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Gas chromatography coupled with mass spectrometry-based metabolomics for the classification of tempe from different regions and production processes in Indonesia

Adinda Darwati Kadar,[1,2] Pingkan Aditiawati,[2] Made Astawan,[3] Sastia Prama Putri,[1,2,*] and Eiichiro Fukusaki[1]

Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan,[1] Department of Biotechnology, School of Life Sciences and Technology, Institut Teknologi Bandung, Jl. Ganesha 10, Bandung 40132, Indonesia,[2] and Department of Food Science and Technology, Faculty of Agricultural Technology and Engineering, Bogor Agricultural University, IPB Darmaga Campus, PO Box 220, Bogor 16002, Indonesia[3]

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Tempe, a fermented soybean originally from Indonesia, is an excellent protein source with high nutritional quality. Differences in the production process and unique fermentation condition in different regions result in varieties of tempe. Despite its high cultural and economic values, there are very few studies on the characterization of tempe based on the differences of production process and geographical origin. Metabolomics is a powerful tool assessing food quality, food safety, and determination of origin and varietal differences. In this study, metabolomics is applied for the study of Indonesian tempe obtained from various regions and different production processes. Seventeen samples were collected from 6 different cities in Java Island, which were produced by local tempe crafters (traditional), semi-modern industry and modern industry. Untargeted metabolomics by gas chromatography coupled with mass spectrometry (GC/MS) was implemented to discriminate various kinds of tempe and identify metabolites that are associated with these differences. Results showed that tempe produced in different places clustered together according to the cities and their production category. Sugars and amino acids groups were found to be primary compounds that contributed to this result. This is the first report that address the metabolic differences between different varieties of tempe from different regions and production processes. The knowledge from this study is important for future development of tempe production.

[**Key words:** Food metabolomics; Indonesian tempe; Metabolite profile; Untargeted metabolomics; Gas chromatography coupled with mass spectrometry]

Tempe is fermented soybean food originated from Indonesia which described as a white soybean cake (1,2). Tempe production started in the 16th century on the island of Java (3), where it remains a staple source of protein until today. At present, tempe together with tahu (Indonesian tofu), industry ranging from home to larger-scale industry are in demand of 70% of total Indonesian soybean supply (4). It was also reported that an average of 8.5 kg of soybean is consumed by Indonesian per person per year (5). This makes tempe a very important local product with high cultural as well as economic value for Indonesia. In 2013, Indonesia had successfully published the International standard of tempe as CODEX 313r-2013 in which the basic knowledge of tempe product and production were explained. This document also featured more detailed information about tempe, including the correct spelling of tempe that is often misspelled in many previous reports as tempeh. It is mentioned in point 7.2 of the CODEX document that tempe should be referred to as tempe and not tempeh. Although older scientific and non-scientific reports written in English often used tempeh instead of tempe to avoid confusion with the Tempe state in the United States of America, it is necessary from this point forward to consistently use the correct term of tempe based on the CODEX document as the international standard for quality assurance of tempe.

In general, tempe fermentation is done by inoculating *Rhizopus* spp. (6) to cooked dehulled soybean. Based on a manuscript written by National Standardization Agency of Indonesia (SNI 3144:2015) the production of tempe has to follow a certain standard of safety and product characteristics. However, each region in Indonesia maintains their production procedure of tempe based on the indigenous knowledge that is passed through generations. Nonetheless, in general, there are 4 different production procedures that are performed in 4 biggest tempe producing city in Indonesia (Table 1).

Unique production processes might result in different characteristics such as physical appearance, taste (7), and chemical composition (8). Thus, tempe that were produced by different method in different region were thought to have distinct characters. Although there were many studies of tempe have been performed (9–11), very few studies were focused on the characterization of various kinds of tempe. In particular, there is no publication that attempted to compare distinct characters of tempe based on their global metabolome profile.

* Corresponding author at: Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel./fax: +81 6 6879 7416.
E-mail address: sastia_putri@bio.eng.osaka-u.ac.jp (S.P. Putri).

