

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

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21 **ABSTRACT**

22 Non-targeted metabolomic analyses employing nuclear magnetic resonance- and gas  
23 chromatography/mass spectrometry-based techniques were applied for an in-depth  
24 characterization of cabbage vinegar, an original agricultural product made from cabbage  
25 harvested in Tsumagoi, Japan. Water-soluble and volatile metabolite profiles of cabbage  
26 vinegar were compared with those of various vinegars: rice vinegar, grain vinegar, apple  
27 vinegar, and black vinegar (Japanese *kurozu* made of brown rice). Principal component  
28 analysis (PCA) of the water-soluble metabolites indicated that cabbage vinegars belonged  
29 to an isolated class by the contributions of fructose, pyroglutamic acid, choline, and  
30 methiin (*S*-methylcysteine sulfoxide). Regarding the volatile compounds, the PCA data  
31 represented that rice, black, and apple vinegars were characterized by most of the  
32 dominant volatiles, such as acetate esters, alcohols, ketones, and acids. Cabbage and grain  
33 vinegars were included in the same class although these two vinegars have different  
34 flavors. Orthogonal partial least squares-discrimination analysis exhibited the differences  
35 in volatile compound profile between cabbage and grain vinegars, revealing that cabbage  
36 vinegars were characterized by the presence of sulfides (dimethyl sulfide, dimethyl  
37 disulfide, and dimethyl trisulfide), nitriles (allyl cyanide and 4-methylthio-butanenitrile),  
38 3-hexene-1-ol, and crotonic acid. The time-course changes in these highlighted  
39 compounds during the acetic acid fermentation of cabbage vinegar suggested that  
40 pyroglutamic and crotonic acids were produced through fermentation, whereas choline,  
41 methiin, sulfides, nitriles, and 3-hexene-1-ol were derived from cabbage, suggesting the  
42 key role of these compounds in the unique taste and flavor of cabbage vinegar.

43

## 44 INTRODUCTION

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

53 Recently, the development of novel processed food products of cabbage (*Brassica*  
54 *oleracea* var. *capitata*) has been in demand in Tsumagoi, which is the largest cabbage-  
55 producing area in Japan. However, its intense sulfur odor, known to result from the  
56 generation of sulfides via degradations of sulfur-containing compounds (7), has been a  
57 problem for developing novel cabbage processed foods. Many of the cabbage processed  
58 products investigated so far have not obtained positive sensory evaluations owing to the  
59 strong odor elicited through during the cabbage processing procedures, such as squeezing  
60 and cooking. For the solution of this problem, we previously developed a novel vinegar  
61 using cabbage harvested in Tsumagoi through a traditional surface acetic acid  
62 fermentation system. The cabbage vinegar fermentation reduced the levels of sulfides and  
63 provided a positive sensory evaluation for its mild flavor, resulting in the successful  
64 development of a novel product (8). In the previous study, to compare the quality of  
65 cabbage vinegar with that of grain and fruit vinegars, acidity, pH, and levels of  
66 components (sugars, amino acids, and ethanol) were examined. As a result, fructose (Fru),  
67 glucose (Glc), and sorbitol, along with 20 amino acids, were detected by high-  
68 performance liquid chromatography (HPLC) and an amino acid analyzer, respectively,

69 and the higher level of Fru was highlighted as characteristic of cabbage vinegar (8). This  
70 finding suggested that the higher Fru content had potential impact on the taste of cabbage  
71 vinegar. However, the targeted analyses provided only limited compositional information  
72 about the cabbage vinegar. It is possible that the raw cabbage contains various unique  
73 metabolites, such as sulfur and flavor compounds of *Brassica* vegetables (9), but it  
74 remains unclear whether those metabolites contribute to the compositional characteristics  
75 of cabbage vinegar. Especially, determining the volatile compound profile of cabbage  
76 vinegar is essential to explain its unique, mild flavor, which possibly results from  
77 cabbage-derived compounds, including sulfides and sulfur-containing metabolites.

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

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

94 differentiating the cabbage vinegar from other vinegars.

95

## 96 **MATERIALS AND METHODS**

### 97 *Materials and sample preparation*

98       Eighteen vinegars subjected to metabolomic characterization are listed in Table 1.  
99 Six cabbage vinegars differ in the production lot. As conventional vinegars, 12  
100 commercial products produced by different manufacturers were purchased at a local  
101 supermarket in Isesaki, Japan. For the time-course analysis on compositional changes  
102 during acetic acid fermentation, the cabbage vinegar was prepared as reported in the  
103 previous study (Fig. S1) (8). Briefly, cabbages cultivated in Tsumagoi were harvested in  
104 July 2016 and pressed in a mechanical juicer to obtain fresh juice. After heating the juice  
105 to 85°C for 30 min, 423 mL of the juice was mixed with 27 mL of ethanol to supply the  
106 substrate for acetic acid fermentation, and was inoculated with 50 mL of bacterial  
107 suspension of *Acetobacter pasteurianus* NBRC 3284 (NITE Biological Resource Center,  
108 Japan). The cabbage vinegar fermentation was conducted at 30°C in a static surface  
109 fermentation for 28 days and the fermentation products were collected on the days 0, 7,  
110 14, 21, and 28. For preparation of the bacterial suspension, the strain NBRC 3284 was  
111 precultured in 10 mL of NBRC 804 medium (0.5% polypeptone, 0.5% yeast extract, 0.5%  
112 glucose, and 0.1% MgSO<sub>4</sub>·7H<sub>2</sub>O) at 30°C with rotary shaking for 3 days. The preculture  
113 was then transferred to 100 mL of YPGD medium (0.2% polypeptone, 0.2% yeast extract,  
114 0.2% glucose, and 0.2% glycerol) supplemented with 0.2% ethanol and 1.0% acetic acid  
115 and incubated at 30°C with rotary shaking for 3 days. After washing them with distilled  
116 water, the cells were resuspended with 100 mL of distilled water and used as bacterial  
117 suspension.

### 118 *NMR spectroscopy*

119 Water-soluble compounds in the vinegar samples were analyzed by NMR  
120 spectroscopy. In advance of the NMR analysis, the vinegar samples were lyophilized  
121 three times to avoid the severe chemical shift fluctuations by the high acetic acid  
122 concentration, that exceeded the buffering capacity of a standard buffer for high-  
123 sensitivity NMR measurement (20). The dried residue obtained by lyophilizing 325  $\mu$ L  
124 of vinegar sample was dissolved in 650  $\mu$ L of 100 mM potassium phosphate buffer (pH  
125 7.0) in deuterium oxide ( $D_2O$ , 99.9%, Cambridge Isotope Laboratories, Andover, MA)  
126 containing 1 mM of sodium 2,2-dimethyl-2-silapentane-5-sulfonate- $d_6$  (DSS- $d_6$ ,  
127 Cambridge Isotope Laboratories). The solution was subsequently centrifuged at  $21,500 \times$   
128  $g$  for 5 min at room temperature ( $25^\circ C$ ), and the supernatant was transferred into 5.0 mm  
129 O.D.  $\times$  103.5 mm NMR tubes (Norell, Landisville, NJ).

