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Metabolic profiling of millet (*Panicum miliaceum*) using gas chromatography–time-of-flight mass spectrometry (GC-TOFMS) for quality assessment

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Abstract

Gas-chromatography coupled with time-of-flight mass spectrometry (GC-TOFMS) was used to determine the diversity among primary metabolites and phenolic acids in three varieties of millet (*Panicum miliaceum* L.). Three cultivars of millet seeds were germinated in a greenhouse, and the seedlings were transferred to the field and allowed them to grow for a period of 4 months. A total of 48 metabolites were identified from millet, including 43 primary metabolites and five phenolic acids. The metabolite profiles were subjected to principal component analysis (PCA) and partial least-squares discriminate analysis (PLS-DA) to evaluate the differences among varieties. PCA and PLS-DA fully distinguished the three varieties tested. Joongjuk millet was separated from the other varieties based on the high levels of metabolites, and appears to be a good candidate for future breeding programs because of its high phenolic acids levels. This GC-TOFMS-based metabolic profiling approach is a viable alternative method for evaluating food quality.

Keywords: metabolic profiling; millet; *Panicum miliaceum*; phenolic acid; principal component analysis.
Abbreviations: GC-TOFMS gas chromatography time-of-flight mass spectrometry; IS-internal standard; MSTFA-*N*-methyl-*N*-trimethylsilyltrifluoroacetamide; PCA-principal component analysis; TMS-trimethylsilyl.

Introduction

Millet (*Panicum miliaceum* L.) is a cereal plant cultivated for its grain, mostly in Asia and North America. It is one of the world's oldest cultivated crops that may be used both as grain and for forage. Millet can be successfully grown in a wide range of environmental conditions, being better adapted than most crops to hot, dry regions, and is particularly valuable in semiarid regions because of its short growing season. It can tolerate drought and intense heat or avoid these conditions by growing to maturity very quickly. Millet plays an important role in the economies of many developing countries in the Old World because it is consumed directly as human food. However, millet in the western world is grown primarily for birdseed, livestock feed, hay, or as an emergency catch crop (Casey and Lorenz, 1977). Phenolic compounds are secondary plant metabolites, and their type and content in grain depends on a number of factors such as the type of cereal, variety, part of the grain, climatic conditions, and cultivation practices, among others (Shahidi and Naczk, 2004). The potency of phenolic compounds to act as antioxidants arises from their ability to donate hydrogen atoms via hydroxyl groups on benzene rings to electron-deficient free radicals, and in turn form resonance-stabilized and less reactive phenoxyl radicals. Plant phenolics may also act as reducing agents, singlet oxygen quenchers, and metal chelators, thus motivating the wide characterization of the genotypic diversity of phenolic compounds in millet. Chandrasekara and Shahidi (2010) reported a wide range of phenolic compound concentrations and antioxidant capacities in soluble as well as bound fractions of millet. Therefore, the nutritional value of millet is dependent upon its metabolic profile, including the types and amounts of natural phenolic compounds present, which warrant evaluation in order to determine the best varieties for human consumption. However, to the best of our knowledge, phenolic acid concentrations in various varieties of Korean millet have not been reported. Plant identification and differentiation at the species, population and individual genotype levels is of major importance for plant scientists and breeders. Consumers are aware of the need for a constant supply of phytochemical-containing foods for antioxidant support and disease prevention (Kim et al., 2013a). Metabolomics facilitates the classification of samples of diverse biological status, origin, or quality, employing chemometrics such as principal component analysis (PCA), hierarchical clustering analysis (HCA), and partial least square-discriminate analysis (PLS-DA). The primary application of metabolomics in plants includes screening mutant collections (Messerli et al., 2007), quality control and assessment of food and crop products (Pongsuwan et al., 2007; Jumtee et al., 2009), and the development of traditional medicines (Tarachiwin et al., 2008a, b). Metabolite profiling combined with chemometrics has already been employed to direct breeding strategies for improving and optimizing the balance of food components. A combination of gas chromatography and mass spectrometry

