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Doctoral Dissertation

The metabolomics-based approach of *tempe* as a baby food product ingredient

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List of Abbreviations

(*in alphabetical order*)

ANOVA	Analysis of variance
CATA	Check-all-that-apply
FAO	Food & Agriculture Organization
GC	Gas chromatography
LC	Liquid chromatography
MS	Mass Spectrometry
MSTFA	N-Trimethylsilyl-N-methyl trifluoroacetamide
PCA	Principal Component Analysis
OPLS-DA	Orthogonal projection to latent structures with discriminant analysis
WHO	World Health Organization

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Chapter 1

General introduction

1.1 Tempe

Tempe is known as Indonesian traditional fermented food commonly produced from soybean. *Tempe* is recognized as food with many benefits with high and good quality protein content and recognized as cheap yet nutritious food in Indonesia (Astawan et al. 2013). Moreover, *tempe* has recently gained popularity globally due to its taste characteristics, which are called meat-like characteristics that can fulfil the satisfaction of a vegetarian person (Ahnan-Winarno et al., 2021).

Based on the CODEX regional standard for tempe in 2018, *tempe* should have a white color with a compact texture that is not easy to crumble. The flavor and aroma characteristic of fresh *tempe* should be meaty, mushroom-like, nutty and free from ammonia smells. Without any additive and foreign matters such as small stone and husk, the minimum protein composition of *tempe* should be around 15% (w/w), with the maximum moisture content and lipid content are 65% (w/w) and 7 % (w/w), respectively. The maximum crude fiber content is 2.5% (w/w).

Tempe words have come from the process where half-cooked beans (originally soybean) are bound together with the help of microorganisms to be a white-compact cake. The process of making tempe is much influenced by the activity of certain microorganisms, such as *Rhizopus oligosporus, R. oryzae, R. stolonifer, R. arrhizus, R. formosaensis, Mucor spp,* yeast,

lactic acid bacteria, and different gram-negative bacteria. However, the main fungus of tempe production is *R. oligosporus* (Hachmeister and Fung 1993).

As Indonesian indigenous food, *tempe* has been consumed in many ways, such as stir-fries, fried, grilled, etc. Based on a report by (Shurtleff & Aoyagi 2011), *tempe,* as a staple food in Indonesia since 300 years ago, has been produced not only from soybean but also from varieties of raw materials such as beans, nuts, tofu waste and grains or even coconut milk or milk press cake (*tempe bongkrek)* (Karyadi & Lukito, 1996).

1.1.1 Non-soy legume *tempe*

Tempe from other raw materials has been known by Indonesian since the mid-1070s, and it was reported by (Shurtleff & Aoyagi, 2007). The utilization of non-soy legumes as *tempe* ingredients is expected to be increased by its demand and legume availability. A previous study showed that the potency of non-soy legumes as *tempe* ingredients in *tempe* productions contributes to improving nutritional content and functionality. Therefore, non-soy legumes in Indonesia have a promising potential to be utilised.

Some research shown that the physical properties and the nutrient profile of legumes showed great potential to be utilized as raw ingredients for *tempe* productions (Puspitojati et al. 2019; Radiati 2016). For example, the cowpea bean is suitable as a raw ingredient for *tempe* production due to its bean size, which is similar to the soybean (Haliza, Purwani, & Thahir, 2007). As a seed, cowpeas in Indonesia are mainly utilized as an additional vegetable cooked with jack fruit stew and vegetables with coconut soup or mixed with other ingredients for porridge*, bakpia*, and rice cake. Through the fermentation process, cowpea is gaining some attributes that give it the potential to be utilised as an ingredient for *tempe* (Haliza, Purwani, & Thahir, 2007). According to Wardiah,

Samingan; & Putri in 2016, cowpea *tempe* contains p-coumaric acid and ferulic acid, which are expected to be the most potent antioxidant. This ferulic acid in cowpea *tempe* can suppress blood pressure and glucose level. Red kidney bean is one of the various commodities of local legumes well known in Indonesia. Fermentation can increase the nutrient and digestibility profile of red kidney beans (Maryam, 2016).

On the other hand, mung beans were also found to have greater soluble protein. Another bean, such as jack bean, was also used as *tempe* material. The fermentation of jack bean increased a bioactive compound with high ACE inhibitory activity after fermentation by Rhizopus *sp.* molds (Pupitojati, Indrati, Cahyanto, & Marsono, 2019).

Each legume has differences in size, color, peels, and weight. The size of beans influences the fermentation process; hence, the quality of *tempe* will also be determined by the size of the beans. As shown in Table 3, cowpea has a similar weight to soybean. The 50 pieces of cowpea beans were comparable with 50 pieces of soybean which are 7.0 and 8.0 grams, respectively. The similarity of the weights seemed to be the advantage of cowpea being used for *tempe* ingredients (Haliza, Purwani, & Thahir, 2010)

There have been many kinds of research related to the utilisation of non-soy legumes as raw ingredients for *tempe*. Further processing into *tempe* showed that the nutrient content of each non-soy legume was improved and compared to soybean *tempe,* even shows greater functionalities. However, the yield of each non-soy legumes *tempe* is lower than soybean tempe but still comparable (Astuti et al. 2000).

1.2 *Tempe*-based baby food

Baby foods or complementary foods are generally given to babies in the range of 6-24 months and breast milk. In developing and underdeveloped countries, baby food preparation still lacks scientific guidelines. The baby food was prepared only high in carbohydrates and lack of other nutrients(Osundahunsi and Aworh 2002). Based on the guidelines, the baby food must consider some issues such as types and amount of food given and provide essential micro and macronutrients. Improper food selection and feeding practices will lead to several problems, such as malnutrition in children under five years old.

Energy requirements for a weaning infant ranged from 414 kJ/kg per day for a 4- to 5-month-old to 397 kJ/kg for an 8- to 9-month-old (FAO.1973; WHO.1985). If the net protein utilization (NPU) value is greater than 80, the protein advisory group of the United Nations System recommends a minimum percentage w/w protein of 15.0 %. If the NPU value is between 60 and 80, a minimum level of 20% is required in all baby supplementary foods. Then, the recommended fat intake is up to 10% as long as it does not compromise the food's keeping qualities; linoleic acid should be at least 1%, and ash should not exceed 5 g (Kluvitse 1999).

Gathering all these facts together, the selection of baby foods with optimal nutritional values is a must. In addition, based on (CODEX Alimentarius 1991), for complementary feeding, baby food must be cost-effective, affordable, locally available, and practical for low-income populations, many of which are susceptible to malnutrition and obesity.

In Indonesia, one of the malnutrition problems is stunting. Indonesia is 4th rank in the world that suffers from stunting. Stunting, or short-for-age, is due to inadequate nutrient intake and absorption compared to needs. The inadequacy of nutrient intake does not just affect linear

growth, but also other processes, such as development of the brain and the immune system. Furthermore, the brain of a young child who interacts little with the surrounding environment due to tiredness caused by anemia develops fewer neuron connections resulting in a different brain structure and lower brain weight compared to a child that has been able to develop to full potential. The outcome of brain development by the age of two years, in which nutrition plays a large role, determines to a large extent a person's mental capacity for the rest of life (Hoddinott 2008; Victora 2008).

As mentioned earlier, the fermentation process in *tempe* improved the nutritional quality of legumes. The pervious study used *tempe* and other staple utilisation as weaning foods at costs and space affordable by mothers could be prepared by remote villages or communities in Tanzania and Nigeria (Osundahunsi and Aworh 2002)

In Indonesia, *tempe* has been further studied as a baby food product, such as instant porridge as baby food. The purpose is to address the nutritional security of the rural and urban poor; this *tempe* could also be an appropriate choice for the elite who will benefit from its functional properties (Puteri., et.al 2018). Another study also stated that eating fermented soybean could give more beneficial effects on decreasing gastrointestinal digestive and absorptive capacity problems (Nout and Kiers 2005).

Iron absorption increased in soybean *temp* and iron absorption increased in soybean *tempe* (Astuti 1992) (Cui et al. 2012). Kustyawati et al., (2020) reported adequate vitamin BI2 production by tempe manufacture; this may be important in the management of anaemia. This

evidence has aided the study of the formulation and development of nutritious weaning foods from tempe, and it has gotten a lot of attention in many developing countries.

Besides all the benefits of legumes as the raw material of *tempe,* there are some drawbacks that the amount of the nutritional quality of legumes also depends on the presence of antinutritional factors. Phytic acid is widely distributed in legume seeds, and it accounts for about 78% of the total phosphorus in pulses (Radiati 2016). Phytates interact with proteins, reducing their solubility and availability (Shurtleff and Aoyagi 2011). Low absorption of minerals has been associated with a high intake of phytic acid and dietary fibre (Brouns 2022). However, this problem can be solved by the fermentation process in *tempe*.

Tempe-based baby foods could play a role as sources of readily available nutrients for the prevention and management of malnutrition and diarrhoea. The product will be high nutritive value, easily digestible, acceptable, well-tolerated, and preferably should have additional nutrients anti-diarrhoeal properties.

1.3 Metabolomic approach

Metabolomics is an omics study that investigates the metabolites produced by living materials in order to distinguish differences in systems and explain changes in metabolites caused by environmental changes or processes (Putri et al. 2013b; Hanifah et al. 2018). Metabolomics has been used in a wide range of research, from medical to food science studies, due to the advantages of comprehensively analyzing many metabolites.

Food metabolomics has been used in food research to differentiate samples based on origin or cultivar, evaluate stress response, optimize the post-harvest process, monitor changes during growth or fermentation, and predict quality. (Putri et al. 2019)

The chromatography method was used in the metabolomics study to separate metabolites in a matrix based on several parameters such as molecular weight and retention time. The library will then identify that information in order to obtain information on each metabolite. Metabolomics can be divided into two types based on their target metabolites: targeted metabolomics and non-targeted metabolomics. When we want to focus on specific metabolites such as amino acids, carbohydrates, and so on, we use targeted metabolomics. Simultaneously, non-targeted metabolomics is used when we want to analyze all metabolites obtained from biological sample separation, regardless of whether they can be annotated by the library. (Putri et al. 2019; Ikram et al. 2020).

Nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (LC-MS) are several instrument that can be used for metabolomics study (Putri et al. 2015). The GC-MS is one of the commonly used instruments in food metabolomics. It has been applied to various types of food, such as soy sauce, cheese, and coffee (Jumhawan et al. 2013; Yamana et al. 2020). The GC-MS metabolite profiling has several advantages, including its robustness, stability, low cost, and ease of use. As a result, this method is widely used to rapidly characterize and distinguish small hydrophilic molecules in foods. (Putri et al. 2019). The LCMS is also used in metabolomics to profile targeted soluble compounds such as amino acids and unknown metabolites. The derivatization process, however, is not required for this analysis, and LC-MS analysis could be used for more comprehensive metabolite detection.

The GC-MS and LC-MS results were then processed for annotation using multivariate analysis to provide a clear understanding of the data analysis (Putri and Fukusaki 2014). In metabolomics studies, the principal component analysis (PCA) is a common multivariate analysis method. We were able to obtain the general metabolite profile in the samples using PCA.

Following that, statistically significant metabolites were identified as discriminant candidates using orthogonal projection to latent structures with discriminant analysis (OPLS-DA) (Putri et al. 2013a)

1.4 Metabolomics of *tempe*

In 2011, and 2016, several studies on the use of non-soy products as *tempe* ingredients have been conducted (Radiati 2016; Shurtleff and Aoyagi 2011). To determine the effect of raw material properties on *tempe* and its sensory properties, one study has been conducted. They used soy flour that was made from crushed *tempe* to improve nutritional intake (Romulo and Surya 2021). The nitrogen content of *tempe* was used in that study to calculate total protein content using the Kjeldahl method. The sensory test, total fat and total fiber were determined to investigate the sensory profile of each legume's *tempe*. Those methods were recognized as the conventional method in food analysis. However, the results showed no clear distinct differences in each samples (*tempe*). As a result, a comprehensive study, specifically a metabolomic study, is required to identify a key compound that differentiates the various legume tempes.

We used metabolomics in this study to thoroughly examine the metabolite profile of legume *tempe*, which can be useful for further utilizing legume *tempe* as a secondary product. Furthermore, the effect of process and treatment was investigated using metabolomics to investigate metabolite changes during processing and treatment.

1.5 Objective and strategy

This research aims to find the most suitable *tempe* materials for baby food products ingredients from various legumes by using a metabolomic approach. As the first strategy, metabolomics was used to analyze the metabolites profile in each legume tempe comprehensively. The sensory evaluation was conducted to find the general attributes of legume *tempe*. The selected legume *tempe* samples were analyzed for their protein digestibility and amino acid profile. Lastly, the drying and mixing process optimisation was carried out to choose the best treatment and process to develop ready-to-use product ingredients from *tempe* for baby food products.

1.6 Thesis outline

This thesis is divided into five chapters. Chapter 1 describes the general introduction of *tempe*, various types of *tempe*, recent trends in *tempe* based baby food products and how it can be used as a potential raw ingredient to develop nutrient dense baby food products. The previous study on evaluating nutrient content in *tempe*, how metabolomics could be used as a useful method to reveal the complete metabolites profile of legume *tempe* and the objective of this study is also presented in this chapter.

Chapter 2 explained the metabolites difference between various legume *tempe* before and after fermentation, various legumes were purchased in Indonesia and produced to become a *tempe* in Japan and Indonesia to find out the effect of environmental conditions on the metabolites profile of each legume *tempe*. In addition, sensory analysis was also performed to see the specific attributes of each legume *tempe*.

Chapter 3 focused on the investigation of the in-vitro protein digestibility of each legume *tempe* to give a general overview of protein quality. Then, based on the metabolites profile in chapter 2, selected legume *tempes* were analyzed with other *tempe* samples produced from locally found legumes in the Japanese market by using GC-MS and LC-MS analysis to find out the general metabolites and amino acids profile of each legume *tempe*.

Chapter 4 discussed the various optimization methods for producing ready-to-use *tempe*-based ingredients for baby food products. The GC-MS-based metabolomics study was performed to analyze the effect of different drying treatments which are oven-dried, freeze-dried and freeze crushed drying methods on the metabolites profile of legume *tempe*. Different drying temperature and mixing processes were also done to select the most suitable processes for the production of *tempe*-based ready-to-use ingredients.

Chapter 5 presents the conclusion of this study and future perspectives were proposed for the development *tempe* based baby food products.

Chapter 2

GC-MS-based metabolites profiling of legume *tempe* and its sensory evaluation result

2.1 Introduction

Tempe is a traditional fermented food from Indonesia made from soybean *(Glycine max (*L.*)* Merr.) and fermented by the microorganism *Rhizopus sp.* Because of its low cost and ease of preparation*, tempeh* was recognized as a cheap protein food source from plants (Astawan., et.al 2014). In Indonesia, the demand of *tempe* has recently increased in Indonesia. *Tempe* consumption in Indonesia in 2015 was 0.146 kg per capita per week, according to data from the Indonesian Statistical Center (BPS 2015). This statistic was higher than the weekly beef and chicken consumption, which were 0.009 and 0.121 kg, respectively. The increasing popularity of *tempe* in Indonesia is due to a greater understanding of the benefits of eating plant-based foods to increase protein intake and avoid health problems(Wikandari et al. 2020)

Tempe is made in Indonesia from soybeans and other raw materials such as tofu waste, peanut oilcake, and other legumes (Wikandari et al. 2020; Radiati 2016). According to a previous study, people in Central Java, Indonesia, have consumed more non-soy *tempe* and tofu waste *tempe* than soybean tempe since the 19th century (Shurtleff and Aoyagi 2011). The legumes from which *tempe* was prepared varied due to the availability of legumes in Indonesia (Wikandari et al. 2020). The protein content of locally grown beans such as mungbean (*Vigna radiata* (L.) R. Wilczek), cowpea (*Vigna unguiculata* (L.) Walp.), and red kidney bean (*Phaseolus vulgaris* L.) differed significantly from soybean, according to a recent study(Wikandari et al. 2020). The nutrient content of each legume *tempe*, however, was comparable to that of soybean *tempe* once processed. The fermentation processes used to produce *tempe*, according to the report, were sufficient to

increase its total protein content (Astuti et al. 2000). Another study on the use of soybean *tempe* as a rich protein product discovered that, while soybean *tempe* is high in amino acids, it is low in sulfur-containing amino acids. Consider combining soybean *tempe* with another amino acid-rich material, such as a cereal or legume, to obtain a complete amino acid profile(David Owens and Owens 2014; Astuti et al. 2000; Kustyawati et al. 2020).

Several studies have been conducted to investigate the use of non-soy products as *tempe* ingredients (Radiati 2016; Shurtleff and Aoyagi 2011; Dinesh Babu et al. 2009). Before standardizing low-cost press cake *tempe* from soy flour to improve diet, one study was conducted to determine the effect of raw material properties on *tempe* and its sensory properties (Dinesh Babu et al. 2009). In that study, the nitrogen content of *tempe* was used to calculate the total protein content using the Kjeldahl method. To investigate the sensory profile of each legume's *tempe*, crude fat and crude fiber determination, as well as sensory evaluation, were performed. However, the differences between each legume *tempe* were not clear using these methods, and the results were sometimes inconsistent when compared to another report.

Metabolomics refers to the comprehensive study of metabolites produced by living things. Metabolite profiling using gas chromatography-mass spectrometry (GC-MS) is a common technique in food metabolomics. It has been used in many different foods, including soy sauce, cheese, and coffee (Yamana et al. 2020; Putri et al. 2019; Ochi et al. 2012). GC-MS metabolite profiling has several advantages, including its dependability, stability, low cost, and ease of use. As a result, this technique has been widely used to quickly identify and differentiate small hydrophilic compounds in foods (Putri et al. 2019). In order to maintain the quality and grade of Indonesian speciality coffee from various species and geographical origins, GC-MS-based metabolomic investigations were conducted (Putri et al. 2019). Another study was carried out

using the same methodology to compare the metabolite composition of *tempes* produced in various Indonesian locations using different procedures (Romulo and Surya 2021). These studies discovered differences in the metabolite profiles of various cultivars, locations, and processing techniques.

Sensory evaluation has recently been combined with metabolomics to investigate primary metabolites in foods that are associated with human sensory perception (Yamana et al. 2020; Ochi et al. 2012). This method has been useful in confirming the benefits of metabolomic studies in discovering the unique metabolite profile in food and its relationship with the taste of food products. A study that correlates the metabolite profile of legumes *tempe* with its sensory evaluation, on the other hand, is still unknown. As a result, this study was carried out to investigate the metabolite and sensory profile of *tempe* derived from various legumes that are locally available in Indonesia, as well as its relationship to sensory analysis results.

2.2 Materials and methods

2.2.1 Legume *Tempe* Samples

We used jack bean, red kidney bean, cowpea bean, and soybean-based tempe samples. Each bean, referred to as a locally grown legume, was bought from the local Indonesian market. Table S1 includes Figure 2.1 and a list of the samples. The examples of *tempe* were all made in Indonesia and Japan. In December 2019, tempe was created in Japan at Osaka University's Laboratory of Bioresource Engineering (metabolomics). In March 2020, *tempe* was prepared in Rumah Tempe Indonesia (RTI) in Bogor, West Java, Indonesia

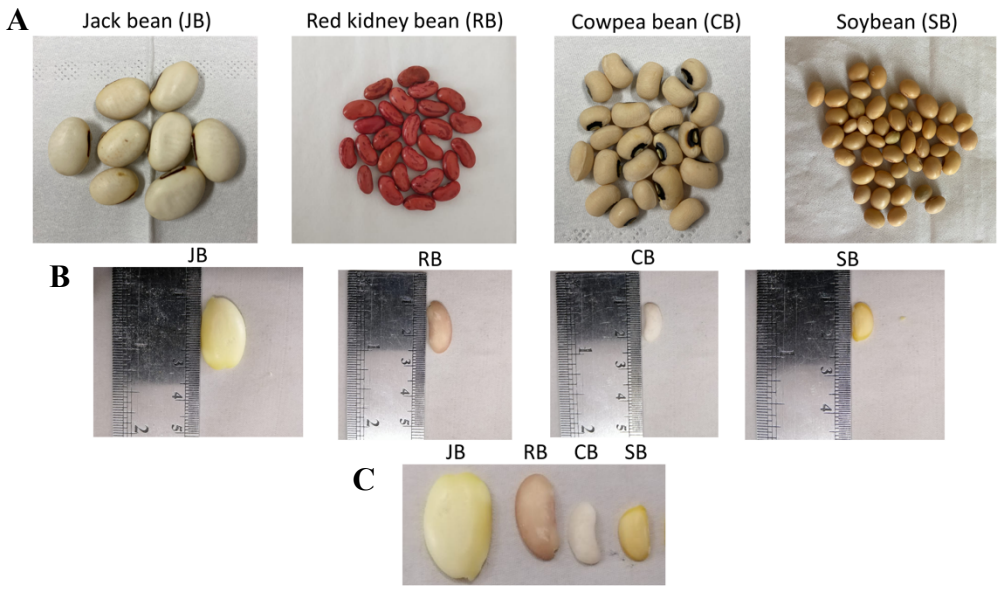


Figure 2.1. Legume in use. (A) unpeeled legume. (B) Peeled legume. (C) Size comparison between legumes. JB: Jack bean; SB: Soybean; RB: Red kidney bean; CB: Cowpea bean.

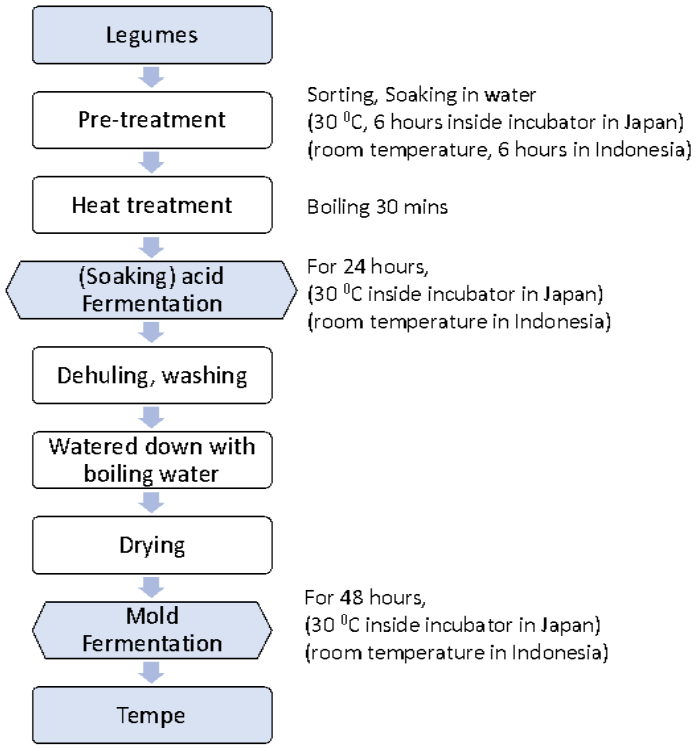
IGenerally, the RTI method was used to create each tempe (Fig. 2.2). Each legume was weighed at 250 grams and steeped in two times as much tap water as there were legumes for an overnight period. The legumes are then cooked in tap water for 30 minutes or until they are soft, followed by another night of soaking. The bean was then dehulled, rinsed in boiling water, and fermented using the tempe starter Raprima® for two to three days at room temperature with the main fungal strain utilized being *Rhizopus* spp. Japan's manufacturing of tempe uses a significantly modified version of the technique. For the Indonesian-produced *tempes,* however, the soaking and mold fermentation procedures were carried out at ambient temperature (26–32 °C). Each *tempe* was produced using water from its specific producing location

For sensory evaluation (Check-All-That-Apply (CATA), hedonic test), and metabolomic analysis, all Indonesian-produced *tempe* samples were used. The samples created in Japan, on the other hand, were only used for metabolomic research.

