Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–10, 2018

www.elsevier.com/locate/jbiosc

Nuclear magnetic resonance- and gas chromatography/mass spectrometry-based metabolomic characterization of water-soluble and volatile compound profiles in cabbage vinegar

Satoru Ishihara,[1] Takashi Inaoka,[2] Toshihide Nakamura,[2] Keitarou Kimura,[2] Yasuyo Sekiyama,[2] and Satoru Tomita[2,*]

Gunma Agriculture Technology Center, 493 Nishi-obokata, Isesaki, Gunma 379-2224, Japan[1] and Food Research Institute, National Agriculture and Food Research Organization (NARO), 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan[2]

Received 30 November 2017; accepted 2 February 2018
Available online xxx

Non-targeted metabolomic analyses employing nuclear magnetic resonance- and gas chromatography/mass spectrometry-based techniques were applied for an in-depth characterization of cabbage vinegar, an original agricultural product made from cabbage harvested in Tsumagoi, Japan. Water-soluble and volatile metabolite profiles of cabbage vinegar were compared with those of various vinegars: rice vinegar, grain vinegar, apple vinegar, and black vinegar (Japanese *kurozu* made of brown rice). Principal component analysis (PCA) of the water-soluble metabolites indicated that cabbage vinegars belonged to an isolated class by the contributions of fructose, pyroglutamic acid, choline, and methiin (*S*-methylcysteine sulfoxide). Regarding the volatile compounds, the PCA data represented that rice, black, and apple vinegars were characterized by most of the dominant volatiles, such as acetate esters, alcohols, ketones, and acids. Cabbage and grain vinegars were included in the same class although these two vinegars have different flavors. Orthogonal partial least squares-discrimination analysis exhibited the differences in volatile compound profile between cabbage and grain vinegars, revealing that cabbage vinegars were characterized by the presence of sulfides (dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide), nitriles (allyl cyanide and 4-methylthiobutanenitrile), 3-hexene-1-ol, and crotonic acid. The time-course changes in these highlighted compounds during the acetic acid fermentation of cabbage vinegar suggested that pyroglutamic and crotonic acids were produced through fermentation, whereas choline, methiin, sulfides, nitriles, and 3-hexene-1-ol were derived from cabbage, suggesting the key role of these compounds in the unique taste and flavor of cabbage vinegar.

[**Key words:** Cabbage vinegar; Metabolomics; Nuclear magnetic resonance spectroscopy; Headspace solid phase micro-extraction; Gas chromatography/mass spectrometry; Sulfides; Isothiocyanates]

Vinegar is an important food product, which is globally manufactured from various agricultural materials (1,2). Major ingredients are grain and fruit. Rice and malt vinegars are popular grain vinegars and are mainly produced in East Asian countries (Japan, Korea, Taiwan, and China) and Britain, respectively. Apple and grape are common ingredients for the fruit vinegars widely produced in Europe and the United States. Besides these major vinegar products, various kinds of vinegars are also produced utilizing local agricultural specialties. In Japan, onion, marmelo, white asparagus, and purple sweet potato have been utilized to develop new vinegar products (3–6).

Recently, the development of novel processed food products of cabbage (*Brassica oleracea* var. *capitata*) has been in demand in Tsumagoi, which is the largest cabbage-producing area in Japan. However, its intense sulfur odor, known to result from the generation of sulfides via degradations of sulfur-containing compounds (7), has been a problem for developing novel cabbage processed foods. Many of the cabbage processed products investigated so far have not obtained positive sensory evaluations owing to the strong odor elicited through during the cabbage processing procedures, such as squeezing and cooking. For the solution of this problem, we previously developed a novel vinegar using cabbage harvested in Tsumagoi through a traditional surface acetic acid fermentation system. The cabbage vinegar fermentation reduced the levels of sulfides and provided a positive sensory evaluation for its mild flavor, resulting in the successful development of a novel product (8). In the previous study, to compare the quality of cabbage vinegar with that of grain and fruit vinegars, acidity, pH, and levels of components (sugars, amino acids, and ethanol) were examined. As a result, fructose (Fru), glucose (Glc), and sorbitol, along with 20 amino acids, were detected by high-performance liquid chromatography (HPLC) and an amino acid analyzer, respectively, and the higher level of Fru was highlighted as characteristic of cabbage vinegar (8). This finding suggested that the higher Fru content had potential impact on the taste of cabbage vinegar. However, the targeted analyses provided only limited compositional information about the cabbage vinegar. It is possible that the raw cabbage contains various unique metabolites, such as sulfur and flavor compounds of *Brassica* vegetables (9), but it remains unclear whether those metabolites contribute to the compositional

* Corresponding author. Tel.: +81 29 8388066; fax: +81 29 8387996.
E-mail address: satorutomita@affrc.go.jp (S. Tomita).

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2018.02.003

TABLE 1. Vinegar samples used in this study.