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

141 For the metabolite annotation, 2D NMR spectra including double quantum filtered  
142 correlated spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY),  $^1H$ - $^{13}C$   
143 heteronuclear single quantum coherence (HSQC), and  $^1H$ - $^{13}C$  heteronuclear multiple-

144 bond connectivity (HMBC) were measured. Metabolite signals were annotated using the  
145 SpinAssign program (21, 22), NMRPipe, and NMRDraw (23), as described previously  
146 (24). Public NMR spectral databases, Human Metabolomics Database  
147 (<http://www.hmdb.ca/>) (25), and the Biological Magnetic Resonance Data Bank  
148 (<http://www.bmrb.wisc.edu/>) (26), were used to increase the credibility of our annotations.  
149 When appropriate, signals were assigned by spiking with standard metabolites.

#### 150 *GC/MS analysis*

151 The volatiles present in the sample were extracted by headspace solid phase micro-  
152 extraction (HS-SPME) under the conditions described by Iijima *et al.* (27), using an AOC-  
153 5000 autosampler (Shimadzu, Kyoto, Japan). A 20-mL glass vial containing 2 mL of  
154 sample was maintained at 50°C for 10 min, and volatiles in the headspace were extracted  
155 by inserting a DVB/CAR/PDMS fiber (2 cm, Supelco, Bellefonte, CA, USA) for 20 min  
156 at 50°C with intermittent agitation at 250 rpm. The volatiles absorbed on the SPME fiber  
157 were injected into a GCMS-QP2010 Ultra (Shimadzu) by thermal desorption at 250°C  
158 for 3 min and were separated on a Rtx-WAX capillary column (60 m × 0.25 mm, f.t. 0.25  
159 μm, Restek, Bellefonte, PA, USA). Helium was used as carrier gas at a column flow rate  
160 of 2.0 mL/min. The oven temperature was increased from 40°C (hold for 5 min) to 180°C  
161 at a rate of 5°C/min, and to 230°C at a rate of 10°C/min. The final temperature was  
162 maintained for 5 min. Electron impact (EI) mass spectra were obtained under the  
163 following conditions: ionization voltage, 70 eV; ion source temperature, 230°C;  
164 quadrupole temperature, 150°C; mass range,  $m/z$  33–350; detector voltage, 1.0 kV; and  
165 scan speed, 3.15 scans/s. During the analysis, the filament was turned off for 90 s (runtime  
166 21.0–22.5 min) to cut excessive ions derived from the acetic acid.

167 Tentative metabolite identification was carried out by similarity search of mass  
168 spectra based on the NIST mass spectral library (NIST11) through the GCMSsolution



169 software (Shimadzu). Retention index (RI) in the NIST Chemistry Webbook  
170 (<http://webbook.nist.gov/chemistry/>) was also referred. RI was calculated using a mixture  
171 of aliphatic hydrocarbons (C6–C20; Sigma-Aldrich, St. Louis, MO, USA).

## 172 *Multivariate analysis*

173 The <sup>1</sup>H NMR spectra from 18 vinegar samples were processed using the TopSpin  
174 software (ver. 3.5, Bruker BioSpin). Bucket tables were generated using the Amix  
175 software (ver. 3.9.14, Bruker BioSpin). For the non-targeted multivariate analysis,  
176 datasets were generated by subdividing the spectra (10.00–0.50 ppm) into integrated  
177 regions (buckets) of 0.04 ppm each. The integrated data were then normalized to the  
178 integrated area of the internal standard (DSS-d<sub>6</sub> at 0.00 ppm). Nine buckets at 5.08–4.72  
179 ppm were excluded on the basis that they contained residual water signals. This created  
180 229 buckets in total. The GC/MS data were processed through baseline correction and  
181 peak alignment using the MetAlign software (28). The resulting retention time (RT) and  
182 *m/z* matrix was integrated to the dataset for the multivariate analysis using the AIoutput  
183 software (29). This created a dataset comprising of 375 GC/MS peaks.

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

189

190

## 191 **RESULTS**

### 192 *NMR based metabolomics*

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

194 PCA. First, PCA was carried out using the datasets containing all variables from the NMR  
195 spectra (Fig. 1A). The first and second principal component (PC1 and PC2) represented  
196 60.9% and 21.6% of the total variance, respectively. The score plots showed class  
197 separations of the 18 samples, and the following four classes were indicated by the Ward's  
198 hierarchical clustering method: class I (R1, B1, and B2), class II (G1–G3), and class III  
199 (R2, R3, B3, A2, and A3) were separated along the PC1 axis, and class IV (C1–C6, and  
200 A1) was along the PC2 axis (Fig. 1A). The loading plot indicated that PC1 was primarily  
201 explained by the buckets containing signals of Glc, 2,3-butanediol (BDO), and lactic acid  
202 (LacA), whereas PC2 was explained by those of Fru (Fig. 1A). The result showed that all  
203 cabbage vinegar samples belonged to class IV and shared the common characteristic of  
204 higher Fru level and lower Glc amount. Although the PCA data clarified the contribution  
205 of the dominant metabolites among the vinegar samples, that of the low-abundance  
206 metabolites remained uncharacterized with this approach.

207 Subsequently, to investigate the contributions of the low-abundance metabolites to  
208 cabbage vinegar, a multistep PCA (30) was performed after excluding the buckets  
209 containing the dominant compound signals, as shown in Fig. 1A (Glc, Fru, BDO, and  
210 LacA). The generated PCA model showed 31.6% and 25.1% of the total variance for the  
211 PC1 and PC2, respectively. Unlike the result of the first PCA shown in Fig. 1A, the  
212 multistep PCA score plot showed that the samples were clearly separated depending on  
213 the vinegar type (Fig. 1B). The PC1 and PC2 principally highlighted the characteristics  
214 of black vinegars and cabbage vinegars, respectively (Fig. 1B). The loading plot  
215 explained that black vinegars were characterized by higher levels of branched chain  
216 amino acids (BCAAs), glycerol, and alanine (Ala), whereas cabbage vinegars were  
217 characterized by substantial levels of pyroglutamic acid (Glp), choline, and methiin (Fig.  
218 1B). Malic acid (MalA) indicated by PC1 was contained at high concentration in apple

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

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

#### 227 *GC/MS based metabolomics*

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

244 (6.6 min, 97%)] (Fig. S2).

245 Although the PCA data showed that the cabbage and grain vinegar samples  
246 belonged to the same class, these two vinegars clearly differ from each other in  
247 ingredients and sensory properties. We therefore expected the presence of characteristic  
248 compounds in these two vinegars and performed OPLS-DA to clarify the detailed  
249 compositional difference between cabbage and grain vinegars. The generated OPLS-DA  
250 model provided cumulative regression coefficient ( $R^2$ ) and cumulative cross-validation  
251 coefficient ( $Q^2$ ) values of 0.951 and 0.852, respectively. The orthogonal PLS component  
252 1 of the model explained 24.2% of the total variance. To identify responsible peaks for  
253 characterizing the cabbage vinegar, S-plot was examined, which visualizes the OPLS-DA  
254 loadings and extracts potential variables contributing to discrimination of two classes (31).  
255 The S-plot indicated the presence of candidate metabolites responsible for cabbage  
256 vinegar. The data and metabolite annotations are presented in Fig. 3B.