.

Each *tempe* sample was lyophilized overnight using a freeze-dryer at the final step of manufacturing (Fig. 2.2B), and each sample was divided into four pieces that each measured 3x3 cm to fit within a 50 mL tube. The Indonesian tempe samples were lyophilized, put in an insulated bag with ice, and transported to Japan. Following that, both the samples made in Japan and Indonesia were shock-powdered with multiple beads twice for 10 seconds, and then they were both stored at -30°C for subsequent examination. Additionally, three grams of each legume sample were lyophilized in a freeze-dryer over night, powdered using a multi-bead shocker for 10 seconds four times, and stored at -30°C for future analysis.

A



B

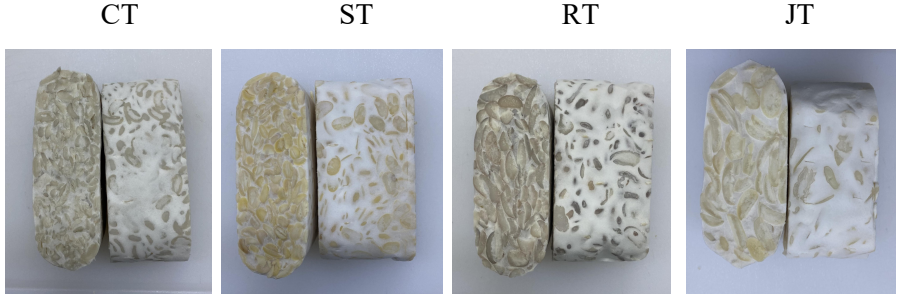


Figure. 2.2 Flow chart of *tempe* production and legume *tempe* product. (A) Flow chart of *tempe* production. (B) Legume tempe at the final stage of fermentation. Left: middle part of legume *tempe*, right: surface part of legume *tempe*. CT: Cowpea bean tempe; ST: Soybean *tempe*; RT: Red kidney bean *tempe*; JT: Jack bean *tempe*

2.2.2 Reagents

From Genpure, ultrapure water was obtained (Thermo Scientific, Osaka, Japan). From Fujifilm Wako Pure Chemical Industries, Ltd., ribitol and pure pyridine were bought (Osaka, Japan). Kanto Chemical Co., Inc. supplied the methanol for the GC-MS (Tokyo, Japan). Kishida Chemical Co., Ltd. supplied the chloroform for the GC-MS. We bought methylamine hydrochloride from Sigma-Aldrich Japan (Tokyo, Japan). From GL Sciences, trifluoroacetamide (MSTFA) and an alkene mixture (C9-C40) were purchased (Tokyo, Japan)

2.2.3 Hydrophilic, low-molecular-weight compound derivatization for GC-MS analysis

One milliliter of a mixture of methanol, ultrapure water, chloroform, and 200 L/mL ribitol as internal standard was added to the 2-mL microfuge tube along with the 10 mg of each powdered tempe sample. After that, the mixture was freeze-dried and lyophilized. The next step was to add 100 µL of methoxyamine in pyridine (20 mg methoxyamine/mL pyridine). The mixture was then subjected to a 90-minute oximization incubation at 30°C in a shaker incubator (Eppendorf Ltd., Hamburg, Germany). After oximization, silylation was carried out by adding 50 µL of MSTFA and incubating for 30 minutes at 37 °C. The final step was transferring the solution to a GC-MS vial for analysis. Three separate biological samples from each legume were examined in this investigation.

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2.2.4 GC-MS Conditions

GC-MS QP 2010 Ultra (Shimadzu, Kyoto, Japan) was utilized for the analysis. The apparatus was equipped with a GL Sciences Inert-Cap 5 MS/NS column. With a flow rate of 1.12 mL/min and a linear speed of 39 cm/s, helium was used as the carrier. With an injection temperature of 230°C and split mode 25:1 (v/v), the sample was introduced into the GC-MS. In the column, the temperature was maintained at 80°C for 2 minutes before rising at a rate of 15°C/min to 330°C, where it was maintained for 6 minutes. The transfer lines and ion source were each at 200 and 250 degrees Celsius. Ions were produced using electron ionization (EI) at a voltage of 0.94 kV. Then, 6.67 scans per second were used to record mass spectra over the mass (m/z) range

2.2.5 GC-MS Data Analysis

The GC-MS solution software program was used to convert the GC-MS data to the AIA format (Shimadzu, Kyoto, Japan). Peak alignment, filtering, and annotation were done using MS-DIAL version 4.00 and the GC-MS-5MP Library (Riken, Kanagawa, Japan).

The assigned peak intensities for each identified metabolite were adjusted against the ribitol peak intensity as an internal reference. According to level 1 metabolite analysis, which was acquired by examining at least two genuine standards under identical experimental settings as the samples, the substances were detected (Sumner et al. 2007). Then, the metabolites were validated using in-house library input via MS Dial (GL-Sciences DB (InertCap 5MS-NP, Kovats RI MSP file) that is available online (Ikram et al. 2020).

Additionally, each annotated metabolite was filtered by choosing those that displayed a relative standard deviation (RSD) within the quality control (QC) samples that was less than 30%.

Using SIMCA P+ ver. 13.0, principal component analysis (PCA) was carried out. To display the data, Umetrics' 13.0.3 package (Umea, Sweden) was utilized.

2.2.6 CATA-Sensory evaluation

Check-All-That-Apply (CATA) test was used to assess the sensory quality of each legume *tempe*. Bogor Agricultural University did a sensory evaluation. Twelve professional panelists, including students and researchers who had studied *tempe*, assessed the qualities of each legume *tempe* in the CATA test. Students from Bogor Agricultural University's Food Technology Department made up the entire sensory panel. Indonesians who were familiar with the *tempe* samples served on the panel. Panelists that are able to show sensory tests based on training and expertise in food sensory testing have been chosen as expert panelists (*tempe*). As a result, they can perform reliable and reproducible sensory evaluations (International Standard 2007). The test questionnaire is shown in Fig. S6.

For the CATA testing, cube-shaped samples of raw and fried *tempe* were prepared (Adawiyah et al. 2019). The samples were given out in a plastic cup bearing a unique three-digit random number on the label. For each test, samples were prepared in a single testing session along with a cup of mineral water to cleanse the palate following each sample test. The panelists had 15-20 minutes to test the sample and complete the questions (Adawiyah et al. 2019). The characteristics of the samples used in the CATA test were created from earlier work on temporal sensory evaluation. The day before the test, they were the subject of a group discussion with qualified panelists. The panelist was requested to review all characteristics pertaining to each sample that was made (King and Meiselman 2010).

2.2.7 Statistical analysis

Using SIMCA-P+ version 13, normalized data from the GC-MS data analysis were scaled by autoscaling and then converted to PCA (Umetrics, Umea, Sweden). The goal of the transformation was to make a metabolite under analysis smaller so that patterns, clusters, and outliers could be quickly found (Ikram et al. 2020).

In addition to PCA, analysis of variance (ANOVA) and post hoc tests were employed to examine the mean value of the outcomes of the hedonic sensory evaluation (TUKEY). The CATA data was also examined using XLSTAT 2018 to ascertain the sensory characteristics of legume *tempe*.

2.3 Result and Discussion

2.3.1 GC-MS-based metabolite profiling of legumes before and after become a *tempe*

To investigate the metabolite variations in the samples, GC-MS-based metabolite profiling was done on aqueous extracts of different legumes both before and after fermentation. The samples used in the dataset were grown locally in Indonesia and included soybean, red kidney, cowpea, and jack beans. These legumes were prepared utilizing a *tempe* starter throughout the fermentation process to create *tempe* in Indonesia and Japan at various production scales, in various environments, and using slightly changed procedures. Comparing four legumes before and after fermentation with an internal library generated 83 chemicals that were tentatively annotated (RI and mass spectra). These annotated chemicals *include* amino acids, organic acids, sugars, and other substances (Table S2). Additionally, our samples included annotations for substances identified by prior studies on *tempe* metabolomics, including genistein, daidzein, and nine important amino acids (Kadar et al. 2018).

PCA was used as an unsupervised analysis to examine the effect of fermentation in different production locations on each metabolite profile of 83 annotated metabolites from various legumes and legume *tempes* produced in Japan and (Fig. 2.3). According to the PCA results, the samples were separated along PC1 and formed two distinct clusters. With a 36.3 percent variance, all legumes were clustered in negative PC1, while *tempes* were clustered in positive PC1. Sugars and amino acids are recognized as important metabolites in separating legumes before and after fermentation (Fig. 2.3). This finding indicates that the fermentation process used by R. oligosporus in *tempe* production increased its amino acid content (Kadar et al. 2018; Nout and Kiers 2005). The proteolytic enzyme activity of Rhizopus sp., which breaks long-chain molecules of proteins into shorter fragments (amino acids), causes an increase in the number of amino acids after fermentation (Nout and Kiers 2005; Widaningrum et al. 2017).

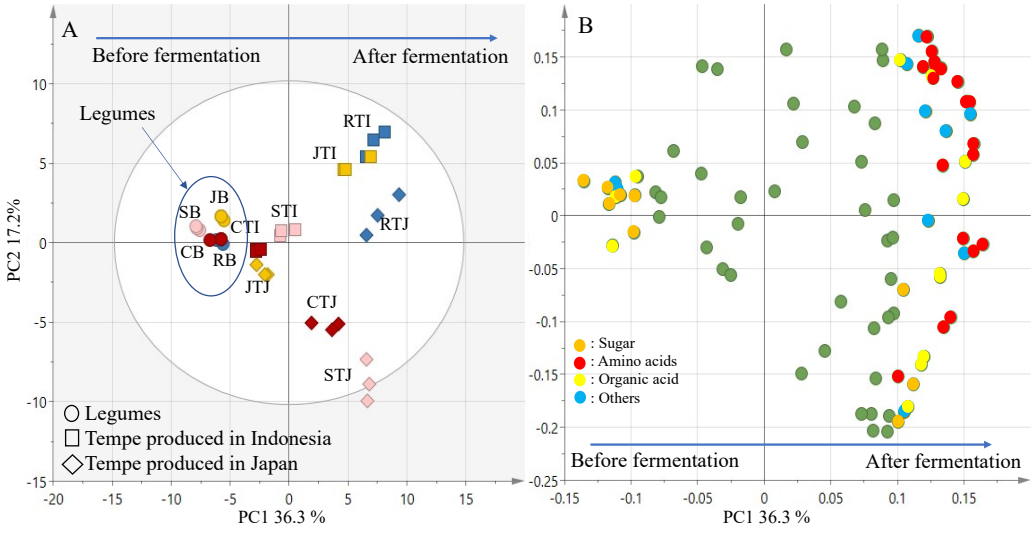


Figure. 2.3. GC-MS principal component analysis (PCA) results of legume samples before and after become *tempe,* produced in different places (Japan and Indonesia). **A.** Score plot. Points represent legume samples (SB: soybean, RB: red kidney bean, CB: cowpea bean, and JB: jack bean), and different shapes represent different sample forms and production places (I: Indonesia;

J: Japan). **B.** Loading plot of the GC-MS analysis result. With a loading factor of more than ± 0.1, Orange represent sugar compounds, red represents amino acid compounds, yellow represents organic acid compound, and blue indicates others.

A sugar domain was discovered in the negative region of PC1. This finding indicated that before fermentation, legumes contain a variety of sugars such as sucrose, sorbose, and psicose (Table S3). The sugars were then reduced primarily due to their utilisations by microorganisms during fermentation (Widaningrum et al., 2017). As a result, *tempe* products have a higher amino acid content than non-fermented legumes (Astuti et al., 2000).

Furthermore, the results revealed a separation tendency based on production location (Japan or Indonesia) along PC2, with a 17.2 per cent variance (Fig. 2.3A). In the following analysis, samples were separated based on production location to visualize the characteristics of each legume *tempe.*

2.3.2 GC-MS metabolite profiling of *tempes* sample produced in different places from various legumes based on GC-MS analysis

To visualize the essential metabolites that differentiate t*empes* produced in Japan and Indonesia, we used GC-MS analysis followed by PCA on lab-scale *tempe* samples produced in Japan with a slightly modified production process and large-scale *tempe* samples produced in the Indonesian *tempe* industry (Rumah *Tempe* Indonesia). Table S4 provides a summary of the *tempe's* production conditions.

The PCA revealed that the different production locations (Japan and Indonesia) were visualized using a slightly modified method based on PC1 with a 30.35% variance (Fig. 2.4). Soybean, cowpea bean and red kidney bean *tempe* samples produced in Japan are clustered on the positive part of PC1. Meanwhile, Indonesian soybean, cowpea bean, and jack bean *tempe* samples were clustered on the negative side of PC1. These findings suggest that different fermentation

locations and slightly different production processes resulted in different metabolite profiles for each legume *tempe*. This result was consistent with a previous soybean tempe metabolite profile study conducted in several Indonesian regions (Kadar et al., 2020). However, environmental conditions and slightly different production methods have no significant impact on some legumes,

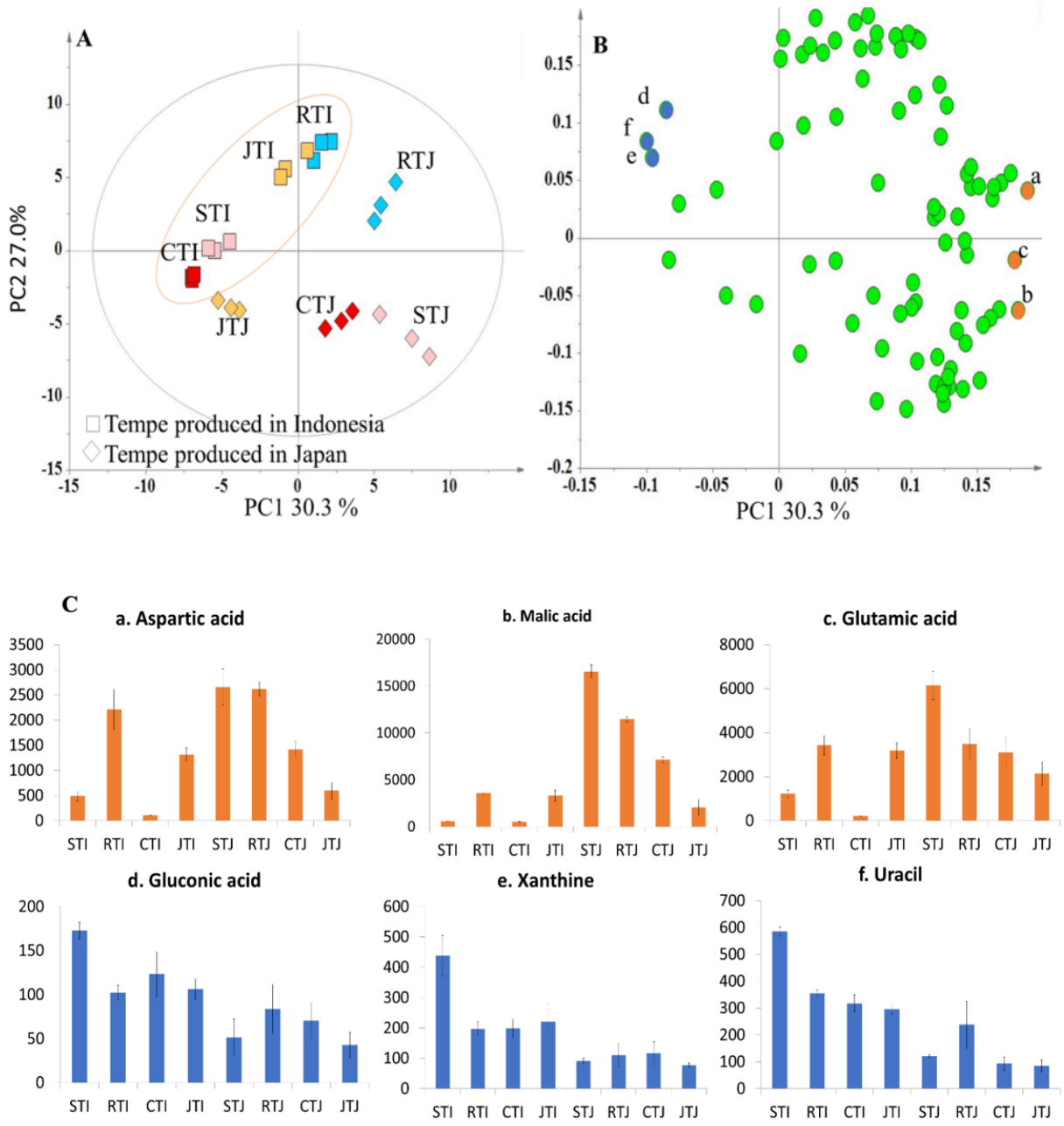


Figure. 2.4 Result of GC-MS-based principal component analysis (PCA) and bar graph of legume *tempe* samples produced in two different places. **A**. GC-MS based score plot of *tempe*. Points represent legume types (ST: Soybean *tempe*; RT: Red kidney bean *tempe*; CT: Cowpea bean *tempe*; and JT: Jack bean *tempe*), and J; I and different shapes represent production place, which is Japan and Indonesia **B.** GC-MS based loading plot of *tempe* produced in Indonesia and Japan. In the PC1, the major metabolites are lettered as follows: **a**: aspartic acid; **b**: malic acid; **c**: glutamic acid; **d:** gluconic acid; **e**: xanthine; **f**: uracil. **C.** Bar graph of major metabolites. The vertical axis represents relative intensity. The horizontal axis represents samples. The error bar shows the standard deviation from three biological replicates in *tempe* samples.

According to the loading plot, amino acids, organic acids, and sugars were the metabolites contributing to the separation along PC1 (Fig. 2.4.B, C). The loading plot in PC1 revealed that amino acids such as aspartic acid and glutamic acid contributed to the separation of soybean, cowpea bean, and red kidney bean tempes produced in Japan. Furthermore, due to lactic acid fermentation during the soaking process, an organic acid group, such as malic acid, was discovered to be a significant metabolite in tempe produced in Japan (Table S5). Organic acids, on the other hand, such as gluconic acid, were generally significant in Indonesian tempes. Purine derivatives, such as xanthine, have also been discovered to contribute to tempe production in Indonesia (Fig. 2.4C).

The metabolite profile of tempe produced in Japan and Indonesia, particularly soybean and cowpea bean *tempe*, was discovered to differ between production locations due to a variety of factors including production scale, water, and environmental conditions.. While the storage condition and shipping condition of legumes were at room temperature inside of vacuum-sealed plastic packaging and shipped for 3 days to Japan before producing into *tempe* were not the factors

that could affect the metabolites profile. Based on (Ikegwu et al. 2010) the structure of the seed of a legume consists of the *endosperm* and *embryo* which protect by a pericarp (outer wall) and testa. The testa is a selective layer that protects the embryo from water and air, which will not affect the metabolites profile of the legume. Therefore, legumes can be stored in room temperature with sealed plastic packaging. In addition, the problem of post-harvest of legumes is only pest damage, the storage condition with vacuum-sealed is enough to prevent contamination. In *tempe* production process. The outer layer of legumes was dehulled after soaking. Therefore, we could say that the initial condition of legumes is the same.

In Indonesia, the environmental conditions for tempe production differ from those in Japan. In Japan, the samples were fermented at a controlled fermentation temperature of 30°C inside an incubator, whereas in Indonesia, the samples were fermented at room temperature. The controlled temperature was used because the room temperature, which was approximately 11°C-14°C with a 60% humidity in December 2019 in Japan, was insufficient for the optimal growth of the tempe starters.

In contrast, according to data from the Indonesian Agency for Meteorological, Climatological, and Geophysical Geophysics' official website (https://www.bmkg.go.id; available online April 2020, in Indonesian), samples were produced in Indonesia in March 2020 at 28°C-32°C with a humidity of 90% in the fermentation room without any temperature control. This temperature was identified as a suitable temperature for the growth of the major tempe fungus *Rhizopus* spp. (Kadar et al. 2018; Sparringa et al. 2002). The rate of degradation of carbohydrates and complex proteins could be affected by differences in production temperature. Other researchers have investigated the effect of temperature, water content (Aw), and the atmosphere on the mycelia growth of *tempe* fungi. The study discovered that tempe fungi are more sensitive

to temperature changes in higher humidity environments and have the highest sensitivity to environmental fluctuations such as humidity and temperature (Han and Nout 2000). As a result, soybean and cowpea bean tempe from Indonesia had different metabolite profiles than tempe from Japan, particularly in amino acid profiles. Another study supported the conclusion that environmental factors influence the flavor and quality of the fermented beverage *Baiiu* (Chinese liquor) (Pang et al. 2018). Furthermore, the scale of production may have a different effect on the metabolites.

In addition to the previously mentioned data, PC1 data revealed that each legume has a different metabolite profile in different production locations. Soybean and cowpea bean tempe samples from Japan and Indonesia were assigned to separate clusters (Fig. 2.4A). However, the PCA revealed that red kidney bean tempe produced in different locations was clustered together, as was jack bean tempe. Meanwhile, the PCA revealed that each legume responded differently to fermentation conditions, resulting in distinct metabolite profiles.

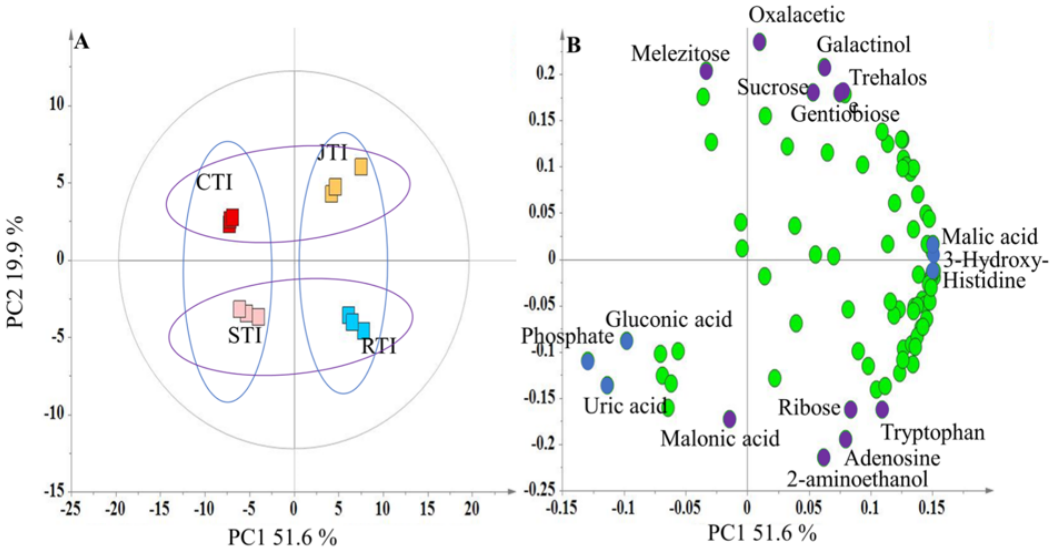
According to this finding, environmental conditions, a slightly modified method of production, and legume variation may all have an impact on the metabolite profile of legume tempe produced in Japan and Indonesia. Therefore, we next analyzed the *tempes* produced in Indonesia to determine the metabolite differences between various legume *tempes*.

