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Application of a metabolomic method combining one-dimensional and two-dimensional gas chromatography-time-of-flight/mass spectrometry to metabolic phenotyping of natural variants in rice☆

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Abstract

We have developed a comprehensive method combining analytical techniques of one-dimensional (1D) and two-dimensional (GC × GC) gas chromatography-time-of-flight (TOF)–mass spectrometry. This method was applied to the metabolic phenotyping of natural variants in rice for the 68 world rice core collection (WRC) and two other varieties. Ten metabolites were selected as metabolite representatives, and the selected ion current of each metabolite peak obtained from both techniques were statistically compared. Our method of combining 1D- and GC × GC-TOF/MS is useful for the metabolic phenotyping of natural variants in rice for further studies in breeding programs.

Keywords: Rice core collection; GC × GC; Two-dimensional chromatography; Gas chromatography; Metabolic phenotyping

1. Introduction

Metabolomics, an important field of "-omics" studies, has been developed since the end of the 20th century [1–3]; currently, the application of its methodology extends to various branches such as toxicology [4,5], human epidemiological and clinical studies [6,7], and hidden metabolic phenotypes in functional genomic studies of plants [8–11] or microbes [12–14]. One of the goals of metabolomics is to detect all metabolites (defined as the metabolome) in a biological sample with high accuracy in terms of quality and quantity. In order to achieve this goal, a variety of techniques, including gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS), capillary electrophoresis–mass spectrometry (CE–MS), and nuclear magnetic resonance (NMR) are applied to carry out metabolite detection to the maximum extent possible. Of these, GC–MS is one of the most widely used techniques and a key technology for metabolic profiling because of its long history in analyzing low molecular weight compounds [15–17]. The GC–MS data show highly repeatable mass spectral fragmentation on electron impact (EI) ionization, and unsurpassed chromatographic reproducibility and resolution, although the technique needs further improvement, e.g. with regard to optimization of the chromatographic matrix for the metabolites of interest. In addition, the GC–MS technology platforms are relatively less expensive than other instrumentation modalities such as CE–MS, LC–MS, or LC–NMR. In the GC–MS technology, the gas chromatography-time-of-flight/mass spectrometry (GC-TOF/MS) analysis provides information not only regarding the known metabolites but also regarding unknown metabolites; this is attributable to a unique combination of high resolution gas chromatography with a rapid and sensitive time-of-flight mass analyzer [18]. The advantages of GC-TOF/MS analy-

Abbreviations: GABA, 4-aminobutyric acid; IAA, indole-3-acetic acid

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sis are a relatively high reproducibility, and the possibility to high-throughput analysis due to its rapid spectra accumulation times. Furthermore, the high mass spectra similarities over peak, makes the use of mathematic algorithms for deconvolution of closely overlapping peaks possible. Both commercial and non-commercial software have are available (ChromaTOF, AMDIS [19], or Jonsson et al. [20]). The software can separate co-eluted peaks mathematically, even when the overlapping peaks are not completely separated by chemical chromatography. Thus, the GC-TOF/MS technique is now applied widely for high-throughput analysis in the field of metabolomics.

Two-dimensional gas chromatography GC × GC-TOF/MS is a novel approach for enhancing the GC resolution, and it has great advantages in increasing the resolution and peak capacity over the one dimensional separation method. The GC × GC-TOF/MS for metabolomics has been applied for complex metabolite profiles from mouse spleen [21]. This is the first report for the use of a technique which significantly enhances metabolite resolution. Currently, the GC × GC-TOF/MS technique has been applied in the analysis of volatile compounds [22,23] and also in the analysis of metabolite mixtures from mouse tissue, yeast cells, and human urine and serum as the comprehensive GC × GC-TOF/MS analysis [24–27].

When non-targeted metabolic profiling data are subjected to multivariate statistical analysis such as principal component analysis (PCA) and partial least square-discriminate analysis (PLS-DA) toward the obtained data, a high throughput and high accuracy can be achieved for clustering according to the vectors of numerous metabolites.

In order to develop a technique for studies in plant metabolomics, we have developed a combined method, namely, 1D- and GC × GC-TOF/MS. We applied this technique in the non-targeted metabolic profiling of brown rice seeds from the world rice core collection (WRC). The WRC is a representative set of Asian cultivated rice, *Oryza sativa* L., comprising 69 varieties selected from 3000 accessions stored in the Genebank of the National Institute of Agrobiological Sciences (NIAS), Japan, classified based on DNA polymorphism [28]. The WRC covers ca. 90% of the DNA polymorphisms detected in the original population and can be used as a convenient set to survey the genetic diversity of rice. Therefore, the metabolic phenotyping of the WRC varieties gives us an insight on the representative metabolite diversity of rice natural variants. This report describes a metabolomic method combining 1D-GC-TOF/MS and GC × GC-TOF/MS analyses and an example of its application to focus on the differences in the characteristics of data obtained from 1D-GC/MS and GC × GC/MS measurements of the WRC samples. This study presents the first report of the initial metabolic phenotyping in brown rice seeds of the WRC.

2. Experimental

2.1. Samples

Twenty-five rice seeds for each of the 68 WRC cultivars [28] and var. Dahonggu and Pokkari were sown on April 19, 2005, at a rice field in NIAS, Tsukuba, Japan. Seeds were harvested independently for each cultivar after 40 days, starting from the day on which the first panicle of rice was observed. The seeds were threshed from the panicles manually and then collected by each cultivar, after they were dried at 30 °C for three days. All seeds in the husks were stored at 5 °C under dark conditions until analysis. For each cultivar, 100 seeds were selected according to the average weight and length of seeds. After separating the husks from the seeds, the brown rice seeds obtained were bulked and crushed by using a Retsch mixer mill MM301 at a frequency of 20 Hz^{-1} for 2 min at 4 °C. Successively, the obtained powder was divided into four pools for metabolic phenotyping. One hundred milligrams of each material was extracted with extraction buffer [methanol/chloroform/water (3:1:1, v/v/v)] at a concentration of 100 mg/ml and containing 10 stable isotope reference compounds. Each isotope compound was adjusted to a final concentration of 15 ng/μl for each 1-μl injection [20,29,30]. After centrifugation, a 200-μl aliquot of the supernatant was drawn and transferred into a glass insert vial. The extracts were evaporated to dryness in an SPD2010 SpeedVac® concentrator from ThermoSavant (Thermo electron corporation, Waltham, MA, USA).

