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Comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry reveals the correlation between chemical compounds in Japanese sake and its organoleptic properties

Kei Takahashi,[1,*] Fumie Kabashima,[2] and Fumihiko Tsuchiya[2,§]

National Research Institute of Brewing, 3-7-1 Kagamiyama, Higashi-hiroshima, Hiroshima 739-0046, Japan[1] *and LECO Japan Corporation, 2-13-4 Shiba, Minato-ku, Tokyo 105-0014, Japan*[2]

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Japanese sake is a traditional alcoholic beverage composed of a wide variety of metabolites, which give it many types of tastes and flavors. Previously, we have reported that medium-chain fatty acids contribute to a fatty odor in sake (Takahashi, K., et al., J. Agric. Food Chem., 62, 8478–8485, 2014). In this study, we have reanalyzed the data obtained using two-dimensional gas chromatography coupled with time-of-flight mass spectrometry. The relationship between the chemical components in sake and specific organoleptic properties such as off-flavor and quality has been explored. This led to the identification of the type of chemical compounds present and an assessment of the numerous candidate compounds that correlate with such organoleptic properties in sake. This research provides important fundamental knowledge for the sake-brewing industry.

[**Key words:** Organoleptic property; Aroma; Off-flavor; Japanese sake; Rice wine; Comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry; Metabolomics; Ethyl caproate; Dipeptide]

Japanese sake, a traditional fermented alcoholic beverage, is produced from polished rice (*Oryza sativa* Japonica sp.), rice mold (*Aspergillus oryzae*), and sake yeast (*Saccharomyces cerevisiae*) by simultaneous saccharification and alcohol fermentation (1,2). Thus, sake contains a variety of metabolites derived from these ingredients and microorganisms, which often vary depending on the brewing process, the brewing conditions, techniques employed, and storage conditions (3–8), similar to the winemaking process (9–11).

Many terms describe the characteristic tastes and flavors of sake, including ethyl caproate flavor (12), fatty odor (5,7,13), sweet caramel-like odor, roast odor, *hineka* odor (14,15), raw-*hineka* odor (similar to *mureka* odor (16)), yeast-debris odor, sulfur compound odor (17), diacetyl odor (18), umami taste (19), bitterness (5), and astringent taste. These organoleptic properties could be due to the concentration or combinations of the chemical compounds in the sake. Several studies have identified compounds that confer such organoleptic properties (4,5,14,15,17,18,20). However, the exact relationships among the different compounds and the resulting organoleptic properties remain unclear.

Recent developments in analytical instrumentation and data processing techniques have led to the emergence of two-dimensional gas chromatography (GC × GC) coupled with high-speed scanning time-of-flight mass spectrometry (TOFMS; full abbreviation, GC × GC–TOFMS) as a powerful analytical method for identifying large numbers of volatile metabolites in complex samples. Furthermore, by using a statistical approach that combines the multidimensional data obtained from these analyses – such as the 1st and 2nd retention time, mass spectrum, and replicate variance–we can effectively unravel the nature of the different components present in each sample (5,21–25). Previously, we have used GC–flame ionization detector (FID) and GC × GC–TOFMS systems to demonstrate that an unpleasant fatty odor in sake could be positively correlated with medium-chain fatty acids (MCFAs), MCFA derivatives, long-chain alkanes, and indole-related compounds (5). In addition, an inharmonious bitter taste was positively correlated with some furans, ketones and the ratio of octanoic acid/hexanoic acid. Compared with the loading plot method, which is used with principal component analysis (PCA), this direct approach appears to be highly effective for identifying the chemical compounds that can be correlated with certain organoleptic properties. In this study, we reanalyzed the data obtained using comprehensive GC × GC–TOFMS and identified correlations between the putative chemical compounds and the specific organoleptic properties other than fatty odor and inharmonious bitter taste, which have been previously reported.

MATERIALS AND METHODS

Materials Eleven premium Japanese sake (*ginjo* sake) samples were selected from entries of the National New Sake Awards [National Research Institute of Brewing (NRIB) competition in 2011], as described previously (5).

Methanol (mass spectrometry grade) was purchased from Sigma Aldrich (St. Louis, MO, USA). All other chemicals were purchased from Wako Pure Chemical

* Corresponding author. Tel.: +81 82 420 0812; fax: +81 82 420 0850.
E-mail address: k.takahashi@nrib.go.jp (K. Takahashi).
§ Present address: ThermoFisher Scientific, 3-9 Moriya-cho, Kanagawa-ku, Yokohama, Kanagawa 221-0022, Japan.

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Industries Ltd. (Osaka, Japan). DB-1 and BPX-50 capillary columns were purchased from Agilent Technologies and SGE Analytical Science Japan Inc. (Yokohama, Japan), respectively.

Sensory evaluations Sensory evaluations were performed by 15 well-trained panelists as described in a Japanese government report (26). The taste quality and the flavor quality were evaluated using a 5-point scale.

Identification of specific organoleptic properties (ethyl caproate flavor, *hineka* (14,15), raw-*hineka* odor, yeast debris-like odor, sulfur like odor, pleasant bitter taste, and pleasant astringent taste) was determined based on an assessment of their presence or absence in a sample using a check-all-that-apply (CATA) survey (27,28). The number of notations of the presence of each factor was used as an index of intensity (Table 1).