2.3.3 GC-MS-based metabolite profiling of samples produced in Indonesia

When the analyses were carried out independently for one of the production locations, the PCA revealed that the data separation based on legume variation could be clearly visualized. We chose *tempe* samples from Indonesia for further investigation. This dataset was chosen because Indonesia is considered the standard place of *tempe* production, where the majority of *tempe* production occurs.

Figure 2.4A shows different clusters for each legume, with a 51.6 % variance. Soybean and cowpea bean *tempes* were found to be clustered in negative PC1. Meanwhile, red kidney bean and jack bean *tempe* samples were found to be clustered in positive PC1.

Figure 2.4B depicts the representative metabolites in the PC1 loading plot. Malic acids, 3-hydroxy-3-methyl glucarate, and histidine were the metabolites found in higher concentrations in red kidney bean tempe and jack bean *tempe*. Soybean *tempe* and cowpea bean *tempe*, on the other hand, had higher phosphate, uric acid, and gluconic acid concentrations than soybean *tempe* and cowpea bean *tempe* (Fig. 2.4C). As a result, histidine and malic acid could be candidate marker metabolites for distinguishing non-soy legume *tempes*. This result also showed that red kidney bean tempe and jack bean *tempe* have different amino acid profiles than soybean *tempe*. Phenylalanine, histidine, leucine, isoleucine, and tyrosine were the amino acids found in significantly higher concentrations in red kidney bean *tempe* samples (Table S6). The difference in the degree of softening by heating among the legumes may account for the highest contribution of these amino acids in red kidney bean t*empe* (Koriyama et al. 2018). Koriyama et al. discovered in 2018 that the cooking time required for soybean and red kidney beans to reach an optimum edible value differed (Koriyama et al. 2018). The red kidney bean has the shortest boiling time to achieve the highest edible value, which is nearly half that of the soybean.



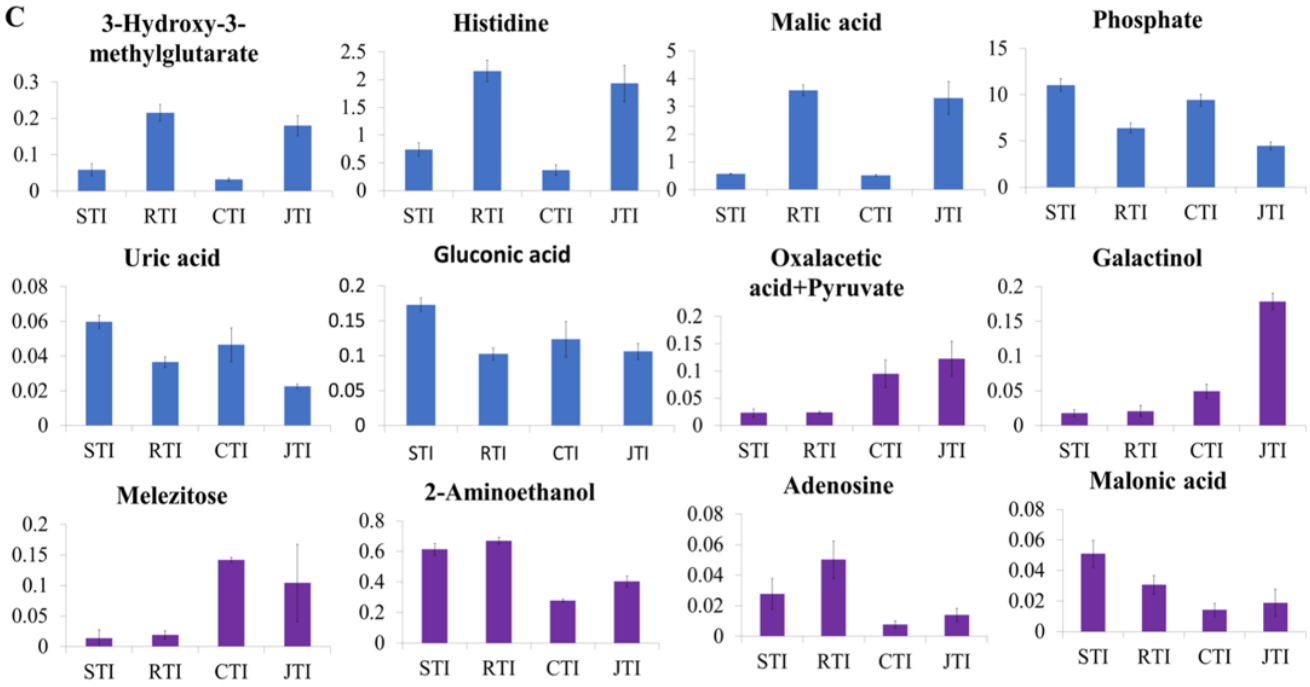


Fig. 2.4. GC-MS-based principal component analysis (PCA) analysis result of *tempe* sample produced only in Indonesia. **A.** GC-MS-based score plot sample. **B.** GC-MS-based loading plot of sample. Points represent legume types (ST: Soybean *tempe*; 2: RT: Red kidney bean *tempe*; CT: Cowpea bean *tempe*; JT: Jack bean tempe and I: Indonesia). In PC1, the major metabolites are 3-hydroxy 3-menthyl glutarate, histidine, malic acid, phosphate, uric acid, and gluconic. Based on PC2, the major metabolites are oxalacetic acid+pyruvate, galactinol, melezitoze, 2-aminoethanol, adenosine, and malonic acid. **C.** Bar graph of major metabolites in PC1 and PC2. The vertical axis represents relative intensity. The horizontal axis represents samples. The error bar is the standard deviation from three biological replicates in *tempe* samples.

For each legume, we used the same water level and boiling time. Each legume had a different level of softness prior to fermentation. Furthermore, according to a previous study, the hardness/softness level of legumes may affect the adaptation time of fungal growth and mycelium

formation in *tempe* (Baumann and Bisping 1995; Puspitojati et al. 2019). The same fermentation time results in different amino acid profiles and contents in each legume (Puspitojati et al., 2019).

Furthermore, *tempe* samples were separated along PC2 with a variance of 19.9 %. The metabolites shown in the PC2 loading plot helped separate soybean *tempe* and red kidney bean *tempe* from jack bean tempe and cowpea bean *tempe*. Soybean *tempe* and red kidney bean *tempe* had higher levels of 2-aminoethanol, adenosine, and malonic acid (Table S7). Cowpea bean *tempe* and jack bean *tempe*, on the other hand, accumulated more oxaloacetic acid + pyruvate, galactinol, and melezitose which is the carbohydrates group. This finding is consistent with previous reports that legumes such as jack bean and cowpea bean had higher carbohydrate concentrations, resulting in the production of melezitose and galactinol during the fermentation process.

2.3.4 The CATA-based sensory evaluation result of each legume *tempe* sample produced in Indonesia

PC1 showed a 51.6 % variance in the metabolite profiles of different legume *tempes* (Fig. 2.5A). PC1 metabolites influence the differences in legume *tempe* made from soybean, red kidney bean, cowpea bean, and jack bean. The CATA test was used to determine the general characteristics of each sample in order to study the variance of metabolites through the sensory attributes of each temperature. The sensory characteristics of each tempe sample were evaluated by a panel of twelve expert panelists.

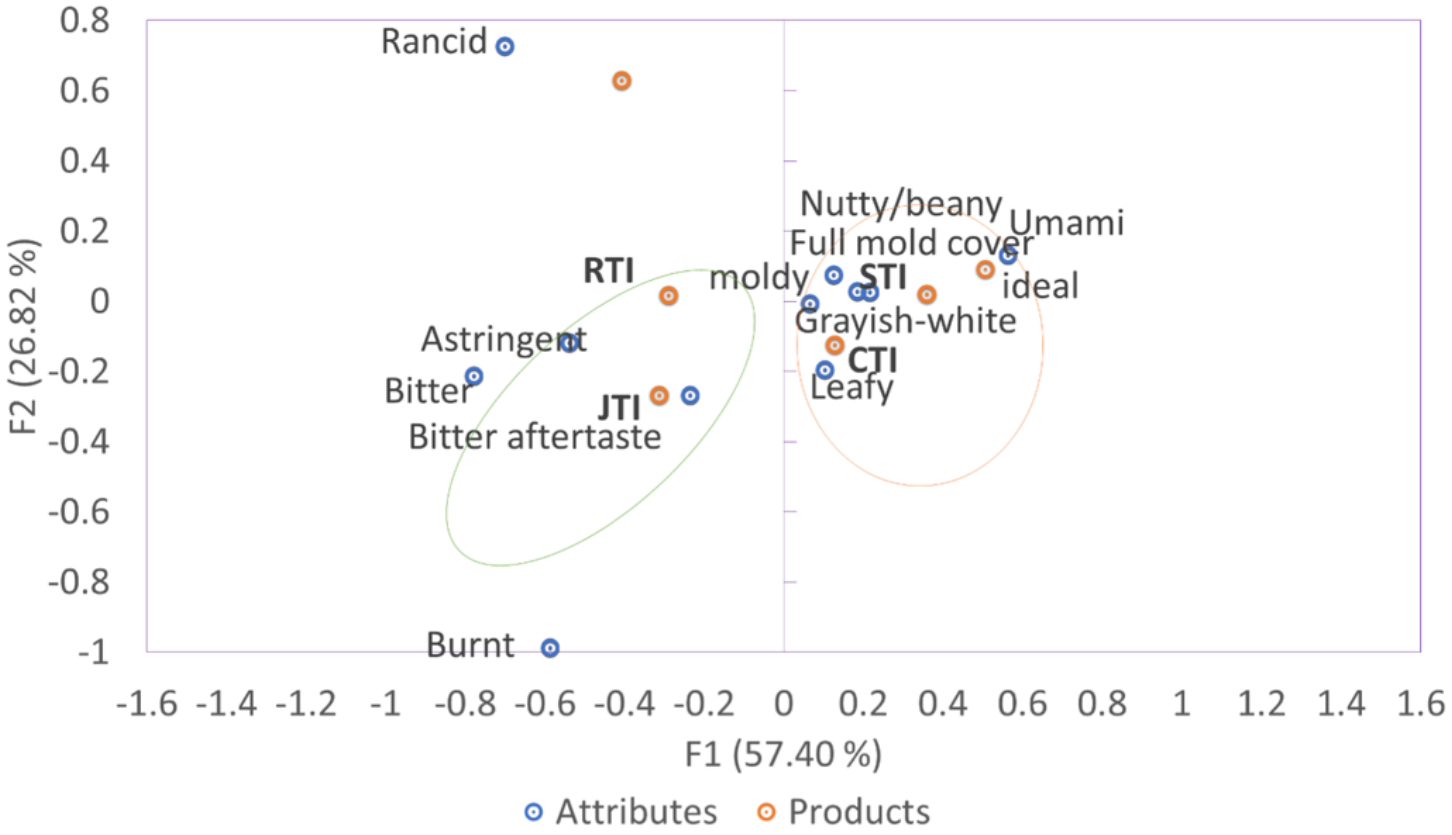


Figure. 2.5. CATA-based sensory evaluation result by expert panelist of *tempe* sample. (ST: soybean tempe; JT: Jack bean tempe; RT: Red kidney bean tempe; CT: Cowpea bean tempe; I: Indonesia and J: Japan)

Based on the non-parametric Cochran's test results for each attribute, the panelists selected soybean *tempe* as an ideal *tempe* with a nutty/beany and umami taste as "ideal" taste attributes and fully covered in white as a "ideal" appearance. However, the cowpea bean *tempe* characteristics were also close to the ideal *tempe* based on panelist preferences, according to the CATA results (Fig. 2.5). This result confirmed the earlier PCA finding that cowpea bean *tempe* and soybean *tempe* were closely clustered together. According to the CATA results, the cowpea bean and soybean *tempe* have a similar metabolomic profile associated with taste, which may be related to an umami taste, as well as similar appearances, being completely covered in mold and having a

greyish-white color. Glutamic acid has been identified as one of the compounds involved in the umami flavor (Ninomiya 2015). According to the loading plot graph, soybean and cowpea bean *tempes* were high in the free amino acid group of glutamic acid, such as pyroglutamic acid. Another sensory preference study found that cowpea bean *tempe* has a high taste preference and is "liked slightly" with a hedonic point of 5, while soybean *tempe* is "liked moderately" with a hedonic point of 6 (Dewi IWR, Anam C, 2010).

The panelists thought the *tempe* from red kidney beans had slightly astringent, bitter, and bitter aftertaste attributes, according to Cochran's test plot. The metabolomic data also support the conclusion that red kidney beans are high in valine, leucine, tryptophan, and phenylalanine, which are amino acids that contribute to food bitterness (Aluko 2017). The jack bean *tempe* was distinguished in the different clusters by panelists due to its strong bitterness and bitter aftertaste. According to the loading plot and bar graph, jack bean is also high in proline, a bitter amino acid. As a result, the panelists rated the jack bean tempe as far from having "ideal" *tempe* characteristics.

Because of its similar sensory profile to soybean *tempe*, which is the most common *tempe* in Indonesia, cowpea bean could be used as a possible alternative soybean replacement as a raw *tempe* material based on this result. Meanwhile, combining soybean *tempe* and red kidney bean *tempe* could help improve *tempe's* amino acid profile.

2.4 Conclusion

This chapter investigated metabolite profiling of legumes before and after fermentation, different production environmental conditions, and their sensory profile. The results revealed that 83 metabolites were annotated from all samples. The samples were separated based on their type (legume and *tempe*) along PC1 with a 36.3 % variance, with sugars and amino acids significantly contributing to the separation, according to principal component analysis (PCA) from GC-MS

analysis. Different production locations resulted in metabolites in the same legume *tempe*. The same soybean and cowpea bean *tempe* samples produced in Japan and Indonesia were clustered differently, according to PC1.and cowpea bean *tempe* samples produced in Japan and Indonesia were clustered differently.

Data from metabolites profiling revealed that each legume tempe has unique metabolite characteristics. According to the PC1 loading plot, soybean and cowpea bean were clustered together with higher levels of gluconic acid, phosphate, and uric acid. Meanwhile, according to PC2, soybean *tempe* and red kidney bean *tempe* were separated from jack bean *tempe* and cowpea bean *tempe*. According to the PC2 loading plot, soybean *tempe* and red kidney bean *tempe* had higher concentrations of 2-aminoethanol, adenosine, and malonic acid. While jack bean *tempe* and cowpea bean *tempe* contained more oxaloacetic acid + pyruvate, galactinol, and melezitose, respectively. The same patterns emerged in sensory evaluation data. Cowpea bean *tempe* has sensory properties similar to soybean *tempe*, according to the sensory results.

These findings provide detailed information on metabolites for each legume *tempe*. The findings also suggest that metabolomics could be used to comprehensively identify differences between *tempes* from different legumes. Based on this data, further research into potential legume *tempe* as an ingredient in baby food products will be carried out.

These findings provide complete metabolites information for each legume *tempe*. The result also gives evidence that metabolomics could be used to identify the differences between tempes from different legumes comprehensively. Based on this information, further study of potential legume *tempe* as an ingredient of baby food products will be conducted.

Chapter 3

In-vitro protein digestibility & amino acids profile of selected legume *tempe*

3.1 Introduction

The recent world trend of plant-based food production has been observed as increased consumption of meat protein substitutes. As a result, many researchers started to study the health benefits and nutritional properties of plant-based food products (Qin et al. 2022). As mentioned in the previous chapter, *tempe* is a plant-based and fermented food from Indonesia. Nowadays, *tempe* has gained popularity due to its meat-like taste, health benefits, and affordability (Ahnan-Winarno et al. 2021).

Tempe is also known to have a high quality of protein digestibility compared to other plant-based protein foods (Astawan et al. 2014). The amino acid content was significantly increased in *tempe* after the fermentation process compared with beans before fermentation (Kadar et al. 2020). The fermentation process has been proved to improve the quality and add more nutrients that are not present in the original product, which is beans (Steinkraus 1994). *Tempe* words come from the process where half-cooked beans (originally soybean) are bound together with the help of *Rhizopus spp* to be a compact cake. Therefore, *tempe* can be made from various legumes.

Many researchers from outside Indonesia started to utilize other legumes to become *tempe*. The study by (Osundahunsi and Aworh 2002) tried to use cowpea beans as *tempe* material to be made into maize-based complementary food in Nigeria. The result stated that higher protein and fat were observed in cowpea *tempe* enriched complementary food than in non-enriched complimentary food. Another study used lupin seeds from Australia to become a *tempe* and natto. It was found that lupin could be used as raw material for *tempe* and natto with significantly higher

protein content, bioavailability, and so on than the lupin seeds before fermentation (Wickramasinghe 2017).

The gas chromatography-mass spectrophotometer (GC-MS) has been used to discriminate small hydrophilic molecules as a robust analysis tool (Putri et al., 2019). Nevertheless, the exploration and nutritional study based on the metabolomics of legume *tempe* would be beneficial in collecting more information on legume *tempe* as a plant-based food product with many health benefits comprehensively. For example, in a previous chapter using GC-MS analysis, red kidney bean *tempe* has been found to have a higher concentration of aspartic acid and histidine than other legumes, which are considered important amino acids in sensory attributes of red kidney bean *tempe*. That study has indicated that *tempe* from various legumes commonly found in Indonesia produced in different places gave different metabolites such as sugar, amino acid, and organic acid composition. This general overview has been helpful for further product development from legume *tempe*.

However, the amino acid profiles based on GC-MS data analysis were not enantioselective, meaning that each amino acid included information from both $_{D}$- and $_{L}$-amino acids. Therefore, it is also essential to study the enantiomer of amino acids due to its role in different biological properties by using LC-MS (Liquid chromatography-mass spectrophotometer), which is often used as a promising tool for profiling $_{D}$- and $_{L}$-amino acids (Taniguchi et al. 2019). Therefore, in this study, various legume *tempe* are subjected to in-vitro protein digestibility, GC-MS and LC-MS analysis to characterize the differences among legume *tempe*. As a result, this study would be more beneficial for developing legume *tempe*-based food products for global markets.

3.2 Materials and Methods

3.2.1 Legume *tempe* samples

Four legumes locally found in the Japanese market and two legumes purchased in Indonesia were chosen as a *tempe* raw materials. Each legume was purchased from the local market in Japan and Indonesia, and the sample list is shown in Table S8. All legumes were produced to become a *tempe* in the Laboratory of Bioresource Engineering (metabolomics), Osaka University, Japan, in January 2021. The legume was weighed and washed before being soaked overnight using tap water. Then, the overnight legume was boiled for about 20 minutes and then soaked again for another night before it was dehulled and rinsed with boiling water. The dehulled legume was dried and inoculated by the *tempe* starter Raprima®, then fermented in the incubator for about 48 hours at 30 ^{0}C until it became a compact cake fully covered by the mold (*tempe*).

All Tempe legumes were cut into four pieces of 3x3 cm size and put inside the 50 mL grinding tube. After that, it was lyophilized overnight using a VD-800F Freeze dryer (Taitec, Saitama, Japan) and powdered using a multi-bead shocker (Yasui Kikai, Osaka, Japan) for 10 seconds, twice. The powdered *tempe* samples were stored at -30^{0}C until analysis.

3.2.2 In-vitro protein digestibility analysis

The pepsin and pancreatic enzyme (Sigma-Aldrich) are used to determine the in-vitro protein digestibility of each legume *tempe* (Puteri et al., 2018). The sample was weight for 1.5 g/ 30 mL ultrapure water, then incubated in the thermo-mixer at 25 °C for 30 minutes. The supernatant was mixed with pepsin solution (pH 1.9) and incubated for 30 min at 37°C. Following the incubation, pH was altered to 7.5 by using NaOH. Pancreatin solution was then added, and incubated for six h at 37 ^{0}C. The mixture was then mixed (1:1) with TCA solution (20 g/100 mL) and centrifuged. The soluble protein obtained from the supernatant was then measured by using the BCA method.

The BSA (bovine serum albumin) was used as standard and oluble protein was compared to total protein.

3.2.3 Extraction and derivatization of legume *tempe* samples for GC-MS analysis

The ten milligrams of each legume *tempe* were extracted by using the solvent mixture of methanol (Wako Chemical, Osaka, Japan), chloroform (Kishida Chemical Co. Ltd, Osaka, Japan), and ultrapure water (Wako Chemical, Osaka, Japan); in the ratio 5:2:2 (v/v) containing 50 µL/mL of internal standard (ribitol (Fujifilm Wako Pure Chemical Industries, Ltd. Osaka, Japan)). The mixture was incubated at 37 ^{0}C, and 1200 rpm for 30 minutes and then centrifuged for 3 min at 4^{0}C. Four hundred microliters of supernatant were mixed with 300 µL of ultra-pure water (Wako Chemical, Osaka, Japan) using vortex and centrifuge for 3 min at 4^{0}C. Two hundred microliters of supernatant were transferred into a new 1.5 mL microtube, and another 200 µL of each supernatant was pooled as a quality control into a 10 mL Eppendorf® tube. Then, the quality control mixture was transferred into another 1.5 mL microtube to be concentrated in the centrifugal concentrator for about 1 hour, together with another supernatant of samples. The concentrated sample was put into a freeze dryer for overnight lyophilisation. After that, the overnight samples were mixed with 100 µL of methoxamine hydrochloride in pyridine (20 mg/mL) and then incubated for about 90 minutes at 30 ^{0}C at Thermo shaker (Eppendorf Ltd., Hamburg, Germany), followed by a silylation process using 50 µL of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) (GL Sciences) to each sample. After that, samples were incubated for 30 minutes at 37^{0}C and transferred to a GC vial for analysis.

3.2.4 GC-MS Analysis and Data Analysis

The GC-MS QP2010 Ultra (Shimadzu, Kyoto, Japan) equipped with an InertCap 5 MS/NP column (GL Sciences) has been used for analysis. Before the analysis, a leak check and tuning of the mass spectrometer were done before analysis. One microliter of prepared samples was injected in split mode, 25:1 (*v*/*v*), with an injection temperature of 230 °C. An alkene mixture (C8-C40) was used and injected as a standard for peak identification. Helium was used as carrier gas. The flow of He was 1.12 mL/min with a linear velocity of 39 cm/s. The column temperature was held at 80 °C for 2 min, increased by 15 °C/min to 330 °C, and then held for 6 min. The transfer line and ion source temperatures were 250 and 200 °C, respectively. Ions were generated by electron ionization (EI) at 0.94 kV. Spectra were recorded at 10,000 u/s (check value) over the mass range *m*/*z* 85−500.