2.2. Derivatization

Methyl oxime derivatives were obtained by dissolving the dry extracts in 30 μl of methoxyamine hydrochroride-HCl (20 mg/ml in pyridine) for 30 h at room temperature. After methoxymation, the sample was trimethylsilylated for 1 h by 30 μl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) with 1% of trimethylchlorosilane (TMCS) at 37 °C with shaking. Subsequently, 30 μl of *n*-heptane was added after silylation. All the derivatization steps were performed in the vacuum glove box VSC-1000 (Sanplatec, Japan) filled with 99.9995% (G3 grade) of dry nitrogen.

2.3. One-dimensional gas chromatography-time-of-flight/mass spectrometry (1D-GC-TOF/MS) analysis

One microliter of each sample was injected in the splitless mode by an CTC CombiPAL autosampler (CTC analytics, Zwingen, Switzerland) into an Agilent 6890N gas chromatograph (Agilent Technologies, Wilmingston, USA) equipped with a 30 m × 0.25 mm inner diameter fused-silica capillary column with a chemically bound 0.25-μl film Rtx-5 Sil MS stationary phase (RESTEK, Bellefonte, USA) for metabolome analysis. Four replicates were subjected to 1D-GC-TOF/MS analysis. Helium was used as the carrier gas at a constant flow rate of 1 ml/min. The temperature program for metabolome analysis started with a 2-min isothermal step at 80 °C and this was followed by temperature ramping at 30 °C to a final temperature of 320 °C, which was maintained for 3.5 min. Data acquisition was performed on a Pegasus III TOF mass spectrometer (LECO, St. Joseph, MI, USA) with an acquisition rate of 30 spectra/s in the mass range of a mass-to-charge ratio of $m/z = 60–800$. Alkane standard mixtures (C8–C20 and C21–C40) were purchased from Sigma–Aldrich (Tokyo, Japan) and were used for calculating the retention index (RI) [31,32]. The normalized response for

the calculation of the signal intensity of each metabolite from the mass-detector response was obtained by each selected ion current that was unique in each metabolite MS spectrum to normalize the peak response, using the method of Kopka et al. [33]. The normalized responses are peak areas corrected using the sample weight and a constant amount of the representative internal standard compound ([$^{13}C_4$]-hexadecanoic acid).

2.4. Two-dimensional gas chromatography-time-of-flight/mass spectrometry (GC × GC-TOF/MS)

The GC × GC-TOF/MS system consisted of the Agilent 6890N gas chromatograph and Pegasus 4D TOF-MS (Leco, St. Joseph, MI, USA). An Rtx-5 Sil MS column containing 5% diphenyl–95% dimethylpolysiloxane as the stationary phase was used as the first column (I.D., 30 m × 0.25 mm and film thickness, 0.25 μl) and an Rtx-50 column containing 50% methyl–50% phenyl polysiloxane was used as the second column (I.D., 1.0 m × 0.18 mm and film thickness, 0.20 μl). Both columns were purchased from Restek (Bellefonte, USA). They were connected using a universal Press-Tight® connector (Restek). A thermal modulator and the first and second ovens were independently controlled by Leco ChromaTOF optimized for Pegasus 4D software version 2.32 (Leco, St. Joseph, MI, USA). The second column was housed within the second oven. A 1-μl portion of each extract was injected in the splitless mode; three replicate injections were made for each biological sample. The temperature of the GC inlet and transfer line was set at 250 °C. The first column was maintained at 70 °C for 2 min and then increased at the rate of 15 °C/min to 320 °C, and this temperature was maintained for 4 min. The second column was initially set at 80 °C and the temperature program followed was the same as that used for the first column. The modulator was operated at 30 °C for a 3.0-s period; this temperature was higher than that of the first GC oven. The carrier gas (helium) was maintained at a constant flow rate of 1 ml/min. Signals within the mass range from *m/z* 60 to 600 were collected at a rate of 100 spectra/s after 273 s of solvent delay. The linear retention time ($RT_{compound\ x}$) was calculated using the RT value of each compound on the first (1st $RT_{compound\ x}$) and second dimensions (2nd $RT_{compound\ x}$) as mentioned below:

$$RT_{compound\ x} = 1st\ RT_{compound\ x} + 2nd\ RT_{compound\ x}$$

The alkane mixtures used in GC × GC-TOF/MS analysis were analyzed to determine the RI. The algorithm was applied in the Leco ChromaTOF software to calculate the RI. The normalized response was then calculated as described by Kopka et al. [33].

2.5. Mass spectral data processing

To compare the response of the metabolite peaks with the same algorithm, the Leco ChromaTOF optimized for Pegasus 4D software version 2.32 (Leco, St. Joseph, MI, USA) was used. Data, including baseline correction, peak deconvolution, and peak annotation for 1D- and GC × GC-TOF/MS were processed. In parallel, non-processed MS data from the 1D-GC-TOF/MS analysis were exported in the NetCDF format to MATLAB software 6.5 (Mathworks, Natick, MA, USA), where all data-pretreatment procedures such as data normalization, baseline correction, and the subsequent data treatments were performed using custom scripts [20] to perform multivariate statistical analysis for metabolite phenotype clustering. The resolved MS spectra obtained from the custom scripts were matched against reference mass spectra by using the National Institute of Standards and Technology (NIST) mass spectral search program for the NIST/EPA/NIH mass spectral library (version 2.0) and our custom mass spectral search software written in JAVA (http://www.metabolome.jp/). Two mass spectral libraries – an in-house metabolite library in PRIMe (Platform for RIKEN Metabolomics, http://prime.psc.riken.jp) and the library in the Golm Metabolome Database (GMD) at CSB.DB [34,35] – were used for the collection of mass spectra obtained by analysis. The extracted MS spectra were finally identified or annotated according to their RI and comparison with the reference mass spectra in the libraries.