(GC-MS) allows for the identification and robust quantification of several hundred metabolites within a single extract. Hydrophilic primary metabolites have been well-studied since there efficient protocols for machine setup, sample preparation and analysis, and chromatogram evaluation and interpretation are available. The primary metabolite profile is closely related to the organism's phenotype and includes important nutritional characteristics (Hoekenga, 2008; Kok et al., 2008). In this study, hydrophilic metabolic profiling (including phenolics) in whole millet grains from three varieties using GC-time-of-flight (TOF)MS coupled with chemometrics was applied to determine the phenotypic variation and to analyze relationships between their contents. Additionally, we identified and quantified the content of phenolic acids of the samples in order to determine and compare the quality of millet varieties. This study provides valuable information about future conventional breeding programs for millet containing phenolic compounds.

Results and discussion

Metabolic profiles among millet varieties

The production of secondary metabolites is tightly associated with pathways of primary or secondary metabolism. Thus, we conducted a comprehensive metabolic phenotyping of primary metabolism in three *Panicum miliaceum* varieties. In this study, low-molecular-weight molecules from grains were identified by GC-TOFMS. ChromaTOF software was used to assist with peak location. Peak identification was performed by comparison with reference compounds and the use of an in-house library. In addition, identification of several metabolites was performed using direct comparison of the sample mass chromatogram with those of commercially available standard compounds obtained by a similar MO/TMS derivatization and GC-TOFMS analysis. In total, 48 metabolites including 19 amino acids, 17 organic acids, eight sugars, three sugar alcohols, and one amine were detected and identified in *Panicum miliaceum* (Fig. 2). The corresponding retention times and their fragment patterns are in agreement with our previous data (Kim et al., 2013b). Five phenolics (ferulic, *p*-hydroxybenzoic, salicylic, *p*-coumaric, and vanillic acids) were identified in the samples. Quantification was performed using selected ions (Fig. 2), and the quantitative calculations of all analytes were based on the peak area ratios relative to that of the IS. Traditionally food component analysis involves identifying food constituents into very broad categories such as proteins, fats, carbohydrates, fiber, vitamins, trace elements, solids, and/or ash. However, with the advent of metabolomics, metabolite profiling combined with chemometrics has been employed to direct breeding strategies to enhance specific desired balances of food components in fresh food which have been identified as being more optimal (Kim et al., 2010). PCA and PLS-DA were used to arrange unsupervised and supervised large, complex datasets, respectively. The quantitative data for the 48 metabolites were subjected to PCA to assess the overall experimental variation and to outline the differences in the metabolite profiles among varieties (Fig. 3). Core primary metabolites provide good metabolite discrimination between genotypes (Tarpley et al., 2005). PCA revealed that the two highest-ranking principal components accounted for 76.3% of the total variance within the data set. The first principal component, accounting for 49.2% of the total variance, resolved the metabolites profiles of the Joongjuk variety from the other two varieties. The second principal component, accounting for 27.1% of the total variance, resolved the Hwangguem variety from the Joongback variety. The PCA results clearly indicated the absence of significant variance within the same variety. Identifying the compounds exhibiting the greatest variance within a population and determining closely related compounds is possible using PCA (Kim et al., 2010). To further investigate the contributors to the principal components, the metabolic loadings in principal component 1 (PC1) and principal component 2 (PC2) were compared. In PC1, the corresponding loadings were positive for all metabolites excluding salicylic acid, trehalose, ethanolamine, glycolic acid, raffinose, and *p*-hydroxybenzoic acid. This result suggests that most metabolites were present in higher amounts in the Joongjuk variety than in the others. In PC2, the variation was mainly attributable to *p*-hydroxybenzoic acid and *p*-coumaric acid, for which the eigenvectors were 0.2694 and 0.2606, respectively. Previous studies have demonstrated the formation of *p*-hydroxybenzoic acid from *p*-coumaric acid (Schnitzler et al., 1992; Sircar and Mitra, 2009). These results provided correlations between metabolites that participate in closely related pathways and demonstrated the robustness of the present experimental system. PLS-DA is a projection method that separates groups of observations by rotating the PCAs such that a maximum separation among classes, here millet varieties, is obtained. PLS-DA could also distinguish between these varieties (Fig. 4). In the first component of the PLS-DA, the variation was mainly attributed to fructose, mannose, aspartic acid, pyroglutamic acid, phenylalanine, vanillic acid, and glutamine. In terms of the composition of the genetic varieties, these results suggest that these metabolites were present in higher amounts in the Joongjuk variety than in the Hwangguem and Joongback variety.

