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

NMR- and GC/MS-based metabolomic characterization of water-soluble and volatile compound profiles in cabbage vinegar

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Key words: cabbage vinegar; metabolomics; nuclear magnetic resonance spectroscopy; headspace solid phase micro-extraction; gas chromatography/mass spectrometry; sulfides; isothiocyanates.

Short title: Metabolomics for characterization of cabbage vinegar

ABSTRACT

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-methylthio-butanenitrile), 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.

INTRODUCTION

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 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) 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).

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 ^{1}H and 125.80 MHz for ^{13}C. For the multivariate analysis, ^{1}H 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), ^{1}H–^{13}C heteronuclear single quantum coherence (HSQC), and ^{1}H–^{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 ^{1}H 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 AIoutput 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).

RESULTS

NMR based metabolomics

The water-soluble metabolite profiles of the 18 vinegar samples were analyzed by

PCA. First, PCA was carried out using the datasets containing all variables from the NMR spectra (Fig. 1A). The first and second principal component (PC1 and PC2) represented 60.9% and 21.6% of the total variance, respectively. The score plots showed class separations of the 18 samples, and the following four classes were indicated by the Ward's hierarchical clustering method: class I (R1, B1, and B2), class II (G1–G3), and class III (R2, R3, B3, A2, and A3) were separated along the PC1 axis, and class IV (C1–C6, and A1) was along the PC2 axis (Fig. 1A). The loading plot indicated that PC1 was primarily explained by the buckets containing signals of Glc, 2,3-butanediol (BDO), and lactic acid (LacA), whereas PC2 was explained by those of Fru (Fig. 1A). The result showed that all cabbage vinegar samples belonged to class IV and shared the common characteristic of higher Fru level and lower Glc amount. Although the PCA data clarified the contribution of the dominant metabolites among the vinegar samples, that of the low-abundance metabolites remained uncharacterized with this approach.

Subsequently, to investigate the contributions of the low-abundance metabolites to cabbage vinegar, a multistep PCA (30) was performed after excluding the buckets containing the dominant compound signals, as shown in Fig. 1A (Glc, Fru, BDO, and LacA). The generated PCA model showed 31.6% and 25.1% of the total variance for the PC1 and PC2, respectively. Unlike the result of the first PCA shown in Fig. 1A, the multistep PCA score plot showed that the samples were clearly separated depending on the vinegar type (Fig. 1B). The PC1 and PC2 principally highlighted the characteristics of black vinegars and cabbage vinegars, respectively (Fig. 1B). The loading plot explained that black vinegars were characterized by higher levels of blanched chain amino acids (BCAAs), glycerol, and alanine (Ala), whereas cabbage vinegars were characterized by substantial levels of pyroglutamic acid (Glp), choline, and methiin (Fig. 1B). Malic acid (MalA) indicated by PC1 was contained at high concentration in apple

vinegars. This PCA data did not show particular characteristics for grain and rice vinegars.

Taken together, it was shown that the cabbage vinegars were characterized by Fru, Glp, choline, and methiin. The changes in the levels of these components during cabbage vinegar fermentation are shown in Fig. 2. A successive increase in the Glp level was observed until the day 28, and the level of glutamine (Gln) decreased inversely with that of Glp. The signal intensity of methiin slightly decreased within the first 7 days and then it was retained until the day 28. The Fru and choline levels showed no marked change during fermentation.

GC/MS based metabolomics

To characterize the volatile component profile of cabbage vinegar, a PCA was performed using the dataset generated from the GC/MS data of the 18 vinegar samples. The PCA score and loading plots are depicted in Fig. 3A. PC1 and PC2 represented 47.6% and 15.6% of the total variance, respectively. The score plots showed a separation on PC1 between the cabbage–grain class and the rice–black–apple class (Fig. 3A). The loading plot indicated that most GC/MS peaks contributed to the latter class (Fig. 3A). Amongst the dominant volatiles, higher levels of the following compounds primarily explained the characteristics of the latter class: acetate esters [isobutyl acetate (8.8 min, 95% MS similarity), isoamyl acetate (12.3 min, 94%), acetoin acetate (20.4 min, 97%), and 2-phenethyl acetate (31.2 min, 96%)], alcohols [isoamyl alcohol (15.3 min, 96%) and 2-phenethyl alcohol (33.5 min, 98%)], ketones [diacetyl (7.7 min, 96%) and acetoin (17.7 min, 98%)], acids [isobutyric acid (25.1 min, 96%) and isovaleric acid (27.6 min, 95%)], and furfural (22.5 min, 95%). This observation suggested that cabbage vinegars had, in common with grain vinegar, a low number of dominant peaks in comparison to the other vinegars. A comparison of the raw GC/MS chromatograms showed that the cabbage and grain vinegars had only two dominant peaks [ethyl acetate (5.5 min, 97%) and ethanol

(6.6 min, 97%)] (Fig. S2).

Although the PCA data showed that the cabbage and grain vinegar samples belonged to the same class, these two vinegars clearly differ from each other in ingredients and sensory properties. We therefore expected the presence of characteristic compounds in these two vinegars and performed OPLS-DA to clarify the detailed compositional difference between cabbage and grain vinegars. The generated OPLS-DA model provided cumulative regression coefficient (R^2) and cumulative cross-validation coefficient (Q^2) values of 0.951 and 0.852, respectively. The orthogonal PLS component 1 of the model explained 24.2% of the total variance. To identify responsible peaks for characterizing the cabbage vinegar, S-plot was examined, which visualizes the OPLS-DA loadings and extracts potential variables contributing to discrimination of two classes (31). The S-plot indicated the presence of candidate metabolites responsible for cabbage vinegar. The data and metabolite annotations are presented in Fig. 3B.

Dunnett's test confirmed that some metabolites highlighted by the OPLS-DA were characteristically contained in the cabbage vinegar, as compared to all of the other vinegars. The average levels of dimethyl sulfide (DMS), allyl cyanide, dimethyl trisulfide (DMTS), crotonic acid, 4-methylthio-butanenitrile, and an unknown peak (34.2 min) in the cabbage vinegar were significantly greater than those in the other four vinegars (Fig. 4). Although dimethyl disulfide (DMDS) and 3-hexene-1-ol did not show statistical significance owing to its varied levels among the cabbage vinegar samples, some samples contained these volatiles at high concentrations, suggesting the potential of these compounds for characterizing cabbage vinegar.

Subsequently, time-course changes in the levels of the eight volatile components were analyzed (Fig. 5). The sulfides (DMS, DMDS, and DMTS) markedly decreased within 7 days, whereas crotonic acid and the unknown peak (34.2 min) clearly increased

during fermentation. Allyl cyanide, 4-methylthio-butenenitrile, and 3-hexene-1-ol did not show significant changes during fermentation.

DISCUSSION

Recent vinegar metabolomic studies based on the NMR method have revealed various potential compounds characterizing different types of vinegars: ethyl acetate, glycerol, methanol, and tartaric acid for wine vinegar (32), Ala for apple vinegar (32), and ethanol, 3-hydroxy-2-butanone, acetate, sugars, and hydroxyl methyl furfural as key factors for the aged balsamic vinegar (33). Additionally, in our previous study, targeted analyses on carbohydrates and amino acids showed that the cabbage vinegar was characterized by Fru (8). In the present study, non-target NMR-based metabolomics provided further information on the characteristics of the water-soluble compound profile of cabbage vinegars and revealed the characteristic accumulations of Fru, Glp, choline, and methiin. Of these, Fru was in agreement with the previous study. Glp, choline, and methiin were proposed as novel characteristic compounds.