2.6. Statistics

The obtained data matrix for the 1D-GC-TOF/MS data was used for statistical analyses, including multivariate analysis as log_{10}-transformed data. Multivariate statistical analysis was performed using the SIMCA-P 11.0 software (Umetrics AB, Umeå, Sweden). The following statistics for the PLS-DA models represent the reliability of each model. The explained variation in the *X*-matrix (R^2X) is the cumulative modeled variation in *X*; the explained variation in the *Y*-matrix (R^2Y) is the cumulative modeled variation in *Y*; and Q^2Y is the cumulative predicted variation in *Y*, according to cross-validation. To perform the statistical analysis for peak areas in Leco ChromaTOF software, we scaled all normalized peak area by 10,000 and then added 1 uniformly, in terms of minimizing the effect of zero-substitution. The statistical Welch's *t*-test was calculated using the *R* package as an open source statistical computing environment (http://www.r-project.org/). Simple comparisons of means of the obtained peak areas were performed by *t*-test. A difference of $p < 0.05$ was regarded to be significant.

3. Results and discussion

3.1. Method development for metabolic profiling in combination with 1D- and GC × GC-TOF/MS analyses

Ultracomplex rice extracts contain up to hundreds of co-eluting compounds that frequently vary in terms of abundance by several orders of magnitude. Compared with 1D-GC-TOF/MS, GC × GC-TOF/MS is greatly advantageous with respect to high resolution and peak capacity. On the other hand, metabolic profiling often requires a high throughput for analysis because studies on metabolomics generally require the analysis of many replicates for each biological data point. In terms of achieving a high throughput, the 1D-GC-TOF/MS technique combined with sophisticated mass spectral deconvolution algorithms is therefore a more powerful technique for rapid metabolite profiling

Table 1
Comparison of the characteristics of 1D-GC-TOF/MS and GC × GC-TOF/MS analyses

	1D-GC-TOF/MS	GC × GC-TOF/MS
Throughput	High	Medium
Resolution	High	Very high
Sensitivity	Medium	High
Cost performance	Low	Medium
Deconvolution	Good	Very good
Data size (ASCII CSV format)	Medium (approx. 200 MB)	Large (approx. 1 GB)

as compared to GC × GC-TOF/MS. Comparison of the characteristics of 1D- and GC × GC-TOF/MS analyses is summarized in Table 1. As a general strategy adopted for the experiment on large-scale samples such as the WRC varieties, 1D-GC-TOF/MS analysis is first carried out and its properties of achieving a high throughput and good reproducibility are exploited for the clustering of varieties by metabolic phenotyping to select a small number of the representative varieties for further analysis. Subsequently, for these selected varieties, a detailed analysis can be carried out by GC × GC-TOF/MS. This protocol of employing 1D-GC-TOF/MS in combination with GC × GC-TOF/MS allows us to draw a comprehensive picture of the metabolic profiles of the WRC.

3.2. *Classification of the WRC by multivariate statistical analysis using 1D-GC-TOF/MS data*

The extracts of brown rice seeds from 68 WRC varieties and two additional varieties, namely, var. Dahonggu and Pokkari, were first analyzed using 1D-GC-TOF/MS to select the representative varieties of metabolic clusters, and these selected varieties were then subjected to a detailed analysis for high-resolution metabolite profiling by using GC × GC-TOF/MS. The 1D-GC-TOF/MS raw data were imported to MATLAB software for peak alignment and extraction of MS spectra as described by Jonsson et al. [20,29,30]. Approximately, 430 extracted MS peaks were obtained in each 1D-GC-TOF/MS analysis by using the hierarchical multivariate curve resolution (H-MCR) technique, which resolves samples simultaneously [20]. In contrast, less than 400 extracted MS peaks were obtained by using Leco ChromaTOF software. Two types of multivariate statistical analyses were performed toward the obtained matrices: the principal component analysis as an unsupervised analysis and the partial least square-discriminate analysis as a supervised analysis. Fig. 1 shows the PLS-DA score scatter plots obtained from the 1D-GC-TOF/MS analysis of the extracts of the brown rice cultivars. Two cultivars, namely, *Oryza sativa* var. Nipponbare and Kasalath, were selected as the controls for the Japonica and Indica varieties, respectively. Clear separations were observed between the varieties of non-glutinous and glutinous rice in Fig. 1. For non-glutinous rice, the score scatter plots of the groups belonging to Radin Goi Sesat, Neang Menh, and Kemasin were clustered very close as shown in the Fig. 1(A). On clustering by PLS-DA, we selected three non-glutinous varieties – Ryou Suisan Koumai, Anjana Dhan, and Urasan 1 – based on the criterion of good separation from the others so that unique metabolic profiles would be revealed for further analysis by GC × GC-TOF/MS.

