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Gas chromatography/mass spectrometry based component profiling and quality prediction for Japanese sake

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Sake is a Japanese traditional alcoholic beverage, which is produced by simultaneous saccharification and alcohol fermentation of polished and steamed rice by *Aspergillus oryzae* and *Saccharomyces cerevisiae*. About 300 compounds have been identified in sake, and the contribution of individual components to the sake flavor has been examined at the same time. However, only a few compounds could explain the characteristics alone and most of the attributes still remain unclear. The purpose of this study was to examine the relationship between the component profile and the attributes of sake. Gas chromatography coupled with mass spectrometry (GC/MS)-based non-targeted analysis was employed to obtain the low molecular weight component profile of Japanese sake including both nonvolatile and volatile compounds. Sake attributes and overall quality were assessed by analytical descriptive sensory test and the prediction model of the sensory score from the component profile was constructed by means of orthogonal projections to latent structures (OPLS) regression analysis. Our results showed that 12 sake attributes [*ginjo-ka* (aroma of premium *ginjo* sake), grassy/aldehydic odor, sweet aroma/caramel/burnt odor, sulfury odor, sour taste, umami, bitter taste, body, *amakara* (dryness), aftertaste, pungent/smoothness and appearance] and overall quality were accurately explained by component profiles. In addition, we were able to select statistically significant components according to variable importance on projection (VIP). Our methodology clarified the correlation between sake attribute and 200 low molecular components and presented the importance of each component thus, providing new insights to the flavor study of sake.

[**Key words:** Japanese sake; Metabolomics; Gas chromatography/mass spectrometry; Orthogonal projections to latent structures; Variable importance on projection]

Many of the low molecular compounds in fermented food are odor and taste-active and they constitute the flavor. Sake is a Japanese traditional alcoholic beverage which is produced by simultaneous saccharification and alcohol fermentation of polished and steamed rice by *Aspergillus oryzae* and *Saccharomyces cerevisiae* (1). This multiple parallel fermentation process is unique to sake brewing and generates various low molecular weight compounds that constitute the complex flavor of sake. A recent study on sensory analysis arranged flavor terminology system with 86 terms representing the attributes independently perceivable in sake (2).

Recently, the declining domestic consumption has become a serious problem in sake industry thus, there is an ever-increasing interest and challenge to increase the variety of sake to stimulate enthusiasm among consumers (3). For example, sake that is rich in fragrance was made by breeding isoamyl acetate/ethyl caproate-high producing yeasts (4,5), while yeasts which have different productivities of organic acids (an important components of taste) have also been bred (6,7). However, information about the relationship between the flavor characteristics and the components necessary for these studies is limited. Moreover, because the amount of export has been increasing for the last decade, a method capable of objectively evaluating the flavor of sake is required.

A lot of effort in the 20th century has focused on the identification of 300 compounds in sake, as well as the contribution of individual components to sake flavor (8–10). However, there are many attributes of sake that cannot be explained by chemical compounds. Nowadays, there has been a growing interest in metabolomics which can detect complex biological changes by examining variation in total metabolite profiles (11,12). In fact, the comprehensive approach of metabolomics has been effectively applied to explain the differences in quality of food samples (13–15).

The purpose of this study was to examine the relationship between the component profile and the attributes of sake. Nonvolatile components were derivatized by oximation and trimethylsilylation while volatile components were extracted by stir bar sorptive extraction (SBSE) or dichloromethane extraction method. Gas chromatography coupled with mass spectrometry (GC/MS) based non-targeted analysis was then employed to obtain the low molecular weight component profile. On the other hand, analytical descriptive sensory test was performed to assess the sensory attributes of sake. Afterward, orthogonal projections to latent structures (OPLS) regression analysis was used to construct the prediction model of the sensory score from component profile and

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statistically significant components were selected according to variable importance on projection (VIP). Result showed that sake characteristics were accurately explained by the component profiles. Furthermore, this methodology was able to clarify the correlations between the each component and the sake attributes and present the candidates for odor/taste-active compounds.

MATERIALS AND METHODS

Samples and reagents Forty various types of sake samples, such as ordinary sake and specially designated sake, were purchased from the market (June 2012, Hiroshima, Japan). The sample descriptions are shown in Table 1.

Ribitol, 3-octanol, pyridine, dichloromethane, ethanol, sodium chloride, ammonium sulfate and sodium sulfate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Methoxyamine hydrochloride was purchased from Sigma–Aldrich Co. LLC. (Milwaukee, WI, USA). *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) and *n*-alkanes (C_6–C_{25}) were purchased from GL Science, Inc. (Tokyo, Japan). $^{13}C_2$-sotolon was synthesized according to (16).