Glp is known to be produced from Gln (and from glutamic acid [Glu] at a slower rate) by a non-enzymatic reaction under acidic condition in the fermentation of soy sauce (34). The NMR signal of Glu on the day 0 of fermentation was barely detected, and had a considerably smaller level than that of Gln (data not shown). In contrast, the Gln level substantially decreased in inverse proportion to the Glp intensity during fermentation (Fig. 2), indicating that Glp was likely converted from the Gln contained in cabbage. Choline is a nutrient abundant in beef, chicken liver, and vegetables, especially in *Brassica* plants such as cauliflower and cabbage (35). The sustained level of choline during fermentation might indicate that choline in the cabbage vinegar was of cabbage-origin and was stable under the condition of acetic acid fermentation. Methiin is a well-known sulfur-

294 containing metabolite typically found in *Brassica* vegetables (36, 37). It has been reported
295 that methiin is degraded and converted into sulfides including DMS, DMDS, and DMTS
296 (38), which are important aroma components of cabbage vinegar as described below. The
297 decrease in the methiin level within the first 7 days of fermentation (Fig. 2) may result
298 from this decomposition process. Intriguingly, although a subsequent decrease in the
299 methiin level was not clear, sulfides were still detectable at very low levels for a long
300 period (Fig. 5). Further investigation on the sulfide production can be valuable for
301 maintaining the mild cabbage-like flavor of the cabbage vinegar product. Overall, these
302 water-soluble compounds highlighted by NMR metabolomics seem to be important
303 characteristics reflecting the composition of raw cabbage. In particular, methiin is a
304 characteristic compound in cabbage vinegar as it was not detected in other vinegars in
305 this study (Fig. S3).

306 The PCA data using the GC/MS dataset revealed that most peaks contributed to the
307 rice, black, and apple vinegar samples, rather than to cabbage vinegar, indicating that the
308 volatile profile of cabbage vinegar was relatively plain. This result seems to reflect that
309 cabbage vinegar is produced by adding alcohol (pure ethanol) as a substrate for the acetic
310 acid fermentation, without an alcohol fermentation process by yeast. Similarly, this is in
311 agreement with the fact that the grain vinegar samples, separated into the same class as
312 cabbage vinegar, were also produced by adding alcohol (Table 1). In the traditional
313 method, vinegar is produced by successive processes of alcohol fermentation followed
314 by acetic acid fermentation. In the alcohol fermentation, yeast produces various flavor
315 metabolites other than ethanol, including isoamyl alcohol, isobutyl alcohol, 2-phenethyl
316 alcohol, and their esters (39). These compounds were the main contributors to the vinegar
317 samples produced through alcohol fermentation in the present study (Fig. 3). Therefore,
318 the lack of the alcohol fermentation process possibly caused the principal difference in

319 the volatile compound profile of cabbage vinegar.

320 Besides, cabbage vinegar was characterized by unique compounds (DMS, DMDS,
321 DMTS, allyl cyanide, 4-methylthio-butanenitrile, 3-hexene-1-ol, and crotonic acid). Of
322 these, sulfides and allyl cyanide decreased during fermentation whereas 4-methylthio-
323 butenenitrile and 3-hexen-1-ol did not show significant change during fermentation.
324 These observations suggested that the source of these compounds was raw cabbage but
325 not bacterial metabolism. Sulfides (DMS, DMDS, and DMTS) are important aroma
326 compounds, and DMS has an odor commonly described as cabbage-like. Although
327 sulfides potentially exercise a negative impact on the flavor of food products owing to
328 their low olfactory thresholds, their levels in cabbage vinegar were markedly reduced
329 through the traditional surface acetic acid fermentation (Fig. 5). Sulfides are known to be
330 products resulting from the degradation of methiin by the catalytic action of cysteine
331 sulfoxide lyases and by heating (38). Methiin was likely the source of the sulfides of the
332 cabbage vinegar, because it is one of the dominant sulfur-containing compounds in
333 cabbage and it was actually detected in the present study by NMR analysis. Similarly,
334 allyl cyanide and 4-methylthio-butanenitrile could be derived from the degradation of
335 sinigrin and glucoerucin, respectively, which are glucosinolates found in cabbage (40, 41).
336 Glucosinolates are degraded into various volatile flavor compounds such as nitriles and
337 isothiocyanates by the action of myrosinase, a well-known enzyme playing a key role in
338 producing the unique flavor of *Brassica* vegetables (42). 3-Hexen-1-ol would also be a
339 contributor for characterizing the cabbage vinegar as it is a flavor-active compound
340 contributing to the green note flavor (a young leaf-like, grassy odor) of cabbage (43).

341 In this study, a comprehensive water-soluble and volatile compound profiling
342 through non-targeted, NMR- and GC/MS-based metabolomics revealed characteristic
343 compounds in cabbage vinegar. Most of the highlighted compounds in the cabbage

vinegar samples were likely to be ingredient-derived metabolites linked to the flavor of cabbage. Therefore, it can be concluded that cabbage vinegar is a unique product, which successfully retains the sensory characteristics of cabbage. The comprehensive compositional information and findings obtained by the metabolomic approach in this study would be useful for the quality control and improvement of cabbage vinegar. Furthermore, they could facilitate the development and evaluation of novel types of vinegars utilizing various local agricultural products.

ACKNOWLEDGEMENT

The NMR analyses were carried out with the technical support of the Advanced Analysis Center at the National Agriculture and Food Research Organization (Tsukuba, Japan). We thank Prof. Yoko Iijima (Kanagawa Institute of Technology) and Dr. Hiroshi Tsugawa (RIKEN Center for Sustainable Resource Science) for their support in the comprehensive volatile analysis.

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FIGURE LEGENDS

Fig. 1. PCA results based on water-soluble compound profiles. Score plot (left) and loading plot (right) of PCA data with (A) full dataset, and (B) data obtained by multistep PCA with selected variables excluding Glc, Fru, BDO, and LacA signals. The variable labelled with multiple metabolites represents the bucket containing overlapped signals of the shown metabolites.

Fig. 2. Changes in the levels of representative water-soluble metabolites in cabbage vinegar during acetic acid fermentation. Data are expressed as relative intensity to DSS-d_6 (0.00 ppm) and shown as means ± *SD* (n = 3).

Fig. 3. Data of GC/MS-based metabolomic analyses based on volatile compound profiles. (A) Score (left) and loading (right) plots of PCA employing all vinegar samples. Labels represent retention time (min) of the originating peaks for indicated variables. (B) S-plot calculated by OPLS-DA (cabbage vs grain vinegar). An overall view of the plot is shown over the enlarged view, and the box with broken line represents the part magnified for the contributors to cabbage vinegar. The labels indicate annotations of representative metabolites, and unannotated compounds are shown as U with retention time (min).

Fig. 4. Peak intensity of representative volatile metabolites characterizing cabbage vinegar samples. Data are shown as means ± *SD* (cabbage vinegar, n = 6; other vinegars, n = 3). Asterisks denote statistical significance against cabbage vinegar (Dunnett's test; $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$).

Fig. 5. Changes in the levels of representative volatile metabolites in cabbage vinegar during acetic acid fermentation. Data are shown as means ± *SD* (n = 3).