Phenolic acid contents in millet varieties

In general, ferulic and *p*-coumaric acids are reported as the major hydroxycinnamic acids in cereals (Harukaze et al., 1999; Rao and Muralikrishna, 2001). Table 1 presents the contents of 6 phenolic acids—*p*-hydroxybenzoic, vanillic, syringic, *p*-coumaric, ferulic, and sinapic acids in soluble and insoluble phenolic fractions of different millet varieties. Ferulic acid represented the greatest proportion of total phenolic acids in millet. Joongjuk millet had the highest total content of phenolic acids, including ferulic, vanillic, syringic, and sinapic acids, as well as higher concentrations of most metabolites compared to the other varieties. The Joongjuk millet had high levels of phenylalanine, a major amino acid donor for the synthesis of phenolic acid, among the three varieties examined in the present study. These results demonstrate that PCA is an excellent tool for easy visualization of complex data.

Materials and methods

Plant materials and chemicals

Three varieties of millet (Joongback, Joongjuk, and Hwangguem) were used in this study (Fig. 1). Millet seeds were germinated in a greenhouse, and the seedlings were transferred to the experimental farm at Chungnam National University (Daejeon, Korea). All three varieties of millet were harvested after 4 months. They were manually hulled and ground to obtain a fine powder using a cyclone mixer mill (HMF-590; Hanil, Seoul, Korea) and a mortar and pestle. The milled powders were kept at –80°C prior to extraction. All chemicals used in this study were of analytical grade. Methanol and chloroform, which were used as extraction solvents, were purchased from J.T. Baker (Phillipsburg, NJ, USA). Salicylic acid, sinapic acid, vanillic acid, butylated hydroxyanisole (BHA), ethyl acetate, ribitol, and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) were obtained from Sigma

Table 1. Contents (μg/g on dry weight basis) of phenolic acids in millet based on variety.[1)]

component (μg/g)	soluble			insoluble			total		
	Joongback	Joongjuk	Hwangguem	Joongback	Joongjuk	Hwangguem	Joongback	Joongjuk	Hwangguem
p-hydroxybenzoic acid	6.54±0.53b	7.87±0.67b	18.27±2.44a	10.89±1.31b	13.0±1.54b	21.88±2.12a	17.44±1.83b	20.89±2.20b	40.16±4.56a
vanillic acid	16.81±0.68c	79.20±3.00a	37.24±3.29b	23.28±1.26c	95.65±10.26a	37.50±1.87b	40.10±1.93c	174.86±13.21a	74.74±5.16b
syringic acid	2.36±0.23c	9.68±0.60a	5.18±0.09b	1.96±0.21b	11.86±1.52a	3.00±0.09b	4.32±0.43c	21.54±2.11a	8.18±0.16b
p-coumaric acid	9.43±0.39b	16.94±2.03a	11.02±0.47b	75.38±9.29b	74.50±5.33b	115.90±6.59a	84.81±9.56b	91.43±7.29b	126.92±6.55a
ferulic acid	46.16±1.60c	214.28±9.26a	76.66±1.82b	233.46±14.41b	324.56±35.32b	351.11±19.48a	279.63±16.00c	538.84±44.45a	427.77±17.72b
sinapic acid	11.73±0.13c	13.14±0.42a	12.63±0.05b	10.59±0.23b	12.96±0.55a	11.06±0.14b	22.32±0.34c	26.11±0.97a	23.69±0.14b

Different letters represent significant ($p < 0.05$) differences between means according to ANOVA combined with Duncan's multiple range test. [1)]Each value represents the mean ± standard deviation (n = 3).