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

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

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

271

## 272 **DISCUSSION**

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

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

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

351

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358



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- 498

499 **FIGURE LEGENDS**

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

505 Fig. 2. Changes in the levels of representative water-soluble metabolites in cabbage  
506 vinegar during acetic acid fermentation. Data are expressed as relative intensity to DSS-  
507 d<sub>6</sub> (0.00 ppm) and shown as means  $\pm$  SD (n = 3).

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

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

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

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

Satoru Ishihara, Takashi Inaoka, Toshihide Nakamura, Keitarou Kimura, Yasuyo Sekiyama, and Satoru Tomita\*

\* Corresponding Author: Satoru Tomita. E-mail satorutomita@affrc.go.jp, Tel +81-(0)29-8388033, Fax +81-(0)29-8387996.

### **Supplementary material description**

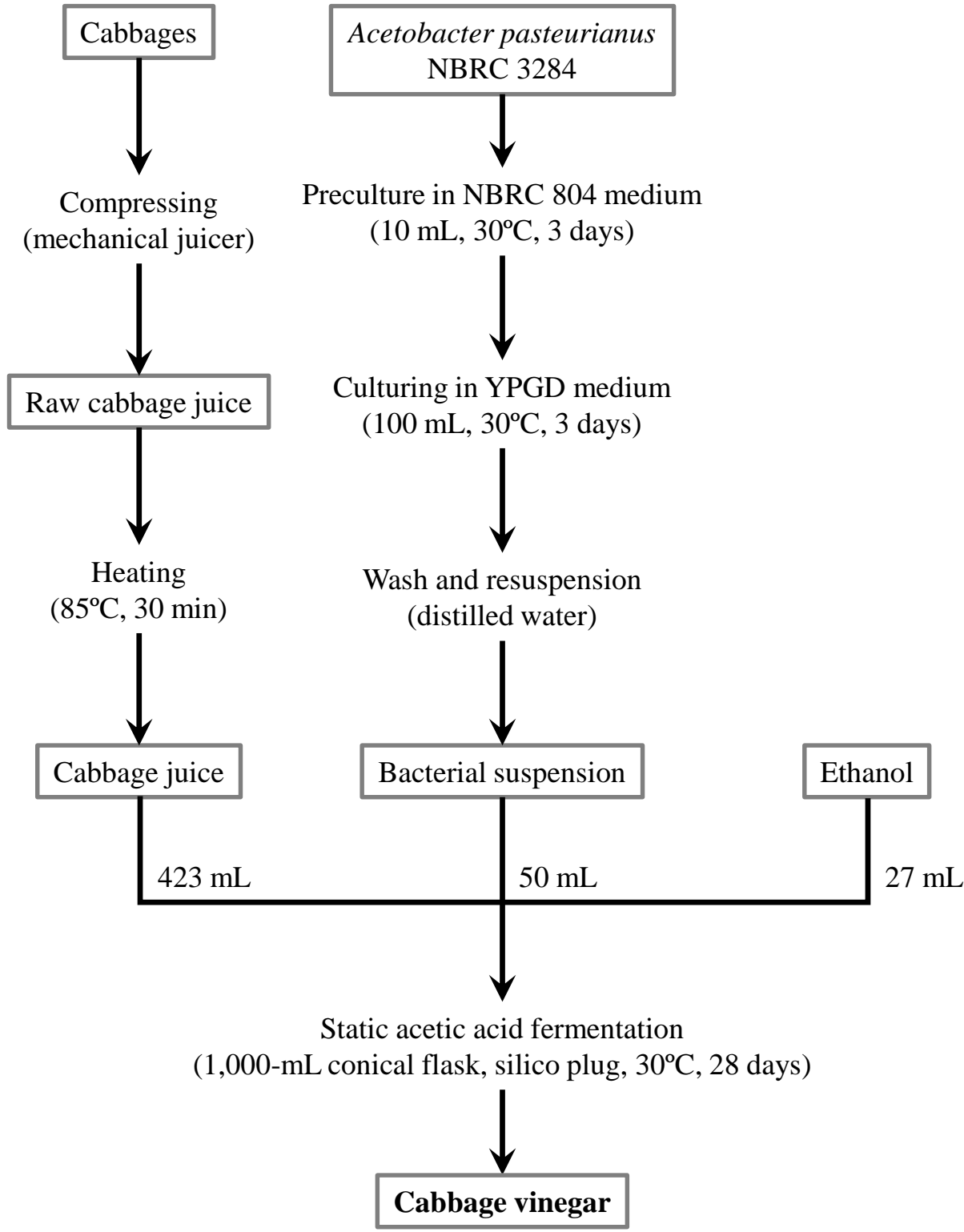
Fig. S1. The manufacturing flow of cabbage vinegar

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  $\pm$  *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.

