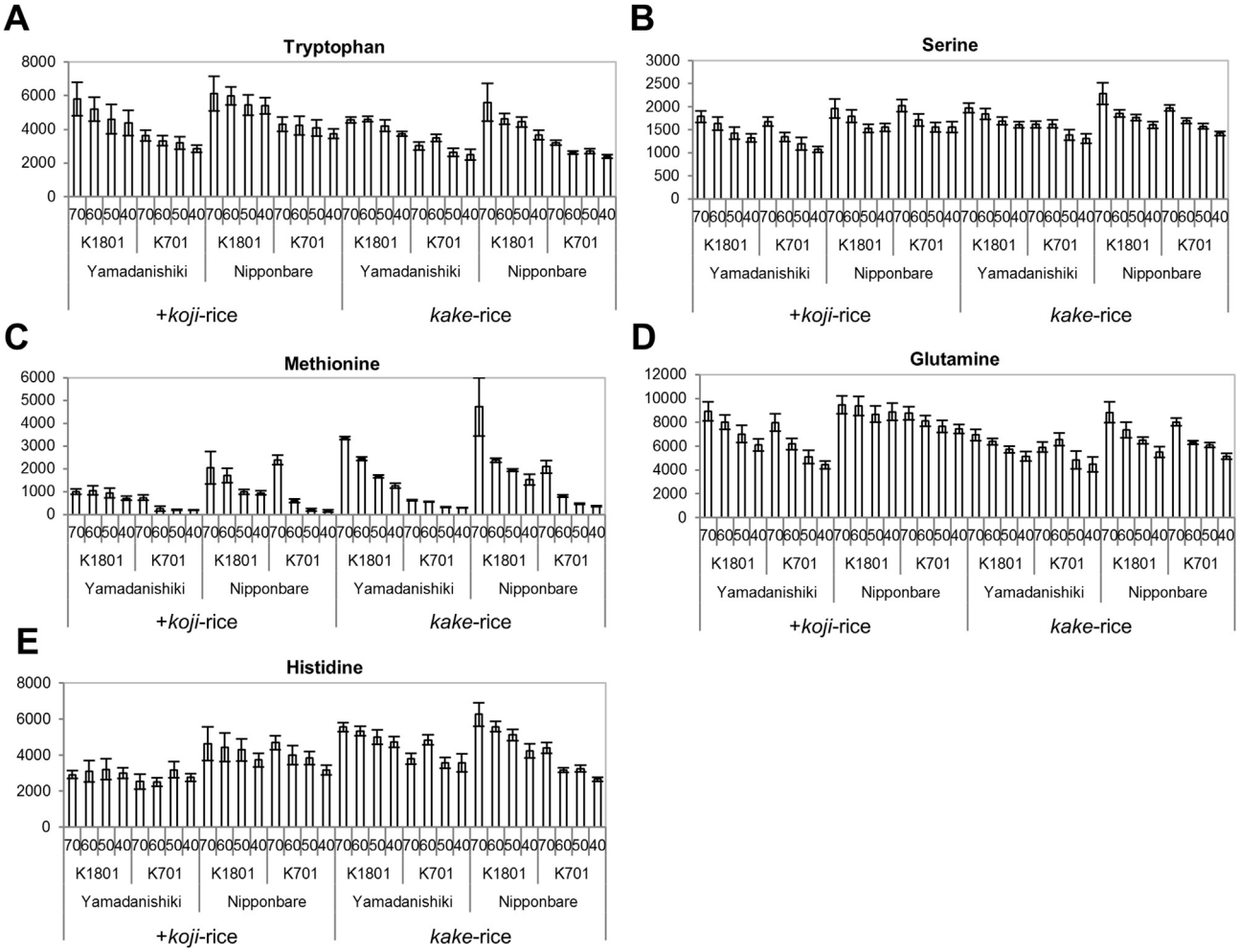

FIG. 5. Effect of sake-making parameters on amino acids. Intensity of (A) tryptophan (RT = 8.52, $m/z = 205.0917$), (B) serine (RT = 1.23, $m/z = 106.044$), (C) methionine (RT = 2.66, $m/z = 150.0572$), (D) glutamine (RT = 1.21, $m/z = 147.0913$), and (E) histidine (RT = 1.17, $m/z = 156.0715$). Each sake-making parameter, rice polishing ratio, yeast strain, and rice cultivar is indicated under the graph.