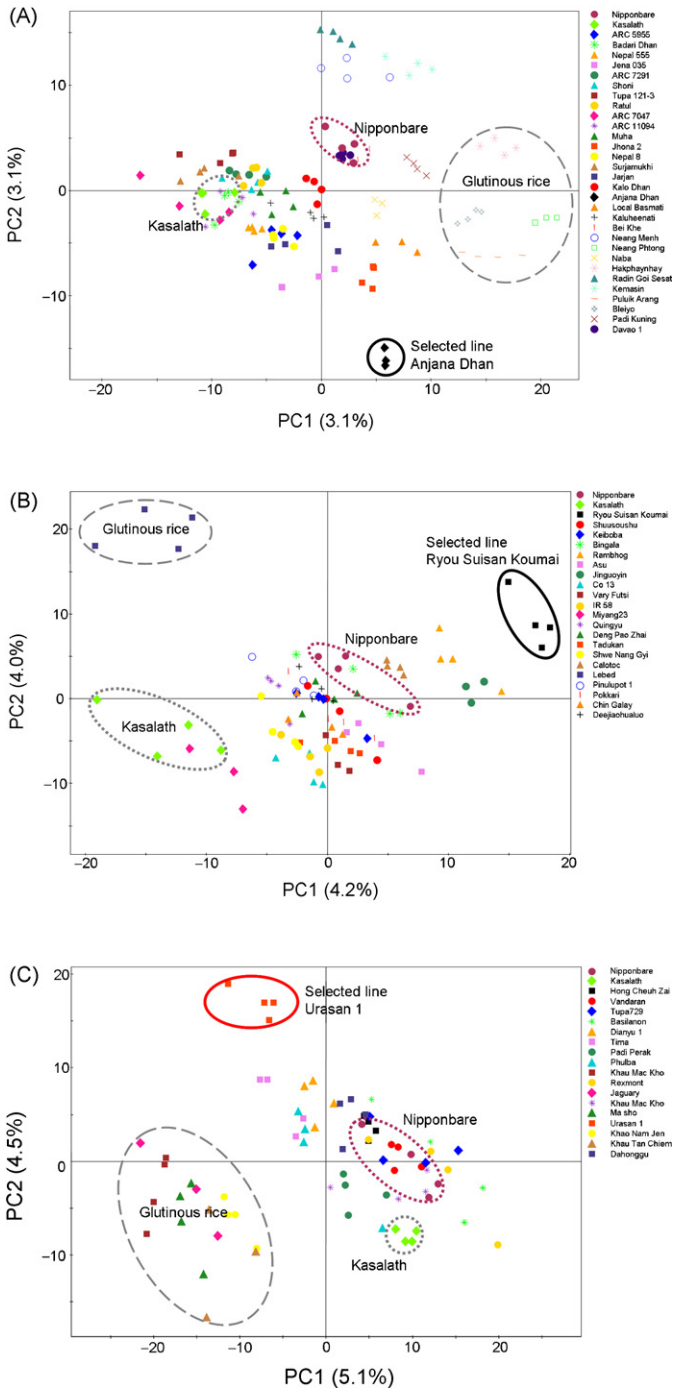

Fig. 1. The PLS-DA score scatter plot for metabolite peaks obtained from the extracts of rice seeds of Nipponbare (brown dot), Kasalath (pale-green diamond), and other core collections. 1D-GC-TOF/MS analysis of WRC rice was performed by dividing the varieties randomly into three batches as shown in (A)–(C). (A) Number of significant components: 14, $R^2X = 0.68$, $R^2Y = 0.38$, and $Q^2Y = 0.13$. (B) Number of significant components: 11, $R^2X = 0.67$, $R^2Y = 0.40$, and $Q^2Y = 0.15$. (C) Number of significant components: 9, $R^2X = 0.69$, $R^2Y = 0.42$, and $Q^2Y = 0.15$. Three varieties, namely, Anjana Dhan, Ryou Suisan Koumai, and Urasan 1, were selected as well-separated representatives from the others. PC: principal component.

The PLS-DA models of the two controls Nipponbare and Kasalath and three selected WRC varieties were created and analyzed as shown in Fig. 2. For example, the loading of the principal components 1 (Anjana Dhan/Nipponbare separation) and 2 (Anjana Dhan/Kasalath separation) in Fig. 2 (A) was selected according to the value of the first weight vector (w^* 1) and second weight vector (w^* 2) together with the 95% confidence intervals calculated using jackknifing [36,37] to extract the loadings, which is a distinguishing factor between the controls and the selected varieties. Fifty-six variables were considered as significant in the separation of Anjana Dhan and Nipponbare, and 29 variables were considered as significant in the separation of Anjana Dhan and Kasalath. Finally, based on the interpretation of the results of the multivariate analysis for each model as shown in the Fig. 2A–C, 10 metabolite peaks (GABA, glycerol-3-phosphate, myristate, fructose, IAA, inositol-1-phosphate, trehalose, alpha-tocopherol, cholesterol, and raffinose) were selected as representatives of metabolites, which contributed to significant differences between the controls and the three varieties in the PLS-DA models. The two controls and the three selected cultivars were further analyzed using the GC × GC-TOF/MS analysis for high-resolution metabolic profiling.