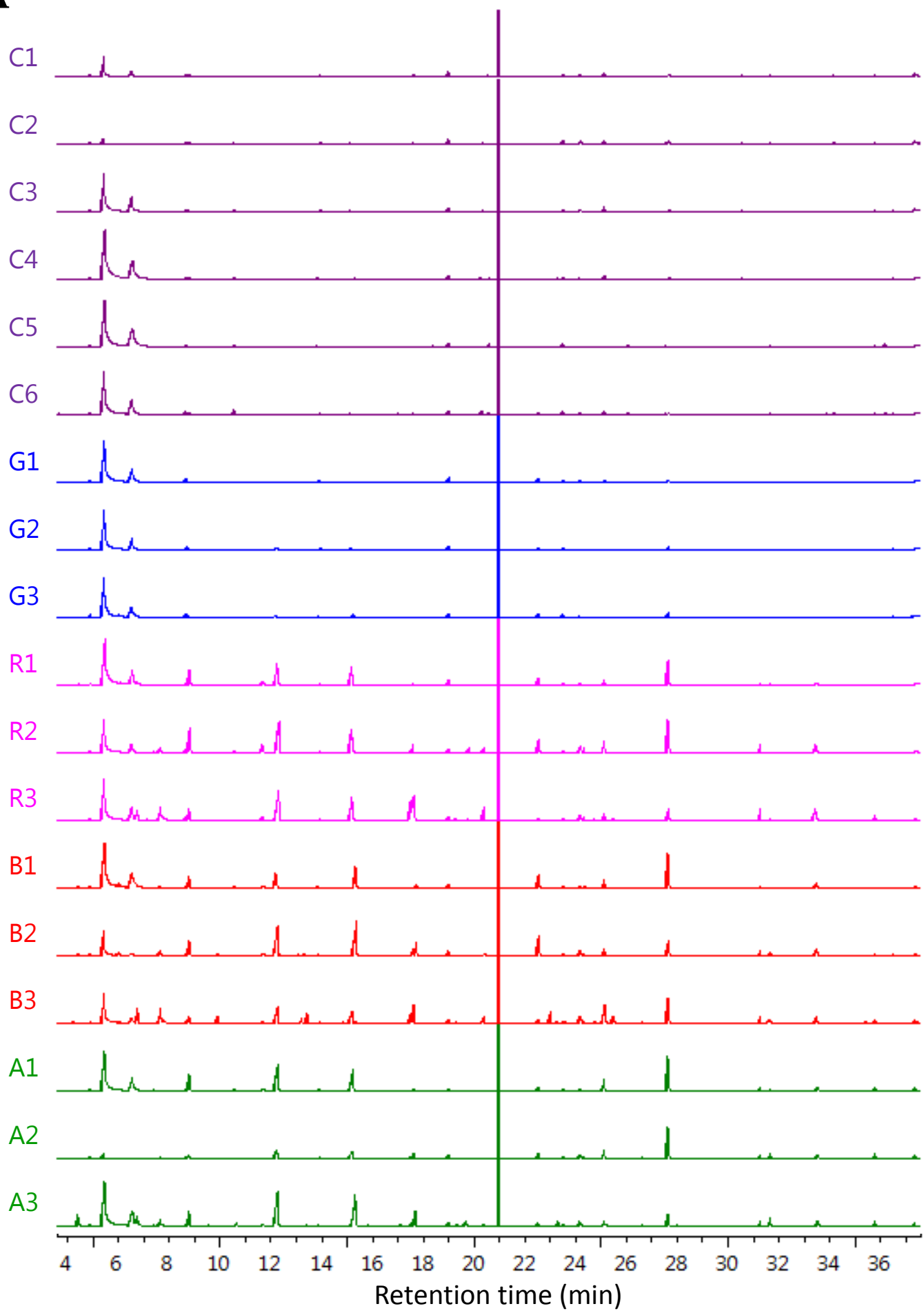


**Fig. S1**



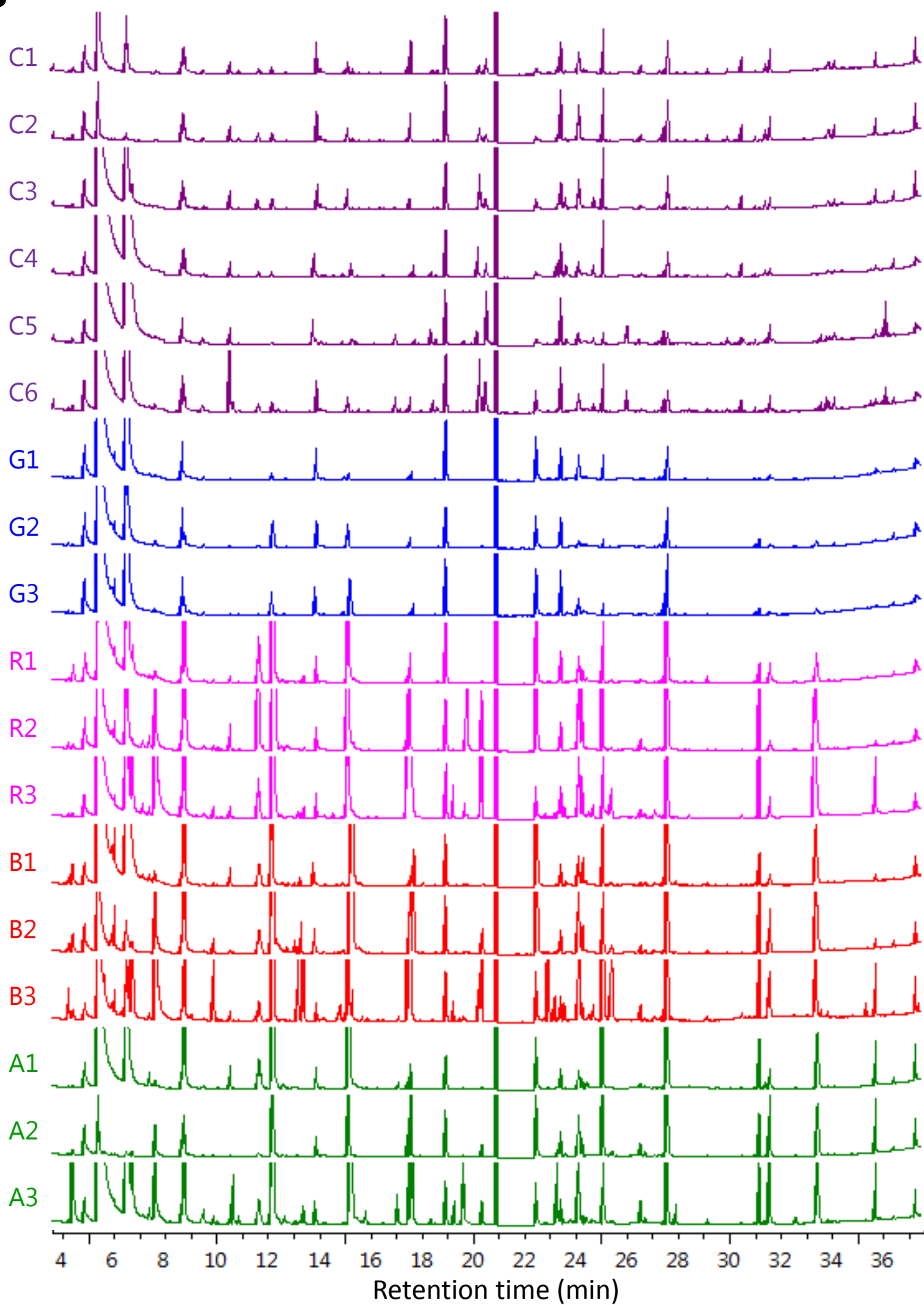
**Fig. S2**

**A**

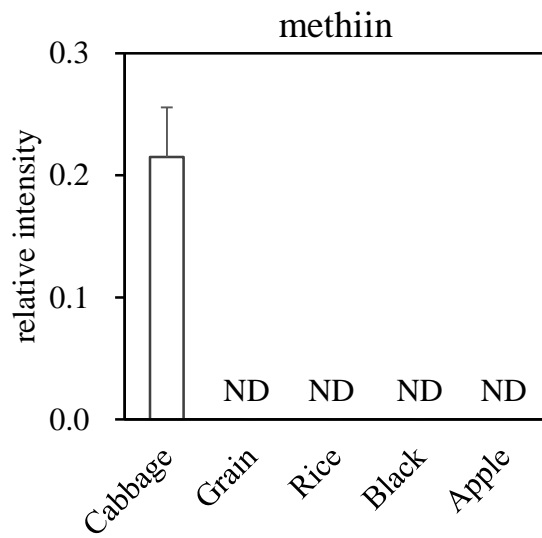
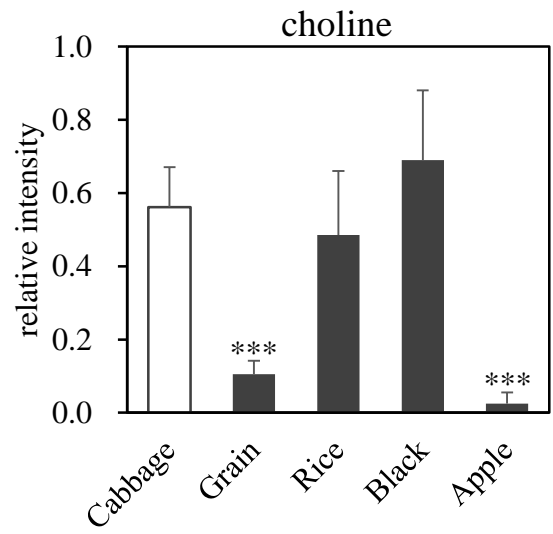
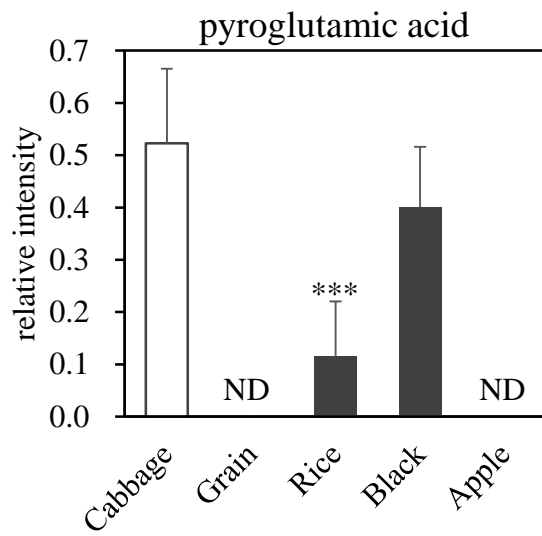


**Fig. S2 (continued)**

**B**



**Fig. S3**



1 **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, <i>sake</i> cake	4.22