Fig 1. Seeds of three varieties (A: Joongback, B: Joongjuk, and C: Hwangguem) of *Panicum miliaceum* L

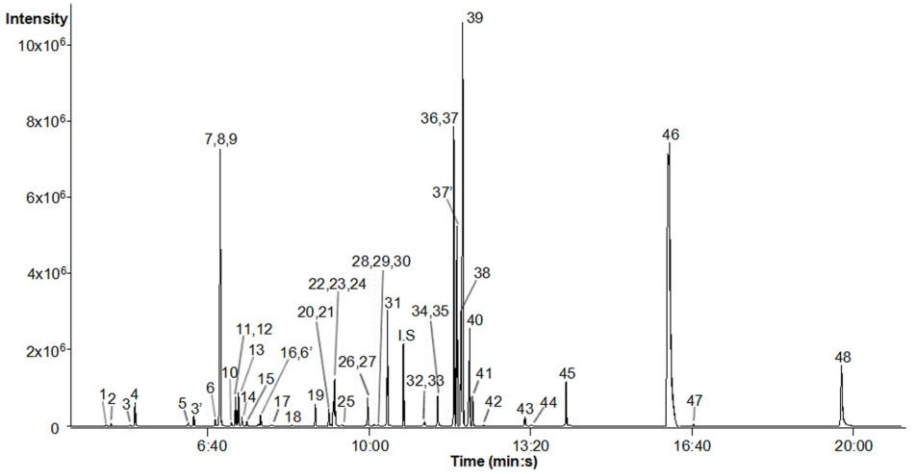

Fig 2. Selected ion chromatograms of metabolites extracted from *Panicum miliaceum* L (Joongjuk variety) as MO/TMS derivatives separated on a 30 m × 0.25 mm I.D. fused silica capillary column coated with 0.25 μm CP-SIL 8 CB low bleed stationary phase. Peak identification: 1 pyruvic acid (retention time, RT: 4.57 min, quantification ion, QI: 174), 2 lactic acid (RT: 4.66 min, QI: 147), 3 valine (RT: 5.06 min, QI: 146), 4 alanine (RT: 5.16 min, QI: 116), 5 glycolic acid (RT: 6.26 min, QI: 147), 3′ valine (RT: 6.38 min, QI: 144), 6 serine (RT: 6.82 min, QI: 116), 7 ethanolamine (RT: 6.90 min, QI: 174), 8 glycerol (RT: 6.92 min, QI: 147), 9 leucine (RT: 6.94 min, QI: 158), 10 isoleucine (RT: 7.16 min, QI: 158), 11 proline (RT: 7.24 min, QI: 142), 12 nicotinic acid (RT: 7.27 min, QI: 180), 13 glycine (RT: 7.30 min, QI: 184), 14 succinic acid (RT: 7.37 min, QI: 147), 15 glyceric acid (RT: 7.47 min, QI: 147), 16 fumaric acid (RT: 7.71 min, QI: 245), 6′ serine (RT: 7.76 min, QI: 204), 17 threonine (RT: 7.99 min, QI: 219), 18 β-alanine (RT: 8.41 min, QI: 174), 19 malic acid (RT: 8.89 min, QI: 147), 20 salicylic acid (RT: 9.16 min, QI: 267), 21 aspartic acid (RT: 9.17 min, QI: 100), 22 methionine (RT: 9.21 min, QI: 176), 23 pyroglutamic acid (RT: 9.26 min, QI: 156), 24 4-aminobutyric acid (RT: 9.29 min, QI: 174), 25 threonic acid (RT: 9.44 min, QI: 147), 26 arginine (RT: 9.94 min, QI: 142), 27 glutamic acid (RT: 9.97 min, QI: 246), 28 phenylalanine (RT: 10.10 min, QI: 218), 29 p-hydroxybenzoic acid (RT: 10.11 min, QI: 223), 30 xylose (RT: 10.18 min, QI: 103), 31 asparagine (RT: 10.38 min, QI: 116), 32 vanillic acid (RT: 11.13 min, QI: 297), 33 glutamine (RT: 11.14 min, QI: 156), 34 shikimic acid (RT: 11.29 min, QI: 204), 35 citric acid (RT: 11.42 min, QI: 273), 36 quinic acid (RT: 11.67 min, QI: 345), 37 fructose (RT: 11.75 min, QI: 103), 37′ fructose (RT: 11.81 min, QI: 103), 38 galactose (RT: 11.89 min, QI: 147), 39 glucose (RT: 11.93 min, QI: 147), 40 mannose (RT: 12.07 min, QI: 147), 41 mannitol (RT: 12.14 min, QI: 319), 42 p-coumaric acid (RT: 12.37 min, QI: 219), 43 inositol (RT: 13.22 min, QI: 305), 44 ferulic acid (RT: 13.33 min, QI: 338), 45 tryptophan (RT: 14.07 min, QI: 202), 46 sucrose (RT: 16.17 min, QI: 217), 47 trehalose (RT: 16.70 min, QI: 191), 48 raffinose (RT: 19.76 min, QI: 217), IS internal standard (ribitol).