NMR- and GC/MS-based metabolomic characterization of water-soluble and volatile compound profiles in cabbage vinegar

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Supplementary material description

Fig. S1. The manufacturing flow of cabbage vinegar

Fig. S2. Chromatograms of SPME-GC/MS analysis of vinegar samples. (A) Stacked raw chromatograms and (B) its magnified view of the same time range. The peak at 21.0 min is an artifact in data drawing due to the range of filament-off (21.0–22.5 min).

Fig. S3. Relative signal intensity of representative metabolites in cabbage vinegar. Data are shown as means ± *SD* (cabbage vinegar, n = 6; other vinegar, n = 3). Asterisks denotes statistical significance against cabbage vinegar (Dunnett's test; ***$p < 0.001$). ND = not detected.

Fig. S1

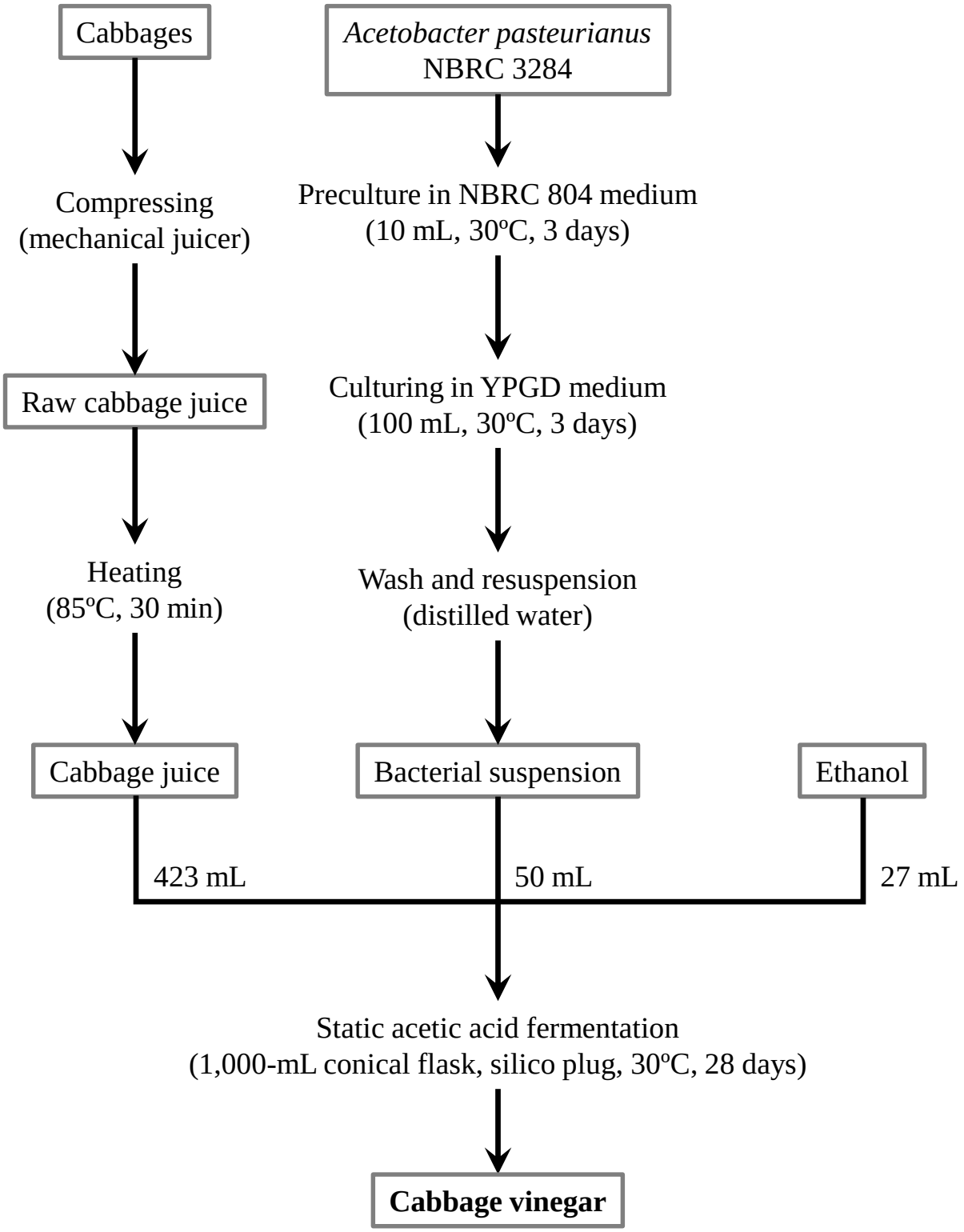

Cabbages
Acetobacter pasteurianus NBRC 3284
Compressing (mechanical juicer)
Preculture in NBRC 804 medium (10 mL, 30ºC, 3 days)
Raw cabbage juice
Culturing in YPGD medium (100 mL, 30ºC, 3 days)
Heating (85ºC, 30 min)
Wash and resuspension (distilled water)
Cabbage juice
Bacterial suspension
Ethanol
423 mL
50 mL
27 mL
Static acetic acid fermentation (1,000-mL conical flask, silico plug, 30ºC, 28 days)
Cabbage vinegar

