JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Changes in the Charged Metabolite and Sugar Profiles of Pasteurized and Unpasteurized Japanese Sake with Storage

Masahiro Sugimoto,*[,†,‡] Miku Kaneko,[†] Hiromi Onuma,[†] Yasuko Sakaguchi,[†] Masayo Mori,[†] Shinobu Abe,[†] Tomoyoshi Soga,[†] and Masaru Tomita[†]

[†]Institute for Advanced Biosciences, Keio University, Tsuruoka, Yamagata 997-0052, Japan

[‡]Graduate School of Medicine and Faculty of Medicine, Kyoto University, Yoshida-Konoe-cho Sakyo-ku, Kyoto 606-8501, Japan

Supporting Information

ABSTRACT: Japanese sake (rice wine) is commonly heat treated (pasteurized) to maintain its quality. In this study, temporal changes in the metabolite profiles of pasteurized and unpasteurized sake were investigated during storage. Metabolomic analyses were conducted for eight sets of pasteurized and unpasteurized sake obtained from single process batches stored at 8 or 20 °C for 0, 1, 2, or 4 months. Capillary electrophoresis time-of-flight mass spectrometry and liquid chromatography tandem mass spectrometry were used to obtain charged metabolite and sugar profiles, respectively. The total amino acid concentration decreased with storage, and the decrease was faster in pasteurized sake than in unpasteurized. The organic acid concentrations were relatively constant in both types of sake. Peptide and glucose concentrations increased and polysaccharide concentrations decreased in unpasteurized sake, while they were relatively constant in pasteurized sake. Rather than stabilizing the sake metabolite profile during storage, pasteurization results in characteristic changes compared to unpasteurized sake.

KEYWORDS: *Sake, Rice wine, Metabolome, Metabolomics, Pasteurized, Unpasteurized, CE-TOFMS, LC-MS/MS*

INTRODUCTION

Japanese sake (rice wine) is a popular alcoholic beverage in Japan that is made from steamed rice by multiple fermentations with rice malt (*Aspergillus oryzae*) and sake yeast (*Saccharomyces cerevisiae*).[1] The sake brewing process starts with the polishing of rice kernels to remove the outer layer of proteins, lipids, and minerals that could adversely affect the aroma and flavor of the sake.[2] The polished rice is separated into two parts, and one part is steamed with the addition of *Aspergillus oryzae* spores and incubated to generate malted rice (*koji*). *S. cerevisiae*, water, and lactic acids are added to the other group (*shubo*). After this processing, the two parts are combined to yield the final mash (*moromi*), and alcohol can be added at this stage. Finally, a clear liquid is obtained by filtration and then bottled with or without pasteurization.

Sake is roughly divided into two types; pasteurized (*hiire*) and unpasteurized (*namazake*). Unpasteurized sake has a fresh flavor but should be consumed soon after production and stored under particular conditions to avoid rapid deterioration of its quality because of its microorganism and enzyme content.[3] Pasteurized sake is more commonly available commercially than unpasteurized sake. Pasteurization of sake inactivates most microorganisms and enzymes, which makes its quality more stable, but the heat treatment results in the loss of the fresh flavor of unpasteurized sake. Pasteurization is usually conducted at ≤65 °C for ≤30 min to avoid production of compounds that contribute to undesirable aromas,[4] and these pasteurization conditions do not result in complete inactivation of microorganisms and enzymes.[5] The taste of pasteurized sake becomes milder after storage for several months, which is preferable for consumption. However, the mechanism for this change in the sake taste with time is still unknown.

Many studies have used profiling techniques to investigate the relationship between sake's components and its characteristics. For example, changes in aroma compounds with long-term storage have been investigated.[6] Amino acids are abundant in sake,[7,8] and the amino acid balance determines the quality of some types of premium sake, such as *junmai* and *ginjo*, which are distinguished by the rice polishing rate and addition of alcohol during production.[9,10] Organic acids are also major compounds in sake.[2,11] Peptides are known to contribute to bitter and unpleasant tastes.[12] In an earlier nontargeted analysis of charged metabolites, we found that amino acids and organic acids were the dominant metabolites in sake, and their concentrations were positively correlated to the sake sourness and turbidity.[13] In addition to these metabolites, sugars are also important in determining the general quality, and especially the sweetness, of sake.[14,15] Therefore, a wide range of molecules should be profiled simultaneously.

The aim of this study was to investigate the metabolite profiles of pasteurized and unpasteurized sake, and their changes with time after storage for several months. Capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) was used for comprehensive analysis of charged metabolites, such as amino acids, organic acids, and short peptides, and liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to obtain sugar profiles. These comprehensive metabolomic analyses revealed characteristic changes for each type of sake.

Received: November 29, 2011
Revised: February 16, 2012
Accepted: February 21, 2012
Published: February 21, 2012

dx.doi.org/10.1021/jf2048993 | J. Agric. Food Chem. 2012, 60, 2586–2593

MATERIALS AND METHODS

Chemicals. Ophthalmic acid and leucinic acid were purchased from Bachem AG (Bubendorf, Switzerland). 2-Amino-2-methyl-1,3-propandiol, 2-amino-2-(hydroxymethyl)-1,3-propanediol, hydroxyatrazine, terephthalic acid, and vanillic acid were obtained from Chem Service (West Chester, PA). Other chemicals used included pseudopelletierine (Fluorochem, Hadfield, United Kingdom), betaine and pelargonic acid, *p*-hydroxymandelic acid (Tokyo Chemical Industry, Tokyo, Japan), tropine, *N*-acetyl-L-histidine and 4-hydroxycinnamic acid (MP Biomedicals Japan, Tokyo, Japan), D-fructose (Research Organics, Cleveland, OH), 6-hydroxynicotinic acid and propionic acid (Kanto Chemical, Tokyo, Japan), glycerol-3-phosphate (Nakalai Tesque, Kyoto, Japan), 6-hydroxyhexanoic acid (Acros Organics, Geel, Belgium), and cysteine-glutathione (APOLLO Scientific, Bredbury, United Kingdom). All other compounds were from Sigma-Aldrich Japan (Tokyo, Japan) or Wako Pure Chemical Industries (Osaka, Japan).

Samples. Sixteen sake samples were obtained from eight commercial companies in Tsuruoka (Yamagata Prefecture, Japan). Seven of these companies provided pasteurized and unpasteurized sake samples from the same process batch, so that the ingredients (e.g., rice, water, yeast, and *koji* (malted rice)) and brewing process conditions (e.g., rice polishing ratio and length of brewing) were the same for each sample. One company provided pasteurized and unpasteurized sake from different process batches, and the rice and polishing ratio were different for these two samples. The samples represented four types of sake according to the rice polishing ratio of rice. These types were *junmai* and special *junmai, junmai-ginjo*, which was made without the addition of alcohol, and special *dai-ginzyo* made with the addition of alcohol.[8] Two of the samples were *nigorisake*, which is not filtered and appears white and cloudy. The sample information is summarized in Table S1 (Supporting Information). The sake samples were stored in the dark at 8 and 20 °C, to represent refrigerator and room temperature storage, respectively. Metabolomic analyses were conducted after heat treatment of samples that had been stored for 0, 1, 2, or 4 months.