Chemical Co. (St. Louis, MO). Methoxyamine hydrochloride was purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). Syringic, *p*-hydroxybenzoic, 3,4,5-trimethoxycinnamic, ferulic, and acetic acid were acquired from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *p*-Coumaric acid was obtained from MP Biomedicals (Solon, OH, USA). Pyridine and *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) with 1% *tert*-butyldimethylchlorosilane (TBDMCS) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

GC-TOFMS analysis of polar metabolites

Metabolite extraction was performed as described previously (Kim et al., 2012). Two hundred milligrams of powdered sample was extracted with 1 ml of 2.5:1:1 v/v/v methanol: water: chloroform. Ribitol (60 μL, 0.2 mg/ml) was added as internal standard (IS). Extraction was performed at 37°C with a mixing frequency of 1200 rpm for 30 min using a thermomixer comfort (Model 5355, Eppendorf AG, Hamburg, Germany). The solutions were then centrifuged at 16,000 × *g* for 3 min. The polar phase (0.8 mL) was transferred into a new tube, 0.4 ml of water was added, and the solution was centrifuged at 16,000 × *g* for 3 min. The methanol/water phase was dried in a centrifugal concentrator (CVE-2000, Eyela, Japan) for 2 h, followed by drying in a freeze dryer for 16 h. Methoxime (MO)-derivatization was performed by adding 80 μl of methoxyamine hydrochloride (20 mg/mL) in pyridine and shaking at 30°C for 90 min. Trimethylsilyl (TMS) etherification was performed by adding 80 μl of MSTFA at 37°C for 30 min. GC-TOFMS was performed using an Agilent 7890A gas chromatograph (Agilent, Atlanta, GA, USA) coupled to a Pegasus HT TOF mass spectrometer (LECO, St. Joseph, MI). Derivatized sample (1 μl) was separated on a 30 m × 0.25 mm I.D. fused-silica capillary column coated with 0.25 μm CP-SIL 8 CB low bleed stationary phase (Varian Inc., Palo Alto, CA, USA). The split ratio was set at 1:25. The injector temperature was 230°C. The helium gas flow rate through the column was 1.0 ml/min. The temperature program was as follows: initial temperature 80°C for 2 min, followed by an increase to 320°C at 15°C/min, and a 10 min hold at 320°C. The transfer line and ion-source temperatures were 250 and 200°C, respectively. The scanned mass range was 85–600 *m/z*, and the detector voltage was set at 1700 V.