The data obtained from GC-MS analysis was converted to an AIA file using the GC-MS solution software package (Shimadzu, Kyoto, Japan). Then, MS-Dial ver. 4.00 software is used for peak alignment, filtering, and annotation using the GC-MS-5MP Library (Riken, Kanagawa, Japan).

3.2.5 Extraction of legume *tempe* samples for LC-MS analysis

The 10 mg of *tempe* powder were weighed in a 1.5 mL Eppendorf tube. Then, 500 µL of 1% trifluoric acid (TFA) (Fujifilm Wako Pure Chemical) in water was added along with 20 µL of $_{DL}$-Alanine-2,2,3,3, -d_4 (internal standard; 40 µmol/mL) (Santa Cruz Biotechnology Inc.). Then, the samples were incubated in the thermomixer incubator (Thermomixer comfort, Eppendorf) at 1200 rpm at 4°C for 30 minutes. After that, the 100 µL chloroform was added and centrifuge at 10,000 rpm at 4°C for 10 minutes.

The 300 μL supernatant was taken and transferred into a 2 mL Eppendorf tube to be mixed with 1400 μL of 70% acetonitrile. The solutions were incubated in the thermomixer incubator for 15 minutes at 1200 rpm at 4°C and centrifuged at 10,000 rpm at 4°C for 10 minutes. Then, the samples were concentrated using a centrifugal concentrator (VC-96R, Taitec) at 150 rpm at 25°C for approximately 120 minutes. Subsequently, 100 μL of the supernatant was diluted with 400 μL 80% acetonitrile/20% ethanol solution. Afterwards, 100 μL of samples were transferred into an LC-MS vial.

3.2.6 LC-MS analysis and data analysis

The LC-MS analysis was performed by using LCMS-8060 (Shimadzu). The condition of the LCMS was as follow: column (CROWNAK® CR-I(+) and CROWNAK ® CR-I(-) (Dicel Corp.) (5 μm, 150 nm x 3.0 nm I.D.); mobile phase (Acetonitrile/Ethanol/Water/Trifluoroacetic acid (ACN/EtOH/H_2O/TFA) = 80/15/5/0.5 (v/v/v/v)); flow rate (0.4 mL/min); injection volume (1 μL); column oven temperature (25 °C); ionization mode (positive); nebulizer gas (3 L/min); heating gas (5 L/min); drying gas (15 L/min); interface temperature (230 °C); DL block temperature (250 °C); heating block temperature (310 °C); Interface voltage (4 kV).

For analysis, the automatic valve switching method described in (Taniguchi et al. 2019) was performed using a high-pressure switching valve FCV-32AH (Shimadzu). From the LC-MS analysis, the chromatogram and mass spectral data were collected. For normalization, the peak area value of each compound was divided by the peak area of $_D$-Alanine-2,2,3,3,-d_4, the internal standard. Metabolites were then annotated using amino acids standards in LabSolutions (Shimadzu). Similarly, PCA was carried out to distinguish the amino acid differences between samples.

3.3 Results and discussion

3.3.1 In-vitro protein digestibility of legume and legume tempe

A general overview of protein quality and metabolites profiling of legume *tempe* has been done by analyzing in-vitro protein digestibility, GC-MS and LC-MS analysis. The samples of legume *tempe* were produced by using the legumes sold in the Japanese market, namely, green soybean, green pea, red kidney bean, white bean, garbanzo, and soybean. The legumes were processed into a *tempe* in the same environmental condition and treatment by using a modified method of Rumah Tempe Indonesia.

Tempe is known as a food with high-quality protein. Moreover, to visualize one parameter of protein quality of each legume *tempe*, we subjected the samples to In-vitro protein digestibility. This analysis was conducted to estimate protein availability at intestinal absorption of various legume *tempe* using multi-enzyme processes.

In-vitro protein digestibility was used to determine the quality of protein in legume *tempe* based on the protein availability in the samples. The in-vitro protein digestibility has been used to predict the protein availability at intestinal absorption, reflecting the utilization efficiency of dietary protein (Ketnawa and Ogawa 2021). Therefore, this analysis is essential to find out whether the legume *tempe* contains protein that is easy to digest or not. The value is obtained from protein which is *tempe* sample which were hydrolyzed by digestive enzymes into amino acids.

The protein can be digested if more peptide bonds are hydrolyzed, and many amino acids are produced. In this study, in vitro protein digestibility using multiple enzyme analyses was performed with conditions as similar as possible to protein digestion in the body.

The percentage of In-vitro protein digestibility of each legume *tempe* is shown in Figure 3.1. The graph shows that the highest protein availability is green soybean *tempe,* white bean *tempe*

and garbanzo *tempe*. On the other hand, the lowest protein availability is beans before fermentation. The result shows that the % protein digestibility of all legume *tempe* samples is 40-90%. Meanwhile, the % in-vitro protein digestibility of legumes is between 5-12%. The different big gap in In-vitro protein digestibility happened due to the fermentation process of *tempe,* which allows the mycelium of *Rhizopus* spp to penetrate layers of cells into a cotyledon of legume. As a result, enzymes such as protease cause the enzymatic degradation of protein into lower molecular weight substances, which are amino acids, were produced (Joye 2019; Kiers 2001). In the form of amino acids, protein could be quickly absorbed by the human body. Therefore, the in-vitro protein digestibility of *tempe* samples is higher than the legume samples before fermentation.

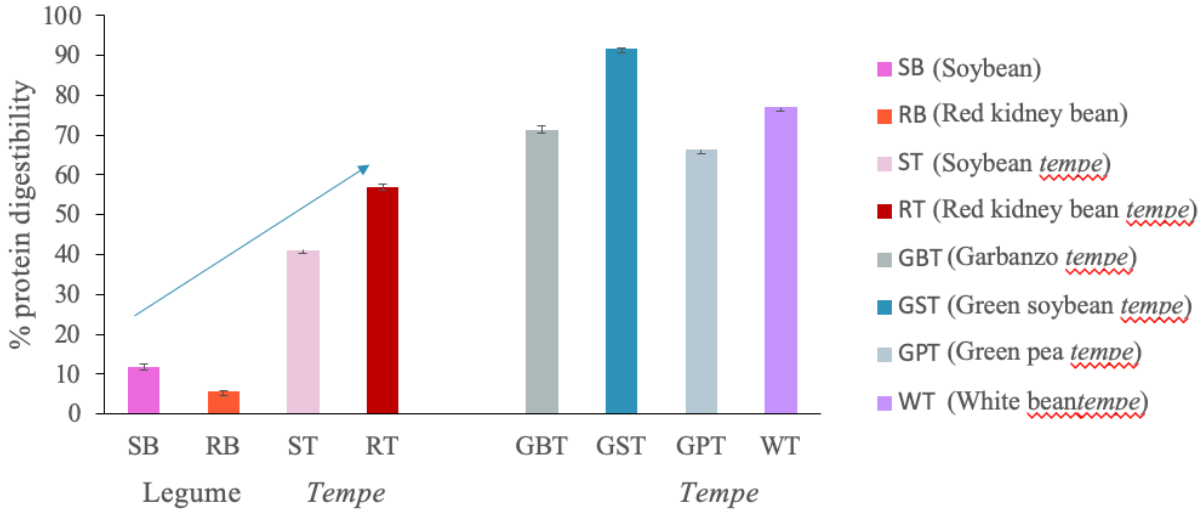


Figure 3.1. In-vitro protein digestibility in percentage. Labelled: ST: Soybean *tempe;* RT: Red kidney bean *tempe*; GST: Green soybean *tempe*; GBT: Garbanzo *tempe;* GPT: Green pea *tempe;* WT: White bean *tempe*; SB: Soybean; RB: Red kidney bean.

In terms of processing technique, in the boiling process, low molecular weight peptides and free amino acids were dissolved, resulted in an increasing the number of available amino acids, makes it easily to be digested and absorbed.

3.3.2 Metabolites profile-based GC-MS analysis of legume *tempe*

Metabolites profiling has been done previously to analyze metabolite changes in legume *tempe* before and after fermentation produced in Japan and Indonesia. This study used metabolites profiling to analyze the metabolites difference and amino acid profiling between legume *tempe* by using GC-MS and LC-MS.

The GC-MS analysis was performed to see the metabolites profile of each legume *tempe*. In order to see the metabolites profile, the PCA was conducted. Based on the score plot, soybean, red bean, and garbanzo *tempe* were clustered together in the negative axis of PC1, meaning that those legumes have similar metabolite profiles. At the same time, white bean and green pea *tempe* have similar metabolites characteristics: a higher amount of amino acids such as glutamic acid and aspartic acid.

Analysis of various legume *tempe* samples was also conducted by GC-MS analysis. Here, we would like to explain the general view of the differences between legume *tempe* aqueous extract. Based on GC-MS analysis, sugar, organic acid, amino acid, and other metabolites were detected. The MSP Library containing RI and EI-MS from our internal experimental data has been annotated with 91 metabolite peaks. The complete list of annotated metabolites of legumes *tempe* is shown in Table S9.

Figure 2A shows the score plot from PCA of *tempe* from various legumes. As seen in Figure 2A, green pea and white bean *tempe* were clustered on the positive part of PC1 with a 51.1% variance. Meanwhile, Soybean, red kidney bean and garbanzo *tempes* were clustered in

negative PC1 and explained by 51.1% of the variance. Figure 2B showed metabolites accumulated in various legume *tempe*. Fumaric acid, aspartic acid and glutamic acid were major metabolites accumulated in the positive axis of PC1. While tryptophan, 3-hydroxy anthranilic acid and 2-aminoethanol were metabolites accumulated in the negative axis of PC1 (Table S10).

Based on PC2, only white bean *tempe* is in the negative axis of PC2. White bean tempe accumulated a higher amount of 5-aminovaleric acid and 3-hydroxyvaleric acid than other legume *tempe*. The 5-aminovaleric acid is a weak gamma-aminobutyric acid (GABA).

Based on a study by Zhao et al., in 2022, 5-aminovaleric acid gradually increases the risk of adverse clinical outcomes in heart failure patients. Meanwhile, the 3-hydroxyvaleric acid is a short-chain fatty acid derived from a valeric acid. As a previous study mentioned, short-chain fatty acids are proven to have a beneficial impact on human health. This finding also aligns with the earlier study stating that the fermentation process significantly increases bioactive compounds such as short fatty acid chains (Annunziata et al. 2020). Legumes, especially white beans, are considered a source of sugar and non-digestible carbohydrates that can be used by microorganisms in the fermentation process to produce short-chain fatty acid compounds such as 3-hydroxyvaleric acid. Previous metabolomics studies also found that legumes before fermentation contain various sugars, such as sucrose and sorbose. Then, during the fermentation process, microorganisms utilize the sugars to be processed into some short fragment compounds such as amino acids and short-chain fatty acids (Erkan et al. 2020).

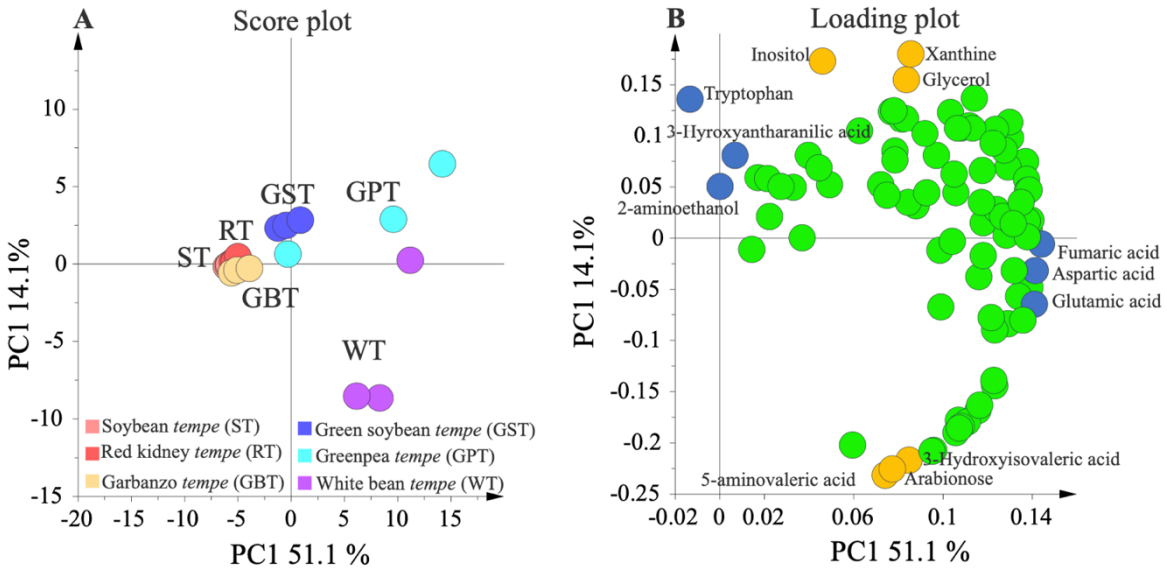


Figure 3.2. Principal component analysis (PCA) of different legume *tempe* based on GC-MS analysis. **A.** Score plot of legumes based on GC-MS analysis. Points indicate *tempe examples* (ST: Soybean *tempe;* RT: Red kidney bean *tempe*; GST: Green soybean *tempe*; GBT: Garbanzo *tempe;* GPT: Green pea *tempe;* WT: White bean *tempe*. **B.** Loading plot of legumes in GC-MS analysis. Blue dots indicate major metabolites in PC1, and yellow dots indicate major metabolites in PC2. Ninety-two annotated metabolites from GC-MS analysis were auto-scaled prior to PCA.

Figure 3. shows the representative metabolites in the loading plot of PC1 and PC2. Metabolites showing higher concentrations in green pea and white bean *tempe* based on PC1 were glutamic acids, aspartic acid and fumaric acid. Conversely, soybean, red kidney bean *tempe* samples had higher tryptophan concentrations based on PC1. Meanwhile, 5-aminovaleric acid, 3-hydroxyisovaleric acid and arabinose were major metabolites accumulated in white bean *tempe* based on PC2. The intensity of each metabolite was normalized using an internal standard, ribitol. Ribitol was chosen due to its stability and nonexistent in the legume *tempe* samples.

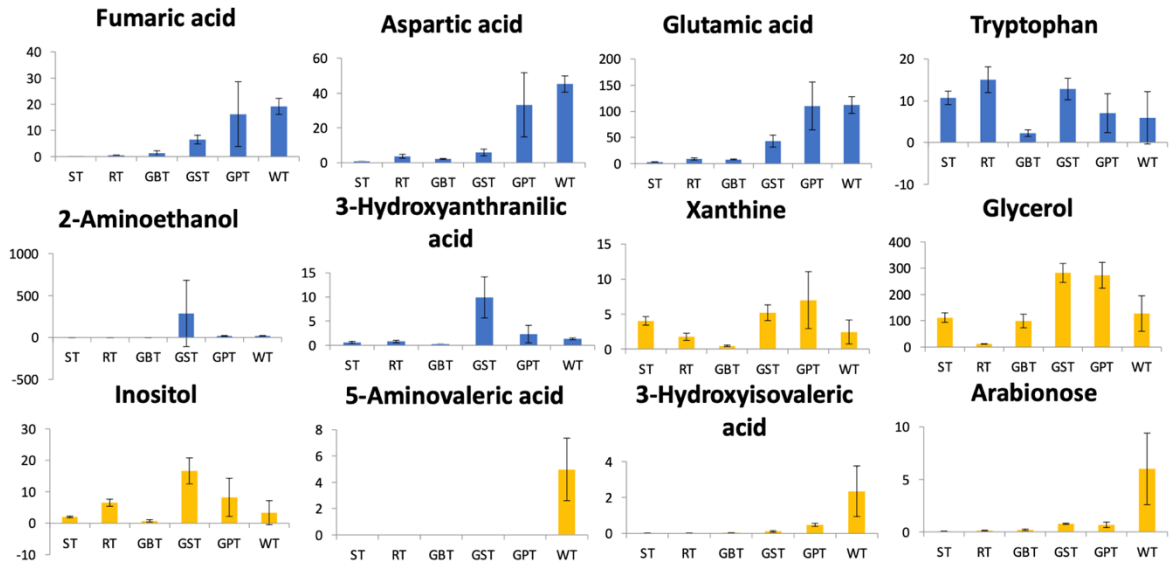


Figure 3.3. Bar graph of major metabolites accumulated in PC1 and PC2 of legume *tempe*. The internal standard normalized the relative intensity of major metabolites. The vertical axis represents the relative intensity of metabolites, and the horizontal axis represents the legume *tempe* sample. Blue colours indicate major metabolites in PC1, and yellow colours indicate major metabolites in PC2. Labelled: ST: soybean *tempe;* RT: red kidney bean *tempe*; GST: green soybean *tempe*; GBT: Garbanzo *tempe;* GPT: Green pea *tempe;* WT: White bean *tempe*

Based on results each legume *tempe* contains nine essential amino acids except arginine (. It is also proved by previous literature that stated *tempe* contains nine essential amino acids. The number of essential amino acids is also correlated with higher biological value of protein in food. Based on a previous study by (Astawan et al. 2022), the biological value (BV) and net protein utilization (NPU) of *tempe* related to protein availability are comparable to casein standard (<99%). The food that has a biological value >70% is considered as food that could provide growth if we consumed it in adequate amount and balance with sufficient energy amount (Astawan et al. 2022). This fact also could fulfil the nutrient requirement, especially protein intake for the baby which is in the recommended percentage w/w protein of 15.0% minimum if the NPU (net protein

utilization) is above 80, but if it is within 60–80, a minimum level of 20% is required in every weaning/infant supplementary food (Kluvitse 1999).

In terms of amino acids profile, based on a previous study in 2014 by Bujang and Taib, pre-treatment and fermentation of *tempe* process up until 24 h increase the amino acid content. The number of amino acid content might not fulfil the amino acid composition of the weaning food blends for infants (0-12 months) based on FAO/WHO is shown in the figure. The infant's diet in the first year of life must contain adequate quantities of amino acids, essential fatty acids such as linoleic and α-linolenic acid, and n-3 and n-6 PUFAs usually come from the mother's milk, not from the weaning foods. Therefore, weaning food can be used as a good source of nutrients to fulfil the baby's needs. However, a total protein *tempe* (13 g/ day) could meet the recommended dietary allowance (RDA) for infants (0-12 months), which is 14 grams/day. However, further study is needed to clarify since this analysis is not covered in this study.

Amino acid (g/ 100g protein	FAO/ WHO
Lysine*	5.80
Histidine *	1.90
Arginine	-
Aspartic acid	-
Threonine *	3.40
Serine	-
Glutamic acid	-
Proline	-
Glycine	-
Alanine	-
Cysteine	2.50
Valine *	3.50
Methionine *	2.50
Isoleucine *	2.80
Leucine *	6.60
Tyrosine	6.30
Phenylanine *	6.30

FAO/WHO (1985)
* Essential Amino Acid

Figure 3.4. The amino acid composition of weaning food for infants (0-12 months) is based on FAO/WHO (1985) in Modu et al. 2010

Amino acids	Pre-treatments			Fermentation time		
	Raw	Soaking	Steaming	18 h	24 h	30 h
Histidine	0.83±0.02^{d}	0.96±0.02^{c}	0.68±0.01^{e}	0.96±0.05^{c}	1.55±0.02^{a}	1.42±0.01^{b}
Isoleucine	1.62±0.04^{e}	1.76±0.01^{d}	1.54±0.03^{f}	1.97±0.01^{c}	3.01±0.01^{a}	2.90±0.01^{b}
Leusine	2.81±0.02^{d}	2.97±0.05^{c}	2.72±0.01^{e}	2.98±0.02^{c}	4.85±0.01^{a}	4.75±0.01^{b}
Lysine	2.03±0.02^{e}	2.15±0.05^{d}	1.92±0.08^{f}	2.32±0.05^{c}	3.67±0.03^{a}	3.29±0.04^{b}
Methionine	0.45±0.01^{d}	0.55±0.03^{c}	0.41±0.01^{e}	0.54±0.01^{c}	1.13±0.01^{a}	1.04±0.03^{b}
Phenylalanine	2.06±0.03^{e}	2.17±0.03^{d}	1.99±0.01^{f}	2.34±0.02^{c}	3.78±0.01^{a}	3.54±0.01^{b}
Threonine	0.59±0.03^{e}	0.63±0.01^{d}	0.52±0.01^{f}	0.76±0.01^{c}	0.92±0.01^{a}	0.87±0.01^{b}
Valine	2.16±0.27^{f}	2.66±0.04^{d}	2.29±0.01^{e}	2.87±0.01^{c}	3.44±0.02^{a}	3.33±0.01^{b}
Essential amino acids	*12.55±0.30^{e}*	*13.85±0.13^{d}*	*12.07±0.19^{f}*	*14.74±0.42^{c}*	*22.35±0.35^{a}*	*21.14±0.13^{b}*
Alanine	1.55±0.02^{d}	1.74±0.04^{c}	1.52±0.02^{e}	1.75±0.01^{c}	2.06±0.04^{a}	1.82±0.01^{b}
Arginine	0.22±0.03^{d}	0.25±0.01^{d}	0.21±0.04^{d}	0.34±0.01^{c}	0.69±0.01^{a}	0.57±0.02^{b}
Aspartic acid	5.20±0.02^{e}	5.41±0.02^{c}	5.08±0.01^{f}	5.36±0.01^{d}	5.86±0.05^{a}	5.71±0.01^{b}
Cystiene	0.69±0.03^{e}	0.84±0.01^{d}	0.61±0.01^{f}	0.97±0.01^{c}	1.44±0.01^{a}	1.32±0.01^{b}
Glutamic acid	8.88±0.10^{bc}	9.30±0.38^{b}	8.61±0.14^{d}	9.00±0.01^{bc}	9.88±0.02^{a}	9.76±0.02^{b}
Glysine	2.04±0.05^{e}	2.40±0.04^{c}	1.89±0.09^{f}	2.28±0.05^{d}	2.75±0.15^{a}	2.55±0.02^{b}
Proline	1.77±0.03^{e}	1.85±0.02^{d}	1.62±0.02^{f}	1.97±0.02^{c}	2.50±0.05^{a}	2.37±0.01^{b}
Serine	0.23±0.02^{e}	0.37±0.01^{c}	0.18±0.01^{f}	0.32±0.01^{d}	0.55±0.01^{a}	0.51±0.01^{b}
Tyrosine	1.51±0.07^{e}	1.65±0.03^{d}	1.35±0.04^{f}	1.76±0.01^{c}	2.39±0.02^{a}	2.21±0.01^{b}
Non-essential amino acids	*22.09±0.22^{e}*	*23.81±0.47^{d}*	*21.07±0.28^{f}*	*23.75±0.26^{c}*	*28.12±0.21^{a}*	*26.82±0.12^{b}*
Total amino acids content	34.64±0.26	37.66±0.30	33.14±0.24	38.49±0.34	50.47±0.28	47.96±0.13

Data are mean ±SD (standard deviation) of n=3
Means with different letter for the same row are significantly different (p<0.05)

Figure 3.5. Amino acid composition of pre-treatment and fermentation process of making *tempe* soybean based on Bujang and Taib in 2014.