	G2		Grains (rice, corn), alcohol, <i>sake</i> cake	4.32
	G3		Rice, alcohol, <i>sake</i> 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

2 \*Japanese traditional vinegar, *kurozu* (*kurosū*)

**fig1**

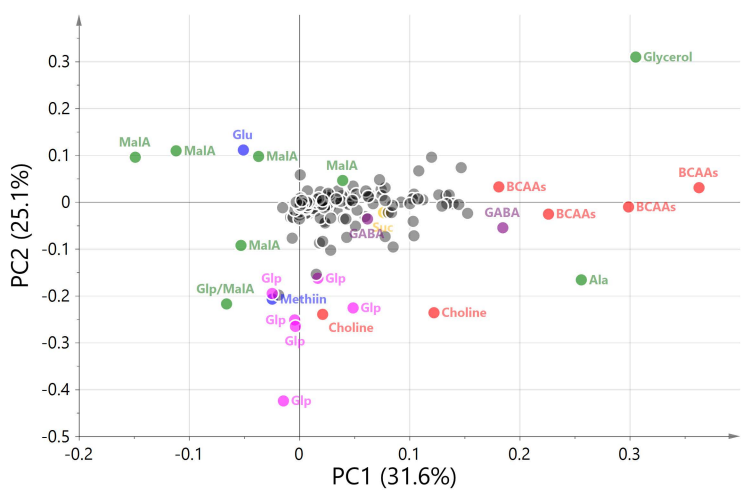
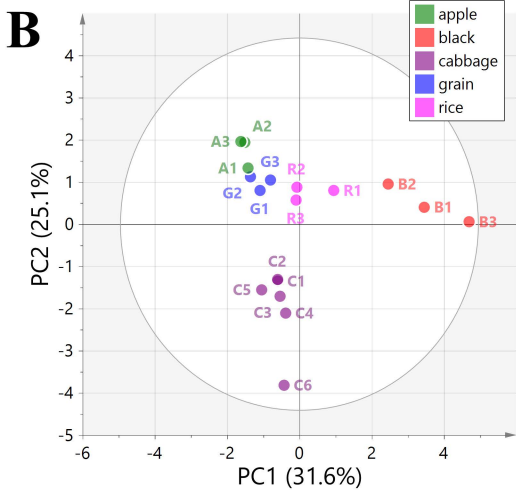
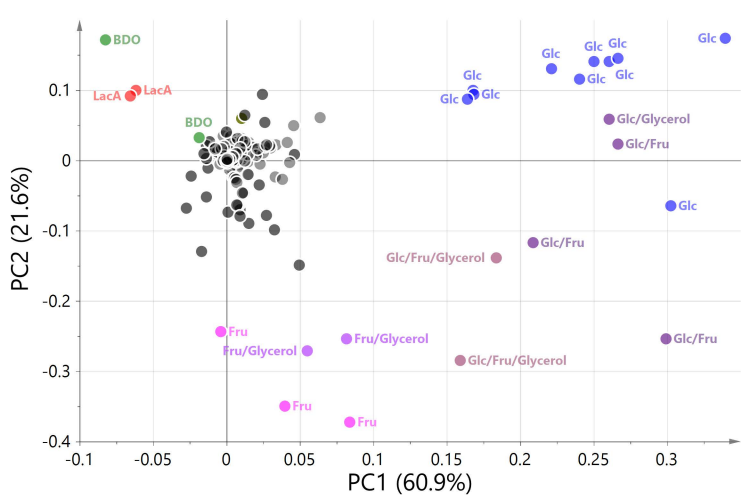
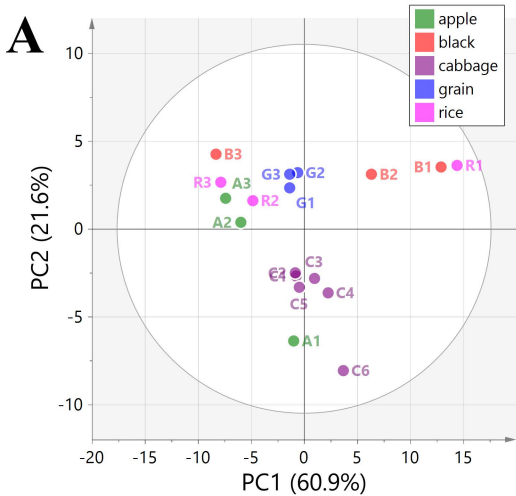