Sample Preparation for CE-TOFMS. An internal standard solution (2 mmol/L in 10 μL of Milli-Q water (Millipore, Billerica, MA)) was added to 10 μL of each sake sample, and the sample was diluted with 80 μL of Milli-Q water. The internal standard solution contained 2 mmol/L each of methionine sulfone and 3-aminopyrrodine as the positively charged (cationic) standards and 2-(*N*-morpholino)ethanesulfonic acid, D-camphol-10-sulfonic acid sodium salt, and trimesate as negatively charged (anionic) standards. The *nigorisake* samples UP3 and PA3 were centrifuged at 9,100*g* for 15 min, filtered through a 5 kDa cutoff membrane filter (Pall, Tokyo, Japan) to remove suspended solids, and the filtrate was immediately analyzed by CE-TOFMS.

Sample Preparation for LC-MS. A 1 mL aliquot of each sake sample was filtered through a DISMIC-13HP syringe filter (Advantec, Tokyo, Japan) and the filtrate was diluted 1:100 with an aqueous solution of MeOH (MeOH/H_2O = 1:1, v/v) immediately before LC-MS analysis.

Instrumental Parameters for CE-TOFMS. The instrumentation conditions used for CE-TOFMS were the same as those described previously[16] with slight modification.[17] CE-TOFMS methods for cationic[16] and anionic[18] metabolites have been described previously. Details for these methods are given in the Supporting Information.

Instrument Parameters for LC-MS/MS. The LC-MS/MS experiments were performed using an Agilent (Santa Clara, CA) 1100-series vacuum degasser, an Applied Biosystems (Carlsbad, CA) API3000 mass spectrometer, and electrospray ionization. Samples were separated on a NH2P-50 4E column (4.6 × 250 mm I.D., Shodex, New York, NY). The initial mobile phase was 80% acetonitrile and 20% Milli-Q water at a flow rate of 0.8 mL/min. The percentage of acetonitrile was 80%, 73%, and 65% at 10, 25, and 35 min, respectively. The post time was 17 min. The temperature of the column oven was 30 °C, and 1.0 μL aliquots were injected onto the column. The turbospray mode was selected in the negative ion mode. The nebulizer gas, curtain gas, collision gas, ion spray voltage, and ion source temperature were 15 psi, 11 psi, 8, −4500 V, and 550 °C, respectively.

The mass spectrometer was run in multiple reaction monitoring mode with unit resolution for both Q1 and Q3 and a dwell time of 80 ms for the multiple reaction monitoring channels. For ribose and arabinose, Q1 and Q3 were m/z 148.9 and 89.1, respectively. The declustering potential (DP), focusing potential (FP), and collision energy (COE) were −41 V, −190 V, and −10 V, respectively. For arabitol, Q1, Q3, DP, FP, and COE were m/z 150.8, m/z 89.1, −36 V, −100 V, and −18 V, respectively. For fructose, mannose, galactose, and glucose, Q1, Q3, DP, FP, and COE were m/z 178.9, m/z 89.0, −26 V, −160 V, and −12 V, respectively. For sorbitol and mannitol, Q1, Q3, DP, FP, and COE were m/z 180.9, m/z 89.0, −41 V, −190 V, and −20 V, respectively. For trehalose, Q1, Q3, DP, FP, and COE were m/z 340.9, m/z 119.1, −61 V, −340 V, and −24 V, respectively. For maltose, Q1, Q3, DP, FP, and COE were m/z 341.3, m/z 160.8, −46 V, −230 V, and −12 V, respectively. For inositol, Q1, Q3, DP, FP, COE were m/z 178.9, m/z 87.0, −36 V, −210 V, and −22 V, respectively. For maltotriose and panose, Q1, Q3, DP, FP, and COE were m/z 503.2, m/z 341.2, −56 V, −310 V, and −14 V, respectively. All data were acquired using Analyst Software (AB Sciex, Framingham, MA).

Data Analysis. File conversion of raw MS data, peak picking, reduction of noise, alignment of the data for multiple samples, and concentration calculations were conducted using our proprietary software.[19] JMP version 9.0.2 (SAS Institute, Cary, NC) and Mev TM4 software (version 4.7.4. Dana-Farber Cancer Institute, Boston, MA)[20] were used for principal component analysis (PCA) and heat map analysis, respectively. The time-dependent changes in the profiles were evaluated by the sum of Euclidean distances of the plots of the first and second principal components (PC) as follows:

$$\sum_{i=1}^{3} \sqrt{(\mathrm{PC1}_i - \mathrm{PC1}_{i+1})^2 + (\mathrm{PC2}_i - \mathrm{PC2}_{i+1})^2} \quad (1)$$

The Pearson correlation coefficient and paired *t*-test (two-tailed) were calculated using GraphPad Prism version 5.04 (GraphPad Software, Inc., San Diego, CA). Statistical significance is indicated by *, $P < 0.005$; **, $P < 0.01$; or ***, $P < 0.001$.

RESULTS

Metabolomic analyses by CE-TOFMS and LC-MS/MS identified 195 metabolites in total over all the samples. Before storage for 4 months, each sake sample contained 140 ± 8 (standard deviation) metabolites on average. Sample 7 contained the most metabolites (157) among the sake samples, which could be attributed to its type (*junmai*) and/or the rice (*Naenuki*) used in its production. Figure 1 depicts the relative differences in the metabolites found in each sample as a heat map. Figure 2 shows the PCA score plots and loading plots for pasteurized and unpasteurized samples stored at 8 and 20 °C. The changes in the total concentrations of amino acids, sugars, organic acids, and peptides with time are shown in Figures 3 and S1 (Supporting Information) for the samples stored at 8 and 20 °C, respectively. The changes in individual metabolite concentrations with time for samples stored at 8 °C are shown in Figures 4 and 5 for pasteurized and unpasteurized samples, respectively. The corresponding data at 20 °C are depicted in Figures S2 and S3 (Supporting Information).