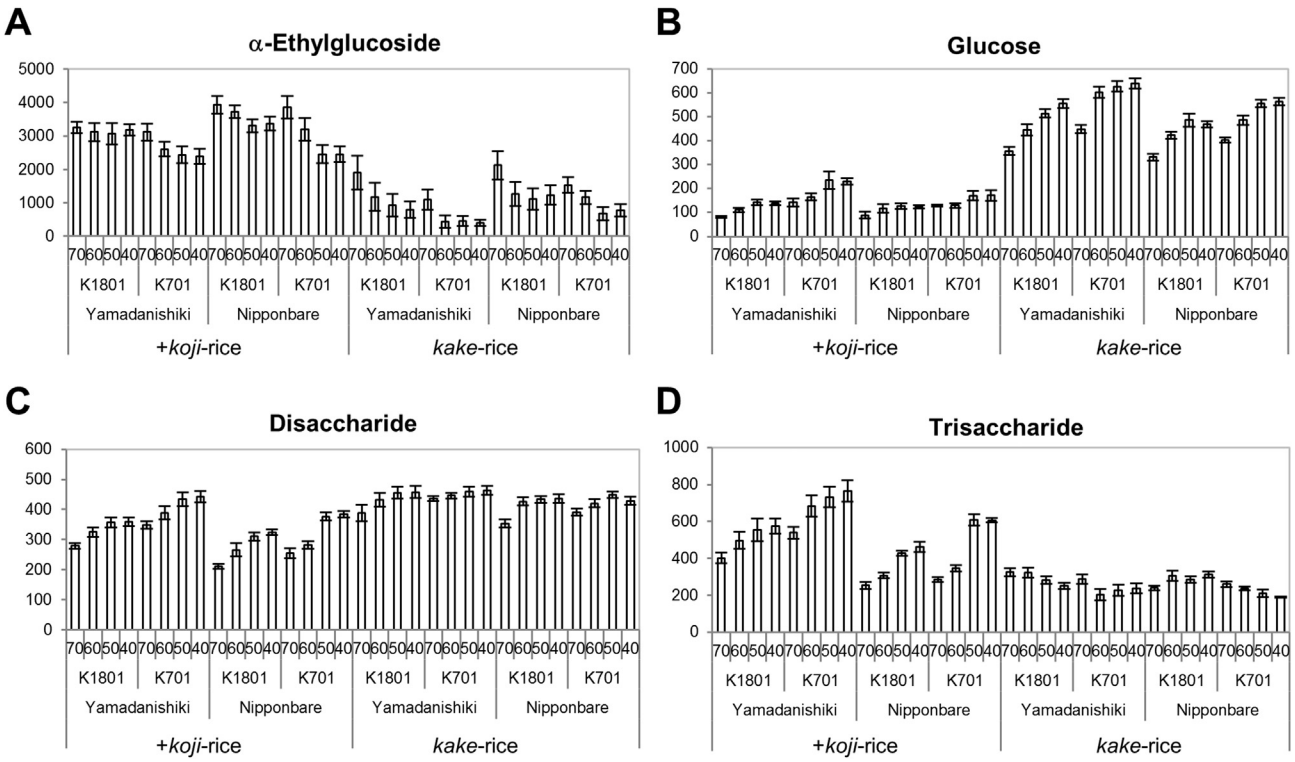

FIG. 6. Effect of sake-making parameters on α-ethylglucoside and saccharides. Intensity of (A) α-ethylglucoside (RT = 5.17, m/z = 209.0976), (B) fragment peak of glucose (RT = 1.34, m/z =109.0283), (C) disaccharide (RT = 1.59, m/z = 343.1224), and (D) trisaccharide (RT = 2.15, m/z =505.1746). Each sake-making parameter, rice polishing ratio, yeast strain, and rice cultivar is indicated under the graph.

ethylglucoside (29,30). Our results of negative correlation supported the possibility that α-glucosidase acts to degrade α-ethylglucoside. Interestingly, the glucose and disaccharide levels showed a negative correlation with α-ethylglucoside ($r = -0.94$ and -0.85, respectively; Fig. 6A–C). Trisaccharides showed rather the same trend (Fig. 6D). A negative correlation between disaccharides and α-ethylglucoside is reasonable because disaccharides are a major substrate of α-glucosidase to produce α-ethylglucoside. However, this observation disagrees with the low α-glucosidase activity under high α-ethylglucoside conditions. The amount of glucose, which is correlated with glucoamylase activity ($r = 0.60$), was also negatively correlated with α-ethylglucoside. Glucose is one of the final products of α-ethylglucoside degradation by α-glucosidase activity and is likely affected by α-ethylglucoside formation. Kang et al. (31) reported a tripeptide, Pro-Phe-Pro [m/z (precursor ion) = 360.191785], which inhibits α-glucosidase activity and is derived from *A. oryzae*. In our experiment, an ion was observed at m/z = 360.208. Therefore, this peptide may have affected α-ethylglucoside formation. Considering the bidirectional activity of α-glucosidase, the formation of α-ethylglucoside is a complex phenomenon, and the existence of other enzymes that contribute to α-ethylglucoside synthesis or degradation should be evaluated in further studies. However, our results reveal new aspects and interesting questions for the study of sake making.

In conclusion, this is the first study to conduct metabolome analysis of Japanese sake using LC-Q/TOF-MS. We developed a sake metabolome analysis method, which can detect 198 compounds. We illustrated the effect of the combined relationship of several sake-making parameters on sake metabolites. No studies have examined the relationship between the sake metabolome and combination of several sake-making parameters at this scale. Our combined analysis revealed that numerous compounds, including amino acids and saccharides, are affected by various sake-making parameters. In some peaks, these parameters showed synergistic effects. The results also revealed that the effect of the rice cultivar of *koji* on the sake metabolome is greater than that of the rice cultivar of *kake*-rice. This has been known experientially, but this study clarified it experimentally for the first time. Our analysis also revealed new aspects of the sake metabolome and enzyme activities in koji. For example, the amount of α-ethylglucoside, which can affect the taste of sake, was negatively correlated with α-glucosidase activity. These results are important for controlling the taste of sake and for understanding the association between enzyme activity and sake metabolites. Furthermore, approximately 80% of peaks remained unidentified in this study. Thus, many unidentified metabolites may have important effects on the taste and quality of sake. To decrease these unknown peaks, we plan to analyze new standard reagents, such as dipeptides, contained in sake using our method. The correlations between unidentified peaks and the combinations of sake-making parameters have already been studied. Thus, it is possible to immediately identify sake-making parameters when important metabolites are identified. However, in this study, we examined limited sake-making parameters. Thus, investigation of unknown sake metabolites and accumulation of data sets for other sake-making parameters are important for developing and optimizing the methods used to generate a novel taste and quality of sake.

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