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TABLE 1. Comparison of tempe production processes between regions in Indonesia.

Step	Bandung (West Java)	Purwokerto (Central Java)	Yogyakarta (Central Java)	Malang (East Java)
Washing	Yes	Yes	Yes	Yes
Soaking 1 (h)	6	24	6	2
Cooking 1	Yes	Yes	Only soaking in boiling water	Only soaking in boiling water
Dehulling	After soaking 2	Yes	Yes	Yes
Soaking 2 (h)	10	Yes	10	10
Cooking 2 (h)	0.5	1	0.5	1
Draining	Yes	Yes	Yes	Yes
Fungal inoculation	Yes	Yes	Yes	Yes
Packaging	Yes	Yes	Yes	Yes
Fermentation	Yes	Yes	Yes	Yes

Source: Tempe Forum Indonesia.

Metabolomics is a relatively new branch of omics that focus on the investigation of variation in total metabolic profile (12). Previous studies has been demonstrated powerful results in authentication (13,14) and discrimination of food products based on production process (15,16), quality (17–20) and geographical origin (21,22). Applying metabolomics study to reveal the uniqueness of tempe based on its metabolic profile might be beneficial to see the general insight of how metabolite profiles are related to certain physical attributes.

Many different machines have been utilized to analyze food samples (12). One of the most useful separations and detection instrument in metabolomics is gas chromatography coupled with a mass spectrometer (GC/MS). This platform is known for its ability to give a very good result and robustness on analysis of esters, fatty acids, alcohols, aldehydes, terpenes, and so on (12). The present research aimed to use GC/MS for analysis of small hydrophilic metabolites in tempe to various characterize tempe by performing untargeted metabolomics research through analyzing of such low molecular weight metabolites. To our knowledge, this is the first publication to report the comparison of metabolite profiles of various tempe.

MATERIALS AND METHODS

Samples used Frozen tempe obtained from Japanese local distributor were used as dummy sample to optimize sample preparation technique. Optimized method then was applied to the real samples. A number of 17 samples from 7 different cities in Java Island, Indonesia were collected in March 2016 as a riped tempe (Table 2, Fig. S1).

Sampling method All samples were obtained on the same day then were quenched together on the sampling day. Samples were cut so that fit to a 50 mL tube. Each sample were put in different tubes for quenching and storage. Quenching was performed by dipping the tubes containing samples to liquid nitrogen for approximately 5 min then were kept in −20°C until transportation day from Indonesia to Japan (2 days). During transportations, quenched samples were kept inside an insulated bag containing dry ice and were subsequently kept in −30°C upon arrival to Japan. All samples were transferred to Japan under material transfer agreement.

Metabolites extraction Metabolites extraction was done by following previously published extraction methods (13) except the weight of samples which was modified to 15 mg. Prior to extraction step, frozen tempe was milled into a fine powder then lyophilized to remove water content. Samples were kept at −30°C until next extraction. Mixed solvent (methanol:chloroform:ultrapure water = 5:2:2) containing internal standard (ribitol 0.2 mg/mL) were added to weighed sample then followed by centrifugation for 3 min at 4°C, 10,000 × g. The supernatant (400 µL) were transferred to a new 1.5 mL microtube, added by 400 µL ultrapure water, and vortexed for 1 min to extract hydrophilic metabolites. Centrifugation was done afterward to separate hydrophilic and hydrophobic fractions. The upper phase (400 µL) from all samples replicates (n = 3) and blank samples were collected at one pool to be used as quality control (QC) samples. Another 400 µL were transferred to new 1.5 mL microtubes to be evaporated using centrifuge concentrator at room temperature then followed by overnight lyophilization.

Derivatization of metabolite extract In this research, oximation and silylation protocol were used to derivatize the metabolites prior to GC/MS analysis. Oximation was done by adding 100 µL of methoxyamine hydrochloride (20 mg/mL in pyridine) (Sigma–Aldrich, St. Louis, MO, USA) to lyophilized samples. The first incubation was done afterward in a shaker incubator (30°C for 90 min, 1200 rpm). After oximation, silylation was conducted by adding 50 µl of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) (GL Science, Tokyo, Japan) then continue to the second incubation (37°C for 90 min, 1200 rpm).