Extraction and analysis of phenolic acids

Soluble (free and esterified) and insoluble (bound) phenolic acids were extracted according to the procedure described by Park et al. (2012) with slight modifications. The powdered samples (0.1 g) were extracted twice by water-based sonication for 5 min at room temperature and incubation at 30°C for 10 min with 1 ml of 85% methanol containing 2 g l^{-1} of BHA. After centrifuging at 13,000 rpm for 10 min at 4°C, the combined extracts and residue were analyzed for the determination of soluble and insoluble phenolic acids,

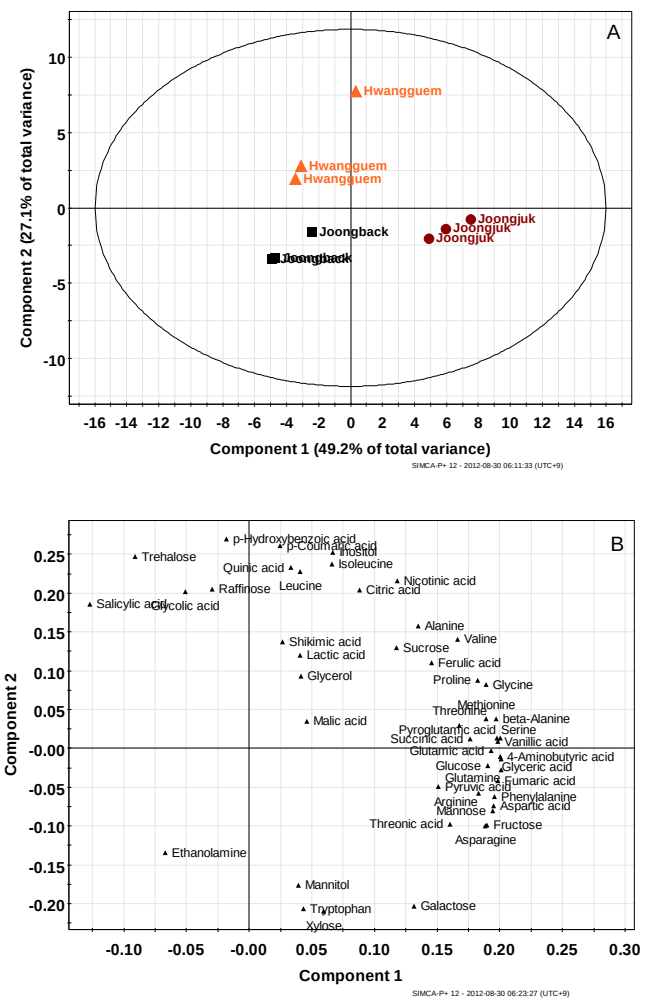

Fig 3. Scores (A) and loading plots (B) of principal components 1 and 2 of the PCA results obtained from polar metabolite data on the three millet varieties.