Fig. S2

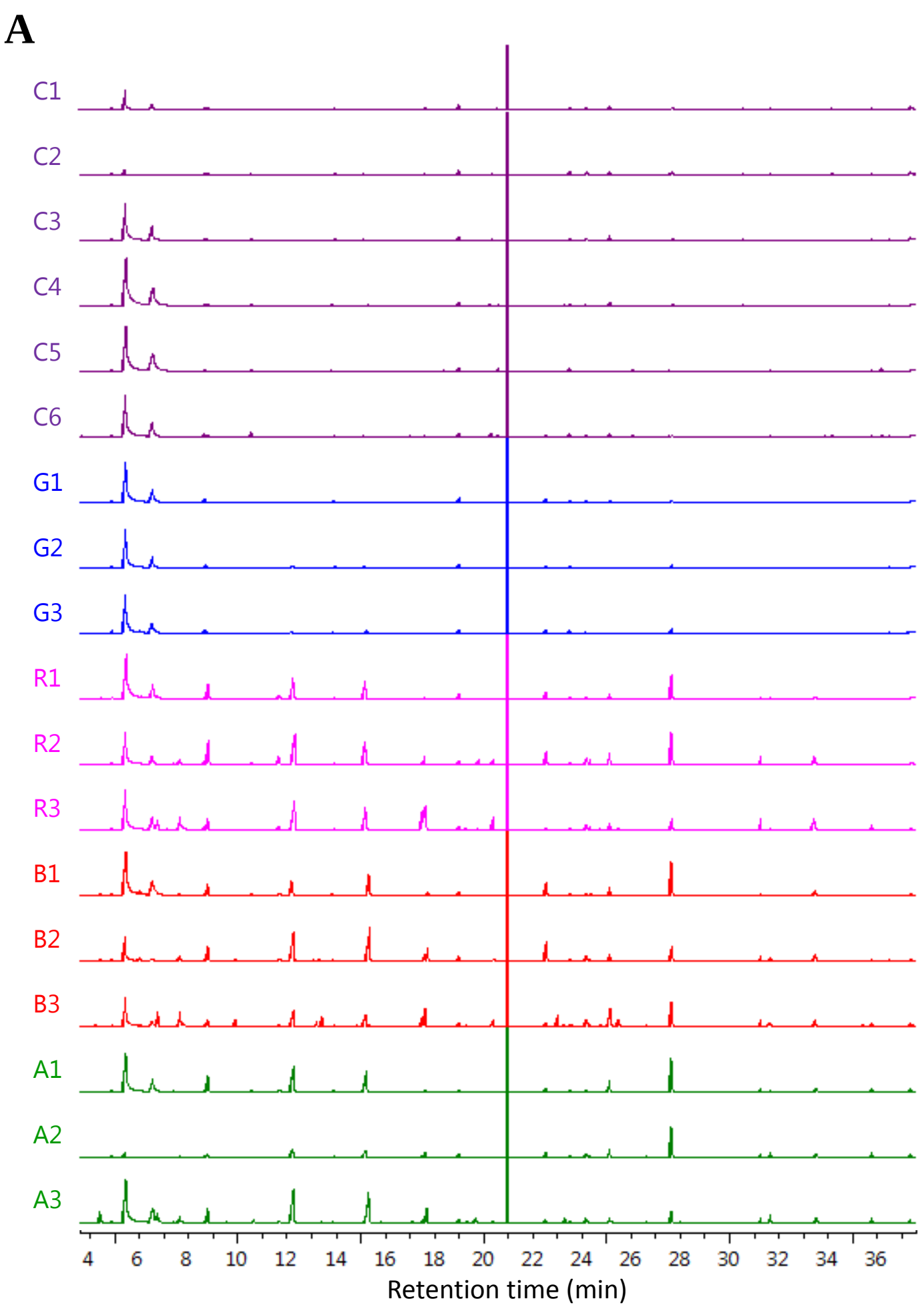

A
C1
C2
C3
C4
C5
C6
G1
G2
G3
R1
R2
R3
B1
B2
B3
A1
A2
A3
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
Retention time (min)

Fig. S2 (continued)

B

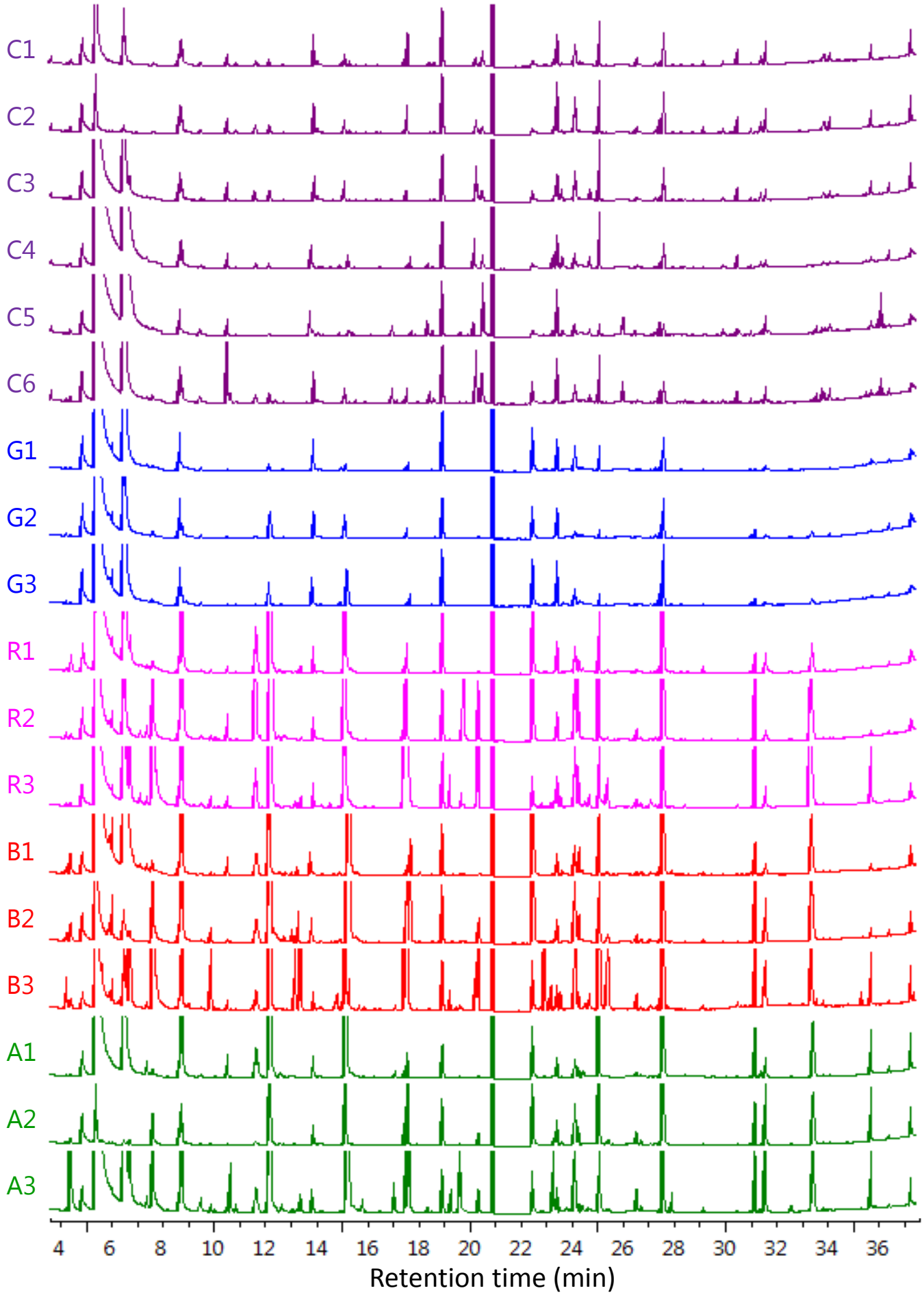
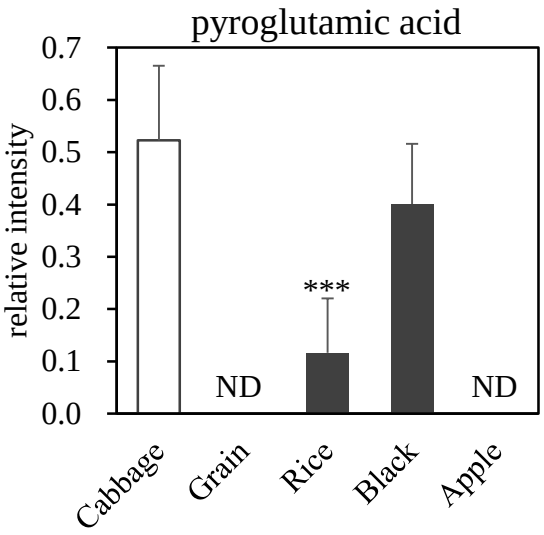
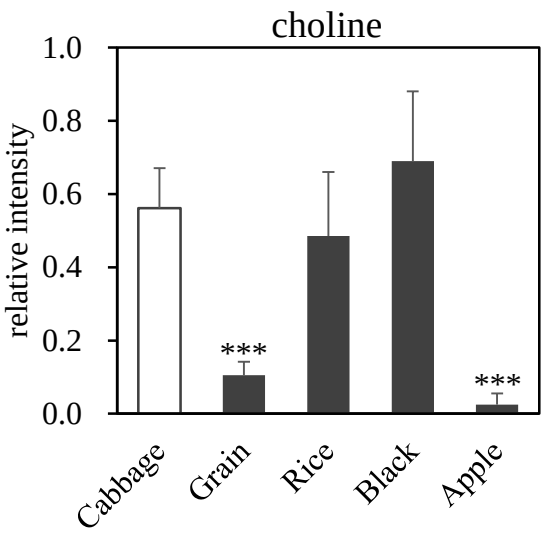