3.3.3 DL-Amino acid profile of legume *tempe* based on LC-MS analysis

GC-MS is used to quantify many metabolites and is recognized as a susceptible and reproducible tool. This non-targeted analysis focuses on polar, low-weight molecular compounds, such as sugars, organic acids, and amino acids. However, it is required some extraction and derivatization processes before analysis. Meanwhile, LC-MS is also used as one of the techniques in metabolomics for profiling targeted soluble, such as amino acids and unknown metabolites. However, the derivatization process is not required for this analysis, and making LC-MS analysis could be used for more extensive metabolite detection.

Table 3.1 List of amino acids for targeted analysis using

D/L-Alanine	D/L-Glutamine	D/L-*allo*-Isoleucine	Proline
D/L-Arginine	D/L-Glutamic acid	D/L-Leucine	D/L-Threonine
D/L-Asparagine	Glycine	D/L-Lysine	D/L-*allo*-Threonine
D/L-Aspartic acid	D/L-Histidine	D/L-Methionine	D/L-Tryptophan
D/L-Cysteine	D/L-Isoleucine	D/L-Phenylalanine	D/L-Tyrosine
			D/L-Valine

The amino acid profiles based on GC-MS data analysis were not enantioselective, indicating that each amino acid included information from D- and L-amino acids. The LC-MS analysis was performed to obtain the DL-amino acid profile of each legume *tempe*. In this analysis, A CROWNPAK CR-I (+) and (-) were used to separate the targeted metabolites simultaneously: D-lysine, L-glutamine, D-threonine, D-*allo*-threonine and D-homoserine, D-isoleucine, D-*allo*-isoleucine.

Figure 3.4A shows the PCA score plot of the legume *tempe* sample. Based on PC1, white bean, soybean, and garbanzo *tempe* were clustered together in the negative axis of PC1 with 44.4% of the variances. As shown in the PCA loading plot in Figure 3.4B, some D-amino acids (D-Leu, D-Phe, D-Tyr, D-Asp, D-allo-Ile, D-Thr, D-Glu, D-Trp) were clustered in the negative axis of PC1. Those D-amino acids were accumulated in white bean *tempe,* shown in Figure 3.5

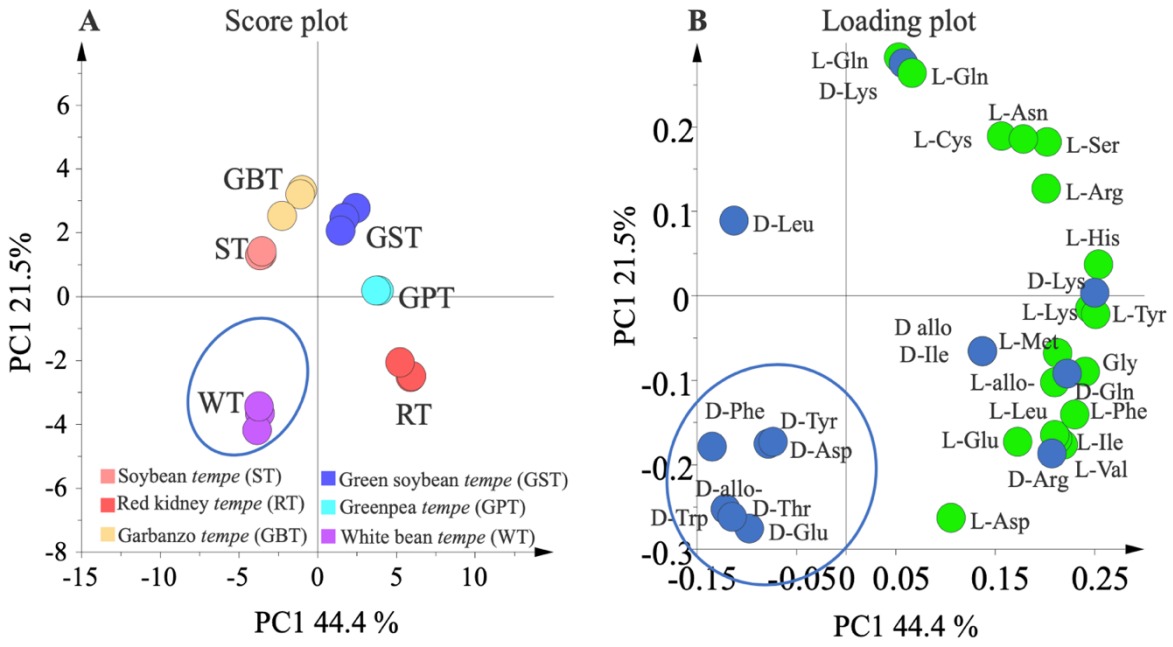


Figure 3.4. Principal component analysis (PCA) of different legume *tempe samples* based on LC-MS. **A.** Score plot of legumes based on LC-MS analysis. Points indicate legume types ST: Soybean *tempe;* RT: Red kidney bean *tempe*; GST: Green soybean *tempe*; GBT: Garbanzo *tempe;* GPT: Green pea *tempe;* WT: White bean *tempe* **B.** Loading plot of legumes in LC-MS analysis. Blue dots indicate D-amino acid; green dots indicate L-amino acids

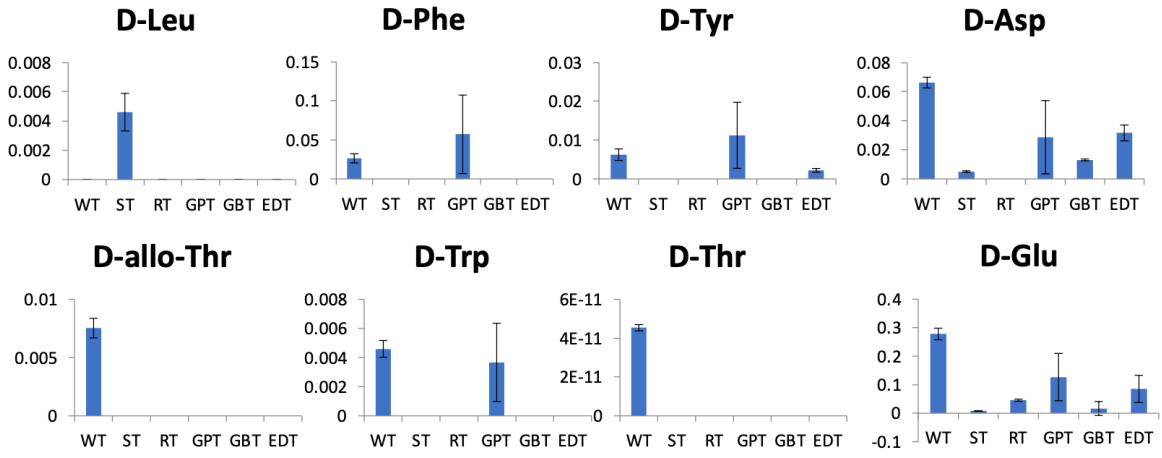


Figure 3.5. Bar graph of the relative intensity of major D-amino acids in legume *tempe* clustered in the negative axis of PC1 based on LC-MS analysis. The internal standard normalised the relative

intensity of the metabolites. The vertical axis represents D-amino acid relative intensity, and the horizontal axis represents the legume *tempe* sample. Labelled: ST: Soybean *tempe;* RT: Red kidney bean *tempe*; GST: Green soybean *tempe*; GBT: Garbanzo *tempe;* GPT: Green pea *tempe;* WT: White bean *tempe.*

The PCA has been performed to access the amino acid profile based on the peak area ratio of targeted metabolites in legume *tempe* (Fig 3.4). In total, 33 compounds were detected, including both $_{D}$- and $_{L}$-forms of amino acids. The PC1 represented 44.4% of total variances, and PC2 represented 21.1% of total variances of samples. Based on PC1, the white bean, soybean and garbanzo *tempe* were clustered together in the negative axis of PC1. In the loading plot, $_{D}$-leucine, $_{D}$-phenylalanine, $_{D}$-tyrosine, $_{D}$- asparagine, and $_{D}$-allo-threonine, $_{D}$-threonine, $_{D}$-tryptophan, and $_{D}$-glutamic acid were found abundance in the negative axis of PC1. Based on a previous study, these $_{D}$-amino acids may contribute to food characteristics (Taniguchi et al. 2019). Another study also reported that $_{D}$-amino acids are important compounds of the peptidoglycan in the bacteria cell wall. They also have a role in producing different tastes and flavors in foods (Marcone et al. 2019). Therefore, the determination of enantioselective amino acid profiles provides more detailed and helpful information than non-enantioselective amino acid profile analysis for further food product development.

Meanwhile, all L-amino acids were higher in the positive axis of PC1. Some $_{D}$-amino acids such as $_{D}$-lysine, $_{D}$-allo-isoleucine, $_{D}$-isoleucine, $_{D}$-arginine, and $_{D}$-glutamine were also accumulated higher in green soybean, green pea and red kidney bean *tempe*. These findings were in line with the previous report that stated fermented food products rich in $_{D}$- amino acids formation, especially $_{D}$-glutamic acid and $_{D}$-asparagine, which have been used as an indicator of ageing due to the fermentation process (Gobbetti et al. 1994).

This result is the first to study various D-amino acids in legume *tempes*. Some D-amino acids exhibited characteristic profiles of each legume *tempe* (white bean, soybean, and garbanzo *tempe* and green soybean, green pea and red kidney bean *tempe*).

3.4 Conclusion

Based on In vitro protein digestibility data analysis, it was found that the fermentation process increases the protein availability at intestinal absorption of various legumes significantly. Based on metabolites data from GC-MS analysis, each legume *tempe* accumulated different types of amino acids as important metabolites; for example, white bean and green pea *tempe* were clustered together based on PCA with a high accumulation of aspartic acid and glutamic acid. At the same time, LC-MS data analysis showed that most D-amino acids were found in white bean *tempe.* As reported in another study, D-amino acids are known as important compounds of the peptidoglycan in the bacteria cell wall. While all L-amino acids were found higher in the positive axis of PC1 accumulated in green pea, garbanzo, and red kidney bean *tempe*. Some D-amino acids such as D-lysine, D-allo-isoleucine, D-isoleucine, D-arginine, and D-glutamine were also accumulated higher in green soybean, green pea, and red kidney bean *tempe*, which may contribute to the better food characteristics such as taste and flavour. In term of the accumulation of essential amino acids, *tempe* samples had nine essential amino acid that is important for growth. Based on those data, legume *tempe* samples are suitable to be processed into a baby food product. However, in the next chapter, we choose red kidney bean and soybean *tempe* for further analysis, due to their availability in Indonesia as the targeted country for the development of baby food products. For further analysis, the changes in metabolites profile of legume *tempe* during processing such as drying and mixing treatment need to be conducted. This study could be used as a basis for data in

profiling metabolites and amino acids in various legume *tempe* for further development of *tempe* based food products.

Chapter 4

Effect of different drying treatments and mixing processes on the metabolites profiling of legume *tempe* powder

4.1 Introduction

As mentioned previously, as a promising ingredient for baby food products, the legume *tempe* samples have different metabolites and amino acid profiles. However, to develop nutrient-dense baby food products, a process that could minimize the nutritional lost need to be found. Drying treatment is one of the techniques of food processing that has several purposes. One of the common purposes is to create new food variances. However, there is a high chance of applying this process leading to nutrient loss. Therefore, the evaluation of different drying treatments and mixing processes needs to be conducted.

Based on the conventional method that targeted only one compound such as protein, various treatments for producing *tempe* give significant differences in food products. Based on the study which was conducted in 2014 by Bujang & Taib revealed that the soaking and steaming processes were given different effects on the amino acid contents of legume *tempe*. The amino acid contents were significantly increased after soaking. While, after steaming processes amino acid contents were significantly decreased. The physicochemical properties resulting from the applied process need to be understood to determine the suitable method.

The oven-drying method is the most popular drying method compared to other methods such as spray-drying, freeze-drying, and vacuum-drying. Each drying method will yield a powder of different properties. Research by (Chen et al. 2021)1 found that freeze-drying is better than spray-drying because the freeze-dried powder has better color and greater aroma scores.

In this chapter, GC-MS based metabolites profiling was performed to find out the effect of different drying treatments and mixing processes on the metabolites profile of legume *tempe*. Gas chromatography was recognized as highly sensitive, with good reproducibility and quantitation for analyzing a lot of metabolites by using one-step extraction (Jumhawan et al. 2015). The result from GC-MS analysis is usually classified by using multivariate analysis. The principal component analysis (PCA) was used widely in metabolomics as an unsupervised method that could explain high throughput metabolite data. Subsequently, orthogonal projection to latent structures with discriminant analysis (OPLS-DA) was used to find statistically significant metabolites as discriminant candidates (Kobayashi et al., 2012).

The OPLS-DA is a model of regression analysis that is usually applied to elaborate the relationship between *X* as a descriptor and *Y* as a response in data matric. The examples of the *X* matrix are chromatographic, or spectroscopic in N samples and mass-to-charge ratio. While the *Y* matrix is often represented by phenotypic properties such as sensory profile, tolerance, toxicity etc. This model could also reveal the important metabolites of the samples (Kobayashi et al. 2012; Jumhawan et al. 2013)

This study will be the first in metabolomics of legume *tempe* that explores the metabolites different in different drying treatments and mixing processes that can be used as a basic selection of drying treatments that retain nutrition content for further product development such as baby food.

4.2 Material and methods

4.2.1 Legume *tempe* samples

Two selected legumes based on previous chapter results were chosen as a *tempe* raw material. Each legume was purchased from Indonesia's local market. All legumes were produced to become a *tempe* in the Laboratory of Bioresource Engineering (metabolomics), Osaka University, Japan and Rumah Tempe Indonesia (RTI). The legume was weighed and washed before being soaked overnight using tap water. Then, the overnight legume was boiled for about 20 minutes and then soaked again for another night before it was dehulled and rinsed with boiling water. The dehulled legume was dried and inoculated by the *tempe* starter Raprima®, then fermented in the incubator for about 48 hours at 30 ^{0}C until it became a compact cake fully covered by the mold (*tempe*).

All Tempe legumes were cut into four pieces of 3x3 cm size and put inside the 50 mL grinding tube. After that, it was lyophilized overnight using a VD-800F Freeze dryer (Taitec, Saitama, Japan) and powdered using a multi-bead shocker (Yasui Kikai, Osaka, Japan) for 10 seconds, twice. The powdered *tempe* samples were stored at -30^{0}C until analysis.

4.2.2 Extraction and derivatization of legume *tempe* samples for GC-MS analysis

The ten milligrams of each legume *tempe* were extracted by using the solvent mixture of methanol (Wako Chemical, Osaka, Japan), chloroform (Kishida Chemical Co. Ltd, Osaka, Japan), and ultrapure water (Wako Chemical, Osaka, Japan); in the ratio 5:2:2 containing 50 µL/mL of internal standard (ribitol (Fujifilm Wako Pure Chemical Industries, Ltd. Osaka, Japan)). The mixture was incubated at 37 ^{0}C, and 1200 rpm for 30 minutes and then centrifuged for 3 min at 4^{0}C. Four hundred microliters of supernatant were mixed with 300 µL of ultra-pure water (Wako Chemical, Osaka, Japan) using vortex and centrifuge for 3 min at 4 ^{0}C. Two hundred microliters

of supernatant were transferred into a new 1.5 mL microtube, and another 200 µL of each supernatant was pooled as a quality control into a 10 mL Eppendorf® tube. Then, the quality control mixture was transferred into another 1.5 mL microtube to be concentrated in the centrifugal concentrator for about 1 hour, together with another supernatant of samples. The concentrated sample was put into a freeze dryer for overnight lyophilization. After that, the overnight samples were mixed with 100 µL of methoxamine hydrochloride in pyridine (20 mg/mL) and then incubated for about 90 minutes at 30 ^{0}C at Thermo-shaker (Eppendorf Ltd., Hamburg, Germany), followed by a silylation process using 50 µL of N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) (GL Sciences) to each sample. After that, samples were incubated for 30 minutes at 37 ^{0}C and transferred to a GC vial for analysis.

4.2.3 GC-MS Analysis and Data Analysis

The GC-MS QP2010 Ultra (Shimadzu, Kyoto, Japan) equipped with an Inert-Cap 5 MS/NP column (GL Sciences) has been used for analysis. Before the analysis, a leak check and tuning of the mass spectrometer were done before analysis. One microliter of prepared samples was injected in split mode, 25:1 (*v*/*v*), with an injection temperature of 230 °C. An alkene mixture (C8-C40) was used and injected as a standard for peak identification. Helium was used as carrier gas. The flow of He was 1.12 mL/min with a linear velocity of 39 cm/s. The column temperature was held at 80 °C for 2 min, increased by 15 °C/min to 330 °C, and then held for 6 min. The transfer line and ion source temperatures were 250 and 200 °C, respectively. Ions were generated by electron ionization (EI) at 0.94 kV. Spectra were recorded at 10,000 u/s (check value) over the mass range *m*/*z* 85−500.

The data obtained from GC-MS analysis was converted to an AIA file by using the GC-MS solution software package (Shimadzu, Kyoto, Japan). Then, MS-Dial ver. 4.00 software is

used for peak alignment, filtering, and annotation using the GC-MS-5MP Library (Riken, Kanagawa, Japan).

4.2.4 Statistical analysis

The obtained metabolites (annotated and unknown) were normalized to ribitol as an internal standard to obtain the normalized peak height information. These normalized peak values were screened using two parameters: (1) relative standard deviation (RSD) below 30%, and (2) the concentration of compounds in the QC samples was more than three times that of the blank samples. Compounds obtained from the screening were then auto-scaled and subjected to multivariate analysis, principal component analysis (PCA), and orthogonal projection to latent structures with discriminant analysis (OPLS-DA), using SIMCA-P version 13 (Umetrics, Umea, Sweden).

4.3 Result and discussion

4.3.1 Metabolites profile of legume *tempe* dried in the different drying processes

In a previous chapter, the differences in metabolites profiling of each legume *tempe* have been analyzed. In this chapter, the metabolites profiling of dried legume *tempe* in different drying treatments was done by using GC-MS analysis. The aqueous extracts of soybean and red kidney bean *tempe* were dried by using an oven dryer, freeze crusher and freeze dryer and then analyzed to investigate the effect of drying treatment on the metabolites profile of legume *tempe*.

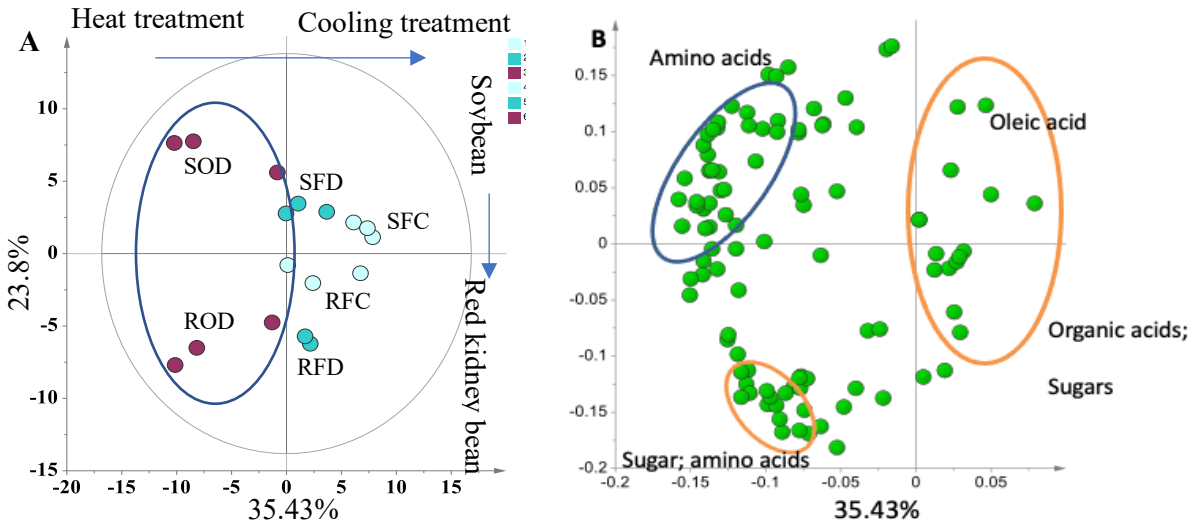


Figure 4.1 Principal component analysis (PCA) of legume *tempe* in different types of drying methods on GC-MS analysis. **A.** Score plot soybean and red kidney bean *tempe* powder dried by an oven, freeze dryer and freeze crusher. Points indicate *tempe* with different drying treatments. **B.** Loading plot of legumes *tempe* in different types of drying methods on GC-MS analysis.

The PCA was used to visualize the information based on sample variances. Based on the result shown in figure 4.1, samples which were dried by oven-drying treatment are clustered in the negative axis of PC1 with 35.43% of variance regardless of the type of legumes. While freeze-dried and freeze crushed samples are clustered in the positive axis of PC1. A total of 102 metabolites were tentatively annotated. Amino acids, organic acids, sugars, and other compounds were found in those 102 tentatively annotated metabolites. The oven-dried samples set were mainly supported by the amino acids. Based on PC1, the freeze-dried and freeze crushed samples were dominated by the sugars and organic compounds. While the oven-dried samples were dominated by amino acids and one fatty acid which is oleic acid (Table S11). The high concentration of oleic acid in soybean *tempe* was predicted. Based on a previous study, the fermentation process especially during the most active phase of fungal growth resulted in an increase in the concentration of oleic acid while palmitic and linoleic acid were decreased (Bisping, et.al. 1993)

The separation based on PC2 projected the differences of legume *tempe*. Red kidney bean *tempe* in all different drying treatments were clustered together on the negative axis of PC2. However, the soybean *tempe* in all different drying treatments were clustered on the positive axis of PC2. In the loading plot of PC2, the soybean *tempe* contained genistein, daidzein, pyroglutamic acid, and glutamine. Even though the differences in the type of legume *tempe* can be seen on the

PC2, the variances of PC1 are higher than PC2, meaning that the drying treatment gives a significant contribution to metabolites profiling.

4.3.2 Metabolites profile of legume *tempe* dried in different oven drying temperature

For further clarification and to find the most suitable drying temperature by using the oven. We subjected three of each legume *tempe* dried at three different temperatures (45 ^{0}C, 50 ^{0}C, 60 ^{0}C) to GC-MS analysis. Based on data in figure 4.2, the PC1 reflected the difference based on the variance of legume *tempe* with 50.7% of variances. While in PC 2, the samples were separated based on oven-drying temperature regardless type of legume *tempe*. The samples that were dried at 60 ^{0}C were clustered in the negative axis of PC2. Meanwhile, the samples were dried at 45 and 50 ^{0}C were clustered at the positive axis of PC2.