GC × GC–TOFMS analysis and data processing GC × GC–TOFMS analysis was performed using a LECO Pegasus 4D Time-of-Flight Mass Spectrometer (LECO, St. Joseph, MI, USA), and data analysis was conducted using ChromaTOF software version 4.50 and the Wiley09 database library as previously described (5). Analyses were performed four times per sake sample, and the first data set obtained for each sample was discarded to avoid possible contamination by carryover compounds from the previous analysis. Thus, data from the subsequent three analyses per sake sample were obtained. These data were screened using ANOVA using JMP version 10.0.2 (SAS Institute Inc., Cary, NC, USA) to identify significant differences between samples. Correlation analyses between a selection of 568 of the putative chemical compounds and organoleptic properties were performed using JMP version 10.0.2 (SAS Institute Inc.).

RESULTS AND DISCUSSION

Correlation of chemical compounds with flavor quality and taste quality In a previous report (5), we performed comprehensive chemical compounds analyses in eleven *ginjo* sake samples using GC × GC–TOFMS, which resulted in obtaining >3000 peaks. After statistical processing of this data using ANOVA to determine specify significant differences between sake samples, these peaks were reduced to 568. Correlation analysis between 568 of the putative chemical compounds and organoleptic properties such as quality and off-flavor, shown in Table 1, was performed. This analysis was first performed to identify associations between sake flavor quality and specific chemical compounds. Some cyclo-dipeptides and amino acid-related compounds appeared to be associated with a high-quality flavor in sake, as shown in Table 2. It is likely that these compounds do not directly contribute to flavor quality because of their low volatility, but they may contribute via an indirect mechanism such as acting as a nutritional source for fermentation or as precursors for flavor components. Generally, the amount of amino acids present has a negative effect on the taste quality of sake, as it increases the unfavorable *zatsumi* taste. Therefore, it is interesting that the presence of certain nitrogen compounds may be necessary for improving the flavor quality of sake.

In contrast, various sulfur-containing compounds and long-chain alkanes were clearly negatively correlated with the flavor quality of sake (Table 2). Various sulfur-containing compounds (thiol compounds) are considered to produce unfavorable flavors in wine, including 2-(thioethyl)ethanol (29), 3-methylsulphanylpropan-1-ol (29), methional (30), and 4-mercapto-4-methyl-2-pentanone (30); these are also considered to be unfavorable compounds in sake. One such sulfur-containing compound, dimethyl trisulfide (DMTS), has been identified as the deterioration odor or *hineka* (14). DMTS is thought to be generated from the sulfur-containing compound, DMTS precursor 1 (1,2-dihydroxy-5-(methylsulfinyl)pentan-3-one) (15). In addition to DMTS-related compounds, there is a strong indication that other sulfur-containing compounds shown in Table 2 give lower flavor quality. In our recent report, long-chain alkanes were identified as the hallmark of the unpleasant fatty odor in sake (5). The results in Table 2 are consistent with our previous findings.

Subsequently, we performed analysis to determine correlations between the taste quality of sake and various chemical compounds. As the correlation coefficient between flavor quality and taste quality was shown to be $R > 0.92$, the estimated chemical compounds were almost identical (Table S1).

These results demonstrate that some dipeptides and amino acid-related compounds show a positive correlation with sake quality in terms of both flavor and taste, whereas sulfur-containing compounds and long-chain alkanes are negatively correlated with both qualities.

Correlation of chemical compounds with ethyl caproate flavor In the sensory evaluation analysis, the number of ethyl caproate notations was used as an index of intensity. Ethyl hexanoate (ethyl *n*-caproate) is thought to be a major contributor to the development of a pleasant apple-like fruity flavor. Interestingly, the chemical compounds identified to positively correlated with ethyl caproate were several cyclo-dipeptides and dipeptides (Table 3), which were also found to be positively correlated with flavor and taste qualities (Tables 2 and S1). The correlation of various dipeptides and sake quality was confirmed using CE-TOFMS analysis (Takahashi, K. and Kohno, H., unpublished results). Amino acids (except for proline) and dipeptides have been found to be more abundant in sake than in wine or beer (31). Dipeptides are produced in the sake mash (*moromi*) as a result of the degradation of rice grain endosperm storage proteins, including glutelin, by rice-mold proteases (31,32). Dipeptides would be imported into cells via the proton-dependent oligopeptide transporter Ptr2 on the yeast plasma membrane (33–35). The imported dipeptides likely act as a nutritional source for fermentation and as precursors for flavor components (36). To determine which dipeptides are present in sake and are associated with taste and flavor quality, further analyses using LC/MS and cellular biological experiments are required.

TABLE 1. The quality score and the number of counts against each sensory properties of *ginjo* sake by 15 panelists.

Sample name	Flavor quality [a]	Taste quality [a]	Ethyl caproate	*Hineka* odor	Raw-*hineka* odor	Yeast debris-like odor	Sulfur like odor	Fatty odor	Pleasant bitter taste	Pleasant astringent taste	Inharmonious bitter taste
A-1	30	34	7	0	0	0	0	0	2	0	0
A-3	40	38	5	0	1	0	0	0	4	4	1
A-9	40	41	1	0	0	0	0	1	4	2	1
A-10	34	33	3	0	0	0	0	3	3	5	2
A-11	31	31	8	0	0	0	0	2	4	0	0
B-4	43	46	1	0	2	1	1	1	2	4	5
B-5	56	49	0	0	1	1	0	0	1	2	5
B-6	71	63	1	3	1	3	4	0	2	0	6
C-2	63	57	2	0	4	4	2	5	0	1	0
C-3	73	63	2	6	3	3	4	5	0	0	0
C-5	52	47	5	0	1	2	0	5	1	1	1

[a] Low score means high quality.