Fig. S3


pyroglutamic acid
relative intensity
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
ND
ND
Cabbage
Grain
Rice
Black
Apple


choline
relative intensity
1.0
0.8
0.6
0.4
0.2
0.0
***
***
Cabbage
Grain
Rice
Black
Apple

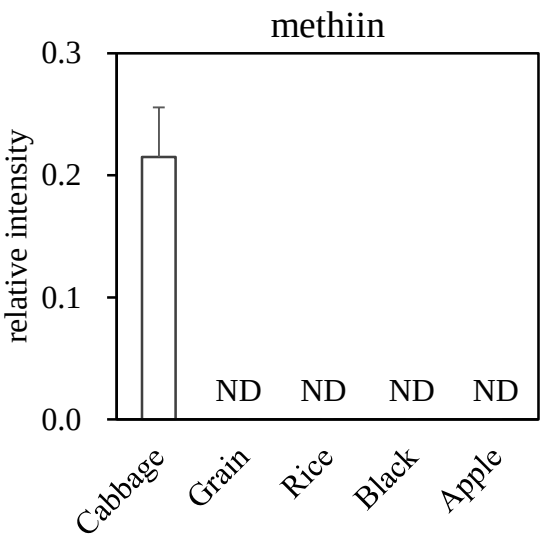

methiin
relative intensity
0.3
0.2
0.1
0.0
ND
ND
ND
ND
Cabbage
Grain
Rice
Black
Apple

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*	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

*Japanese traditional vinegar, *kurozu* (*kurosu*)