fig 2



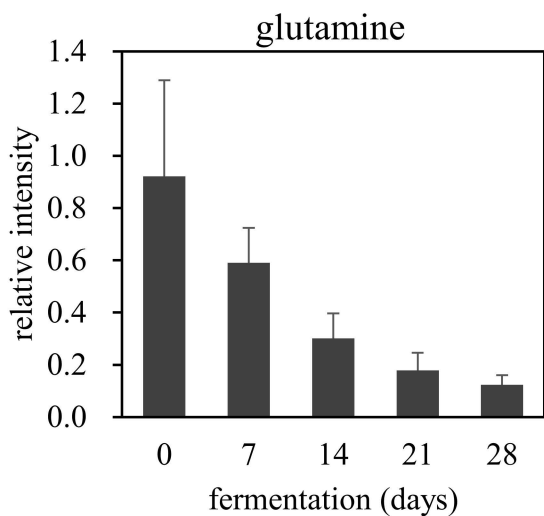
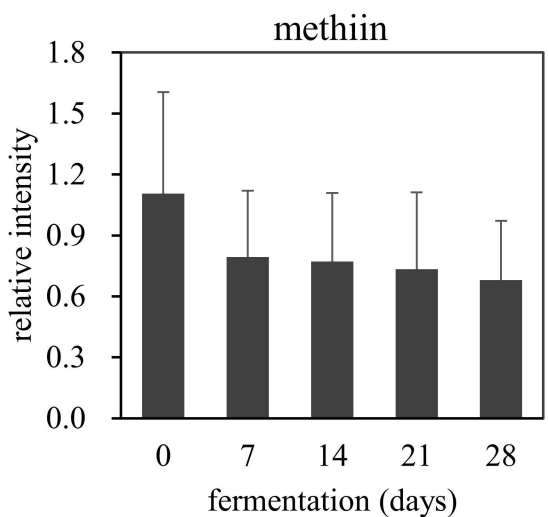
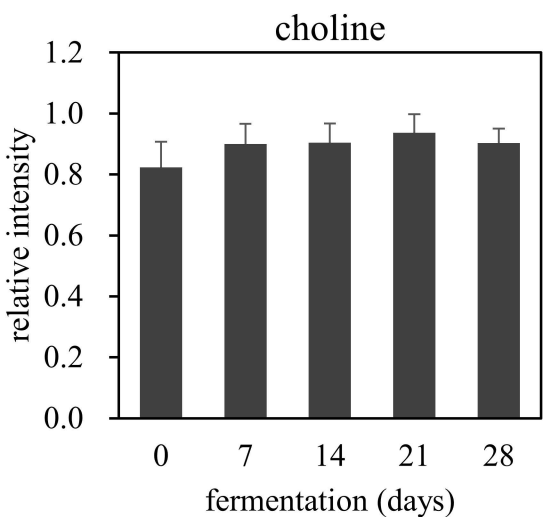
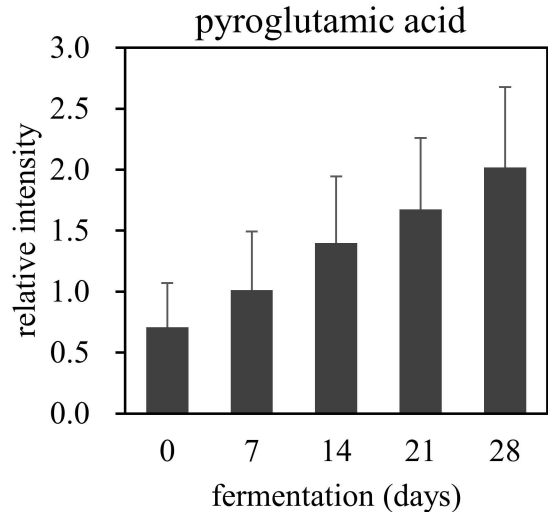
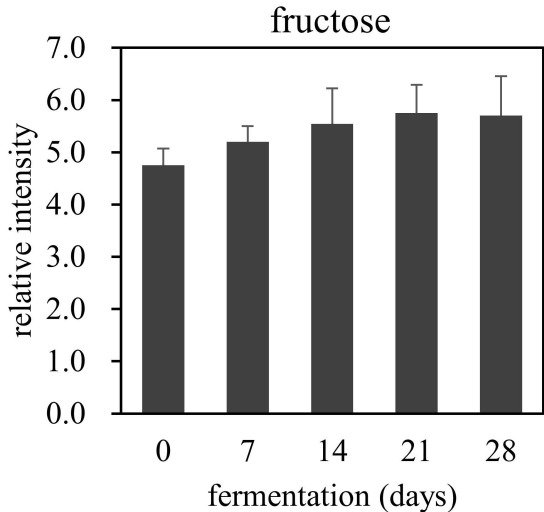


