Analyzing the relationship between the inorganic element profile of sake dilution water and dimethyl trisulfide formation using multi-element profiling

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Dimethyl trisulfide (DMTS) is the main component of *hineka*, an off-flavor generated in sake during storage. *Genshu*, or undiluted sake, is usually diluted with water during *warimizu*, the process of adjusting the alcohol content of sake. In this study, we evaluated how the inorganic element composition of sake dilution water affects the DMTS-producing potential of the sake (DMTS-pp, determined as the DMTS concentration in sake stored at 70°C for 1 week after dilution) using inductively coupled plasma−mass spectrometry (ICP−MS). Partial least squares (PLS) regression analysis was conducted with the ICP−MS data as the explanatory variable and DMTS-pp as the response variable, and the selection of inorganic elements for the construction of the PLS model was performed using variable importance in projection scores. The findings confirmed that some of the compounds containing the inorganic elements extracted from the PLS regression analysis contribute to DMTS-pp.

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[**Key words:** Sake; Dimethyl trisulfide; Water; Multi-element profiling; Partial least squares regression; Inductively coupled plasma−mass spectrometry]

Dimethyl trisulfide (DMTS) is the main component of *hineka*, an off-flavor generated during the storage of sake (1,2). Recent studies on the mechanism of DMTS formation identified precursor compounds of DMTS (3,4), and genes of yeast involved in DMTS formation (5). Other studies have identified factors that affect DMTS production in sake, including the rice content (6) and yeast cell lysis (7,8). Isogai et al. (9) reported that adding vanadium (V) to sake suppresses DMTS formation during storage, suggesting that inorganic elements may affect the generation of DMTS.

Genshu, or undiluted sake, is usually diluted with water during *warimizu*, the process of adjusting the alcohol content of the sake. The dilution water used during *warimizu* becomes part of the final product. Thus, the quality of the dilution water is thought to directly affect the quality of the final sake, including its color (10). Since the inorganic element profiles differ among water samples, the dilution water is expected to influence DMTS formation in sake.

In this study, we evaluated the effect of the inorganic element profile of dilution water on the sake DMTS-producing potential (DMTS-pp, calculated as the DMTS concentration in sake stored at 70°C for 1 week after dilution with water). We also conducted partial least squares (PLS) regression analysis using the inorganic element profile of the dilution water as the explanatory variable and the DMTS-pp of diluted sake as the response variable to clarify the relationship between the inorganic elements present in the dilution water and DMTS formation.

MATERIALS AND METHODS

Samples and reagents *Genshu* [rice variety, *Yamadanishiki*; rice polishing rate, 70%; ethanol content, 19.4% (v/v)] produced by Hakutsuru Sake Brewing Corporation, Ltd. (Kobe, Japan), and pasteurized using the process called *hi-ire* was used in this study. Twelve different types of natural mineral water (A−L) available in Japan were purchased for use as the dilution waters. Ultrapure water for ultra-trace analysis, nitric acid for ultra-trace analysis, DMTS, ethanol, aluminum lactate (purity: >99.9%), nickel standard solution (100 mg/L), zinc chloride (purity: >99.9%), ammonium vanadate(V) (purity: 99.8%), chromium(III) acetate (purity: >99.9%), potassium bromide (purity: 99.8%), barium chloride dihydrate (purity: >99.9%), gallium(III) nitrate *n*-hydrate (purity: >99.0%), bromide ion standard solution (1000 mg/L) and sodium chloride (NaCl) were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Tin(II) chloride (purity: 98.8%) and antimony potassium tartrate trihydrate (purity: 99.5%) were purchased from Sigma−Aldrich (St. Louis, MO, USA). Dimethyl-d_6 trisulfide was obtained from Toronto Research Chemicals (Toronto, Canada). Multi-element standard solution (XSTC-13B) and single element standard solutions (Sn, Sb) were purchased from SPEX CertiPrep (Metuchen, NJ, USA).