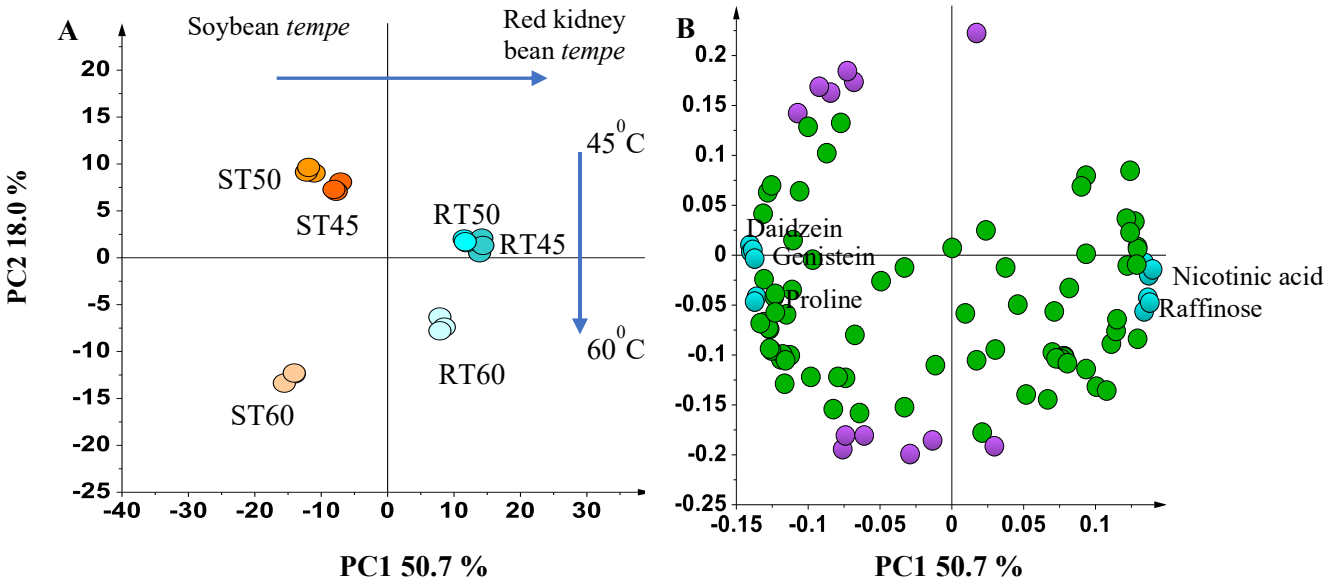


Figure 4.2. Principal component analysis (PCA) of legume *tempe* in different drying treatment temperatures on GC-MS analysis. **A.** Score plot legume *tempe* dried at different temperatures. Points indicate *tempe* powder (ST: Soybean *tempe;* RT: Red kidney bean *tempe*;; dried at 45^{0}C; 50^{0}C; 60^{0}C) **B.** Loading plot of legumes in GC-MS analysis. Blue dots indicate major metabolites in PC1, and purple dots indicate major metabolites in PC2, green dots are other.

However, the amino acid content of each legume dried at different oven temperatures did not significantly change, as shown in **figure 4.3.**

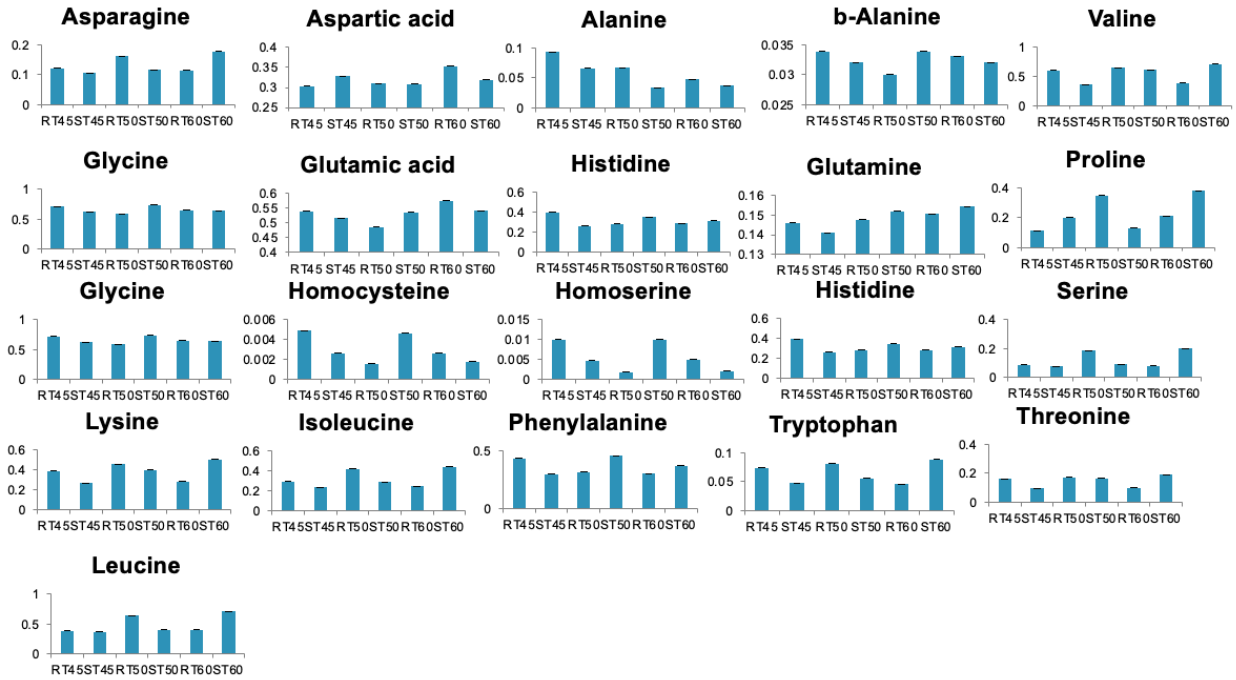


Figure 4.3. Bar graph of amino acid content in legume *tempe* in different drying treatment temperatures by GC-MS analysis. (ST: Soybean *tempe;* RT: Red kidney bean *tempe*;; dried at 45^0C; 50^0C; 60^0C)

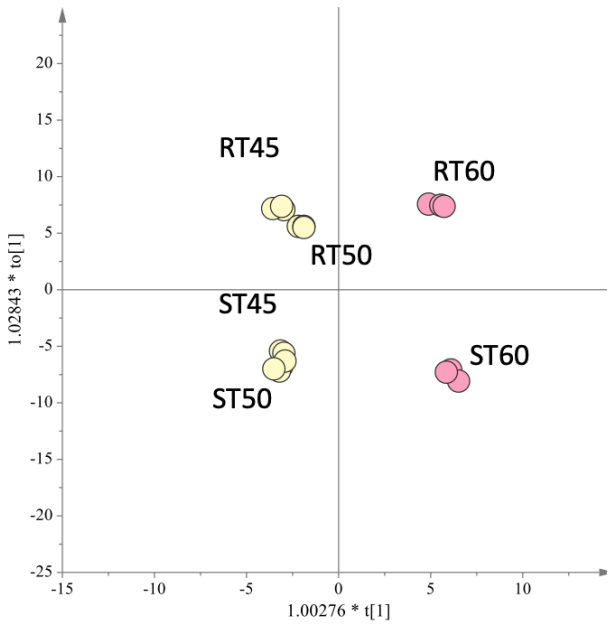


Figure 4.4. OPLS-DA score plot from metabolome data sets of oven-dried legume *tempe* dried at 45; 50 ^{0}C and 60 ^{0}C. Data were analyzed using SIMCA-P+ version 13. Metabolome data was obtained by GC/MS analysis. The yellow color represents oven-dried legume *tempe* dried at 45;50 ^{0}C. The red color represents oven-dried legume *tempe* dried at 60 ^{0}C.

The separation based on the type of legume is greater than the effect of treatment on the metabolites profile of legume *tempe*. Therefore, to observe more in detail, the effect of different temperatures on the metabolite profile of legume *tempe*, we subjected the data to the supervised discriminant analysis (OPLS-DA). The OPLS-DA method was selected due to the advantages of this method in selecting differentiation markers. Metabolites with reliable, and high contribution to the model will possess potentially biochemically interesting characteristics which can be selected as biomarker candidates. The excellent OPLS-DA models need to show R^2 and Q^2 values greater than 0.8(Jumhawan et al. 2013). The candidate metabolites biomarker were selected based on the VIP score which indicates the degree of importance of metabolites during model construction shown in Table S12. The coefficient indicates its correlation between response variables which are drying at 45; 50 ^{0}C and 60 ^{0}C.

Figure 4.4 is the plot of OPLS-DA for oven-dried legume *tempe* dried at 45; 50 ^{0}C and 60 ^{0}C. The OPLS-DA plots showed that oven-dried legume *tempe* dried at 45; 50 ^{0}C was significantly different from that oven-dried legume *tempe* dried at 60 ^{0}C. The metabolites that were affected by the treatment are shown in Table S13.

The metabolites that differentiate the difference between 45^{0}C; 50^{0}C and 60^{0}C drying temperatures were adenosine, uridine, inositol, N- acetyl glucosamine, and adenine. As reported in many reports uridine is one of a compound that is usually used as a dietary supplement that has health benefits for humans (Dobolyi et al. 2011; Zhao et al. 2017). This uridine is a nucleoside and

nucleobases that may be involved in nucleic acid synthesis from the fermentation process. While the N-acetyl glucosamine is the metabolite which is originating from precursor fructose-6-phosphate in glycolysis. This metabolite has an important role in the prevention and treatment of arthritis (Ma et al. 2021).

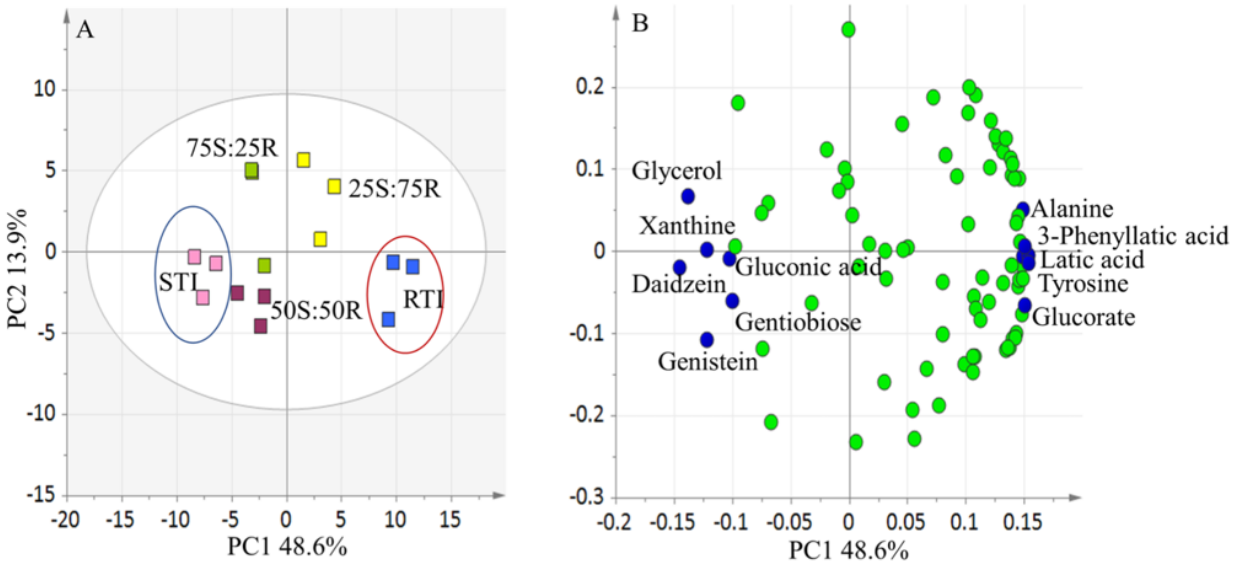
4.3.3 Metabolites difference in mixed *tempe*

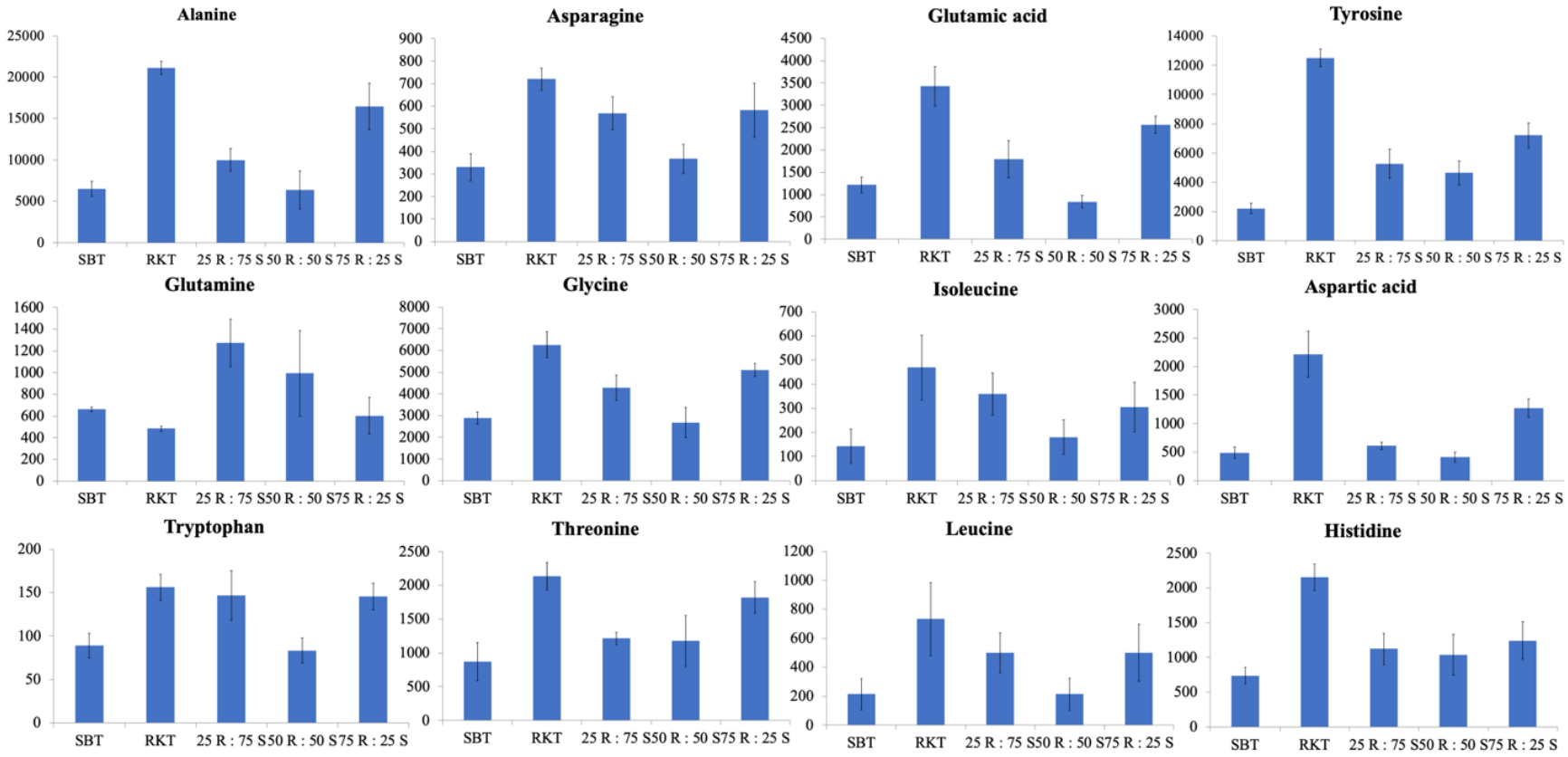
In Indonesia, soybean *tempe* is the most consumed *tempe* compared to other legume *tempes*. However, based on the PCA results, the red kidney bean *tempe* showed an amino acid profile different from the soybean *tempe*. Therefore, the combination of soybean *tempe* and red kidney bean *tempe* might improve the amino acid profile for further development of nutritional baby food.

Another PCA dataset was used to analyze the differences in the metabolite profiles between single legume *tempe* and mixed *tempe* (soybean and red kidney bean) (Fig. 4.5). The soybean and red kidney bean *tempes* powder were mixed in different ratios to determine the optimum combination ratio related to the amino acid profile and other metabolites that might be improved after combination treatment.

The PCA showed that PC1 with a 48.6% variance and PC2 with a 13.9% variance could be used to visualize the metabolomic profile differences between mixed legume and single legume *tempe* samples produced in Indonesia (Fig. 4.5B). Based on PC1, the separation of soybean and red kidney beans was clearly observed. Daidzein, genistein, xanthin, glycerol, and gluconic acid were found to positively contribute to soybean *tempe*, located in negative PC1 (Table S14).. This finding was similar to a previous study, which stated that daidzein and genistein are isoflavones commonly found in soybean products (Liu et al. 2018; Chadha et al. 2017). The positive part of PC1 mostly described the metabolites present in the red kidney bean *tempe*, including alanine, 3-

phenyl lactic acid, lactic acid, tyrosine, and glucarate. Additionally, nicotinic acid (Vit B3) content was found to be highest in red kidney bean *tempe* compared with other legume *tempes*.




Alanine
Asparagine
Glutamic acid
Tyrosine
Glutamine
Glycine
Isoleucine
Aspartic acid
Tryptophan
Threonine
Leucine
Histidine
SBT
RKT
25 R : 75 S
50 R : 50 S
75 R : 25 S

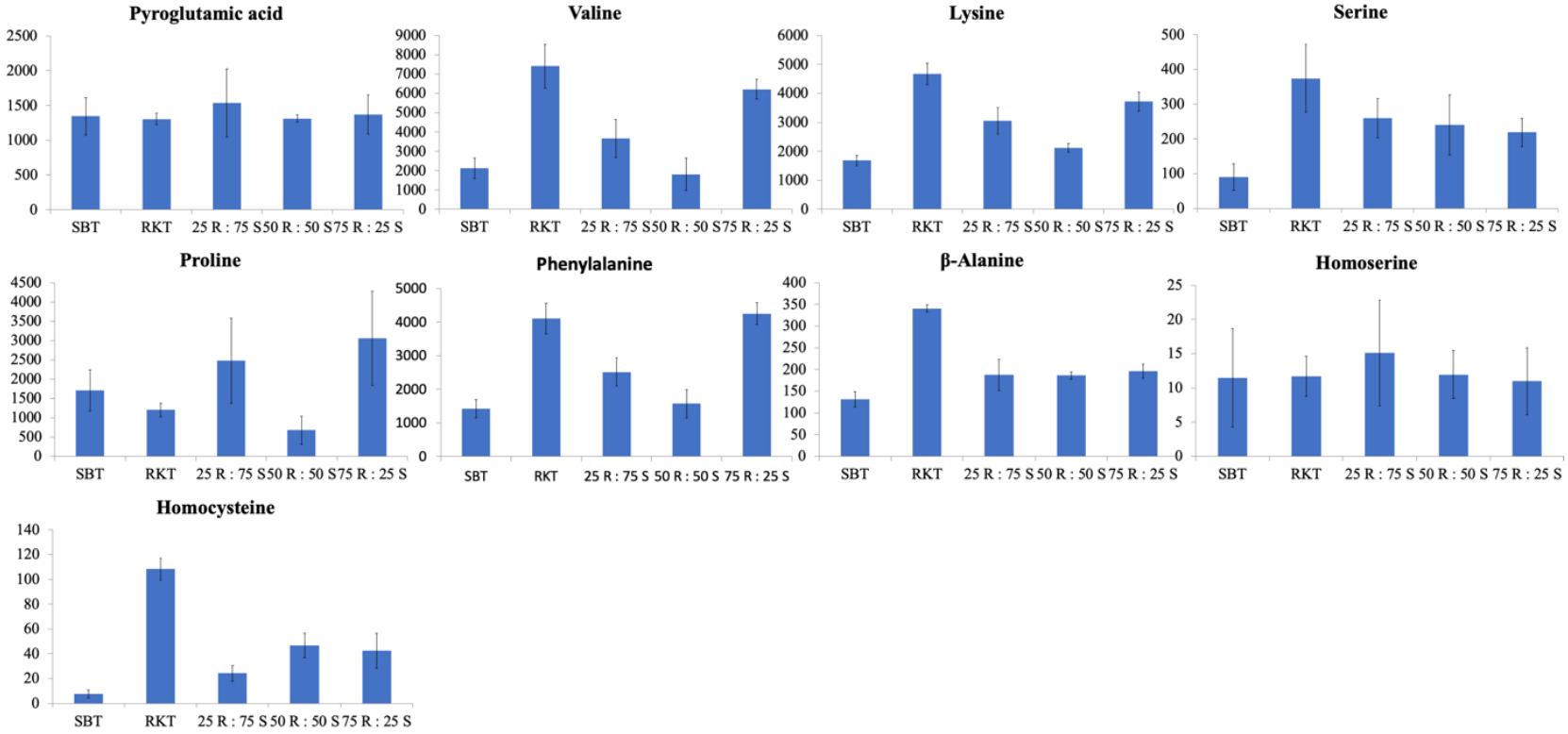

Pyroglutamic acid
2500
2000
1500
1000
500
0
SBT
RKT
25 R : 75 S
50 R : 50 S
75 R : 25 S
Valine
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
SBT
RKT
25 R : 75 S
50 R : 50 S
75 R : 25 S
Lysine
6000
5000
4000
3000
2000
1000
0
SBT
RKT
25 R : 75 S
50 R : 50 S
75 R : 25 S
Serine
500
400
300
200
100
0
SBT
RKT
25 R : 75 S
50 R : 50 S
75 R : 25 S
Proline
4500
4000
3500
3000
2500
2000
1500
1000
500
0
SBT
RKT
25 R : 75 S
50 R : 50 S
75 R : 25 S
Phenylalanine
5000
4000
3000
2000
1000
0
SBT
RKT
25 R : 75 S
50 R : 50 S
75 R : 25 S
β-Alanine
400
350
300
250
200
150
100
50
0
SBT
RKT
25 R : 75 S
50 R : 50 S
75 R : 25 S
Homoserine
25
20
15
10
5
0
SBT
RKT
25 R : 75 S
50 R : 50 S
75 R : 25 S
Homocysteine
140
120
100
80
60
40
20
0
SBT
RKT
25 R : 75 S
50 R : 50 S
75 R : 25 S

Figure 4.5. Principal component analysis (PCA) score plot and bar graph of mix *tempe,* soybean *tempe,* and red kidney bean *tempe* produced in Indonesia based on GC-MS. **A**. Score plot of mix *tempe,* soybean *tempe,* and red kidney bean**. B.** Loading plot of mix *tempe*, soybean *tempe,* and red kidney bean *tempe.* Points indicate legume types (STI: soybean *tempe*; RTI: red kidney bean *tempe;* 25R:75S: Mix *tempe (*25% of red kidney bean tempe with 75% soybean *tempe);* 50R:50S: mix *tempe* (50% red kidney bean *tempe* with 50% soybean *tempe);* 75R:25S: mix *tempe* (75% red kidney bean *tempe* with 25% soybean *tempe).* **C.** Bar graph of the amino acid content of mix *tempe,* soybean *tempe,* and red kidney bean *tempe.* The vertical axis indicates relative intensity. The horizontal axis indicates samples. The error bar shows the standard deviation from three technical replicates, in *tempe* produced in Japan and Indonesia, contributing to PC1.

Based on the bar graph shown in Fig. 4.5.C, we were able to increase the levels of amino acids that are commonly found to be low in soybean *tempe* by combining soybean and red kidney beans at a ratio of 75% and 25%, respectively.

Another treatment that might help in developing legume tempe-based baby food products was different mixing processes. In this mixing process, we try to mix the soybean *tempe* and red kidney bean *tempe* in several ways. First, we mixed each legume *tempe* before crushing while in another treatment we mixed each legume *tempe* after it became a powder.