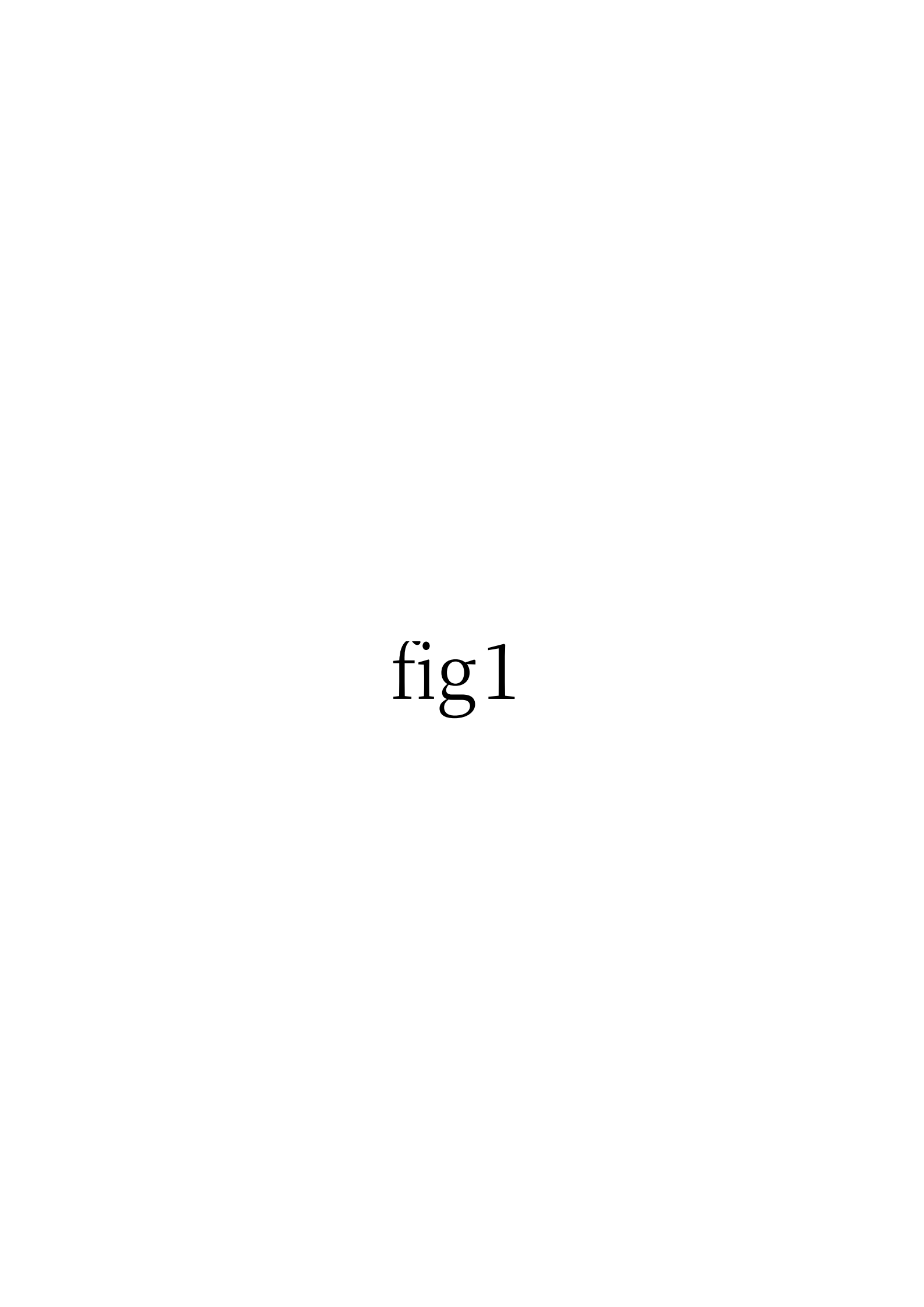


fig1

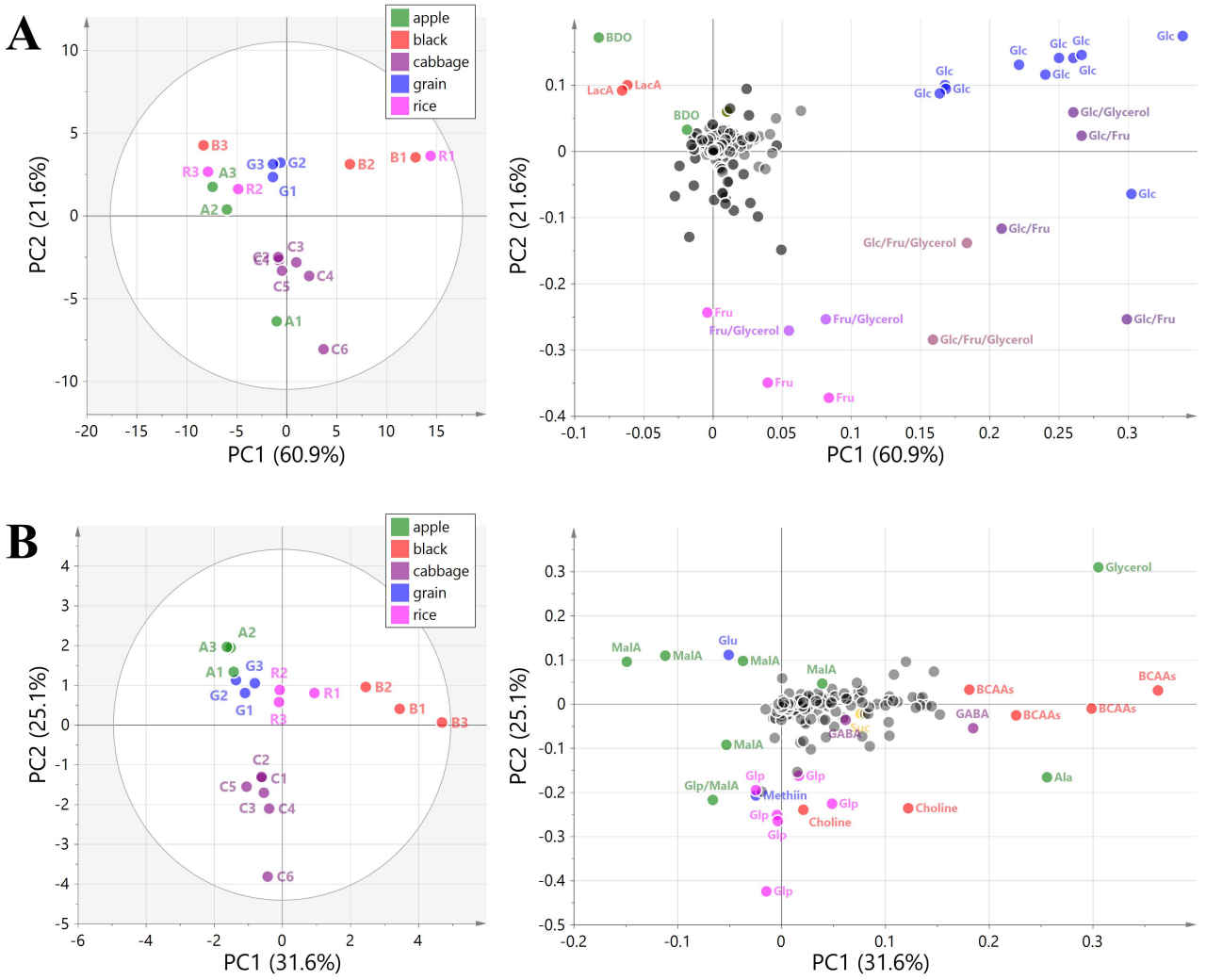
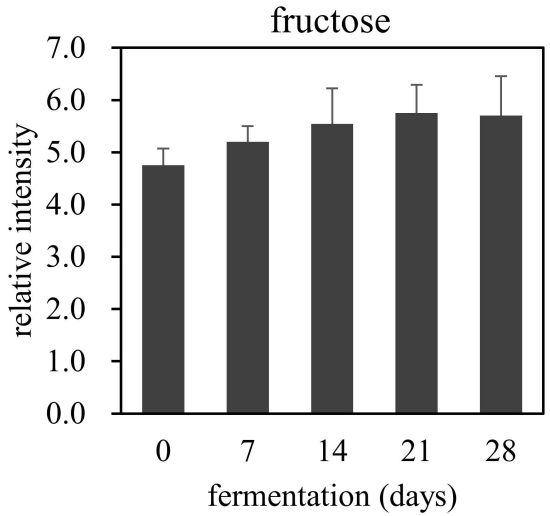
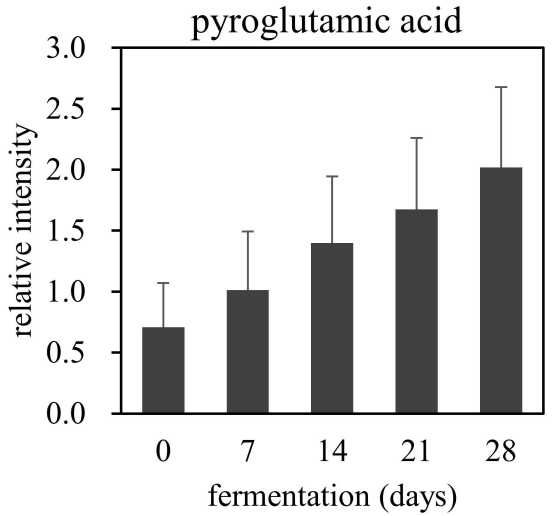
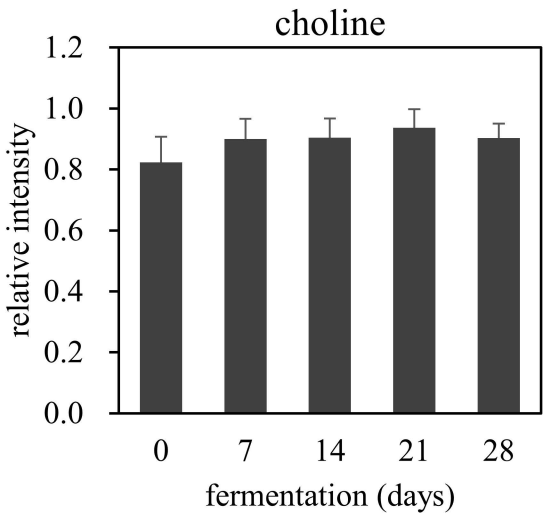
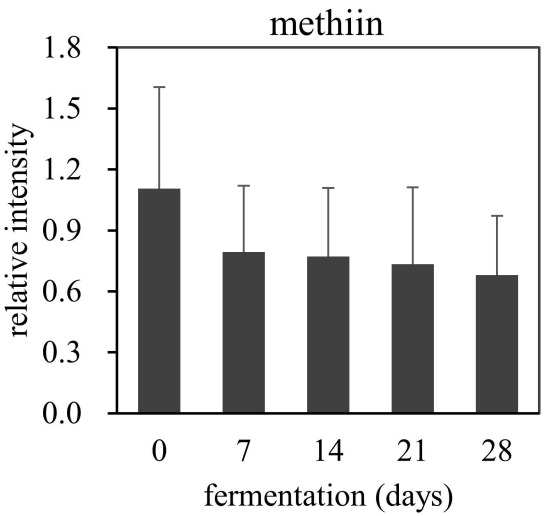

A
apple
black
cabbage
grain
rice
PC1 (60.9%)
PC2 (21.6%)
B
PC1 (31.6%)
PC2 (25.1%)
Glycerol
BCAAs
GABA
Ala
Choline
Glp
Glp/MalA
Methiin
MalA
Glu
Fru
Fru/Glycerol
Glc/Fru
Glc/Fru/Glycerol
Glc/Glycerol
BDO
LacA

fig 2


fructose
relative intensity
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
7
14
21
28
fermentation (days)


pyroglutamic acid
relative intensity
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
7
14
21
28
fermentation (days)


choline
relative intensity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
7
14
21
28
fermentation (days)


methiin
relative intensity
1.8
1.5
1.2
0.9
0.6
0.3
0.0
0
7
14
21
28
fermentation (days)