Analysis of DMTS-pp The *genshu* was diluted with each mineral water (A−L) to obtain a 15.0% (v/v) ethanol concentration, and the diluted samples were stored at 70°C for 1 week. The DMTS-pp of each sample after storage was determined using a combination of headspace solid-phase microextraction (HS-SPME) and gas chromatography/mass spectrometry (GC/MS) as follows. The ethanol concentration of the sake sample (10 mL) was adjusted to 10.0% (v/v) with water purified using a Direct-Q 3 UV system (Merck Millipore, Burlington, MA, USA) and added to a 20-mL glass vial. NaCl (3 g) along with 50 μL of dimethyl-d_6 trisulfide (0.2 mg/L in 10% ethanol) as an internal standard were then added to the sample, and the vial was stoppered with a screw cap. For HS-SPME, a 50/30-μm divinylbenzene/carboxen/poly (dimethylsiloxane)-coated fiber (Sigma−Aldrich) was used to extract volatile compounds. The sample was equilibrated at 35°C for 5 min and extracted for 30 min.

After HS-SPME, the fiber was inserted into the injection port of a GCMS-QP 2010 Plus (Shimadzu Co., Kyoto, Japan) instrument equipped with a InertCap Pure-WAX column (0.25 mm × 30 m, 0.25 μm; GL Science, Inc., Tokyo, Japan) and an AOC-6000 (Shimadzu Co.) autosampler for 2 min to desorb the analytes. Injection was performed in splitless mode at an injection temperature of 250°C. The flow rate of

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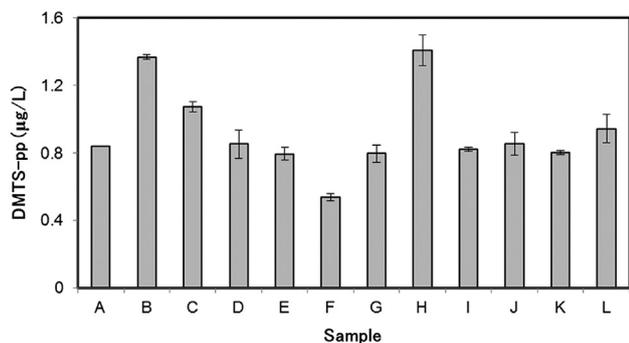*E-mail address:* yoshihiro-tamada@hakutsuru.co.jp (Y. Tamada).

FIG. 1. DMTS-pp of sake diluted with different natural mineral waters. Error bars represent the standard deviation ($n = 3$).

the carrier gas (He) was 1.96 mL/min. The column temperature was first held at 40°C for 2 min, increased by 30°C/min to 90°C, increased to 110°C at 3°C/min, increased to 250°C at 30°C/min, and finally held at 250°C for 5 min. The ion source temperature was 200°C, and ions were formed by electron ionization at 70 eV. The mass spectra were obtained in single-ion monitoring mode (m/z 79, 82, 126, and 132). The peak areas of DMTS and the internal standard were calculated from the extracted ion chromatogram at m/z 126 and 132, respectively. The DMTS concentration in each sample was determined from the calibration curve in the concentration range of 0.05−2 μg/L. All samples were analyzed in triplicate ($n = 3$).

Analysis of the inorganic element profile of natural mineral water Inductively coupled plasma−mass spectrometry (ICP−MS; Agilent 7700×, Agilent, Santa Clara, CA, USA) was used to semi-quantitatively analyze inorganic elements in the natural mineral waters used as dilution waters in this study. Each natural mineral water (19.8 mL) and 200 μL of nitric acid for ultra-trace analysis were added into a 20-mL acid-cleaned perfluoroalkoxy alkane (PFA) bottle for ICP−MS analysis under the following conditions: radio-frequency power, 1.55 kW; radio-frequency matching, 1.8 V; carrier gas flow, 1.03 L/min; extract 1, 0 V; extract 2, −165 V; gas mode, He; and integration time, 0.1 s. All samples were analyzed in triplicate ($n = 3$).

Multivariate analysis Principal component analysis (PCA) and PLS regression were performed using Aloutput software (11). Before multivariate analysis, all inorganic element profiles were standardized by the auto-scaling method to ensure that the average value and variance among all water samples were 0 and 1, respectively.