Metabolites profiling by using GC/MS instrument Metabolites profiling was done by utilizing GC/MS-QP2010 Ultra (Shimadzu, Kyoto, Japan) equipped with Inert Cap 5MS/NP column; 0,25 mm x 30 m, 0,25 µm, (Varian.Inc, Palo Alto, CA, USA) and autosampler AOC-20i/s (Shimadzu, Kyoto, Japan). Machine conditions were set based on previously published work on coffee metabolomics (13). In addition, injection of a standard alkane mixture (C8-C40) were done before samples'. The retention time data of each peak in the mixture were then used as a reference for tentative identification.

Sensory evaluation by hedonic rating test employing semi-trained panelist Hedonic rating test was done to representative tempe obtained from West Java, Central Java and East Java, namely Bandung and Bogor (West Java), Purwokerto (Central Java), and Surabaya (East Java). This test was performed at Department of Food Science and Technology, Bogor Agricultural University, Indonesia by involving 75 students as semi-trained panelists. The panelists were asked to measure their preferences to the samples. Evaluated attributes were aroma, texture, appearances, and overall score. Each attribute were scored based on 7-point scaling: 1, dislike very much; 2, dislike; 3, dislike moderately; 4, neutral; 5, like moderately; 6, like; and 7, like very much.

Two different data sets were used in this test. The first data set for this analysis was to reveal the effect of the region of origin while the second one was set to test the effect of the production process (Table S1). Furthermore, quantitative measurement of hardness was done to same samples by using penetrometer installed at the same department. Statistical analysis of all obtained data was analyzed by means of ANOVA followed by Duncan different test ($p = 0.05$).

Data processing Obtained data from GC/MS analysis (raw data) were processed using GC/MS Solution software package provided by Shimadzu Corporation (Kyoto, Japan). This included file format convert to analytical data interchange protocol (ANDI, cdf). Peak alignment and tentative annotation were performed by using metAlign (downloadable freeware: http://www.wageningenur.nl/) and Aloutput version 1.30 (downloadable freeware: http://prime.psc.riken.jp/) respectively. Tentative annotation of metabolites was carried based on an in-house library in Aloutput and NIST library (NIST11/2011/EPA/NIH) in GC/MS Solution package.

Multivariate analysis SIMCA-P+ version 13 was used to conduct principal component analysis (PCA) with unit variance scaling (UV scaling) to previously processed data. There is no transformation for the data set used in this analysis.

RESULTS

Optimization of extraction methods for tempe analysis Modification of extraction methods was done by using tempe that were obtained from Japanese local retailer. Following the protocol based on the previously published study (14), the step-

TABLE 2. List of samples.

Region	Sample code	Production category
West Java		
Bandung 1	ba	Semi-modern
Bandung 2	bb	Semi-modern
Bogor-Jakarta 1	bja	Semi-modern
Bogor-Jakarta 2	bjb	Semi-modern
Bogor-Jakarta 3	bjc	Traditional
Central Java		
Purwokerto 1	pa	Traditional
Purwokerto 2	pb	Traditional
Yogyakarta 1	ya	Traditional
Yogyakarta 2	yb	Semi-modern
Yogyakarta 3	yc	Semi-modern
East Java		
Malang 1	ma	Traditional
Malang 2	mb	Traditional
Malang 3	mc	Traditional
Surabaya 1	sa	Traditional
Surabaya 2	sb	Modern
Surabaya 3	sc	Traditional
Surabaya 4	sd	Traditional

Data set 1: data set to reveal the effect of region of origin; all traditional tempe samples were used. Data set 2: data set to reveal the effect of production processes; samples sa, sb, sc, sd, ya, yb, ya, bja, and bjb were used.

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