Sample	ID	Food labeling		Acidity (%)
		Product	Ingredients	
Cabbage vinegar	C1	Brewed vinegar	Cabbage, alcohol	4.49
	C2		Cabbage, alcohol	4.47
	C3		Cabbage, alcohol	4.49
	C4		Cabbage, alcohol	4.20
	C5		Cabbage, alcohol	4.52
	C6		Cabbage, alcohol	4.64
Grain vinegar	G1	Grain vinegar	Grains (wheat, rice, corn), alcohol, *sake* cake	4.22
	G2		Grains (rice, corn), alcohol, *sake* cake	4.32
	G3		Rice, alcohol, *sake* cake	4.16
Rice vinegar	R1	Rice vinegar	Rice	4.44
	R2		Rice	4.52
	R3		Rice	4.19
Black vinegar[a]	B1	Rice black vinegar	Brown rice	4.43
	B2		Brown rice	4.44
	B3		Rice	4.26
Apple vinegar	A1	Apple vinegar	Apple juice	5.00
	A2		Apple juice	4.44
	A3		Apple	4.28

[a] Japanese traditional vinegar, *kurozu* (*kurosu*).

characteristics of cabbage vinegar. Especially, determining the volatile compound profile of cabbage vinegar is essential to explain its unique, mild flavor, which possibly results from cabbage-derived compounds, including sulfides and sulfur-containing metabolites.

With the advances in analytical techniques in recent years, metabolomics, which is an approach combining non-targeted comprehensive metabolite analysis with multivariate analysis, has been widely applied to food science (10,11). For example, the metabolomic approach was employed in studies on fermented foods and beverages, e.g., studies on the flavor characteristics of cheese (12,13), umami taste of soy sauce (14–16), and quality prediction of *sake* (Japanese rice wine) (17). Additionally, vinegar metabolomics studies were also carried out, successfully providing metabolic markers for genuine balsamic vinegar to warrant protected geographic indication (PGI) (18) and clarifying the compositional variety among the commercial and traditional vinegars (19). Thus, metabolomics is expected as a powerful method to achieve comprehensive compositional characterization of cabbage vinegar.

In this study, non-targeted metabolomic analyses were applied to cabbage vinegar and other vinegars produced from various raw materials to clarify the characteristics of cabbage vinegar. Herein, we compare their water-soluble and volatile metabolite profiles obtained by nuclear magnetic resonance (NMR) and gas chromatography/mass spectrometry (GC/MS) analyses and describe the unique metabolites responsible for differentiating the cabbage vinegar from other vinegars.

MATERIALS AND METHODS

Materials and sample preparation Eighteen vinegars subjected to metabolomic characterization are listed in Table 1. Six cabbage vinegars differ in the production lot. As conventional vinegars, 12 commercial products produced by different manufacturers were purchased at a local supermarket in Isesaki, Japan. For the time-course analysis on compositional changes during acetic acid fermentation, the cabbage vinegar was prepared as reported in the previous study (Fig. S1) (8). Briefly, cabbages cultivated in Tsumagoi were harvested in July 2016 and pressed in a mechanical juicer to obtain fresh juice. After heating the juice to 85°C for 30 min, 423 mL of the juice was mixed with 27 mL of ethanol to supply the substrate for acetic acid fermentation, and was inoculated with 50 mL of bacterial suspension of *Acetobacter pasteurianus* NBRC 3284 (NITE Biological Resource Center, Japan). The cabbage vinegar fermentation was conducted at 30°C in a static surface fermentation for 28 days and the fermentation products were collected on the days 0, 7, 14, 21, and 28. For preparation of the bacterial suspension, the strain NBRC 3284 was precultured in 10 mL of NBRC 804 medium (0.5% polypeptone, 0.5% yeast extract, 0.5% glucose, and 0.1% $MgSO_4 \cdot 7H_2O$) at 30°C with rotary shaking for 3 days. The preculture was then transferred to 100 mL of YPGD medium (0.2% polypeptone, 0.2% yeast extract, 0.2% glucose, and 0.2% glycerol) supplemented with 0.2% ethanol and 1.0% acetic acid and incubated at 30°C with rotary shaking for 3 days. After washing them with distilled water, the cells were resuspended with 100 mL of distilled water and used as bacterial suspension.

NMR spectroscopy Water-soluble compounds in the vinegar samples were analyzed by NMR spectroscopy. In advance of the NMR analysis, the vinegar samples were lyophilized three times to avoid the severe chemical shift fluctuations by the high acetic acid concentration, that exceeded the buffering capacity of a standard buffer for high-sensitivity NMR measurement (20). The dried residue obtained by lyophilizing 325 μL of vinegar sample was dissolved in 650 μL of 100 mM potassium phosphate buffer (pH 7.0) in deuterium oxide (D_2O, 99.9%, Cambridge Isotope Laboratories, Andover, MA, USA) containing 1 mM of sodium 2,2-dimethyl-2-silapentane-5-sulfonate-d_6 (DSS-d_6, Cambridge Isotope Laboratories). The solution was subsequently centrifuged at 21,500 ×*g* for 5 min at room temperature (25°C), and the supernatant was transferred into 5.0 mm O.D. × 103.5 mm NMR tubes (Norell, Landisville, NJ, USA).