Analysis of the effects of compounds containing each inorganic element on DMTS-pp Aluminum lactate (Al), zinc chloride (Zn), tin(II) chloride (Sn), ammonium vanadate(V) (V), chromium(III) acetate (Cr), potassium bromide (Br), antimony potassium tartrate trihydrate (Sb), barium chloride dihydrate (Ba), and gallium(III) nitrate n-hydrate (Ga) were individually dissolved in ultrapure water for ultra-trace analysis to obtain a concentration of 300 μg/L for each element. For Ni, a 100-mg/L standard solution was diluted with ultrapure water to 300 μg/L. The element composition of each solution was analyzed as described above. The concentration of each element (Al, Ni, Zn, Sn, V, Cr, Br, Sb, Ba, and Ga) was calculated by preparing calibration curves (0−500 μg/L) using standard solutions. *Genshu* samples were diluted with solutions of each inorganic element to obtain a 15.0% (v/v) ethanol concentration. A sample diluted with ultrapure water was used as a control. Each diluted sake sample was stored at 70°C for 1 week, and DMTS-pp was determined for each sample using the method described above.

Measurement of the pH value The pH value of each diluted sake sample was monitored using a pH meter (D-52, Horiba, Kyoto, Japan).

RESULTS AND DISCUSSION

DMTS-pp values of diluted sake samples The DMTS-pp of each sake sample diluted with natural mineral water (A−L) is shown in Fig. 1. The DMTS-pp values of the 12 samples differed by up to 2.6 times, indicating that the type of dilution water strongly affected the DMTS-pp of the sake. Since no difference was found in the pH value of each diluted sample (Table S1), it was considered that pH did not have a significant influence on DMTS-pp.

Inorganic element profiles of natural mineral waters Sixty-four inorganic elements were detected in the samples by ICP−MS (Table S2). To identify differences between data sets, the inorganic element profile data for each natural mineral water were subjected to PCA (Fig. 2, Tables S3 and S4). It was found that samples A and I contained a large amount of rare earth elements. Clusters were formed for each sample, confirming that the inorganic element profile depended on the type of mineral water.

Predictive model for DMTS-pp To analyze the relationship between the inorganic element profiles of natural mineral water and the DMTS-pp of diluted sake, PLS regression was conducted
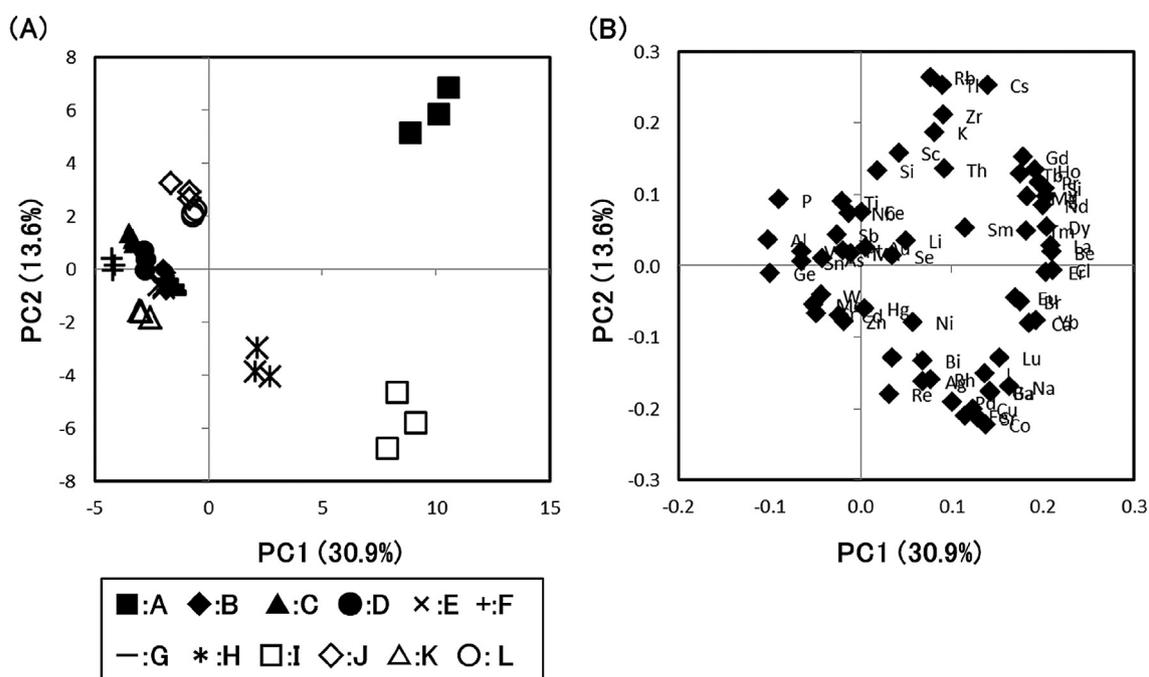