DISCUSSION

Metabolite Profiles before Storage for 4 Months. The metabolites found in each pair of pasteurized and unpasteurized sake samples from the same company were similar (Figure 1), and the sample pairs had high correlation coefficients (R >

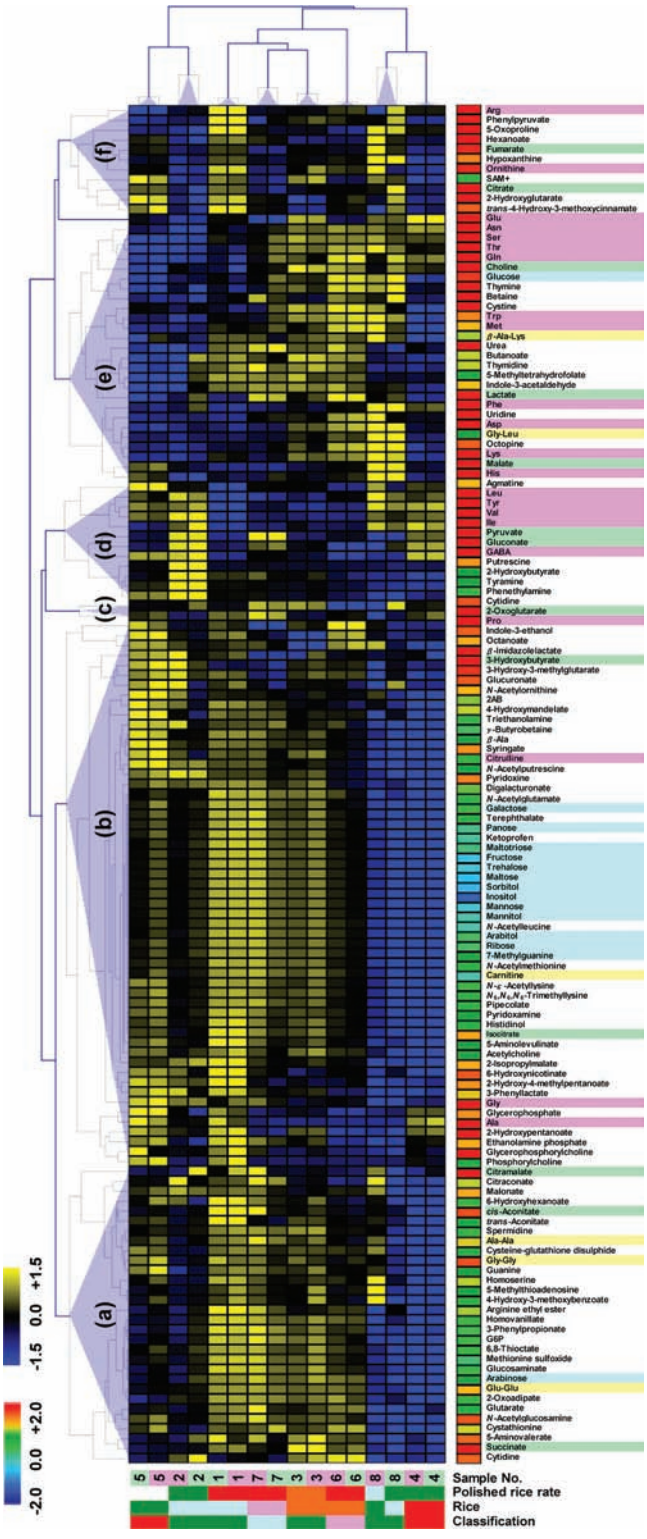

Figure 1. Heat map showing the metabolite profile before storage. All quantified concentrations were normalized to Z-scores for each sake sample, and the values were then further normalized to Z-scores for each metabolite (blue–black–yellow heat map). Log_{10} concentration values are also visualized (blue–green–red heat map). Clustering was conducted based on Pearson correlation for both metabolites and samples. Labels a–f indicate prominent metabolite clusters. Pink and green indicate the pasteurized and unpasteurized samples, respectively (Table S1, Supporting Information). In the color panels for sake classification, blue, pink, green, and red indicate *junmai*, special *junmai*, *junmaai-ginzyo*, and *junmai-dai-ginzyo* sake, respectively. In those for rice, blue, brown, red, green, and pink indicate *Miyama-nishiki*, *Dewano-sato*, *Kame-no-o*, *Yamada-nishiki*, and *Naenuki* rice, respectively. In those for the rice polishing rate (%), blue, green, brown, and red indicate 40, 50, 55, and 60%, respectively. The metabolites are colored pink for amino acids, blue for sugars, green for organic acids, and yellow for peptides. Metabolites that were detected in less than four sake samples were eliminated to reduce the complexity of the heat map.

0.949, $P < 0.0001$) (Table S1, Supporting Information). The samples were not clustered by sake type. For example, *junmai-dai-ginzyo* samples were at either end of the heat map. Samples prepared from *Dewano-sato* rice (samples 3 and 6) were aligned as neighbors on the heat map. Samples prepared with rice polishing rates of 55% and 60% (samples 1, 7, 3, and 6) showed similar features, with a higher concentration of metabolites in clusters a and b, which included all sugars except for glucose. It should be noted that a rice polishing rate of 60% indicates that 40% of the rice is removed. With higher removal of rice (i.e., lower rice polishing rates) fewer proteins are present for fermentation, which is expected to reduce the amount of glucose in the sake samples. However, sample 8 (rice polishing rate = 40% pasteurized and 50% unpasteurized) contained more glucose than other samples, and sample 6 (rice polishing rate = 60%) contained a relatively high amount of glucose. These results indicate that the glucose concentrations cannot be explained simply by the rice polishing rate. Only glucose was included in cluster e, and the other sugars were in clusters a and b, which indicates that the glucose profile is independent of the other sugars. Clusters d and e included most of the amino acids and were not characteristic for sake type, rice, or the rice polishing rate.

Changes in the Metabolic Profiles during Storage. PCA score plots and loading plots are shown in Figure 2. After storage at 8 °C, the mean Euclidean distances of PC1 and PC2 from 0 to 4 months calculated using eq 1 were 5.61 ± 1.60 (unitless) for the pasteurized samples and 7.10 ± 1.65 for the unpasteurized samples ($P = 0.1134$) (Figures 2A,B). These results indicate that the changes in the metabolite profiles of the unpasteurized samples with storage were generally larger than those in the pasteurized samples. This difference was more obvious after storage at 20 °C when the values were 7.43 ± 1.31 for pasteurized samples and 13.0 ± 1.82 for unpasteurized samples ($P < 0.0001$) (Figures 2C,D). Visual inspection of the loading plots showed most metabolites existed at PC1 ≥ 0 (Figures 2E–H). This distribution bias was more obvious in the plots after storage at 8 °C (Figures 2E,F) than in those at 20 °C (Figures 2G,H). Only arginine existed at PC1 < 0 and PC2 < 0 after storage at 8 °C (Figures 2E,F), which implies that the arginine showed different patterns compared to the other amino acids during storage. Under the storage at 20 °C, glycine, citrulline, and γ-aminobutyric acid were located at PC1 < 0 in the pasteurized samples (Figures 2G) and asparagine and alanine were located far from the other amino acids in the unpasteurized samples (Figures 2H). This indicates that a large change in the amino acid balance occurs during storage rather than a simple change in the overall concentration. Plots of the organic acids and sugars were homogenously distributed, while the plots of the peptides, except for glutamate–glutamate, were located closed to each other in all loading plots (Figures 2E–H). This indicates that the changes for these peptides occur