NMR spectra were recorded on an Avance-500 spectrometer (Bruker BioSpin, Karlsruhe, Germany) equipped with a CryoProbe that fits 5 mm diameter NMR tubes (CPBBO, Bruker BioSpin), and an automatic sample transfer unit (SampleJet, Bruker BioSpin) using the automated software IconNMR (Bruker BioSpin). The NMR spectra were acquired at 298 K, operating at frequencies of 500.23 MHz for ^1H and 125.80 MHz for ^{13}C. For the multivariate analysis, ^1H NMR spectra were collected using the Bruker pulse program lc1prf2, which uses solvent pre-saturation to reduce the residual acetic acid and water signals. The following acquisition parameters were used: spectral width, 20 ppm; acquisition mode, digital quadrature detection; offset frequency, 1.82 ppm (acetic acid) and 4.70 ppm (water); proton 90° pulse, 13.5 μs; relaxation delay, 4 s; and number of scans, 256.

For the metabolite annotation, 2D NMR spectra including double quantum filtered correlated spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY), ^1H–^{13}C heteronuclear single quantum coherence (HSQC), and ^1H–^{13}C heteronuclear multiple-bond connectivity (HMBC) were measured. Metabolite signals were annotated using the SpinAssign program (21,22), NMRPipe, and NMRDraw (23), as described previously (24). Public NMR spectral databases, Human Metabolomics Database (http://www.hmdb.ca/) (25), and the Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu/) (26), were used to increase the credibility of our annotations. When appropriate, signals were assigned by spiking with standard metabolites.

GC/MS analysis The volatiles present in the sample were extracted by headspace solid phase micro-extraction (HS-SPME) under the conditions described by Iijima et al. (27), using an AOC-5000 autosampler (Shimadzu, Kyoto, Japan). A 20-mL glass vial containing 2 mL of sample was maintained at 50°C for 10 min, and volatiles in the headspace were extracted by inserting a DVB/CAR/PDMS fiber (2 cm, Supelco, Bellefonte, CA, USA) for 20 min at 50°C with intermittent agitation at 250 rpm. The volatiles absorbed on the SPME fiber were injected into a GCMS-QP2010 Ultra (Shimadzu) by thermal desorption at 250°C for 3 min and were separated on a Rtx-WAX capillary column (60 m × 0.25 mm, f.t. 0.25 μm, Restek, Bellefonte, PA, USA). Helium was used as carrier gas at a column flow rate of 2.0 mL/min. The oven temperature was increased from 40°C (hold for 5 min) to 180°C at a rate of 5°C/min, and to 230°C at a rate of 10°C/min. The final temperature was maintained for 5 min. Electron impact (EI) mass spectra were obtained under the following conditions: ionization voltage, 70 eV; ion source temperature, 230°C; quadrupole temperature, 150°C; mass range, *m*/*z* 33–350; detector voltage, 1.0 kV; and scan speed, 3.15 scans/s. During the analysis, the filament was turned off for 90 s (runtime 21.0–22.5 min) to cut excessive ions derived from the acetic acid.

Tentative metabolite identification was carried out by similarity search of mass spectra based on the NIST mass spectral library (NIST11) through the GCMSsolution software (Shimadzu). Retention index (RI) in the NIST Chemistry Webbook (http://webbook.nist.gov/chemistry/) was also referred. RI was calculated using a mixture of aliphatic hydrocarbons (C6–C20; Sigma–Aldrich, St. Louis, MO, USA).

Multivariate analysis The ^1H NMR spectra from 18 vinegar samples were processed using the TopSpin software (ver. 3.5, Bruker BioSpin). Bucket tables were generated using the Amix software (ver. 3.9.14, Bruker BioSpin). For the non-targeted multivariate analysis, datasets were generated by subdividing the spectra (10.00–0.50 ppm) into integrated regions (buckets) of 0.04 ppm each. The integrated data were then normalized to the integrated area of the internal standard (DSS-d_6 at 0.00 ppm). Nine buckets at 5.08–4.72 ppm were excluded on the basis that they contained residual water signals. This created 229 buckets in total. The GC/MS data were processed through baseline correction and peak alignment using the MetAlign software (28). The resulting retention time (RT) and *m*/*z* matrix was integrated to the dataset for the multivariate analysis using the Aloutput software (29). This created a dataset comprising of 375 GC/MS peaks.

Principal component analysis (PCA), orthogonal partial least squares-discriminant analysis (OPLS-DA), and hierarchical clustering analysis were performed using the SIMCA software (ver. 14, Umetrics, Umeå, Sweden). Pareto scaling was applied to PCA and OPLS-DA as described previously (30). The models generated by OPLS-DA were evaluated by leave-one-out cross-validation and permutation test (n = 999).

Please cite this article in press as: Ishihara, S., et al., Nuclear magnetic resonance- and gas chromatography/mass spectrometry-based metabolomic characterization of water-soluble and volatile..., J. Biosci. Bioeng., (2018), https://doi.org/10.1016/j.jbiosc.2018.02.003