FIG. 2. PCA of inorganic element profile data: (A) score plot and (B) loading plot.
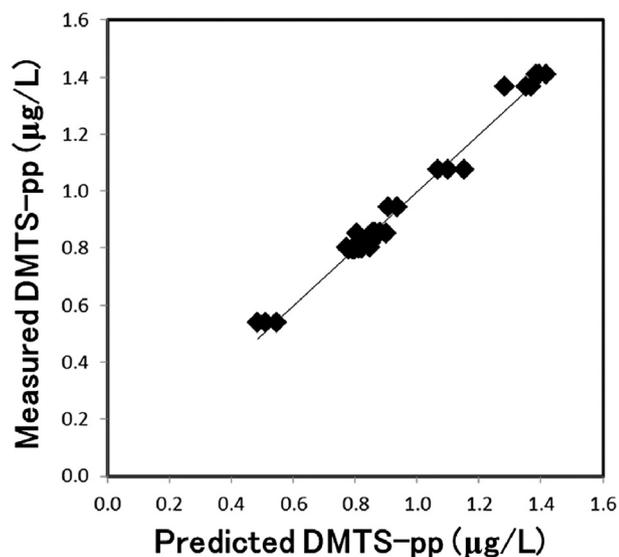
FIG. 3. Relationship between predicted and measured DMTS-pp based on the PLS model.

TABLE 1. List of important inorganic elements that influence variables used to create prediction model of DMTS-pp.

Inorganic element	VIP	Coefficient
Al	2.15	−0.299
Ni	1.85	0.100
Zn	1.76	−0.176
Sn	1.75	−0.159
V	1.74	−0.146
Cr	1.70	−0.126
Br	1.69	0.220
Sb	1.51	−0.308
Ba	1.32	0.010
Ga	1.28	0.001

with the ICP−MS data as the explanatory variable and DMTS-pp as the response variable. The obtained predictive model is shown in Fig. 3; the vertical and horizontal axes show the measured and predicted values, respectively, and there were six PLS components. The R^2 value, which is an index of the model's linearity, was 0.983. The Q^2 value, which is an index of the predicted performance and was calculated by cross validation in the dataset (cross-validated R^2), was 0.902, indicating an excellent model [$Q^2 > 0.5$ is regarded as a good model, and $Q^2 > 0.9$ as an excellent model (12)]. These results indicate that the DMTS-pp of sake can be accurately modeled using the inorganic element profiles of water.

Subsequently, the inorganic elements important for constructing the predictive model were identified on the basis of variable importance in projection (VIP) scores, which were calculated from the effect of each inorganic element on DMTS-pp in the model. The top 10 inorganic elements based on VIP values are shown in Table 1, in which a positive or negative sign on the coefficient of regression for DMTS-pp indicates a positive or negative relationship with DMTS-pp, respectively. As shown in Table 1, V was found to be negatively related to DMTS-pp, in agreement with the literature (9).