dx.doi.org/10.1021/jf2048993 | J. Agric. Food Chem. 2012, 60, 2586–2593

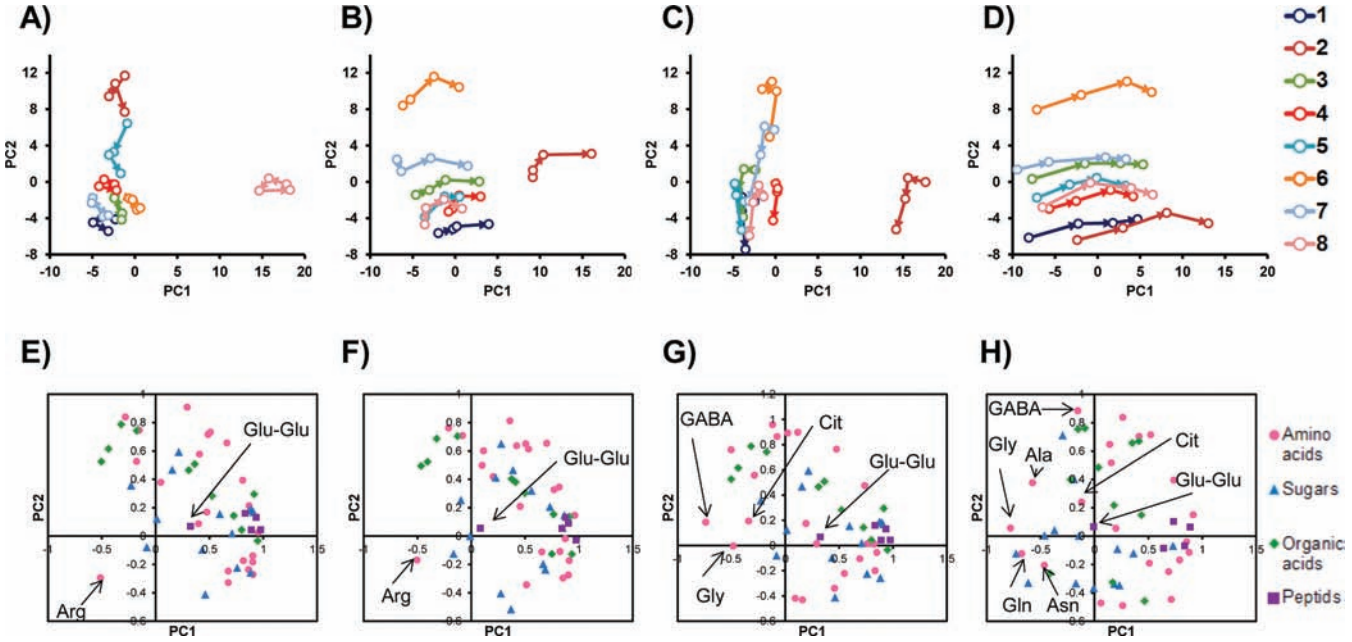

Figure 2. Principal component analysis score plots for (A) pasteurized samples stored at 8 °C, (B) unpasteurized samples stored at 8 °C, (C) pasteurized samples stored at 20 °C, and (D) unpasteurized samples stored at 20 °C. The first and second principal components accounted for (A) 43.1% and 20.7%, (B) 26.7% and 20.7%, (C) 38.9% and 21.1%, and (D) 29.6% and 20.4% of the variance. The labels from 1 to 8 indicate the sake sample number. Corresponding loading plots were visualized in panels E–H. The dots in pink are for amino acids, blue for sugars, green for organic acids, and purple for peptides. All metabolites were used for this analysis, but only metabolites in these four classes were visualized in the loading plots to reduce the complexity.

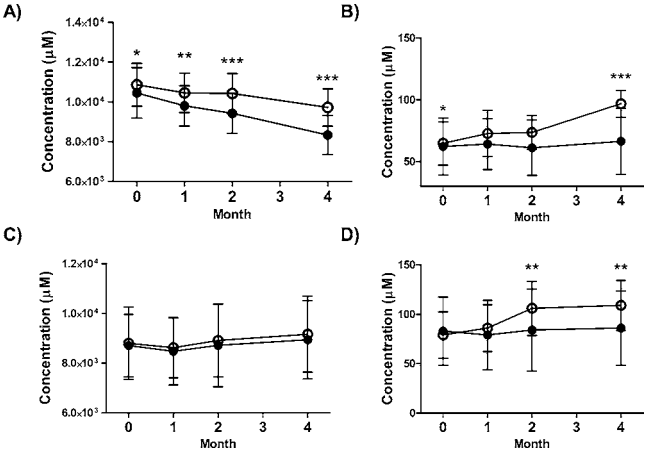

Figure 3. Changes in the total concentrations of amino acids (A), sugars (B), organic acids (C), and peptides (D) with time. Filled and open circles indicate the results for pasteurized and unpasteurized samples, respectively. All samples were stored at 8 °C. Corresponding data for samples stored at 20 °C are shown in Figure S2 (Supporting Information). Error bars indicate the standard deviations. Cysteine was not included in the amino acid profiles because it was detected in only a few samples and could be unstable in CE-TOFMS analysis. γ-Aminobutyric acid, citrulline, and ornithine were included instead.

over similar time-courses during storage and that they are independent of the storage temperature or pasteurization.

Amino Acids Profile. The average total amino acid concentration before storage in the pasteurized sake samples was significantly higher than that in the unpasteurized samples (P = 0.025) (Figure 3A). After storage at 8 °C, the total concentrations in both the pasteurized and unpasteurized samples gradually decreased. The difference between the pasteurized and unpasteurized samples increased after storage for 4 months (P < 0.001). This indicates the amino acid concentrations decreased at a faster rate in the pasteurized samples than the unpasteurized samples. After storage at 20 °C, a similar decreasing trend to that at 8 °C was observed, but slight increases in the amino acid concentrations were observed in the unpasteurized samples stored for 1 or 2 months (Figure S1A, Supporting Information).