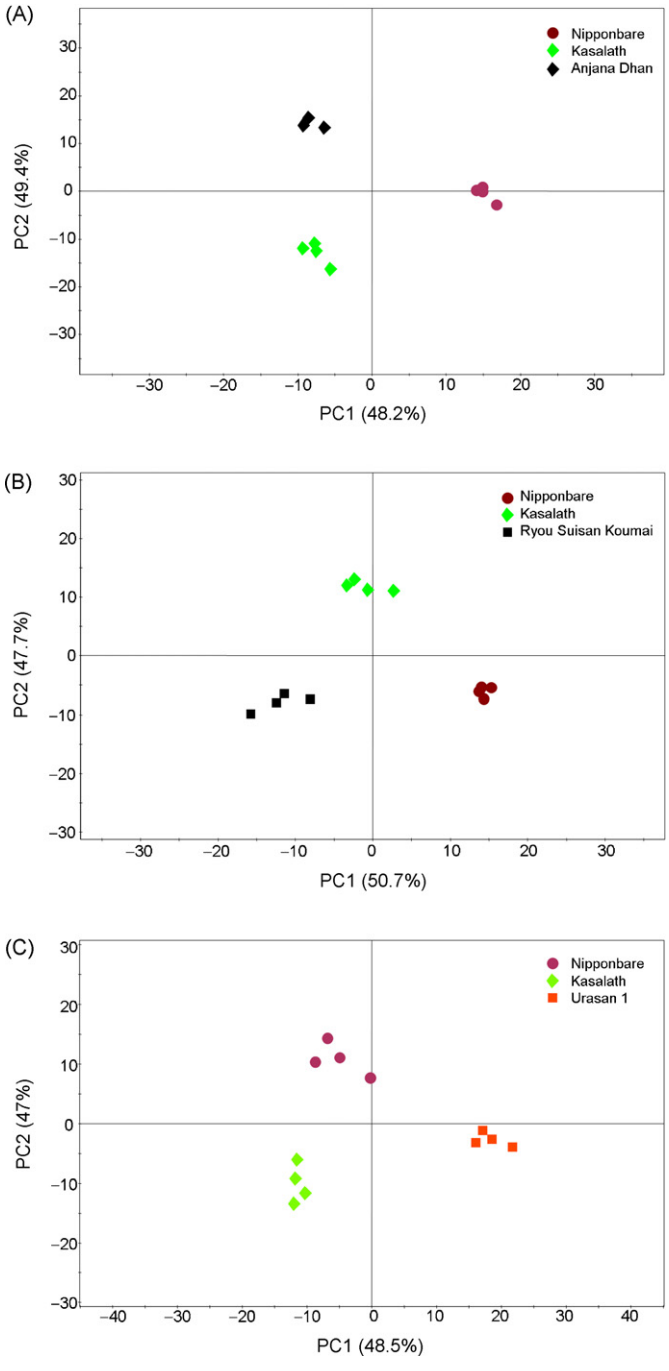

Fig. 2. The PLS-DA score scatter plot for metabolite peaks obtained from the extracts of rice seeds of Nipponbare (brown dot), Kasalath (pale-green diamond), and the following selected varieties: (A) Anjana Dhan (black diamond), number of significant components: 3, $R^2X = 0.75$, $R^2Y = 1.00$, and $Q^2Y = 0.99$. (B) Ryou Suisan Koumai (black box), number of significant components: 3, $R^2X = 0.68$, $R^2Y = 0.99$, and $Q^2Y = 0.95$. (C) Urasan 1 (red box), number of significant components: 3, $R^2X = 0.72$, $R^2Y = 0.99$, and $Q^2Y = 0.95$.

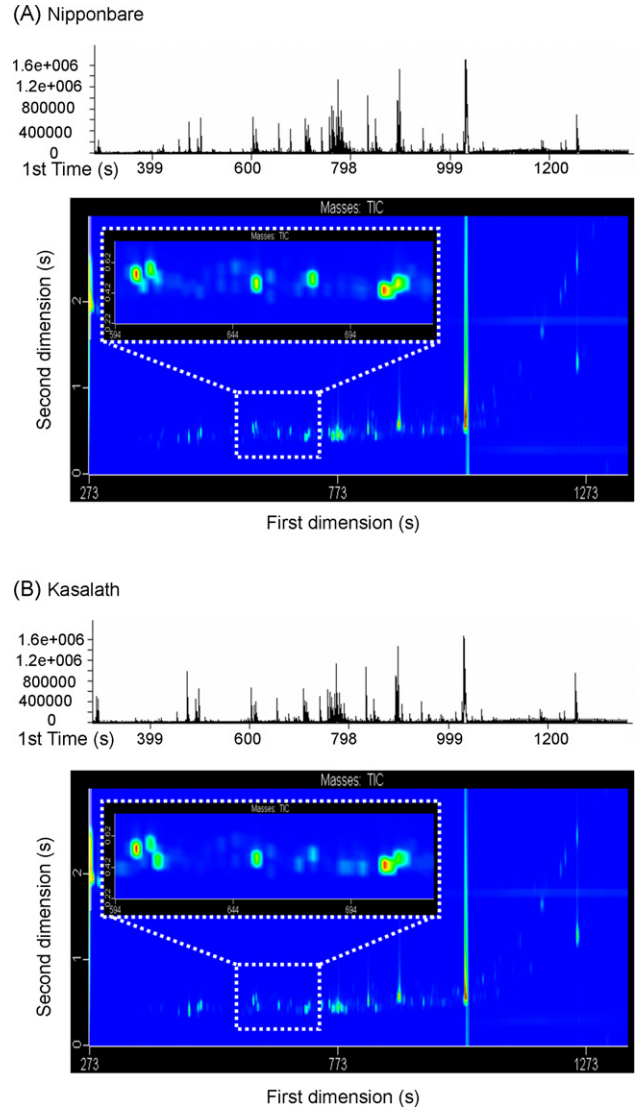

Fig. 3. Modulated chromatogram (above) and the corresponding two-dimensional contour plot (below) for (A) Nipponbare and those of (B) Kasalath. The *x*-axis in the modulated chromatogram indicates the retention time (s) of the first column and that of the second column. The *y*-axis in the modulated chromatogram represents the peak intensity of the TIC.

3.3. GC × GC-TOF/MS analysis for high-resolution metabolic profiling

The GC × GC-TOF/MS analysis enabled the detection of approximately 620 peaks from rice extracts of the WRC cultivars due to its higher resolution ability as compared to 1D-GC-TOF/MS with H-MCR method [20,29]. Fig. 3 exemplifies the separation patterns for the extracts of Nipponbare and Kasalath by using GC × GC-TOF/MS. Each total ion chromatogram (TIC) in the modulated chromatogram and the corresponding two-dimensional contour plot showed a slightly different separation pattern, indicating that both the Japonica and Indica rice varieties possess unique metabolites composition, although a majority of peaks were nearly similar with their intensities. A detailed analysis of the obtained total ion chromatogram of the three selected cultivars showed that the patterns were slightly different. For example, Ryou Suisan Koumai was classified as an Indica rice and it showed a separation pattern similar to that of Kasalath. This result suggested that rice varieties classified as closely related by analysis of DNA polymorphisms exhibit similar metabolite compositions.