fig 3

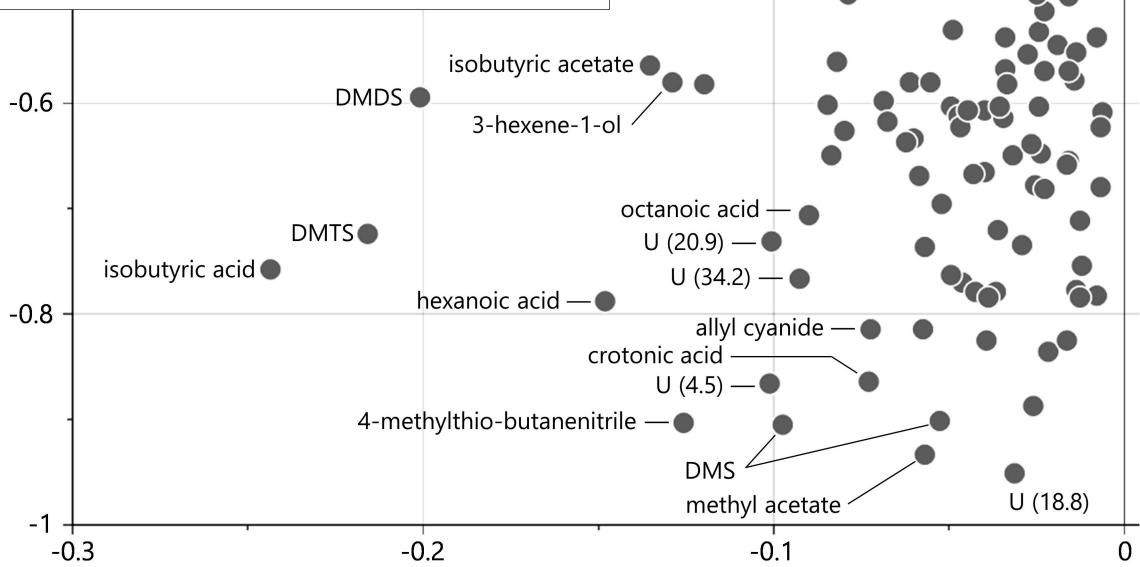
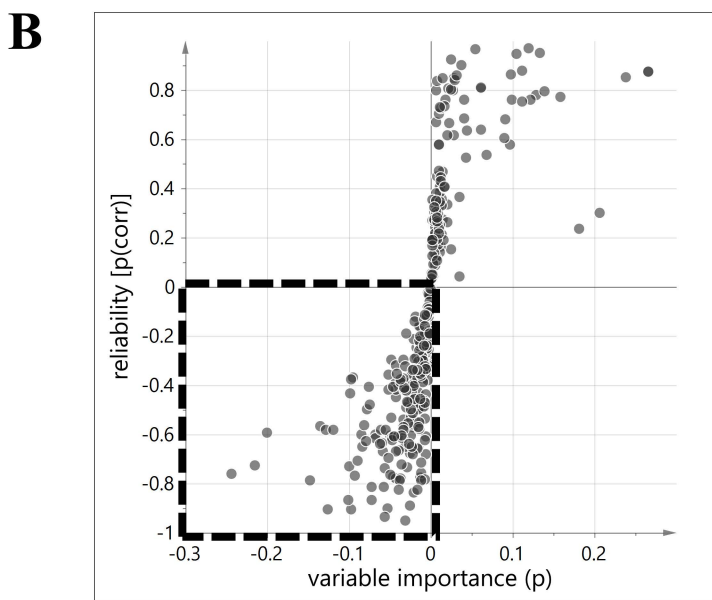
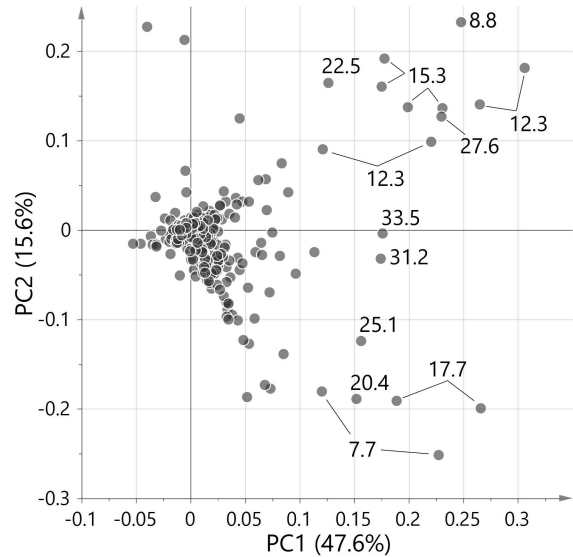
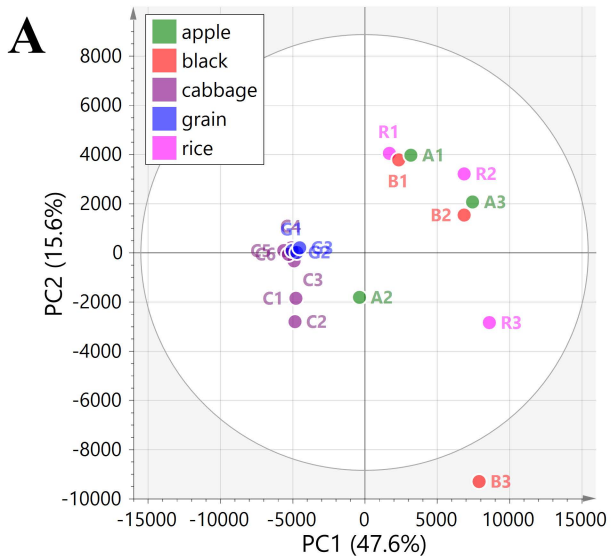


fig 4

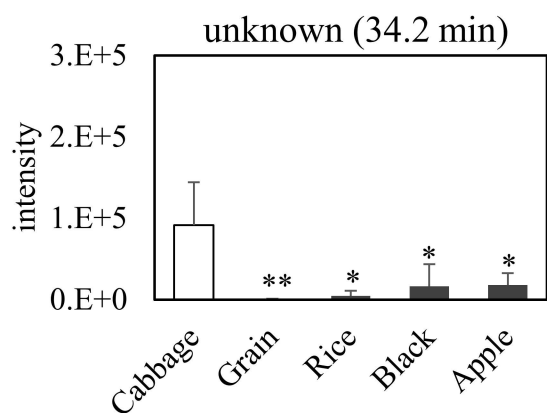
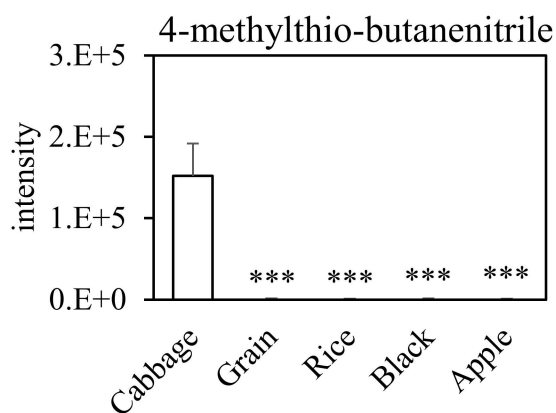
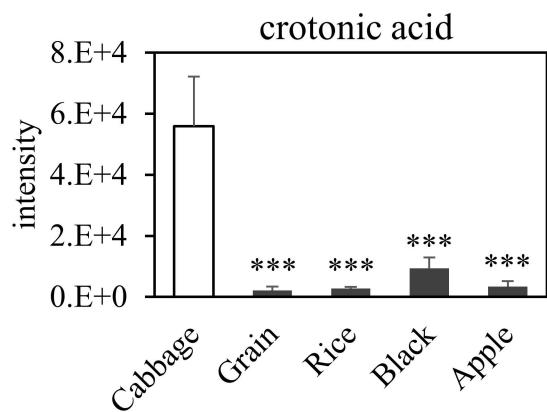
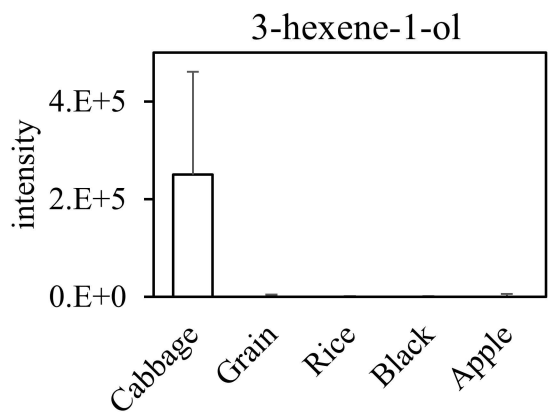
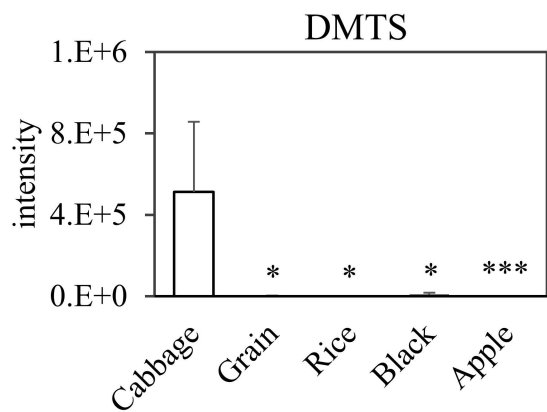
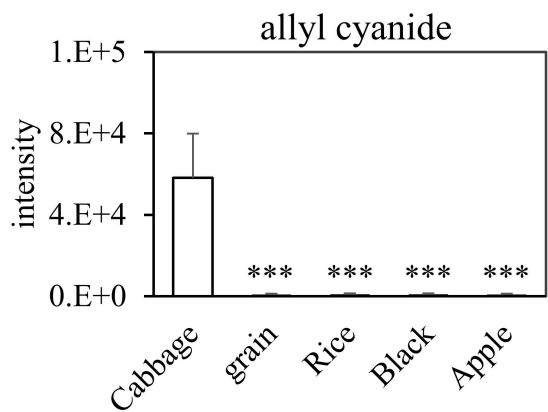
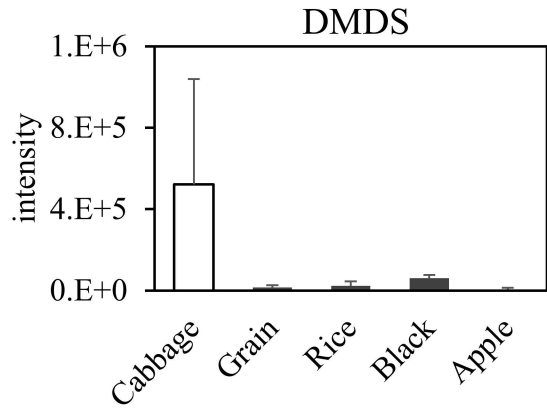
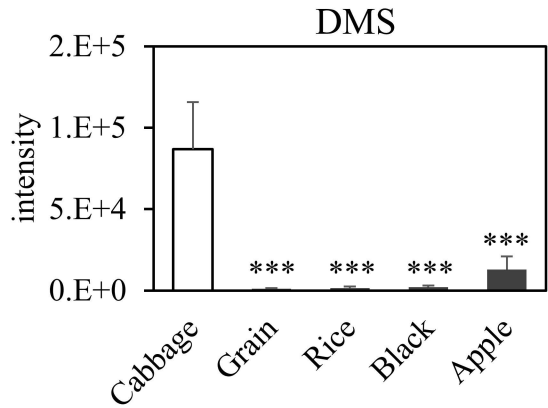
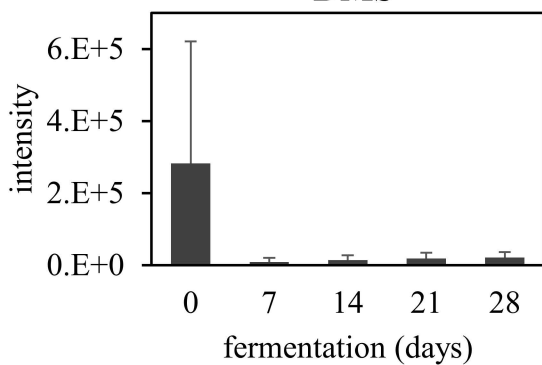
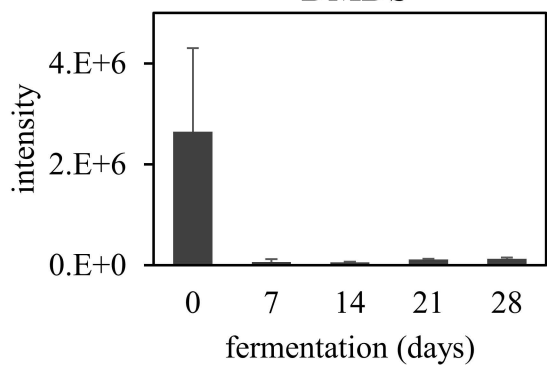