After storage at 8 °C (Figures 4A and 5A), the concentrations of alanine, glycine, and leucine were high and showed significant differences between 0 and 4 months storage (>500 μmol/L, P < 0.001) for both pasteurized and unpasteurized sake. In the pasteurized sake, the concentrations of proline and glutamic acid were also high and showed significant differences between 0 and 4 months storage (P < 0.001). Among the amino acids showing relative high concentrations, the concentration of arginine was almost constant over the storage period (P = 0.0940). Similar trends were observed after storage at 20 °C (Figures S2A and S3A, Supporting Information). A similar gradual decreasing trend in the total amino acid concentration has been observed in Chinese rice wine,[21] and this change could be attributed to amino–carbonyl reactions.[6] However, the faster decrease in pasteurized samples cannot be explained by this reaction alone. Binding of free amino acids and proteins could also contribute to this decrease. The decreases in histidine (P = 0.038), threonine (P = 0.056), tryptophan (P = 0.067), and methionine (P = 0.38) during the storage of unpasteurized sake were faster than those in pasteurized sake (histidine, P = 0.015; threonine, P = 0.0004; tryptophan, P = 0.0002; and methionine, P = 0.0001). It has been reported that methionine degradation could result in the formation of methional and then dimethyl trisulfide, which gives the sake an undesirable aroma.[22] Therefore, these results indicate that the aroma of pasteurized

dx.doi.org/10.1021/jf2048993 | J. Agric. Food Chem. 2012, 60, 2586–2593

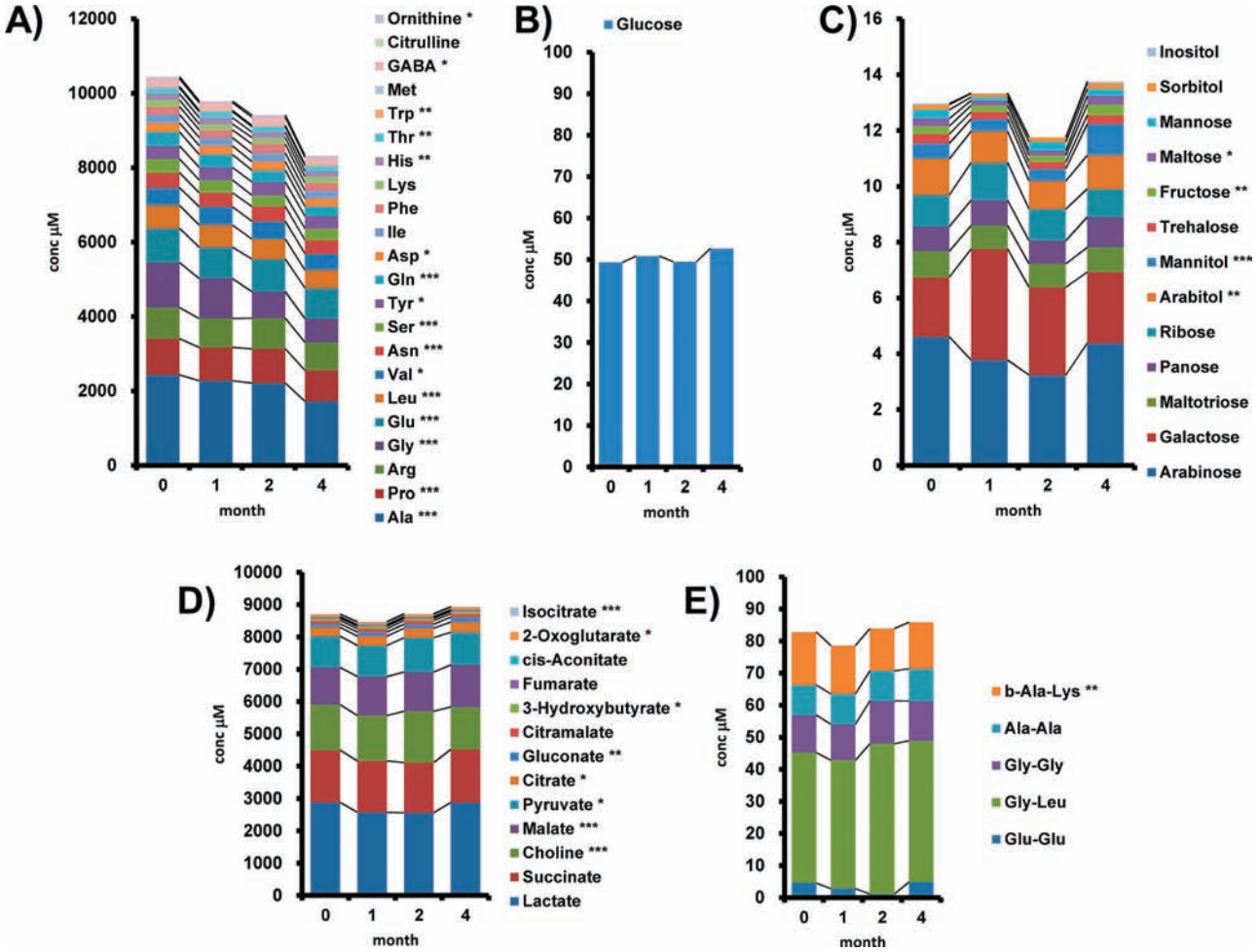

Figure 4. Changes in the concentrations of individual metabolites for amino acids (A), glucose (B), sugars except for glucose (C), organic acids (D), and peptide (E) with time in pasteurized samples stored at 8 °C. Corresponding data for samples stored at 20 °C are shown in Figure S2 (Supporting Information). Statistical differences between storage for 0 and 4 months were assessed.

sake will be more stable during storage than that of unpasteurized sake.

Sugars Profile. Before storage, the total sugar concentration in the unpasteurized sake samples (64.8 μmol/L) was significantly higher than that in the pasteurized samples (62.29 μmol/L, P = 0.0111) (Figure 3B). The total sugar concentration in the unpasteurized sake samples increased after storage at 8 °C for 4 months (Figure 3B), while that in the pasteurized samples was almost constant. Consequently, the difference between the unpasteurized (96.8 μmol/L) and pasteurized samples (66.4 μmol/L) increased and became more significant (P = 0.0007). Similar trends were observed after storage at 20 °C for 4 months, but the difference between the unpasteurized and pasteurized samples was larger (unpasteurized, 95.1 μmol/L; pasteurized, 52.9 μmol/L; P = 0.0009) (Figure S1B, Supporting Information).