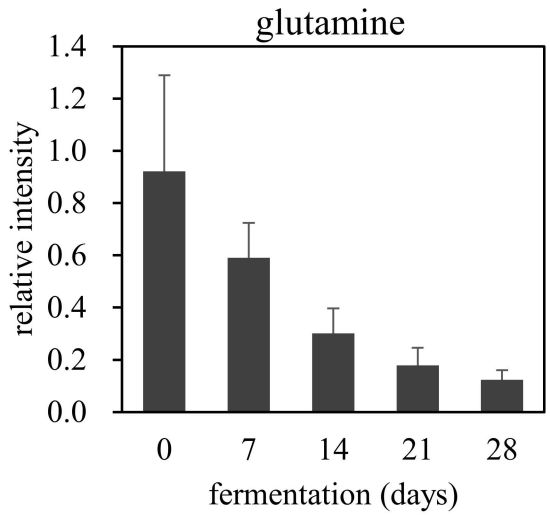

glutamine
relative intensity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
7
14
21
28
fermentation (days)

fig 3

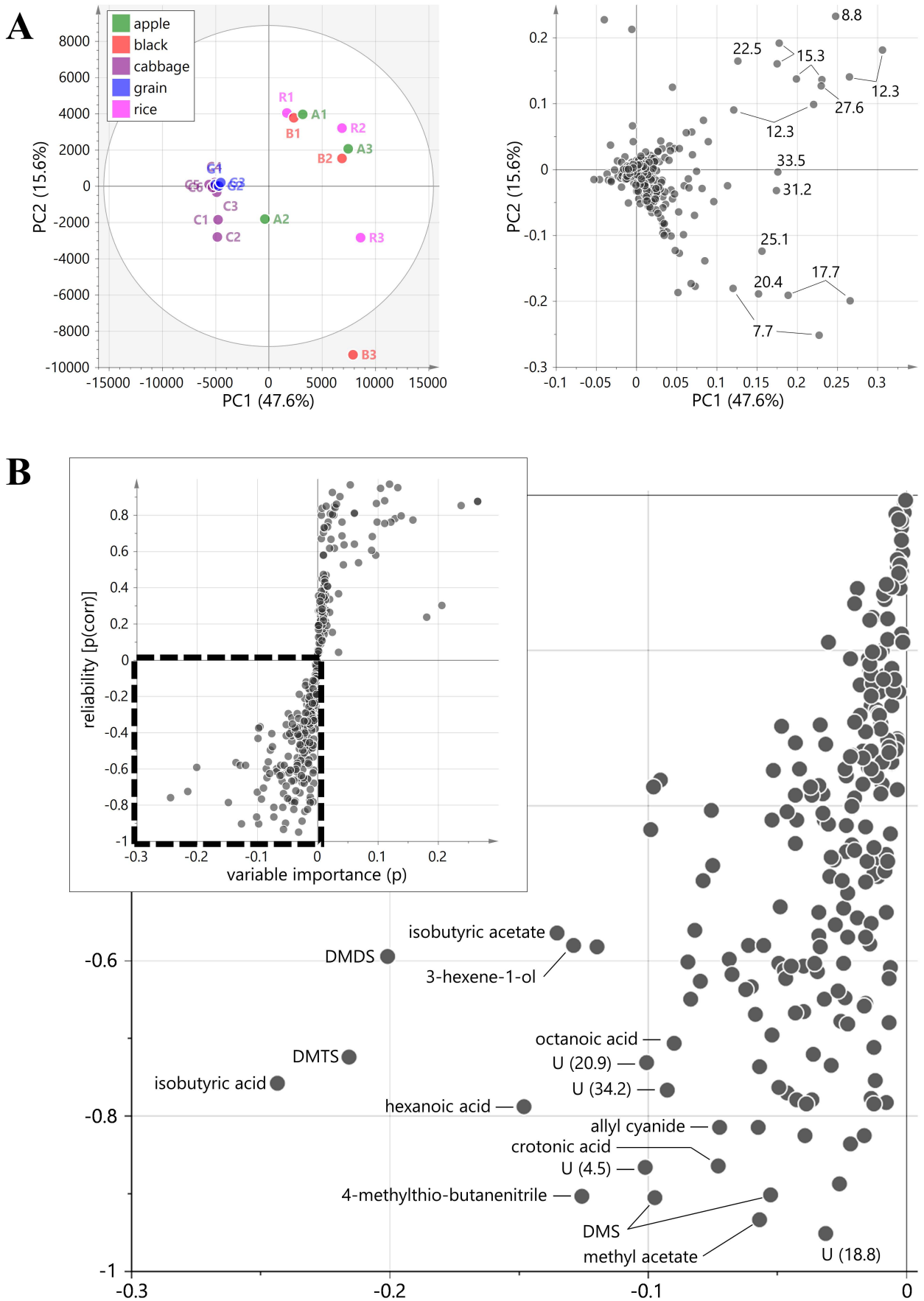

A
apple
black
cabbage
grain
rice
PC2 (15.6%)
PC1 (47.6%)
R1
A1
B1
R2
A3
B2
G1
G2
G3
C3
C1
A2
C2
R3
B3
8.8
22.5
15.3
12.3
27.6
12.3
33.5
31.2
25.1
17.7
20.4
7.7
B
reliability [p(corr)]
variable importance (p)
isobutyric acetate
DMDS
3-hexene-1-ol
octanoic acid
DMTS
U (20.9)
isobutyric acid
U (34.2)
hexanoic acid
allyl cyanide
crotonic acid
U (4.5)
4-methylthio-butanenitrile
DMS
methyl acetate
U (18.8)