fig 5

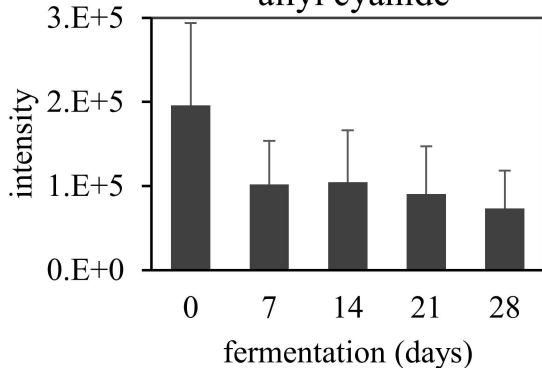
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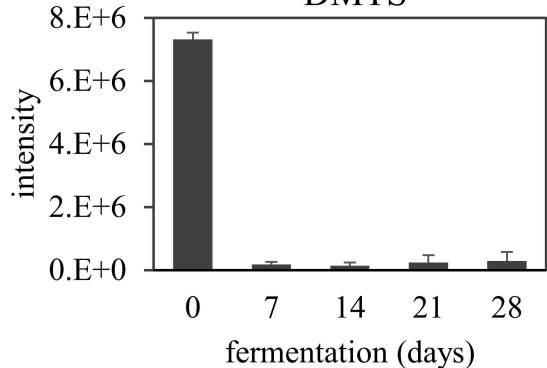
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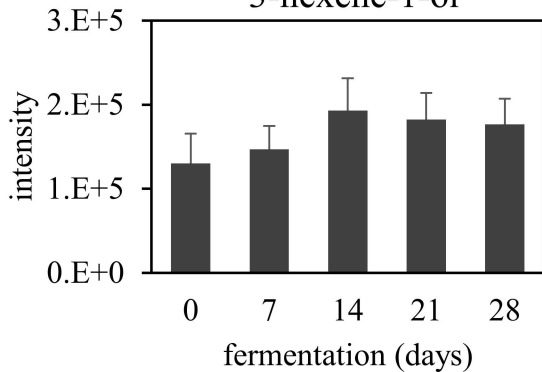
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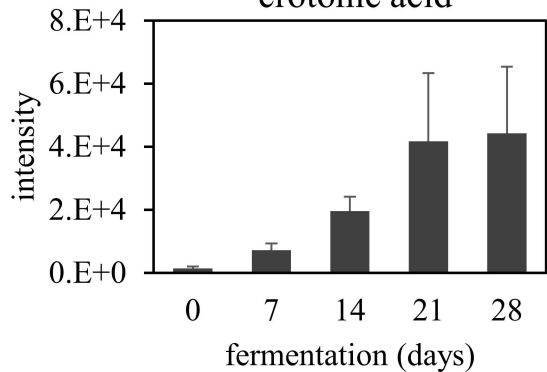
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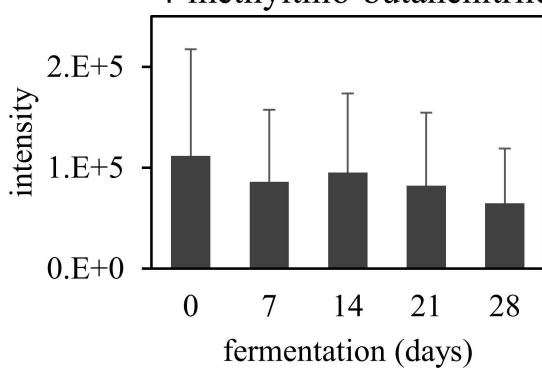
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crotonic acid



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unknown (34.2 min)