Among the free sugars, glucose was dominant (pasteurized 77.6 ± 5.83%, unpasteurized 79.1 ± 5.28%), and the concentrations of panose and maltose were low (Figures 4B,C and 5B,C). These results are consistent with an earlier sugar profiling study.[14] During storage for 4 months at 8 °C, the glucose concentration was almost constant in pasteurized sake (P = 0.126) and increased significantly in unpasteurized sake (P = 0.00029). Over the same period, the concentration of arabitol decreased (P = 0.0012), while the concentrations of mannitol (P = 0.00037), fructose (P = 0.0030), and maltose (P = 0.044) increased significantly in pasteurized sake. In unpasteurized sake, the concentrations of maltotriose (P = 0.0016), panose (P = 0.0000028), and ribose (P = 0.034) decreased, while the concentrations of mannitol (P = 0.000051), fructose (P = 0.0013), and sorbitol (P = 0.044) increased. After storage at 20 °C, similar trends were observed, except for the concentration of glucose in pasteurized sake, which increased up to 1 month and subsequently decreased but not significantly (Figures S2B,C and S3B,C, Supporting Information). Our observations are consistent with those of Tanimoto et al., who found that the concentration of glucose increased and that of isomaltose decreased in unpasteurized sake after storage for 6 months, while the concentrations of these sugars were almost constant in pasteurized sake.[3] These sugars are mainly derived from starch in rice, which is converted by the rice mold, and yeast then metabolizes the products. The glucose and maltose were not metabolized. Therefore, the sugar profile strongly depends on the yeast and rice. However, the profile changes are more pronounced in unpasteurized sake than in pasteurized sake.

Organic Acid Profiles. The total concentrations of organic acids in the pasteurized and unpasteurized samples were similar and almost constant over the 4 month storage period at both 8 and 20 °C (Figure 2C and Figure S1C, Supporting

dx.doi.org/10.1021/jf2048993 | J. Agric. Food Chem. 2012, 60, 2586–2593

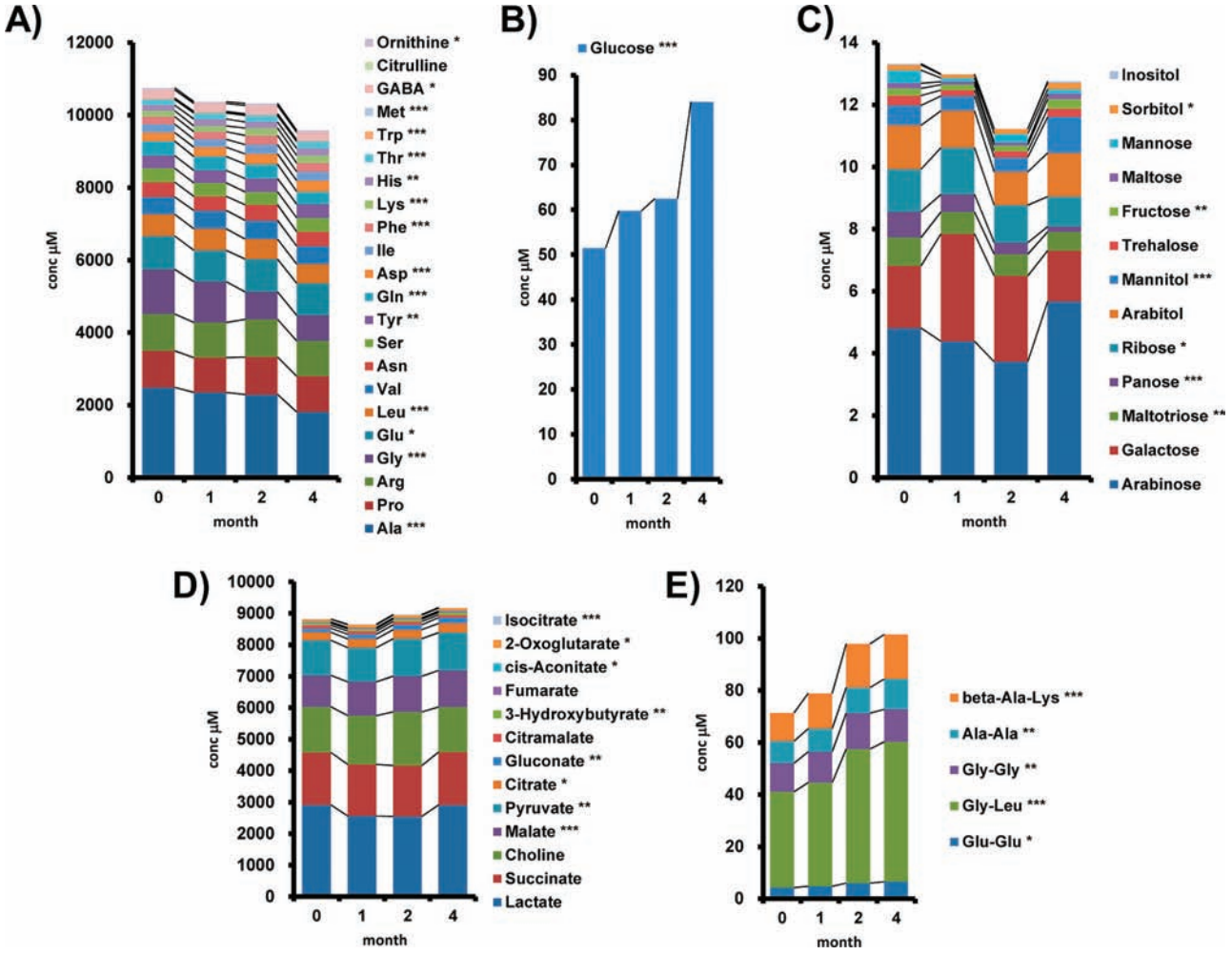

Figure 5. Changes in the concentrations of individual metabolites for amino acids (A), glucose (B), sugars except for glucose (C), organic acids (D), and peptide (E) with time in unpasteurized sake stored at 8 °C. Corresponding data for samples stored at 20 °C are shown in Figure S3 (Supporting Information). Statistical differences between storage for 0 and 4 months were assessed.

Information). Surprisingly, the concentrations of individual metabolites, including lactate (33.3 ± 2.77% pasteurized, 33.5 ± 2.06% unpasteurized), succinate (18.8 ± 1.98% pasteurized, 18.8 ± 1.72% unpasteurized), choline (16.2 ± 1.45% pasteurized, 16.9 ± 1.73% unpasteurized), malate (13.1 ± 4.54% pasteurized, 12.2 ± 2.98% unpasteurized), and pyruvate (10.7 ± 7.31% pasteurized, 10.6 ± 7.05% unpasteurized) (Figures 4D and 5D), were similar for the pasteurized and pasteurized samples. The concentrations of the individual metabolites increased and decreased with storage, but the metabolite balance was almost constant during storage (Figures 4D and 5D; Figures S2D and S3D, Supporting Information). This meant that the overall features of the organic acid profile of the sake that were obtained during brewing remained after pasteurization.

Most of the organic acids in sake come from the yeast, and while their concentrations are lower than those in wine,[23] they are the second most abundant charged metabolites in sake after amino acids.[13] Malate and succinate give sake a pleasant taste.[24,25] In both pasteurized and unpasteurized sake, the concentration of malate increased significantly ($P < 0.01$), while that of succinate was almost constant ($P > 0.69$) after storage at either temperature (Figures 4D and 5D; Figures S2D and S3D, Supporting Information). Although the contributions of these organic acids to the flavor are well-known, the time-dependent changes in their profiles were small for both storage conditions and types of samples. The quantified profile obtained in this study and the relative intensities of individual organic acids are needed to evaluate the contributions of these small changes during storage.