Based on the results shown in figure 4.6, there are no significant differences between the two methods of mixing. The mixed legume *tempe* powder was clustered based on the ratio of mixing. The *tempe* powder of 25% soybean mixed with 75% of red kidney bean that was mixed before became a powder was clustered together with *tempe* powder of 25% soybean mixed with 75% of red kidney bean that was mixed after became a powder.

Based on a previous study, the mixing process could affect the nutrient content of infant formula, the difference between wet and dry mixing significantly affected not only the nutritional properties but also the rheological, and emulsifying properties (Rodríguez Arzuaga et al. 2021). However, in this study, there is no significant change in mixing processes. This phenomenon might happen due to the mixing processes that began after the drying processes which were recognized as quenching methods. In the future, the optimization of the drying process needs to be conducted for further clarification and investigation.

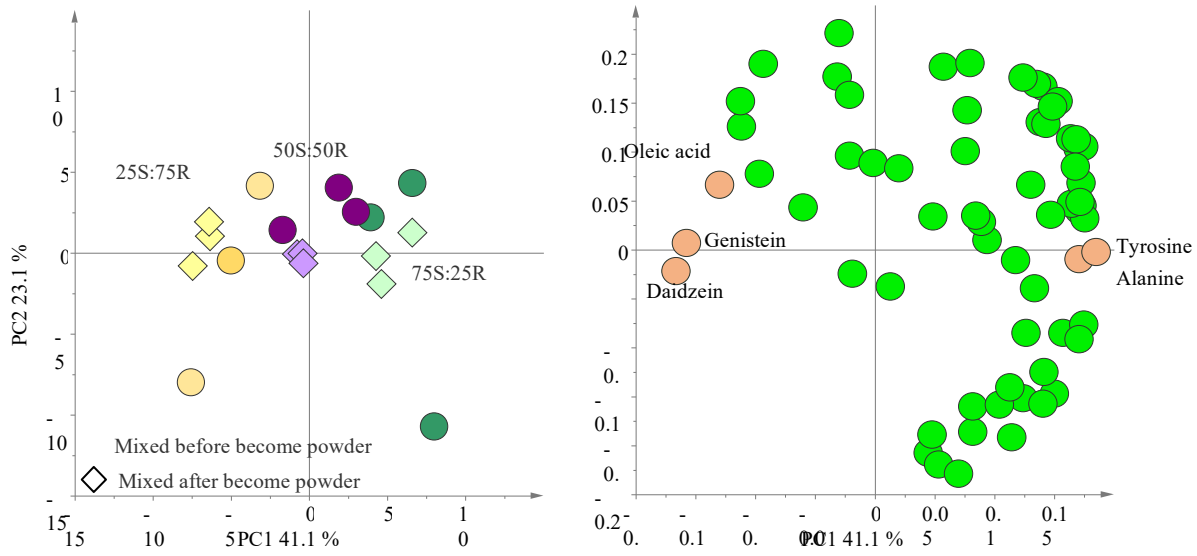


Figure 4.6 Principal component analysis (PCA) of legume *tempe* and mixed *tempe* powder based on GC-MS analysis. **A.** Score plot legume *tempe* and mixed *tempe* powder. Points indicate *tempe* and mixed *tempe* powder (ST: Soybean *tempe;* RT: Red kidney bean *tempe*; 75S:25R: *tempe* powder 75% soybean mixed with 25% red kidney bean *tempe*; 50S:50R: *tempe* powder 50% soybean mixed with 50% red kidney bean *tempe*; 25S:75R: *tempe* powder 25% soybean mixed with 75% red kidney bean *tempe* powder. **B.** Loading plot of legumes in GC-MS analysis. Blue dots indicate major metabolites in PC1, and yellow dots indicate major metabolites in PC2. Ninety-two annotated metabolites from GC-MS analysis were auto-scaled prior to PCA.

4.4 Conclusion

This study shows that oven-drying treatment was able to keep more metabolites compared with the freeze-drying and freeze-crushing methods. The more metabolites accumulated the more nutrient can be kept for further utilization of legume *tempe* as *a* baby food product ingredient. Among three of the most common drying temperature, the 60^0C oven-drying temperature accumulated more metabolites compared with 45 and 50^0C. The important metabolites that contribute to the various drying treatment are adenosine, uridine, inositol, N- acetyl glucosamine, and adenine. Red kidney bean *tempe* has amino acid profiles that are different from those of soybean *tempe*. Furthermore, the combination of soybean and red kidney bean *tempe* could improve the levels of some amino acids that were found to be present at low levels in both *tempe* samples. In further analysis, there is no significant difference in metabolites profile of legume *tempe* that was mixed before and after becoming a powder. This combination of mixed *tempes* could also improve the amino acid profile of *tempes* made from a single legume.

Chapter 5

Conclusion

This research showed that the metabolomics study can be used to comprehensively reveal the profile of metabolites in various legume and legume *tempe* samples produced in different environmental conditions. As potential candidate of baby food product ingredient with complete macro and micronutrients, the metabolite changes before and after fermentation were also captured in this study. The fermentation process is more likely significantly improved the nutritional content of legumes and it is also improved the protein quality based on increasing the percentage of protein digestibility of legume *tempe* compared with legumes before fermentation. The metabolomics also revealed that some $_{D}$-amino acids such as $_{D}$-lysine, $_{D}$-allo-isoleucine, $_{D}$-isoleucine, $_{D}$-arginine, and $_{D}$-glutamine were accumulated higher in green soybean, green pea, and red kidney bean *tempe*, which may contribute to the better food characteristics such as taste and flavor. Based on these results, we selected soybean *tempe* and red kidney bean *tempe* which are the most commonly found legume *tempe* in Indonesia to be subjected into other treatments.

The application of different treatments was done to observe the effect of food processing in the metabolites profile of legume *tempe*. The different drying treatments such as oven-dried, freeze-dried and freeze-crushed were give different metabolites profile to each legume *tempe*. However, the oven-dried process accumulated more diverse metabolites compared with freeze-crushing and freeze-drying methods, especially amino acids. Therefore, we subjected the samples to different oven-drying temperatures to observe whether the different temperatures of drying which are 45, 50 and 60 ^{0}C affected the metabolites changes in legume *tempe*. The application of 45 and 50 ^{0}C was found not significantly different. However, the application of drying treatment of 60^{0}C was significantly different from 45 and 50^{0}C. More metabolites were observed in samples

which were dried at 60^0C. The OPLS-DA result showed that the metabolites that differentiate the difference between 45^0C; 50^0C and 60^0C drying temperatures were adenosine, uridine, inositol, N- acetyl glucosamine, and adenine. In term of amino acid profile, each drying temperature not significantly change the amino acid content of each legume *tempe*. Therefore, as a common method for food drying treatment, the oven-dried at 60 ^{0}C for 6 hours is selected as the suitable method to make legume *tempe* powder. In addition, as one of the strategies to develop nutrient dense baby food products, the combination of two legume *tempe* was performed. The result showed that the combination of red kidney and soybean *tempe* improved the amino acid profile of the *tempe* powder. However, the metabolites profile was observed same statistically regardless of the way of mixing, which is mixed before becoming a powder or after becoming a powder.

As previously mentioned in the general introduction, the need for good quality with high nutrient dense baby food products is important to be solved especially in the undeveloped and developing countries to tackle the malnutrition problem in the children under 5 years old. The *tempe*-based baby food product from red kidney bean and soybean *tempe* oven-dried at 60^0C for 6 hours can be used as one promising solution to solve that issue. Therefore, based on this study further optimization and formulation of *Tempe*-based baby food products is easy to be conducted both on the pilot plan or industrial scale.

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List of publications

Original Paper

Rahmawati D, Astawan M, Putri SP, and Fukusaki E. Gas chromatography-mass spectrometry-based metabolite profiling and sensory profile of Indonesian fermented food (*tempe*) from various legumes. *Journal of Bioscience and Bioengineering*. **132,** 487-495 (2021)

Conference

1. Della Rahmawati, Made Astawan, Sastia Prama Putri, Eiichiro Fukusaki. Metabolomics study of different mixing processes of mixed *tempe* powder (Indonesian fermented food) from

soybean and red kidney bean. 73rd Annual Meeting-The Society for Biotechnology, Japan (2021). Oral presentation

2. Della Rahmawati, Made Astawan, Sastia Prama Putri, Eiichiro Fukusaki. GC-MS based metabolites profiling and sensory profiling of *tempe* from different legumes. Metabolomic Society (MetSoc) (2020) – Poster presentation

Supplementary Tables and Figures

Table S1. List of samples

Sample code	Legume origin	Sample type	Production place
Sample set I			
SB	Indonesia	Soybean	-
RB		Red kidney bean	-
CB		Cowpea bean	-
JB		Jack bean	-
Sample set II			
STJ	Indonesia	Soybean tempe	Japan
RTJ		Red kidney bean tempe	Japan
CTJ		Cowpea bean tempe	Japan
JTJ		Jack bean tempe	Japan
STI	Indonesia	Soybean tempe	Indonesia
RTI		Red kidney bean tempe	Indonesia
CTI		Cowpea bean tempe	Indonesia
JTI		Jack bean tempe	Indonesia
Sample set III			
25R:75S	Indonesia	25% red kidney bean and 75% soybean	Indonesia
50R:50S		50% red kidney bean and 50% soybean	Indonesia
75R:25S		75% red kidney bean and 25% soybean	Indonesia

Table S2. List of annotated metabolites (II)

No	Amino acid	Sugar	Organic acid	Other
1	Alanine	Fructose 6-phosphate	2-Aminoadipic acid	2-Aminoethanol
2	Asparagine	Galactinol	2-Aminobutyric acid	2-Hydroxypyridine
3	Aspartic acid	Galactose	3-Hydroxy-3-methylglutarate	3,4-Dihydroxybenzoate
4	ß-alanine	Gentiobiose	3-Hydroxyanthranilic acid	Adenosine
5	Glutamic acid	Glucose	3-Phenyllatic acid	4-Hydroxyphenethyl alcohol
6	Glutamine	Lactinol	4-Aminobutyric acid	Daidzein
7	Glysine	Maltotriose	4-Hydroxybenzoic acid	Genistein
8	Histidine	Melezitose	4-Hydroxyphenyl pyruvate	Glucarate
9	Homocysteine	Melibiose	4-Hydroxyphenylacetic acid	Glucoronate
10	Homoserine	Psicose	α-Ketoglutaric acid	Glucono-1,5-lactone
11	Isoleucine	Ribose	Allantoin	Glycerol
12	Leucine	Sorbitol	Fumaric acid	Inositol
13	Lysine	Sorbose	Galacturonic acid	Oxalate
14	Phenylalanine	Sucrose	Gluconic acid	Phosphate
15	Proline	Threonic acid	Glyceric acid	Putrescine
16	Serine	Trehalose	Isocitric acid	Propyleneglycol
17	Threonine	Xylitol	Lactic acid	Thymine
18	Tryptophan	Xylonic acid	Malic acid	Uracil
19	Tyrosine		Malonic acid	Urea
20	Valine		Nicotinic acid	Xantine
21			Oxalacetic acid	
22			Pipecolic acid	
23			Pyroglutamic acid	
24			Succinic acid	
25			Uric acid	

Table S3. Metabolite characteristic of legume before and after fermentation based on PC1

No	Metabolites	p[corr] PC1
1	Histidine	0.171166
2	Serine	0.1635
3	Alanine	0.161765
4	3,4-Dihydroxybenzoate	0.158883
5	Lysine	0.157093
6	3-Hydroxy-3-methylglutarate	0.156662
7	Succinic acid	0.155926
8	Glycine	0.155574

9	Threonine	0.15539
10	2-Aminoadipic acid	0.155066
11	Tyrosine	0.147693
12	Glutamic acid	0.14755
13	4-Hydroxyphenylacetic acid	0.140207
14	Proline	0.139321
15	2-Aminobutyric acid	0.13902
16	Nicotinic acid	0.13814
17	Valine	0.134222
18	Isoleucine	0.129234
19	4-Hydroxyphenyl pyruvate	0.12848
20	2-Hydroxypyridine	0.12807
21	Glyceric acid	0.127868
22	Leucine	0.127228
23	Malic acid	0.125672
24	Glucuronate	0.123693
25	Phenylalanine	0.123231
26	Homocysteine	0.12075
27	Fructose 6-phosphate	0.120319
28	Uric acid	0.116856
29	4-Hydroxyphenethyl alcohol	0.115345
30	Thymine	0.113747
31	Trehalose	0.109837
32	Ribose	0.109474
33	Threonic acid	0.108734
34	Asparagine	0.107237
35	Galactose	0.103441
36	Maltotriose	0.10307
37	4-Aminobutyric acid	0.101516
38	Fumaric acid	0.100847
39	Oxalacetic acid	0.100718
40	Pyroglutamic acid	0.100451
41	α-Ketoglutaric acid	0.100398
42	Glucose	0.0994045
43	Aspartic acid	0.0957878
44	Glycerol	0.0913164
45	Glutamine	0.089714
46	3-Phenyllactic acid	0.0889739
47	Putrescine	0.0889404

48	3-Hydroxyanthranilic acid	0.0880009
49	Lactic acid	0.0872069
50	Uracil	0.0844002
51	Urea	0.0805261
52	ß-alanine	0.0776747
53	Xanthine	0.0756795
54	Xylitol	0.0668921
55	Oxalate	0.062961
56	Genistein	0.0496361
57	Daidzein	0.031646
58	2-Aminoethanol	0.0259667
59	Adenosine	0.01757
60	Tryptophan	0.0122299
61	Propyleneglycol	0.00624884
62	Galacturonic acid	-0.0190142
63	Pipecolic acid	-0.0208233
64	Phosphate	-0.0246687
65	4-Hydroxybenzoic acid	-0.0316902
66	Melibiose	-0.0459124
67	Homoserine	-0.0497668
68	Inositol	-0.0522355
69	Lactitol	-0.0721992
70	Allantoin	-0.0818356
71	Malonic acid	-0.0825677
72	Gentiobiose	-0.0866312
73	Gluconic acid	-0.100946
74	Galactinol	-0.101193
75	Melezitose	-0.102536
76	Glucarate	-0.113882
77	Xylonic acid	-0.116286
78	Sorbitol	-0.116803
79	Glucono-1,5-lactone	-0.118501
80	Isocitric acid	-0.118827
81	Psicose	-0.122361
82	Sorbose	-0.123418
83	Sucrose	-0.142205

Table S4. Tempe production condition in Indonesia and Japan

Production condition	Production place	
	Indonesia	**Japan**
Month of production	March 2020	December 2019
Humidity, average room temperature	90%, 28-32^{0}C	60%, 11-14^{0}C
Water source	Tap water in Indonesia	Tap water in Japan
Pre-treatment (Soaking)	At room temperature	At 30^{0}C, inside incubator
Acid fermentation (Soaking)	At room temperature	At 30^{0}C, inside incubator
Mold fermentation	At room temperature	At 30^{0}C, inside incubator

Table S5. Metabolite characteristic of legume tempe produced in Japan and Indonesia based on PC1

No	Metabolites	p[corr] PC1
1	Aspartic acid	0.18788
2	Malic acid	0.18086
3	Glutamic acid	0.17816
4	Histidine	0.175448
5	ß-Alanine	0.168357
6	Allantoin	0.166862
7	Serine	0.162792
8	3-Hydroxy-3-methylglutarate	0.161296
9	Asparagine	0.160151
10	Glyceric acid	0.154268
11	Isocitric acid+Citric acid	0.151924
12	Glucarate	0.151518
13	Propyleneglycol	0.145435
14	2-Hydroxypyridine	0.145207
15	α-Ketoglutaric acid	0.142417
16	Glycine	0.142205
17	Fructose 6-phosphate	0.141246
18	Nicotinic acid	0.140731
19	Pyroglutamic acid	0.138928
20	Xylonic acid	0.138329
21	Pipecolic acid	0.135078
22	Melibiose	0.134487
23	Melezitose	0.130122
24	Glutamine	0.129209

25	Thymine	0.128038
26	2-Aminoadipic acid	0.126755
27	Galacturonic acid	0.126104
28	Trehalose	0.125106
29	Maltotriose	0.124811
30	Uric acid	0.123958
31	Succinic acid(or anhydride)	0.122443
32	Alanine	0.121197
33	Fumaric acid	0.121084
34	4-Hydroxybenzoic acid	0.119615
35	3-Hydroxyanthranilic acid	0.118907
36	Sucrose	0.117133
37	2-Aminobutyric acid	0.117078
38	Threonine	0.106317
39	Glycerol	0.104666
40	Lysine	0.103528
41	Galactose+Glucose	0.10333
42	4-Hydroxyphenylacetic acid	0.103148
43	Ribose	0.101494
44	Glucose	0.100564
45	Tyrosine	0.098057
46	Urea	0.0964657
47	Xylitol	0.0922494
48	Putrescine	0.0919951
49	Proline	0.0909423
50	3,4-Dihydroxybenzoate	0.0894811
51	Valine_2TMS	0.0882451
52	Daidzein	0.0780481
53	Oxalacetic acid+Pyruvate	0.0749469
54	Psicose +Tagatose	0.0741672
55	Isoleucine	0.0741291
56	Homocysteine	0.0731646
57	Oxalate	0.0712905
58	Phenylalanine	0.0675386
59	Glucuronate	0.0634629
60	Tryptophan	0.0619736
61	Leucine	0.0575492
62	Genistein	0.0557934
63	Adenosine	0.0434755

64	Malonic acid	0.0430719
65	4-Hydroxyphenyl pyruvate	0.0427094
66	4-Aminobutyric acid	0.0331643
67	4-Hydroxyphenethyl alcohol	0.0279863
68	Threonic acid	0.0237801
69	Gentiobiose	0.0232544
70	2-Aminoethanol	0.0186845
71	Lactic acid	0.0175513
72	Sorbitol	0.0160366
73	Inositol	0.00318721
74	3-Phenyllactic acid	0.0011445
75	Homoserine	-0.00198159
76	Galactinol	-0.0170004
77	Phosphate	-0.0398108
78	Lactitol	-0.0468412
79	Sorbose	-0.0753049
80	Glucono-1,5-lactone	-0.0830503
81	Uracil	-0.085288
82	Xanthine	-0.0959108
83	Gluconic acid	-0.099773

Table S6. Metabolites characteristic of legume *tempe* produced in Indonesia based on PC1

No	Metabolites	p[corr] PC1
1	3-Hydroxy-3-methylglutarate	0.151537
2	Histidine	0.1515
3	Malic acid	0.150483
4	3,4-Dihydroxybenzoate	0.149926
5	Phenylalanine	0.149581
6	Tyrosine	0.149026
7	Glutamic acid	0.149002
8	Asparagine	0.147755
9	Fumaric acid	0.147527
10	Threonine	0.146733
11	Glycine	0.145993
12	Lysine	0.145044
13	Alanine	0.144852

14	4-Hydroxyphenethyl alcohol	0.143554
15	Xylitol	0.142274
16	2-Aminoadipic acid	0.141436
17	Aspartic acid	0.140856
18	Propyleneglycol	0.13853
19	Inositol	0.138355
20	Serine	0.137792
21	Lactic acid	0.137533
22	Valine	0.136005
23	4-Hydroxyphenylacetic acid	0.135091
24	4-Hydroxybenzoic acid	0.134802
25	Homocysteine	0.134717
26	Leucine	0.134457
27	ß-alanine	0.133899
28	Isoleucine	0.133059
29	4-Hydroxyphenyl pyruvate	0.131829
30	Fructose 6-phosphate	0.128807
31	2-Hydroxypyridine	0.126604
32	3-Phenyllactic acid	0.126464
33	Putrescine	0.126129
34	Isocitric acid	0.125999
35	Galacturonic acid	0.125735
36	Threonic acid	0.124811
37	4-Aminobutyric acid	0.123425
38	Glyceric acid	0.122865
39	Melibiose	0.119563
40	Urea	0.118956
41	Xylonic acid	0.115671
42	Glucuronate	0.113953
43	2-Aminobutyric acid	0.113703
44	Pipecolic acid	0.111909
45	Tryptophan	0.109244
46	Succinic acid	0.108982
47	Glucarate	0.104808
48	Sorbitol	0.0977226
49	α-Ketoglutaric acid	0.0935526
50	Pyroglutamic acid	0.0900349
51	Ribose	0.0837748
52	3-Hydroxyanthranilic acid	0.0815952

53	Adenosine	0.0796995
54	Homoserine	0.0789861
55	Lactitol	0.0769079
56	Gentiobiose	0.0753075
57	Proline	0.0700681
58	Sorbose	0.0649644
59	Galactinol	0.0621664
60	2-Aminoethanol	0.0617394
61	Glutamine	0.054972
62	Sucrose	0.0531327
63	Allantoin	0.0392516
64	Thymine	0.038673
65	Maltotriose	0.0322977
66	Nicotinic acid	0.0222218
67	Psicose	0.0146573
68	Galactose	0.0138253
69	Oxalacetic acid	0.010139
70	Glucose	-0.00409142
71	Oxalate	-0.00515796
72	Malonic acid	-0.0143144
73	Glucono-1,5-lactone	-0.0293001
74	Melezitose	-0.0333182
75	Trehalose	-0.0355977
76	Xanthine	-0.0561695
77	Genistein	-0.0618376
78	Uracil	-0.0643976
79	Daidzein	-0.0687234
80	Glycerol	-0.0705662
81	Gluconic acid	-0.0978284
82	Uric acid	-0.113582
83	Phosphate	-0.129145

Table S7. Metabolites characteristic of legume *tempe* produced in Indonesia based on PC2

No	Metabolites	p[corr] PC2
1	Oxalacetic acid+Pyruvate	0.234728
2	Galactinol	0.207461

3	Melezitose	0.204227
4	Lactitol	0.18164
5	Sucrose	0.180157
6	Gentiobiose	0.179181
7	Homoserine	0.178754
8	Trehalose	0.175692
9	Psicose +Tagatose	0.154505
10	Succinic acid(or anhydride)	0.138281
11	Threonic acid	0.129593
12	Isocitric acid+Citric acid	0.129407
13	Glucono-1,5-lactone	0.126246
14	Glucuronate	0.12437
15	Maltotriose	0.121628
16	Sorbose	0.115276
17	3-Phenyllactic acid	0.108935
18	α-Ketoglutaric acid	0.101918
19	Fructose 6-phosphate	0.10107
20	Putrescine	0.0983547
21	Homocysteine	0.0980074
22	4-Hydroxyphenyl pyruvate	0.0934033
23	Inositol	0.0703728
24	Melibiose	0.0607892
25	Alanine	0.049178
26	Fumaric acid	0.0433462
27	Oxalate	0.0399089
28	Thymine	0.036587
29	4-Hydroxybenzoic acid	0.0322979
30	Glycine	0.016692
31	Malic acid	0.0166033
32	2-Aminobutyric acid	0.0161811
33	Glucose	0.0121279