Nonvolatile component analysis Twenty microliters of sake was dispensed into a 1.5 mL micro tube. As an internal standard, ribitol (60 μL, diluted in deionized water to 0.2 mg/mL) was added and mixed with the sample. Then, vacuum centrifugation was performed for 2 h (Spin Dryer Standard VC-96R, TAITEC Co. Ltd., Tokyo, Japan; Dry Vacuum Pump DTC-41, ULVAC, Inc., Kanagawa, Japan; Freeze Trap VA-500R, TAITEC Co. Ltd.), followed by freeze-drying overnight (Freeze Dryer VD-800F, TAITEC Co. Ltd.). All samples were analyzed in triplicates ($n = 3$).

Oximation and trimethylsilylation were used for derivatization. First, methoxyamine hydrochloride (100 μL, 20 mg/mL in pyridine) was added to the dried sample and the mixture was incubated at 30°C for 90 min (Thermomixer comfort, Eppendorf Co. Ltd., Tokyo, Japan). MSTFA (50 μL) was then added and the mixture was incubated at 37°C for 30 min.

TABLE 1. List of sakes studied.

No.	Designation	Sample ID	Rice variety	Polishing rate of rice (%)	Region
1	*Daiginjo*	D1	*Yamadanishiki*	35	Fukushima
2	*Daiginjo*	D2	*Yamadanishiki*	35	Akita
3	*Daiginjo*	D3	*Yamadanishiki*	35	Akita
4	*Daiginjo*	D4	*Yamadanishiki*	40	Iwate
5	*Daiginjo*	D5	*Yamadanishiki*	40	Nagano
6	*Daiginjo*	D6	*Yamadanishiki*	40	Shizuoka
7	*Ginjo*	G1	*Yamadanishiki*	50	Yamagata
8	*Daiginjo*	D7	*Yamadanishiki*	50	Gunma
9	*Daiginjo*	D8	*Yamadanishiki*	50	Toyama
10	*Ginjo*	G2	*Yamadanishiki*	59	Fukushima
11	*Honjozo*	H1	*Yamadanishiki*	60	Gunma
12	*Ginjo*	G3	*Yamadanishiki*	60	Hyogo
13	*Honjozo*	H2	*Yamadanishiki*	70	Hyogo
14	*Honjozo*	H3	*Yamadanishiki*	70	Hyogo
15	Ordinary	O1	*Yamadanishiki*	70	Ishikawa
16	*Junmaidaiginjo*	JD1	*Yamadanishiki*	35	Kyoto
17	*Junmaidaiginjo*	JD2	*Yamadanishiki*	35	Yamagata
18	*Junmaidaiginjo*	JD3	*Yamadanishiki*	35	Yamagata
19	*Junmaidaiginjo*	JD4	*Yamadanishiki*	40	Iwate
20	*Junmaidaiginjo*	JD5	*Yamadanishiki*	40	Niigata
21	*Junmaidaiginjo*	JD6	*Yamadanishiki*	40	Kochi
22	*Junmai* (*Junmaiginjo*)	JG1	*Yamadanishiki*	50	Yamagata
23	*Junmaiginjo*	JG2	*Yamadanishiki*	50	Gunma
24	*Junmaiginjo*	JG3	*Yamadanishiki*	50	Shizuoka
25	*Junmai*	J1	*Yamadanishiki*	60	Yamagata
26	*Tokubetsu junmai*	TJ1	*Yamadanishiki*	60	Ishikawa
27	*Junmaiginjo*	JG4	*Yamadanishiki*	60	Hyogo
28	*Junmai*	J2	*Yamadanishiki*	70	Kyoto
29	*Junmai*	J3	*Yamadanishiki*	70	Hyogo
30	*Junmai*	J4	*Yamadanishiki*	70	Hyogo
31	*Junmaidaiginjo*	JD7	*Yamadanishiki*		Yamaguchi
32	*Junmaidaiginjo*	JD8	*Yamadanishiki*		Yamaguchi
33	Ordinary	O2			Hyogo
34	Ordinary	O3			Hyogo
35	Ordinary	O4			Kyoto
36	Ordinary	O5			Hyogo
37	Ordinary	O6			Kyoto
38	Ordinary	O7			Kyoto
39	Ordinary	O8			Hyogo
40	Ordinary	O9			Kyoto

GC/MS analysis was performed on a GCMS-QP 2010 Ultra (Shimadzu Co., Kyoto, Japan) equipped with a CP-SIL 8 CB low-bleed column (0.25 mm × 30 m, 0.25 μm, Agilent technologies Inc., Atlanta, GA, USA) and an AOC-20i/s (Shimadzu Co.) as an autosampler. System control and data acquisition were conducted using the GCMS solutions ver.2.7.1 software. The derivatized sample (1 μL) was injected in split mode, 25:1 (v/v), with an injection temperature of 230°C. The carrier gas (He) flow was 1.12 mL/min. The column temperature was held at 80°C for 2 min, increased by 15°C/min to 330°C, and then held for 6 min. The transfer line and ion source temperatures were 250 and 200°C, respectively. Ions were generated by electron ionization (EI) at 70 eV. Mass spectra were recorded at 20 scan/s over the mass range m/z 85 − 500.