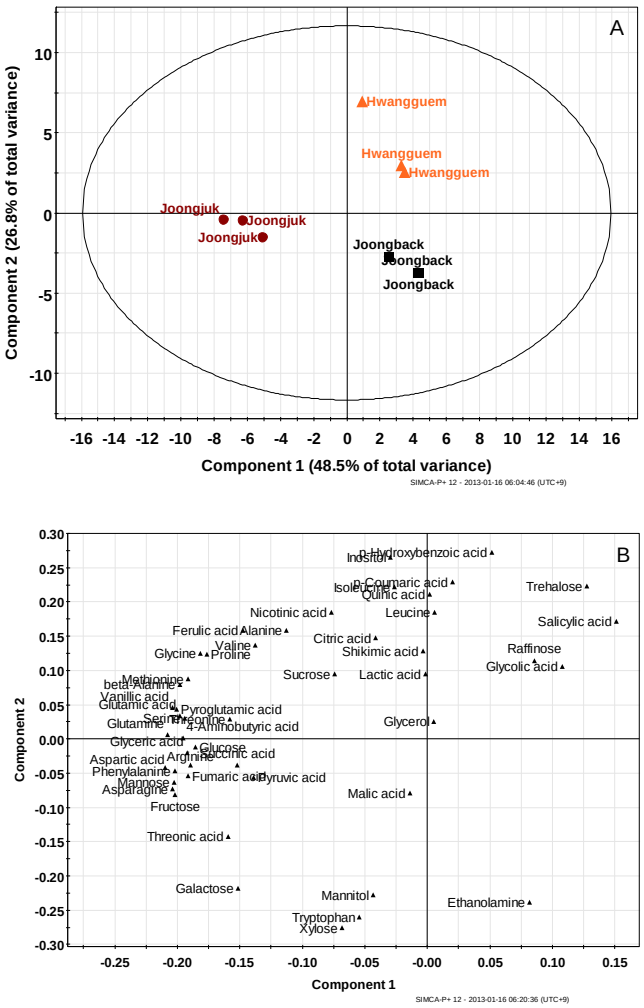

Fig 4. Scores (A) and loading plots (B) of principal components 1 and 2 of the PLS-DA results obtained from polar metabolite data on the three millet varieties.

respectively. 50 µl of 3,4,5-trimethoxycinnamic acid (100 µg ml^{-1}) was added as IS, followed by hydrolysis of the mixture with 1 ml of 5 N NaOH at 30°C under nitrogen gas for 4 h. Each hydrolyzed sample was adjusted to pH 1.5–2.0 with 6 M HCl and then extracted with ethyl acetate and evaporated in a centrifugal concentrator. For derivatization, 40 µl of MTBSTFA containing 1% TBDMCS and 40 µl of pyridine were added to the dried extracts, followed by incubation at 60°C for 30 min at a mixing frequency of 1200 rpm using a thermomixer comfort. The phenolic acid was analyzed by GC-TOFMS as described above. Each derivatized sample (1 µl) was separated on the same column as described above with a split ratio of 10:1. The injector temperature was 230°C, and the flow rate of helium gas through the column was 1.0 ml min^{-1}. The temperature was set at 150°C and maintained for 2 min, followed by a 15°C/min oven temperature ramp to 320°C, which was held for 10 min. The column effluent was later introduced into a Pegasus HT TOF mass spectrometer (LECO, St. Joseph, MI, USA). The transfer line and the ion-source temperatures were 250°C and 200°C, respectively. The detected mass range was 85–700 *m/z*, and the detector voltage was set at 1700 V. For quantification purposes, standard stock solution of six phenolics (ferulic, *p*-coumaric, *p*-hydroxybenzoic, sinapic, syringic, and vanillic acid) and 3,4,5-trimethoxycinnamic acid as IS were prepared in methanol at 100 µg/mL. Calibration samples ranging from 0.01 to 10.0 µg ml^{-1} were prepared by mixing individual stock solutions of the six phenolic acid standards. After drying, each sample was subjected to soluble and insoluble phenolic acid extraction. Calibration curves were constructed by linear regression of the peak area ratio of individual standards relative to the peak area of the IS.

Statistical analysis

All analyses were performed at least in triplicate. The experimental data were analyzed by analysis of variance (ANOVA), and significant differences among the means were determined by Duncan's multiple-range test (SAS 9.2, SAS Institute, Cary, NC, USA). Relative quantification data acquired from GC-TOFMS was subjected to PCA and PLS-DA (SIMCA-P version 12.0; Umetrics, Umeå, Sweden) to evaluate the relationships in terms of similarity or dissimilarity among groups of multivariate data. The PCA and PLS-DA output consisted of score plots to visualize the contrast between different samples and loading plots to explain the cluster separation. Unit variance scaling was applied to the data without any transformation. In conclusion, we identified 48 polar metabolites in *P. miliaceum* and applied PCA and PLS-DA to the metabolite profiles obtained for 3 varieties. PCA and PLS-DA fully distinguished these varieties, suggesting that

reasonable score ranges of the components could be used for sample selection according to the correlation between the variables and these two components. Metabolic loading in component 1 suggested that the Joongjuk variety had relatively high phenolic acid levels, indicating that it may provide the best nutritional value among the millet varieties tested. The results of this study suggest that metabolite profiling combined with chemometrics can be used as powerful tool for assessing food quality

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