fig 4

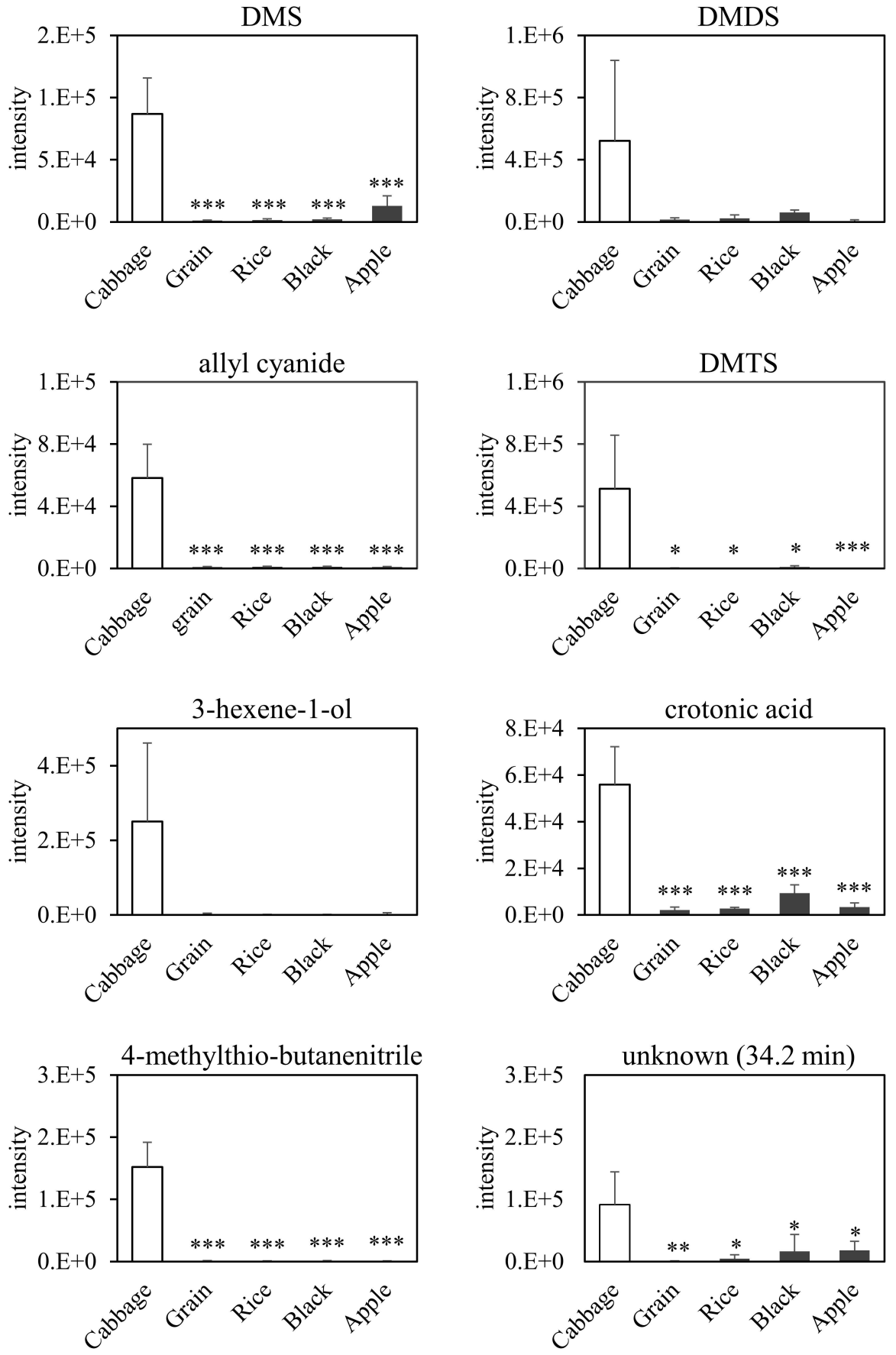
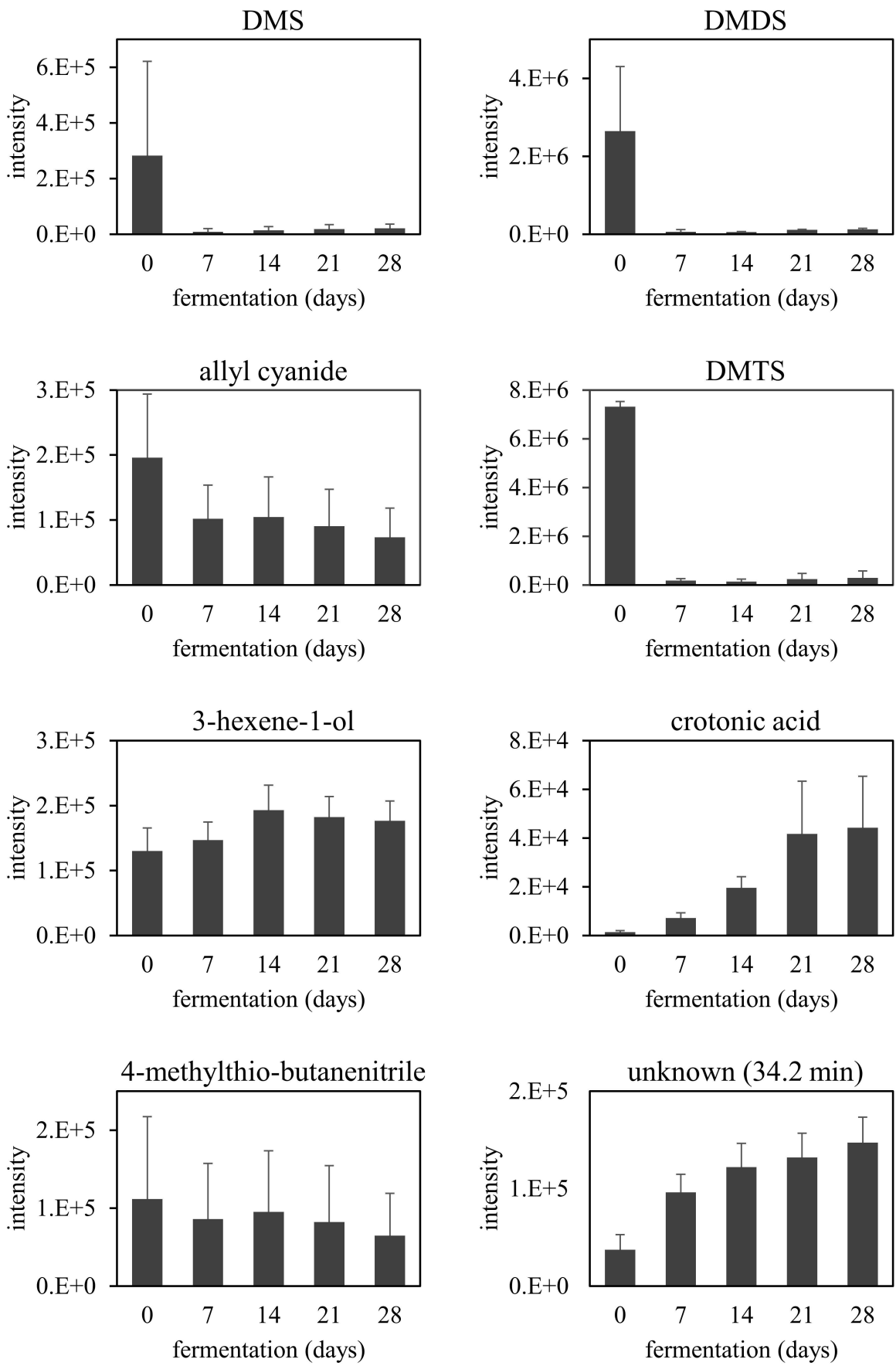

DMS
intensity
2.E+5
1.E+5
5.E+4
0.E+0
***
***
***
***
Cabbage
Grain
Rice
Black
Apple
DMDS
intensity
1.E+6
8.E+5
4.E+5
0.E+0
Cabbage
Grain
Rice
Black
Apple
allyl cyanide
intensity
1.E+5
8.E+4
4.E+4
0.E+0
***
***
***
***
Cabbage
grain
Rice
Black
Apple
DMTS
intensity
1.E+6
8.E+5
4.E+5
0.E+0
*
*
*
***
Cabbage
Grain
Rice
Black
Apple
3-hexene-1-ol
intensity
4.E+5
2.E+5
0.E+0
Cabbage
Grain
Rice
Black
Apple
crotonic acid
intensity
8.E+4
6.E+4
4.E+4
2.E+4
0.E+0
***
***
***
***
Cabbage
Grain
Rice
Black
Apple
4-methylthio-butanenitrile
intensity
3.E+5
2.E+5
1.E+5
0.E+0
***
***
***
***
Cabbage
Grain
Rice
Black
Apple
unknown (34.2 min)
intensity
3.E+5
2.E+5
1.E+5
0.E+0
**
*
*
*
Cabbage
Grain
Rice
Black
Apple

fig 5


DMS
intensity
6.E+5
4.E+5
2.E+5
0.E+0
0
7
14
21
28
fermentation (days)
DMDS
intensity
4.E+6
2.E+6
0.E+0
0
7
14
21
28
fermentation (days)
allyl cyanide
intensity
3.E+5
2.E+5
1.E+5
0.E+0
0
7
14
21
28
fermentation (days)
DMTS
intensity
8.E+6
6.E+6
4.E+6
2.E+6
0.E+0
0
7
14
21
28
fermentation (days)
3-hexene-1-ol
intensity
3.E+5
2.E+5
1.E+5
0.E+0
0
7
14
21
28
fermentation (days)
crotonic acid
intensity
8.E+4
6.E+4
4.E+4
2.E+4
0.E+0
0
7
14
21
28
fermentation (days)
4-methylthio-butanenitrile
intensity
2.E+5
1.E+5
0.E+0
0
7
14
21
28
fermentation (days)
unknown (34.2 min)
intensity
2.E+5
1.E+5
0.E+0
0
7
14
21
28
fermentation (days)