Raw data acquired from GC/MS were exported in the net CDF format. Baseline correction, peak detection and alignment were conducted using the metAlign software (Wageningen UR, The Netherlands, available for free at http://www.pri.wur.nl/UK/products/MetAlign/) (17). The results were exported in the CSV format. Subsequently, the data were read by the AIoutput software (available for free at http://prime.psc.riken.jp/) to make the peak matrix (18). Then, peaks were annotated by comparing the retention time and unique mass spectra with our in-house reference library except α-D-glucosyl glycerol which were annotated with the information from literature (19). The peak intensity was normalized against the ribitol internal standard.

Volatile component analysis combined with stir bar sorptive extraction In this work, we applied the stir bar sorptive extraction (SBSE) method to analyze the volatile components. SBSE, which uses a stir bar coated with poly(dimethylsiloxane) (PDMS), has been widely used for its versatility and sensitivity (20). 10 mL of sake sample which was adjusted to a 10% (v/v) ethanol concentration with pure water was dispensed into a 10 mL glass headspace vial. 2 g of NaCl was added and 3-octanol was added up to 0.5 mg/L as an internal standard. Then, a stir bar (Twister from Gerstel, Mulheim an der Ruhr, Germany, 10 mm length, 0.5 mm layer) was placed in and the mixture was stirred at room temperature (24°C), 125 ×g for 1 h. Stir bar was then removed from the sample, dried with a lint-free tissue, and transferred to a glass thermal desorption tube for GC/MS analysis.

A Gerstel TDS 2 thermodesorption system was used. The desorption tube was introduced into a thermodesorption unit then, the stir bar was thermally desorbed by heating the TDS 2 from 20°C (for 1 min) to 230°C (for 4 min) at 60°C/min. The desorbed components were cryofocused in the CIS4 at −150°C. Afterward, the CIS4 temperature was increased to 250°C at 12°C/s and held for 10 min. The trapped components were injected onto a GC column. Injection was performed in the splitless mode.

GC/MS analysis was performed on an Agilent 6890/5973 GC/MS system equipped with an HP-INNOWax column (0.25 mm × 60 m, 0.25 μm, Agilent technologies Inc.) System control and data acquisition were conducted using the Agilent MSD Chemistation E.02.02.1431 software. The carrier gas (He) flow was 1.0 mL/min. The column temperature was held at 40°C for 5 min, increased by 3°C/min to 240°C, and then held for 15 min. The ion source temperature was 230°C. Ions were generated by EI at 70 eV. Mass spectra were recorded at 4.45 scan/s over the mass range m/z 35 − 350.

Peak detection and calculation of the peak area were conducted using the Agilent MSD Chemistation E.02.02.1431 software. Retention indices of the eluted compounds were calculated on the basis of the standard alkane mixture (C_6–C_{25}). Peaks were annotated by comparing the unique mass spectra with the NIST11 reference library of the National Institute of Standards and Technology and the retention indices with literature data according to the comprehensive retention index database (Nishikawa Keisoku Co., Ltd., Tokyo, Japan) or standard compounds. The peak area was normalized against the 3-octanol internal standard.

Volatile component analysis combined with dichloromethane extraction Polar compounds which were difficult to be extracted by SBSE method were extracted using dichloromethane. 50 μL of internal standard mixture (3-octanol 100 mg/L, $^{13}C_2$-sotolon 5 mg/L in ethanol) was added to 5 mL of sake sample. Ammonium sulfate (0.25 g) and dichloromethane (2 mL) were then added and stirred twice by a mixer for 45 s each. After centrifugation at 1030 ×g for 10 min, the organic layer was transferred to a brown centrifuge tube. Dichloromethane (2 mL) was added to the remaining aqueous layer and extraction was repeated again. The organic layers were combined and centrifuged at 1030 ×g for 10 min to remove the water layer. After dehydration using sodium sulfate, the sample was concentrated to about 30 μL under nitrogen gas stream.

GC/MS analysis was performed on an Agilent 6890/5975 GC/MS system equipped with a DB-WAX column (0.25 mm × 60 m, 0.25 μm, Agilent Technologies Inc.). System control and data acquisition were conducted using the Agilent MSD Chemistation E.012.002.2371431 software. The extracted sample (3 μL) was injected in splitless mode with an injection temperature of 230°C. The carrier gas (He) flow was 2.0 mL/min. The column temperature was held at 40°C for 2 min, increased by 3°C/min to 230°C, and then held for 40 min. The ion source temperature was 230°C. Ions were generated by EI at 70 eV. Mass spectra of 17 compounds were recorded at single ion monitoring (SIM) mode.

Peak detection and calculation of the peak area were conducted using the Agilent MSD Chemistation E.021.020.2371431 software. The peak area was normalized against the 3-octanol or $^{13}C_2$-sotolon internal standard.