After peak-intensity normalization for tissue weight and noise reduction, a normalized response of the selected ion current in each metabolite MS spectrum detected from 1D-GC-TOF/MS analysis and that from GC × GC-TOF/MS analysis was calculated for 10 representative metabolites (Table 2 for Nipponbare). RI data were used to identify the metabolites detected using both techniques. In 1D-GC-TOF/MS analysis, the normalized response had a relative standard deviation (RSD) value from 3 to 49% for the 10 metabolites. The response values obtained using GC × GC-TOF/MS were approximately two times higher than those obtained using 1D-GC-TOF/MS, indicating that the GC × GC-TOF/MS technique enhances the MS signals due to its high-resolution capacity and sharp peaks that result from focusing the compounds in the modulator and then injecting into the second column as a narrow injection band. In addition, metabolite peaks that were enhanced two times higher or more in GC × GC-TOF/MS analysis showed greater similarity than the results of 1D-GC-TOF/MS analysis (e.g. GABA, IAA and alpha-tocopherol). These results indicate that the GC × GC-TOF/MS technique is greatly advantageous in enhancing the peak intensity and resolution over the 1D-GC-TOF/MS technique, although the former technique occasionally exhibits a drawback in terms of less reproducibility and peak accuracy latter.

3.4. Statistical comparison of the metabolite peaks obtained using 1D- and GC × GC-TOF/MS analyses

The 10 metabolite peaks contributing to differences between the Japonica rice variety Nipponbare, the Indica rice variety Kasalath, and three selected varieties were selected based on

Table 2
Comparison of the normalized responses with the signal intensity of selected ions for replicates of Nipponbare obtained from 1D-GC-TOF/MS analysis and those obtained from the GC × GC-TOF/MS analysis

Metabolite	Retention index	First repeat, mean ± SD (RSD %)	Second repeat, mean ± SD (RSD %)	Third repeat, mean ± SD (RSD %)
The normalized response from 1D-GC-TOF/MS analysis[a]				
GABA	1524	0.69 ± 0.06 (8.8)	0.79 ± 0.15 (18.9)	0.64 ± 0.23 (36.1)
Glycerol-3-phosphate	1740	3.06 ± 0.54 (17.6)	3.89 ± 0.65 (16.7)	3.17 ± 1.02 (32.1)
Myristate	1843	0.75 ± 0.06 (7.4)	0.92 ± 0.10 (10.8)	0.90 ± 0.06 (7.0)
Fructose	1850	6.44 ± 0.21 (3.3)	5.85 ± 0.34 (5.8)	4.02 ± 1.34 (33.2)
IAA	1968	0.67 ± 0.03 (3.9)	0.80 ± 0.13 (16.7)	0.73 ± 0.15 (21.1)
Inositol-1-phosphate	2400	0.76 ± 0.12 (15.9)	0.74 ± 0.21 (27.9)	1.02 ± 0.18 (17.9)
Trehalose	2721	0.41 ± 0.04 (9.0)	0.56 ± 0.08 (14.7)	0.48 ± 0.08 (15.9)
Alpha-tocopherol	3153	0.07 ± 0.03 (40.6)	0.10 ± 0.02 (21.3)	0.10 ± 0.02 (17.5)
Cholesterol[b]	3185	6.41 ± 0.64 (9.9)	7.39 ± 1.22 (16.5)	7.60 ± 3.71 (48.8)
Raffinose	3355	0.98 ± 0.04 (4.0)	1.12 ± 0.14 (12.4)	1.10 ± 0.34 (30.7)

Metabolite	Retention index	First repeat, mean ± SD (RSD %)	Second repeat, mean ± SD (RSD %)
The normalized response from GC × GC-TOF/MS analysis[b]			
GABA	1524	2.10 ± 0.78 (37.4)	2.91 ± 0.63 (21.8)
Glycerol-3-phosphate	1743	4.04 ± 0.76 (18.8)	4.26 ± 0.85 (20.0)
Myristate	1842	0.87 ± 0.25 (28.5)	0.96 ± 0.05 (5.5)
Fructose	1857	8.54 ± 3.07 (35.9)	12.18 ± 0.61 (5.0)
IAA	1962	1.49 ± 0.23 (15.6)	2.09 ± 0.19 (9.3)
Inositol-1-phosphate	2401	0.54 ± 0.06 (11.0)	2.26 ± 0.10 (4.6)
Trehalose	2722	0.86 ± 0.28 (32.0)	1.94 ± 0.42 (21.9)
Alpha-tocopherol	3146	0.13 ± 0.05 (40.8)	0.25 ± 0.01 (4.7)
Cholesterol[c]	3166	4.86 ± 0.04 (0.7)	7.49 ± 0.86 (11.5)
Raffinose	3367	2.96 ± 0.58 (19.6)	4.28 ± 0.10 (2.3)

RSD: relative standard deviation; each mean and standard deviation (SD) of the metabolite level for the normalized response is shown. Each normalization response was calculated for the top 10 metabolites that exhibited significant differences in the PLS-DA models.

[a] Four replicates were conducted for each sample repeat by 1D-GC-TOF/MS.

[b] Three replicates were conducted for each sample repeat by GC × GC-TOF/MS.

[c] Cholesterol was a mixture of natural and 25,26,26,26,27,27,27-D_7 labeled peaks.