The effects of compounds containing each inorganic element on DMTS-pp   *Genshu* was diluted with water solutions containing 300 μg/L of the inorganic elements shown in Table 1 and with ultrapure water as a control. The element composition of each water solution is shown in Tables S5 and S6. No difference was found in the pH value of each diluted sample (Table S7). The DMTS-pp values obtained for the diluted sake samples (Fig. 4) suggest that some of the compounds containing each inorganic element contribute to DMTS-pp, even though the influence of a trace amount of impurities (Tables S5 and S6) cannot be disregarded. Among the four inorganic elements that were positively related to DMTS-pp in the model, three of them (Br, Ba, and Ga) were found to significantly increase DMTS-pp. On the other hand, among the six elements negatively related to DMTS-
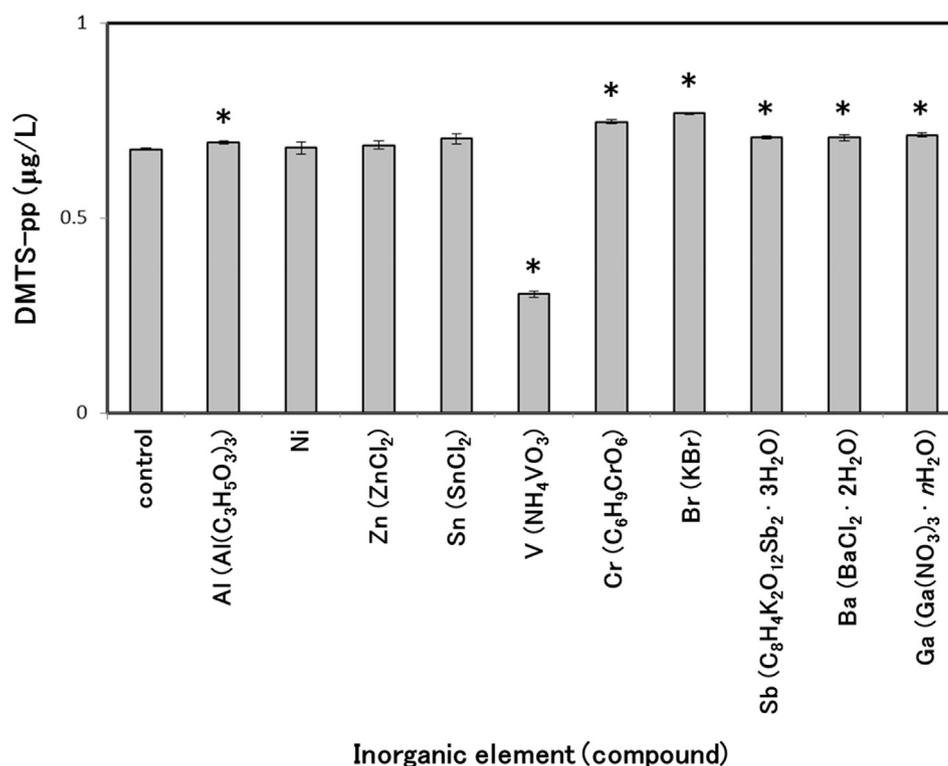
FIG. 4. Influence of each inorganic element on DMTS-pp. Error bars represent the standard deviation ($n = 3$). *$p < 0.01$. Control shows the sample diluted with ultrapure water.

pp in the model, only V (present in ammonium vanadate(V)) was found to significantly reduce DMTS-pp. Since Zn, Sn, and Cr were predominantly contained in water sample F, which had the lowest DMTS-pp and the greatest amount of V, it is possible that Zn, Sn, and Cr have high VIP values in conjunction with V. For this reason, Zn, Sn, and Cr were not consistent with the model. Moreover, even for the same inorganic element, the effect on DMTS-pp may differ depending on the source compound of such element, i.e., the valence number and pairing elements. Thus, further examination is required.

The mechanisms by which the compounds containing the studied inorganic elements affect DMTS-pp remain unknown. Since the *genshu* used in this study had been pasteurized through *hi-ire*, the influence of these inorganic elements on DMTS-pp likely stems from nonenzymatic chemical reactions.

For the first time, this study demonstrated that sake dilution water can affect the DMTS-pp of the sake. The inorganic elements important for the construction of the predictive model were determined from the PLS regression analysis using the inorganic elements data as the explanatory variable and DMTS-pp as the response variable. The results indicate that V is negatively related to DMTS-pp. By diluting *genshu* with water containing specific inorganic elements, we confirmed for the first time that some compounds containing these inorganic elements contribute to the formation of DMTS. It is a new finding to reveal several elements that may increase DMTS-pp. This finding is expected to help elucidate the generation mechanism of DMTS and contribute the development of strategies to control DTMS.

This study is the first to investigate the correlation between the inorganic element profiles of sake dilution water and the characteristics of sake. The inorganic elements in dilution water are thought to contribute to various additional characteristics of sake other than DMTS formation. For example, Fe combines with deferriferrichrysin, which is produced by *Aspergillus oryzae*, resulting in formation of a colored compound (13). Mn and Cu also promote the coloring of sake (14). Other studies have reported the effects of inorganic elements on the flavor of sake. For example, Na, K, Ca, and Mg were reported to have a strong influence on taste, while Cl was found to improve *teri* (brightness) and contribute to richness in flavor (15). Combining comprehensive inorganic element profiles and multivariate analysis to other characteristics of sake has the potential to provide new information on the relationships between inorganic elements and sake characteristics.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2018.11.013.

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