Peptide Profiles. The total peptide concentrations before storage were almost the same for the pasteurized and unpasteurized samples ($P = 0.44$). During storage at 8 °C for 4 months, the total peptide concentration in the pasteurized samples was almost constant, while that in the unpasteurized samples increased ($P = 0.0035$) (Figures 4E and 5E). Similar trends were observed for storage at 20 °C ($P = 0.0032$) (Figures S2E and S3E, Supporting Information)

The amino acids and peptides in sake mainly come from a hydrolysis reaction of proteins and microorganisms in glutinous and wheat *koji*, which is a source of nitrogen during alcoholic fermentation.[26] Several of the individual peptide concentrations increased moderately in pasteurized sake, while they remained almost constant in pasteurized sake. This could be attributed to the presence of active protease in unpasteurized sake but its deactivation by pasteurization. Orosensory effects are known for several peptides. Peptides with 5 to 13 amino acids contribute to an unpleasant taste, such as bitterness, or

dx.doi.org/10.1021/jf2048993 | J. Agric. Food Chem. 2012, 60, 2586–2593

turbidity[12] that is presumably derived from the function of amino acid residues, but these were not included in our profile because we focused on lower weight metabolites. Among the shorter peptides detected, glutamate−glutamate and glycine−leucine produce sour and bitter tastes, while the other detected dipeptides do not contribute to taste.[27]

A sensory evaluation was not conducted in this study, but the metabolite profiles were discussed with respect to known orosensory effects of peptides. In our previous metabolomic profiling study, many metabolites were highly correlated with taste, which made it difficult to identify the contribution of each metabolite.[13] In addition, the intensities of the individual metabolites are different, for example, fructose is sweeter than sucrose, which is sweeter than glucose. Therefore, concentration profiles and a large database of the taste intensities of the relevant metabolites are required to understand the relationship between metabolites and overall taste.

There are several limitations in this study. We did not measure volatile compounds that also contribute to the sensory and flavor properties of sake.[1,6] The high temperature of pasteurization causes suspension of proteins, which increases the turbidity of the sake,[28] but this was not measured in the present study. Another limitation is that chiral metabolites were not differentiated. For example, D-lactic acid is produced by yeast, and the amount of L-lactic acid depends on the concentration in the initial stage of brewing.[2] Integration of such data[2] with these techniques[29] will assist to identify the source of individual metabolites. The lack of a sensory evaluation is also a limitation of this study. However, because of the complexity of the flavor expression of sake,[30] a large number of testers and samples would be required in such an evaluation to obtain consistent scores. This study revealed that profiling metabolites of the just bottled samples is not sufficient to access the quality of the product because the metabolomic profiles change during storage even in pasteurized samples. Metabolomic analyses of the sample with different brewing conditions are necessary in the future to be able to apply these results to brewing process improvement. In addition, to evaluate the quality and the design of the brewing process, the time between the bottling and consumption should be considered.

In summary, we studied the time-dependent profiles of charged metabolites and sugars in pasteurized and unpasteurized sake. Amino acids that were present in high concentrations, such as alanine, proline, and glycine, generally decreased in concentration during storage, while those present in low concentrations increased. Surprisingly, a rapid decrease in the total amino acid concentration was observed in pasteurized sake. The organic acid concentrations were relatively constant in both types of sake. Glucose was the most abundant sugar and had a different profile than the other sugars. Peptide and glucose concentrations increased during storage, while polysaccharide concentrations decreased in unpasteurized sake. By comparison, the concentrations of these metabolites were relatively constant in pasteurized sake. For both pasteurized and unpasteurized sake stored for 4 months, the changes in the metabolite profiles were similar for different types of sake and different storage temperatures.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Instrument parameters for CE-TOFMS; cationic metabolite analysis; anionic metabolite analysis; changes in the total concentrations of amino acids, sugars, organic acids, and peptides with time; changes in the concentrations of individual metabolites for amino acids, glucose, sugars except for glucose, organic acids, and peptides with time in pasteurized samples stored at 20 °C; changes in the concentrations of individual metabolites for amino acids, glucose, sugars except for glucose, organic acids, and peptides with time in unpasteurized samples stored at 20 °C; sample information. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +81-235-29-0528. Fax: +81-235-29-0574. E-mail: msugi@sfc.keio.ac.jp.

Funding

This work was supported by the Yamagata Prefectural Government and the city of Tsuruoka.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the Sake brewer society of Tsuruoka and Regional foundation Shonai industrial promotion center for providing sake samples and their helpful advice.

■ REFERENCES

(1) Moriwaki, H.; Hagiwara, A.; Takasaki, M.; Izumi, F.; Watanabe, A.; Shimizu, R.; Kuribayashi, N.; Totani, Y.; Suzuki, Y. Electrospray ionization-mass spectrometric measurement of sake, a traditional Japanese alcohol beverage, for characterization. *Anal. Sci.* **2010**, *26*, 379−382.

(2) Kodama, S.; Yamamoto, A.; Matsunaga, A.; Matsui, K.; Nakagomi, K.; Hayakawa, K. Behaviors of D- and L-lactic acids during the brewing process of sake (Japanese rice wine). *J. Agric. Food Chem.* **2002**, *50*, 767−770.

(3) Tanimoto, S.; Matsumoto, H.; Fujii, K.; Ohdoi, R.; Sakamoto, K.; Yamane, Y.; Miyake, M.; Shimoda, M.; Osajima, Y. Enzyme inactivation and quality preservation of sake by high-pressure carbonation at a moderate temperature. *Biosci., Biotechnol., Biochem.* **2008**, *72*, 22−28.

(4) Tanimoto, S.; Matsumoto, H.; Fujii, K.; Ohdoi, R.; Sakamoto, K.; Izuwa, S.; Yamane, Y.; Shimoda, M.; Osajima, Y. Thermal inactivation behavior of enzymes in fresh sake during pasteurization. *J. Brew. Soc. Jpn.* **2004**, *99*, 208−214 (in Japanese).

(5) Yamamoto, N.; Saito, T. Pasteurization and storage condition of ginjoshu. *J. Brew. Soc. Jpn.* **1989**, *84*, 496−498 (in Japanese).

(6) Isogai, A.; Utsunomiya, H.; Kanda, R.; Iwata, H. Changes in the aroma compounds of sake during aging. *J. Agric. Food Chem.* **2005**, *53*, 4118−4123.