Table 3
Accumulation of 10 metabolites analyzed using 1D-GC-TOF/MS exhibiting significant differences for loadings in PLS-DA among the selected candidates and Nipponbare or Kasalath

Metabolite	Normalized response, mean ± SD (RSD %)	*X*-fold (/Nipponbare)	*p*-value (/Nipponbare)
Kasalath[a]			
GABA	9.54 ± 0.90 (9.4)	13.7	0.00
Glycerol-3-phosphate	1.75 ± 0.20 (11.3)	0.6	0.00
Myristate	0.56 ± 0.04 (6.3)	0.7	0.00
Fructose	2.36 ± 0.42 (17.7)	0.4	0.00
IAA	0.83 ± 0.06 (6.7)	1.2	0.00
Inositol-1-phosphate	0.63 ± 0.16 (24.8)	0.8	0.23
Trehalose	0.22 ± 0.02 (11.1)	0.5	0.00
Alpha-tocopherol	ND	–	–
Cholesterol[b]	6.01 ± 1.77 (29.4)	0.9	0.57
Raffinose	2.29 ± 0.13 (5.6)	2.3	0.00

Metabolite	Normalized response, mean ± SD (RSD %)	*X*-fold (/Nipponbare)	*p*-value (/Nipponbare)	*X*-fold (/Kasalath)	*p*-value (/Kasalath)
Anjana Dhan[c]					
GABA	8.39 ± 0.34 (4.1)	12.1	0.00	0.9	0.08
Glycerol-3-phosphate	1.54 ± 0.18 (11.5)	0.5	0.00	0.9	0.22
Myristate	0.63 ± 0.03 (4.8)	0.8	0.02	1.1	0.03
Fructose	16.68 ± 0.20 (1.2)	2.6	0.00	7.1	0.00
IAA	0.66 ± 0.00 (0.7)	1.0	0.49	0.8	0.01
Inositol-1-phosphate	0.63 ± 0.09 (13.6)	0.8	0.16	1.0	0.89
Trehalose	0.77 ± 0.10 (12.7)	1.9	0.00	3.5	0.00
Alpha-tocopherol	0.03 ± 0.01 (19.8)	0.5	0.02	–	–
Cholesterol[b]	4.88 ± 1.29 (26.5)	0.8	0.18	0.8	0.36
Raffinose	0.77 ± 0.01 (0.7)	0.8	0.00	0.3	0.00

Metabolite	Normalized response, mean ± SD (RSD %)	*X*-fold (/Nipponbare)	*p*-value (/Nipponbare)	*X*-fold (/Kasalath)	*p*-value (/Kasalath)
Ryou Suisan Koumai[a]					
GABA	37.86 ± 2.04 (5.4)	47.7	0.00	3.8	0.00
Glycerol-3-phosphate	4.09 ± 1.33 (32.5)	1.1	0.90	2.5	0.30
Myristate	0.29 ± 0.01 (4.0)	0.3	0.00	0.5	0.00
Fructose	12.52 ± 0.63 (5.1)	2.1	0.00	5.6	0.00
IAA	1.05 ± 0.03 (2.9)	1.3	0.05	1.0	0.96
Inositol-1-phosphate	0.83 ± 0.07 (8.7)	1.1	0.39	1.2	0.21
Trehalose	0.34 ± 0.02 (6.3)	0.6	0.00	1.1	0.05
Alpha-tocopherol	0.12 ± 0.01 (12.4)	1.2	0.25	–	–
Cholesterol[b]	6.42 ± 0.70 (10.8)	0.9	0.24	0.8	0.22
Raffinose	1.88 ± 0.11 (5.7)	1.7	0.00	0.7	0.00

Metabolite	Normalized response, mean ± SD (RSD %)	*X*-fold (/Nipponbare)	*p*-value (/Nipponbare)	*X*-fold (/Kasalath)	*p*-value (/Kasalath)
Urasan 1[a]					
GABA	56.18 ± 5.91 (10.5)	88.0	0.00	5.9	0.00
Glycerol-3-phosphate	7.42 ± 0.34 (4.6)	2.3	0.01	3.1	0.00
Myristate	2.10 ± 0.05 (2.2)	2.3	0.00	3.6	0.00
Fructose	14.56 ± 0.80 (5.5)	3.6	0.00	6.8	0.00
IAA	0.52 ± 0.02 (4.7)	0.7	0.05	0.5	0.00
Inositol-1-phosphate	1.35 ± 0.13 (9.4)	1.3	0.03	1.9	0.01
Trehalose	0.94 ± 0.08 (9.0)	2.0	0.00	3.2	0.00
Alpha-tocopherol	0.15 ± 0.01 (3.9)	1.4	0.02	–	–
Cholesterol[b]	6.40 ± 3.36 (52.5)	0.8	0.64	1.1	0.90
Raffinose	3.39 ± 0.24 (7.1)	3.1	0.00	1.2	0.04

SD: standard deviation, RSD: relative standard deviation, ND: not detected.
[a] Number of replicates: $n = 4$.
[b] Cholesterol was a mixture of natural and 25,26,26,26,27,27,27-D_7 labeled peaks.
[c] Number of replicates: Anjana Dhan ($n = 3$), Nipponbare and Kasalath ($n = 4$, respectively).

the results of the PLS-DA analysis. Their peak areas obtained from 1D- and GC × GC-TOF/MS analyses were statistically compared as shown in Tables 3 and 4. All metabolite peaks detected by 1D-GC-TOF/MS were detected by GC × GC-TOF/MS analysis. Most of metabolite peaks showed relatively similar tendency between the results from 1D-GC-TOF/MS and 2D-GC-TOF/MS, except for GABA. Interestingly, for 1D-GC-TOF/MS detection, the Indica rice variety Kasalath showed an

Table 4
Accumulation of 10 metabolites analyzed using GC × GC-TOF/MS exhibiting significant differences for loadings in PLS-DA among the selected candidates and Nipponbare or Kasalath