(7) Shen, F.; Niu, X.; Yang, D.; Ying, Y.; Li, B.; Zhu, G.; Wu, J. Determination of amino acids in Chinese rice wine by Fourier transform near-infrared spectroscopy. *J. Agric. Food Chem.* **2010**, *58*, 9809−9816.

(8) Nose, A.; Myojin, M.; Hojo, M.; Ueda, T.; Okuda, T. Proton nuclear magnetic resonance and Raman spectroscopic studies of Japanese sake, an alcoholic beverage. *J. Biosci. Bioeng.* **2005**, *99*, 493−501.

(9) Iwano, K.; Ito, T.; Nakazawa, N. Characteristics of amino acid compositions of Ginjyo-shu, Junmai-shu, Honjyozo-shu, and Futsu-shu. *J. Brew. Soc. Jpn.* **2004**, *99*, 526−533 (in Japanese).

(10) Iwano, K.; Takahashi, K.; Ito, T.; Nakazawa, N. Search for amino acids affecting the taste of Japanese sake. *J. Brew. Soc. Jpn.* **2004**, *99*, 659−664 (in Japanese).

(11) Hauman, I.; Boden, J.; Mainka, A.; Jegle, U. Simultaneous determination of inorganic anions and cations by capillary electro-

phoresis with indirect UV detection. *J Chromatogr., A* **2000**, *895*, 269−277.
(12) Hashizume, K.; Okuda, M.; Numata, M.; Iwashita, K. Bitter-tasting sake peptides derived from the N-terminus of the rice glutelin acidic subunit. *Food Sci. Technol. Res.* **2007**, *13*, 270−274.
(13) Sugimoto, M.; Koseki, T.; Hirayama, A.; Abe, S.; Sano, T.; Tomita, M.; Soga, T. Correlation between sensory evaluation scores of Japanese sake and metabolome profiles. *J. Agric. Food Chem.* **2010**, *58*, 374−383.
(14) Niu, X.; Shen, F.; Yu, Y.; Yan, Z.; Xu, K.; Yu, H.; Ying, Y. Analysis of sugars in Chinese rice wine by Fourier transform near-infrared spectroscopy with partial least-squares regression. *J. Agric. Food Chem.* **2008**, *56*, 7271−7278.
(15) Takenaka, F.; Uchiyama, H.; Imamura, T. Identification of α-D-glucosylglycerol in sake. *Biosci., Biotechnol., Biochem.* **2000**, *64*, 378−385.
(16) Soga, T.; Baran, R.; Suematsu, M.; Ueno, Y.; Ikeda, S.; Sakurakawa, T.; Kakazu, Y.; Ishikawa, T.; Robert, M.; Nishioka, T.; Tomita, M. Differential metabolomics reveals ophthalmic acid as an oxidative stress biomarker indicating hepatic glutathione consumption. *J. Biol. Chem.* **2006**, *281*, 16768−16776.
(17) Sugimoto, M.; Sakagami, H.; Yokote, Y.; Onuma, H.; Kaneko, M.; Mori, M.; Sakaguchi, Y.; Soga, T.; Tomita, M., Non-targeted metabolite profiling in activated macrophage secretion. *Metabolomics*, DOI: 10.1007/s11306-011-0353-9.
(18) Soga, T.; Igarashi, K.; Ito, C.; Mizobuchi, K.; Zimmermann, H. P.; Tomita, M. Metabolomic profiling of anionic metabolites by capillary electrophoresis mass spectrometry. *Anal. Chem.* **2009**, *81*, 6165−6174.
(19) Sugimoto, M.; Wong, D. T.; Hirayama, A.; Soga, T.; Tomita, M. Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles. *Metabolomics* **2010**, *6*, 78−95.
(20) Saeed, A. I.; Sharov, V.; White, J.; Li, J.; Liang, W.; Bhagabati, N.; Braisted, J.; Klapa, M.; Currier, T.; Thiagarajan, M.; Sturn, A.; Snuffin, M.; Rezantsev, A.; Popov, D.; Ryltsov, A.; Kostukovich, E.; Borisovsky, I.; Liu, Z.; Vinsavich, A.; Trush, V.; Quackenbush, J. TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* **2003**, *34*, 374−378.
(21) Shen, F.; Ying, Y.; Li, B.; Zheng, Y.; Zhuge, Q. Multivariate classification of rice wines according to ageing time and brand based on amino acid profiles. *Food Chem.* **2011**, *129*, 565−569.
(22) Isogai, A.; Kanda, R.; Hiraga, Y.; Iwata, H.; Sudo, S. Contribution of 1,2-dihydroxy-5-(methylsulfinyl)pentan-3-one (DMTS-P1) to the formation of dimethyl trisulfide (DMTS) during the storage of Japanese sake. *J. Agric. Food Chem.* **2010**, *58*, 7756−7761.
(23) Ding, M. Y.; Suzuki, Y.; Koizumi, H. Simultaneous determination of organic acids, inorganic anions and cations in beverages by ion chromatography with a mixed-bed stationary phase of anion and cation exchangers. *Analyst* **1995**, *120*, 1773−1777.
(24) Yano, S.; Asano, T.; Kurose, N.; Hiramatsu, J.; Shimoi, H.; Ito, K. Characterization of an α-ketoglutarate-resistant sake yeast mutant with high organic acid productivity. *J. Biosci. Bioeng.* **2003**, *96*, 332−336.
(25) Kubo, Y.; Takagi, H.; Nakamori, S. Effect of gene disruption of succinate dehydrogenase on succinate production in a sake yeast strain. *J. Biosci. Bioeng.* **2000**, *90*, 619−624.
(26) Zhuge, Q.; Li, B.; Liu, X.; Zheng, Y. Comparison study of amino acids in yellow rice wine by high-performance anion-exchange chromatography. *Liquor-Making Sci. Technol.* **2008**, *4*, 108.
(27) Kirimura, J.; Shimizu, A.; Kimizuka, A.; Ninomiya, T.; Katsuya, N. Contribution of peptides and amino acids to the taste of foods. *J. Agric. Food Chem.* **1969**, *17*, 689−695.
(28) Mikami, S.; Nunokawa, Y. Appearance of protein turbidity and pasteurizing conditions in sake. *Hakkokogaku Kaishi* **1978**, *56*, 194−198 (in Japanese).
(29) Tokuoka, M.; Sawamura, N.; Kobayashi, K.; Mizuno, A. Simple metabolite extraction method for metabolic profiling of the solid-state fermentation of *Aspergillus oryzae*. *J. Biosci. Bioeng.* **2010**, *110*, 665−669.
(30) Iwano, K.; Ito, T.; Nakazawa, N. Correlation analysis of a sensory evaluation and the chemical components of Ginjyo-shu. *J. Brew. Soc. Jpn.* **2005**, *100*, 639−649 (in Japanese).