Metabolite	Normalized response, mean ± SD (RSD %)	*X*-fold (/Nipponbare)	*p*-value (/Nipponbare)	*X*-fold (/Kasalath)	*p*-value (/Kasalath)
Kasalath					
GABA	18.17 ± 1.04 (5.7)	6.3	0.00		
Glycerol-3-phosphate	3.28 ± 0.66 (20.1)	0.8	0.18		
Myristate	0.91 ± 0.02 (2.0)	0.9	0.20		
Fructose	7.28 ± 2.07 (28.5)	0.6	0.09		
IAA	3.11 ± 0.15 (4.9)	1.5	0.01		
Inositol-1-phosphate	2.23 ± 0.29 (12.8)	1.0	0.82		
Trehalose	1.34 ± 0.18 (13.7)	0.7	0.10		
Alpha-tocopherol	ND	–	–		
Cholesterol[a]	8.19 ± 0.81 (9.8)	1.1	0.35		
Raffinose	8.69 ± 0.11 (1.3)	2.0	0.00		
Anjana Dhan					
GABA	31.00 ± 3.41 (11.0)	10.7	0.00	1.7	0.01
Glycerol-3-phosphate	3.37 ± 1.16 (34.3)	0.8	0.37	1.0	0.97
Myristate	0.34 ± 0.03 (7.6)	0.4	0.00	0.4	0.00
Fructose	17.13 ± 0.03 (0.2)	1.4	0.01	2.4	0.04
IAA	2.61 ± 0.01 (0.3)	1.2	0.05	0.8	0.02
Inositol-1-phosphate	1.93 ± 0.21 (11.0)	0.9	0.12	0.9	0.23
Trehalose	1.66 ± 0.36 (21.9)	0.9	0.46	1.2	0.28
Alpha-tocopherol	0.30 ± 0.02 (7.9)	1.2	0.04	–	–
Cholesterol[a]	6.91 ± 0.29 (4.2)	0.9	0.37	0.8	0.08
Raffinose	5.23 ± 0.34 (6.6)	1.2	0.02	0.6	0.00
Ryou Suisan Koumai					
GABA	11.99 ± 2.55 (21.3)	4.1	0.00	0.7	0.06
Glycerol-3-phosphate	2.54 ± 0.89 (35.2)	0.6	0.09	0.8	0.29
Myristate	1.07 ± 0.05 (4.9)	1.1	0.07	1.2	0.02
Fructose	23.59 ± 1.89 (8.0)	1.9	0.00	3.2	0.02
IAA	1.65 ± 0.74 (44.9)	0.8	0.34	0.5	0.10
Inositol-1-phosphate	1.98 ± 0.23 (11.5)	0.9	0.17	0.9	0.32
Trehalose	2.20 ± 0.66 (29.8)	1.1	0.62	1.6	0.09
Alpha-tocopherol	ND	–	–	–	–
Cholesterol[a]	8.34 ± 1.82 (21.9)	1.1	0.53	1.0	0.96
Raffinose	3.10 ± 0.65 (20.9)	0.7	0.11	0.4	0.01
Urasan 1					
GABA	38.60 ± 9.73 (25.2)	13.3	0.00	2.1	0.03
Glycerol-3-phosphate	5.28 ± 1.03 (19.5)	1.2	0.25	1.6	0.04
Myristate	2.79 ± 0.12 (4.2)	2.9	0.00	3.1	0.00
Fructose	22.68 ± 6.32 (27.9)	1.9	0.06	3.1	0.01
IAA	1.22 ± 0.25 (20.1)	0.6	0.03	0.4	0.01
Inositol-1-phosphate	3.43 ± 0.18 (5.2)	1.5	0.00	1.5	0.02
Trehalose	2.91 ± 1.50 (51.6)	1.5	0.39	2.2	0.14
Alpha-tocopherol	0.49 ± 0.01 (2.4)	2.0	0.00	–	–
Cholesterol[a]	8.09 ± 1.45 (17.9)	1.1	0.61	1.0	0.88
Raffinose	7.35 ± 2.17 (29.5)	1.7	0.10	0.8	0.37

SD: standard deviation, RSD: relative standard deviation, ND: not detected.
Number of replicates: $n = 3$.
[a] Cholesterol was a mixture of natural and 25,26,26,26,27,27,27-D_7 labeled peaks.

approximate 14-fold increase in the metabolite level of GABA as compared to the Japonica variety Nipponbare (Table 3). The level of GABA in Urasan 1 was 88-fold higher than that in Nipponbare, as assessed using 1D-TOF/MS analysis (Table 3); a 13-fold increase was observed using GC × GC-TOF/MS detection (Table 4), although the selected variety Urasan 1 is considered as a Japonica variety based on DNA polymorphism classification. The nutritional functions of GABA in human health studies are noteworthy, such as release of the growth hormone with remarkable effectiveness, calming,

and soothing effects [38–40]. Our results indicate that GABA accumulation in the WRC varieties varies in terms of the concentration. Rice bran is one of the components of brown rice and is a light-brown layer that covers the white kernel. Rice bran and germ contain nutritionally valuable metabolites such as GABA, vitamins, and antioxidants. Recently, brown rice is receiving increased attention because of its usefulness in human health promotion and disease prevention [41,42]. A comparison of the chromatogram patterns of the WRC varieties revealed that the nutritionally valuable varieties can be selected, possibly revealing a new strategy for rice breeding with respect to human health benefits. Our developed GC-TOF/MS analytical method facilitates detailed metabolite profiling, thereby enabling extraction of the important set of metabolite vectors as the trait of a cultivar. In addition, integration of the information of the metabolic phenotyping trait and other traits, such as visible agricultural phenotypes, would facilitate further improvement in rice breeding programs and the production of useful metabolites in rice.

4. Conclusion

Combining the 1D-GC-TOF/MS and GC × GC-TOF/MS techniques enabled comprehensive profiling of metabolites in a non-targeted fashion, facilitating the selection of nutritionally useful rice varieties. Several metabolites that yielded a clear separation pattern in the PLS-DA models were obtained using the 1D-GC-TOF/MS analysis, and they showed statistically significant differences in GC × GC-TOF/MS detection. It is important to note that the combination of 1D- and GC × GC-TOF/MS renders the profiling data more concrete and accurate than the 1D method alone. Metabolic profiling using a combination of 1D- and GC × GC-TOF/MS techniques opens the possibility of making a significant contribution in the construction of the metabolome database and in agrobiological applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2007.05.002.

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