34	3,4-Dihydroxybenzoate	0.00780031
35	Glutamine	0.00473823
36	Proline	0.0030582
37	Histidine	-0.0124735
38	Phenylalanine	-0.0138765
39	Propyleneglycol	-0.0160535
40	Galactose+Glucose	-0.0180131
41	3-Hydroxy-3-methylglutarate	-0.0191131
42	Glutamic acid	-0.0197092
43	Threonine	-0.027232
44	Tyrosine	-0.0302098
45	2-Aminoadipic acid	-0.0439667
46	Xylonic acid	-0.0451741
47	Asparagine	-0.0454678
48	4-Hydroxyphenethyl alcohol	-0.0490012
49	Lactic acid	-0.0501337
50	4-Hydroxyphenylacetic acid	-0.0516279
51	3-Hydroxyanthranilic acid	-0.0539831
52	Glyceric acid	-0.0543874
53	Leucine	-0.0555538
54	Urea	-0.0611153
55	Lysine	-0.0642221
56	Allantoin	-0.0690716
57	Xylitol	-0.0731205
58	Aspartic acid	-0.0739475
59	Serine	-0.0836124
60	Gluconic acid	-0.0879987
61	Isoleucine	-0.0918069
62	Valine	-0.094455
63	2-Hydroxypyridine	-0.0962875
64	Xanthine	-0.0989731

65	Pyroglutamic acid	-0.09924
66	Glycerol	-0.102349
67	Galacturonic acid	-0.109083
68	Phosphate	-0.109568
69	ß-alanine	-0.112968
70	Sorbitol	-0.115704
71	4-Aminobutyric acid	-0.122659
72	Daidzein	-0.125697
73	Nicotinic acid	-0.128059
74	Genistein	-0.134131
75	Uric acid	-0.13621
76	Pipecolic acid	-0.136998
77	Glucarate	-0.140681
78	Uracil	-0.160332
79	Ribose	-0.162226
80	Tryptophan	-0.16277
81	Malonic acid	-0.172791
82	Adenosine	-0.194801
83	2-Aminoethanol	-0.213883

Table S8. List of samples

Sample code	Legume origin	Sample type	Production place
Sample set I			
SB	Indonesia	Soybean	-
RB		Red kidney bean	-
Sample set II			
ST	Indonesia	Soybean *tempe*	Japan
RT		Red kidney bean *tempe*	Japan
GBT	Japan	Garbanzo *tempe*	Japan
GST	Japan	Green soybean *tempe*	Japan
GPT	Japan	Green pea *tempe*	Japan

Table 9. List of annotated metabolites (III)

No	Amino Acid	Sugar	Organic acid	Other
1	Tryptophan	Xylitol	2-Aminoethanol	4-Hydroxyphenethyl alcohol
2	Methionine	Inositol	3-Hydroxyanthranilic acid	Glucuronate
3	Xanthine	Maltose	Glyceric acid	Daidzein
4	b-Alanine	Arabionose	Malonic acid	2-Hydroxypyridine
5	Phenylalanine	Raffinose	4-Hydroxyphenylacetic acid	Urea
6	Homocysteine	Galactose+Glucose	Isocitric acid+Citric acid	Phosphate
7	Tyrosine	Ribose	Glutaric acid(or anhydride)	5-Aminovaleric acid
8	Threonine	Glucose	Gluconic acid	Tryptamine
9	Proline	Putrescine	Nicotinic acid	Genistein
10	Leucine	Melezitose	3-Hydroxyisovaleric acid	Oleic acid(18:1n-9)
11	Glutamine	b-Lactose	4-Aminobutyric acid	Glycerol
12	Lysine	Trehalose	a-Ketoglutaric acid	Cadaverine
13	Isoleucine	Threonic acid	4-Methyl benzoic acid	Thymine
14	Serine	Gentiobiose	Pyroglutamic acid	Stearic acid(17:0)
15	Asparagine		Lactic acid	Nonanoric acid(9:0)
16	Valine		Uric acid	2-Dehydro gluconate
17	Alanine		Succinic acid(or anhydride)	Oxalate
18	Glycine		Oxalacetic acid+Pyruvate	Uridine
19	Aspartic acid		3-Hydroxy-3-methylglutarate	Meso erythritol
20	Glutamic acid		Malic acid	Tyramine
21			2-Aminoadipic acid	Plamitic acid(16:0)
22			2,6-Pyridinedicarboxylic acid	Myristic acid
23			2-Aminobutyric acid	3-Phenyllactic acid
24			Fumaric acid	Hypoxanthine
25				Adenine
26				n-Butylamine
27				N-a-Acetyl ornithine
28				Heptadecanic acid(17:0)
29				Adenosine
30				Glucono-1,5-lactone
31				Glycolic acid
32				3-Hydroxy-3-methylglutarate
33				Uracil
34				O-Phosphoethanolamine

Table S10. Metabolites characteristic of various legume tempe produced in Japan based on PC1

Var ID (Primary)	p[corr] PC1
Fumaric acid	0.143923
Glutamic acid	0.1411
Aspartic acid	0.140819
2-Aminobutyric acid	0.139134
2,6-Pyridinedicarboxylic acid	0.138866
2-Aminoadipic acid	0.13845
Phosphate	0.138393
O-Phosphoethanolamine	0.13779
Malic acid	0.137611
Uracil	0.137553
Glycine	0.136872
Alanine	0.136664
Threonic acid	0.135688
Trehalose	0.134958
3-Hydroxy-3-methylglutarate	0.133982
Glycolic acid	0.131588
Valine	0.131398
Asparagine	0.129983
Serine	0.129848
Isoleucine	0.129505
Lysine	0.129377
Gentiobiose	0.129146
b-Lactose	0.128746
Glucono-1,5-lactone	0.128315
Glutamine	0.127257
Leucine	0.126374
Adenosine	0.12372
Heptadecanic acid(17:0)	0.123312
Melezitose	0.123254
Proline	0.123104
Oxalacetic acid+Pyruvate	0.12291
N-a-Acetyl ornithine	0.121478
n-Butylamine	0.117967
Adenine	0.117935
Threonine	0.117583
Succinic acid(or anhydride)	0.117097

Uric acid	0.116673
Hypoxanthine	0.115908
3-Phenyllactic acid	0.115834
Tyrosine	0.113847
Putrescine	0.112988
Homocysteine	0.111315
Myristic acid	0.111054
Plamitic acid(16:0)	0.108098
Glucose	0.107818
Tyramine	0.107285
Lactic acid	0.106937
Pyroglutamic acid	0.106364
Meso erythritol	0.106031
4-Methyl benzoic acid	0.105575
Uridine	0.104918
Oxalate	0.10408
2-Dehydro gluconate	0.102897
Ribose	0.0989629
a-Ketoglutaric acid	0.0986117
4-Aminobutyric acid	0.0970317
Nonanoric acid(9:0)	0.0962386
Stearic acid(17:0)	0.0954347
Thymine	0.0924335
Phenylalanine	0.0915985
b-Alanine	0.0878283
3-Hydroxyisovaleric acid	0.0849181
Xanthine	0.0848839
Cadaverine	0.0840519
Glycerol	0.0832749
Oleic acid(18:1n-9)	0.0827519
Nicotinic acid	0.0815227
Galactose+Glucose	0.0785756
Gluconic acid	0.0782491
Raffinose	0.0775999
Arabionose	0.0773675
Genistein	0.0767139
Tryptamine	0.0746284
5-Aminovaleric acid	0.0742789
Glutaric acid(or anhydride)	0.0718601

Urea	0.0625571
Maltose	0.0598298
2-Hydroxypyridine	0.0489362
Inositol	0.0453897
Isocitric acid+Citric acid	0.0442552
4-Hydroxyphenylacetic acid	0.0395585
Daidzein	0.0371782
Malonic acid	0.0326858
Xylitol	0.0274502
Glyceric acid	0.0224521
Methionine	0.0208963
Glucuronate	0.0166726
4-Hydroxyphenethyl alcohol	0.014496
3-Hydroxyanthranilic acid	0.00658955
2-Aminoethanol	0.000130386
Tryptophan	-0.0136969

Table S11. List annotated metabolites of legume *tempe* in different drying treatment based on PC1

Var ID (Primary)	**M1.p[2]**
Genistein	0.175979
Daidzein	0.173242
Putrescine	0.157201
Pyroglutamic acid	0.151179
Thymine	0.149368
Glutamine	0.12993
Oleic acid(18:1n-9)	0.123406
Proline	0.122443
Cadaverine	0.122204
Tryptamine	0.120865
Glycerol	0.11719
Hydroxyproline	0.109437
Glycine	0.108207
2-Aminobutyric acid	0.106788
Glucono-1,5-lactone	0.105227
Hypoxanthine	0.105051
Uric acid	0.103863
2-Aminoethanol	0.102893

4-Aminobutyric acid	0.102565
Melezitose	0.101672
Xanthine	0.101497
Tyramine	0.0999249
Gluconic acid	0.0988103
Serine	0.0970965
Asparagine	0.0877644
Alanine	0.0790562
Inosine	0.073797
Glutamic acid	0.0657639
Uracil	0.0656889
Xylonic acid	0.0655223
Argininosuccinate	0.0646865
Adenine	0.0643706
Lysine	0.0582472
Leucine	0.0480795
Isoleucine	0.0472834
Urocanate	0.0469222
Fructose	0.043956
Lactic acid	0.0439076
Threonine	0.0393389
Uridine	0.0371535
1,3-Propanediamine	0.0361982
Glucosamine	0.0359361
3-Hydroxyanthranilic acid	0.0342814
Histidine	0.0341784
3-Hydroxy-3-methylglutarate	0.0311787
Allantoin	0.0258198
Maltose	0.0218858
Trehalose	0.0218742
Glyceric acid	0.0163735
N-Acetyl glucosamine	0.015641
Threonic acid	0.0148613
Valine	0.0134415
Meso erythritol	0.00207164
Mannose	-0.0042408
Succinic acid(or anhydride)	-0.0045301
2-Dehydro gluconate+Fructose+Sorbose	-0.006589

Malonic acid	-0.0085665
Glucuronate	-0.0102782
Psicose+Tagatose	-0.0119885
Adenosine	-0.0150125
Oxalacetic acid+Pyruvate	-0.0159336
2-Dehydro gluconate	-0.0220024
Phenylalanine	-0.022241
Ribose	-0.0229838
Aspartic acid	-0.0271882
Xylitol	-0.030844
Homoserine	-0.0414181
Arabionose	-0.0453518
a-Ketoglutaric acid	-0.0608369
O-Phosphoethanolamine	-0.0758256
b-Lactose	-0.0775492
Tryptophan	-0.0786095
Lanthionine	-0.0810939
Inositol	-0.0851862
Fructose 6-phosphate	-0.0982088
Oxalate	-0.112746
3-Phenyllactic acid	-0.113112
Xylose	-0.114035
4-Hydroxybenzoic acid	-0.11796
Homocysteine	-0.118796
Urea	-0.119138
Malic acid	-0.120039
4-Hydroxyphenethyl alcohol	-0.124819
Galactose+Glucose	-0.126383
Isocitric acid+Citric acid	-0.128718
Glucose	-0.128861
Tyrosine	-0.130942
Sucrose	-0.133085
Glycolic acid	-0.133215
Glutaric acid(or anhydride)	-0.136422
Nicotinic acid	-0.136428
Epicatechin	-0.137074
2-Aminoadipic acid	-0.143231
Fumaric acid	-0.143483
2-Hydroxypyridine	-0.145314

Melibiose	-0.148122
Raffinose	-0.156415
Glucarate	-0.162321
4-Hydroxyphenylacetic acid	-0.166363
Propyleneglycol	-0.167671
3-Hydroxyisovaleric acid	-0.169172
Galactinol	-0.181567

Table S12. The importance of metabolites during model construction by OPLS-DA

No	Metabolites name	45; 50^0C vs 60^0C dry temp	
		VIP Score	Coefficient
1	Adenosine	2.19667	-0.054284
2	Uridine	2.10961	-0.050212
3	Inositol	2.03327	0.048454
4	N-Acetyl glucosamine	1.9271	-0.048177

Table S13. VIP metabolites (OPLS-DA

Var ID (Primary)	**M1.VIP[1]**	**1.89456 * M1.VIP[1]cvSE**
Adenosine	2.19667	0.791862
Uridine	2.10961	0.328578
Inositol	2.03327	0.380387
N-Acetyl glucosamine	1.9271	0.897698
Adenine	1.89345	0.723198
Glucosamine	1.89235	0.546376
Argininosuccinate	1.78419	0.461379
Fructose	1.76879	0.234572
Fructose 6-phosphate	1.64328	1.29582
Valine	1.56423	0.709923
Threonine	1.49199	0.836818
Asparagine	1.42818	1.01753
Isoleucine	1.42104	0.819938

Leucine	1.41727	0.770345
Threonic acid	1.35838	0.653724
Sucrose	1.34575	1.59674
Epicatechin	1.32703	1.59206
Melibiose	1.32416	1.59322
Ribose	1.29479	1.10339
Xylose	1.2833	1.6259
Serine	1.26987	0.988644
Ornithine	1.25933	0.848182
Glutamic acid	1.25709	1.1894
Tyramine	1.24467	1.09786
Allantoin	1.2225	1.07096
Glucarate	1.1929	1.49526
Galactose+Glucose	1.18286	1.50404
Glucose	1.18129	1.50126
Saccharopine	1.17391	1.01246
Homoserine	1.14603	0.390101
b-Lactose	1.1369	0.525149
Psicose+Tagatose	1.13397	1.44291
3-Hydroxyanthranilic acid	1.12588	0.539243
Tryptophan	1.12221	1.17403
Glucono-1,5-lactone	1.10558	1.04553
Propyleneglycol	1.10206	1.13657
Gluconic acid	1.06684	1.09657
Gentiobiose	1.05741	0.693638
Glutaric acid(or anhydride)	1.04342	0.830454
Xylitol	1.02665	1.30456
Oxalacetic acid+Pyruvate	1.02263	1.04982
Melezitose	1.00863	1.18534
Galactinol	0.944976	1.653
4-Aminobutyric acid	0.878127	0.914876
3-Phenyllactic acid	0.870152	0.853414
Xanthine	0.863644	1.26283
Uric acid	0.799672	1.41723
Oxalate	0.799106	0.868385
Homocysteine	0.793949	0.84326
Thymine	0.786615	1.61848

Proline	0.786262	1.17846
Glycerol	0.780595	1.41109
Xylonic acid	0.776716	1.14458
Pyroglutamic acid	0.771332	1.43279
Alanine	0.72854	1.05526
b-Alanine	0.707381	0.711893
Oleic acid(18:1n-9)	0.663127	1.21832
2-Aminoadipic acid	0.63418	0.842629
Fumaric acid	0.63231	1.51059
Maltose	0.63046	0.737815
3-Hydroxy-3-methylglutarate	0.622158	0.44809
Glycolic acid	0.605716	0.869434
Glutamine	0.545961	1.46213
Glycine	0.510702	1.51607
Lysine	0.503751	0.813327
Inosine	0.499667	0.653978
Cadaverine	0.493929	1.23099
Urocanate	0.428922	0.974287
Lactic acid	0.427295	1.51676
Aspartic acid	0.411475	0.851123
Uracil	0.375537	1.07779
Glucuronate	0.365399	0.939407
Hydroxyproline	0.358329	1.14313
Histidine	0.324259	0.57386
4-Hydroxyphenylacetic acid	0.320104	1.07088
Arabionose	0.305011	0.831984
4-Hydroxybenzoic acid	0.257344	0.503256
Malic acid	0.245074	1.07082
Isocitric acid+Citric acid	0.235824	0.919671
Glycylglycine	0.225857	0.908311
Trehalose	0.218058	0.575759
2-Aminobutyric acid	0.213688	0.624227
Nicotinic acid	0.211838	0.844926
4-Hydroxyphenethyl alcohol	0.202629	0.825718
Genistein	0.201304	0.931785
Glyceric acid	0.190285	0.904552

Hypoxanthine	0.173156	0.823511
Putrescine	0.16575	0.785708
Daidzein	0.155011	0.774357
Raffinose	0.144491	0.812222
2-Aminoethanol	0.14156	0.613399
Tyrosine	0.109388	0.708338
Phenylalanine	0.096921	0.535772
Meso erythritol	0.0548094	0.631644
Urea	0.0288768	0.436875
Theanine	0.0273102	0.52154
3-Hydroxyisovaleric acid	0.00611614	0.499556
Tryptamine	0.00222331	0.411617

Table S14. Metabolites characteristic of mixed tempe and selected legume tempe produced in Indonesia based on PC1

No	Metabolites	p[corr] PC1
1	2-Aminoadipic acid	0.14005
2	2-Aminobutyric acid	0.0301403
3	2-Aminoethanol	0.0316598
4	2-Hydroxypyridine	0.119595
5	3,4-Dihydroxybenzoate	0.120755
6	3-Hydroxy-3-methylglutarate	0.148069
7	3-Hydroxyanthranilic acid	0.00629591
8	3-Phenyllactic acid	0.149376
9	4-Aminobutyric acid	0.12785
10	4-Hydroxybenzoic acid	0.107594
11	4-Hydroxyphenethyl alcohol	0.154272
12	4-Hydroxyphenyl pyruvate	0.14793
13	4-Hydroxyphenylacetic acid	0.134844
14	Adenosine	0.045715
15	α-Ketoglutaric acid	0.0501689
16	Alanine	0.149221
17	Allantoin	0.0668806
18	Asparagine	0.132095
19	Aspartic acid	0.146539
20	ß-alanine	0.145418
21	Daidzein	-0.14559

22	Fructose 6-phosphate	0.0985873
23	Fumaric acid	0.137943
24	Galactinol	0.0461078
25	Galactose	0.0824171
26	Galacturonic acid	0.0804425
27	Genistein	-0.12149
28	Gentiobiose	-0.100039
29	Glucarate	0.150866
30	Gluconic acid	-0.101956
31	Glucono-1,5-lactone	0.0805332
32	Glucose	0.0926065
33	Glucuronate	0.139524
34	Glutamic acid	0.139679
35	Glutamine	-0.06934
36	Glyceric acid	0.106758
37	Glycerol	-0.13828
38	Glycine	0.13892
39	Histidine	0.145641
40	Homocysteine	0.143625
41	Homoserine	-0.00360715
42	Inositol	0.125266
43	Isocitric acid	0.142328
44	Isoleucine	0.121278
45	Lactic acid	0.150707
46	Lactitol	0.00252855
47	Leucine	0.108468
48	Lysine	0.146008
49	Malic acid	0.136229
50	Malonic acid	-0.0976053
51	Maltotriose	-0.0318894
52	Melezitose	0.00810682
53	Melibiose	0.114139
54	Nicotinic acid	0.106506
55	Oxalacetic acid	-0.00151558
56	Oxalate	0.0311208
57	Phenylalanine	0.134208
58	Phosphate	-0.0953493
59	Pipecolic acid	0.148804
60	Proline	-0.00036868

61	Propyleneglycol	0.106177
62	Psicose	-0.0186771
63	Putrescine	0.0720837
64	Pyroglutamic acid	-0.00825489
65	Ribose	0.102307
66	Serine	0.141611
67	Sorbitol	0.132322
68	Sorbose	0.108462
69	Succinic acid	0.10223
70	Sucrose	0.0170213
71	Threonic acid	0.112441
72	Threonine	0.145124
73	Thymine	-0.066874
74	Trehalose	0.0541258
75	Tryptophan	0.102642
76	Tyrosine	0.153635
77	Uracil	-0.0750269
78	Urea	0.0563005
79	Uric acid	-0.0744551
80	Valine	0.140231
81	Xanthine	-0.122093
82	Xylitol	0.143606
83	Xylonic acid	0.0769314

Supplementary Figures

CATA SENSORY ATTRIBUTE

Name :
Age :
Date :

I. Check all the terms that you consider appropriate to describe your ideal tempe

1. Fresh tempe

	Grayish-white colour		Nutty / beany
	Full mold-covered cake		moldy
	Bitter		Sour
	Salty		Rancid
	Fatty		Leafy
	Meat-like aroma		Astringent
	Burnt		Bitter aftertaste
	Umami		Sweet

2. Fried tempe

	Golden brown colour		Nutty / beany
	Compact		Moldy
	Bitter		Sour
	Salty		Rancid
	Fatty		Leafy
	Meat-like aroma		Astringent
	Burnt		Bitter aftertaste
	Umami		Sweet

Sample code	Check all the terms that you consider appropriate to describe sample (fresh)	Sample code	Check all the terms that you consider appropriate to describe sample (fried)
	Grayish-white colour		Golden brown colour
	Full mold-covered cake		Compact
	Bitter		Bitter
	Salty		Salty
	Fatty		Fatty
	Meat-like aroma		Meat-like aroma
	Burnt		Burnt
	Umami		Umami
	Nutty / beany		Sour
	Moldy		Moldy
	Sour		Sour
	Rancid		Rancid
	Leafy		Leafy
	Astringent		Astringent
	Bitter aftertaste		Bitter aftertaste
	Sweet		Sweet

Sample code	Check all the terms that you consider appropriate to describe sample (fresh)	Sample code	Check all the terms that you consider appropriate to describe sample (fried)
	Grayish-white colour		Golden brown colour
	Full mold-covered cake		Compact
	Bitter		Bitter
	Salty		Salty
	Fatty		Fatty
	Meat-like aroma		Meat-like aroma
	Burnt		Burnt
	Umami		Umami
	Nutty / beany		Sour
	Moldy		Moldy
	Sour		Sour
	Rancid		Rancid
	Leafy		Leafy
	Astringent		Astringent
	Bitter aftertaste		Bitter aftertaste
	Sweet		Sweet

Figure S1. Sensory evaluation questionnaire for CATA test

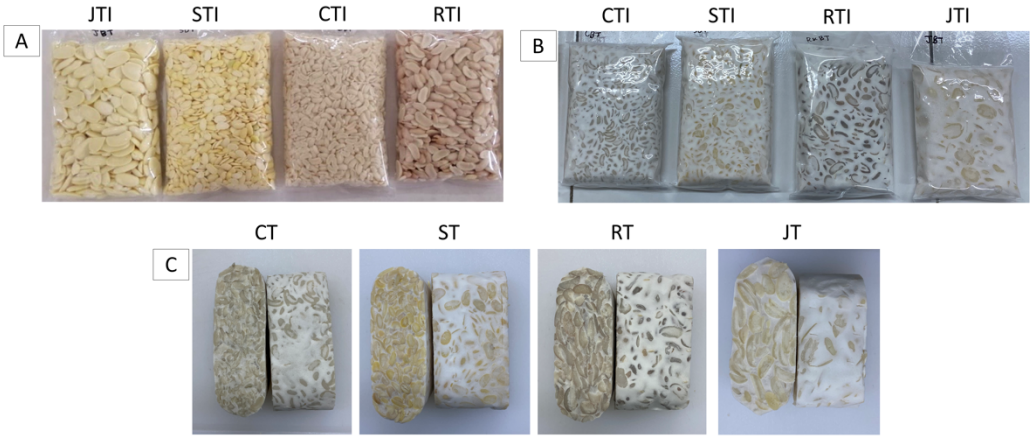


Figure S2. Legume tempe before and after fermentation. A. Legume tempe before fermentation. B. Legume tempe after fermentation. C. Comparison between surface and middle part of legume tempe comparisons after fermentation. JTI: Jack bean tempe; STI: Soybean tempe; RTI: Red kidney bean tempe; CTI: Cowpea bean tempe

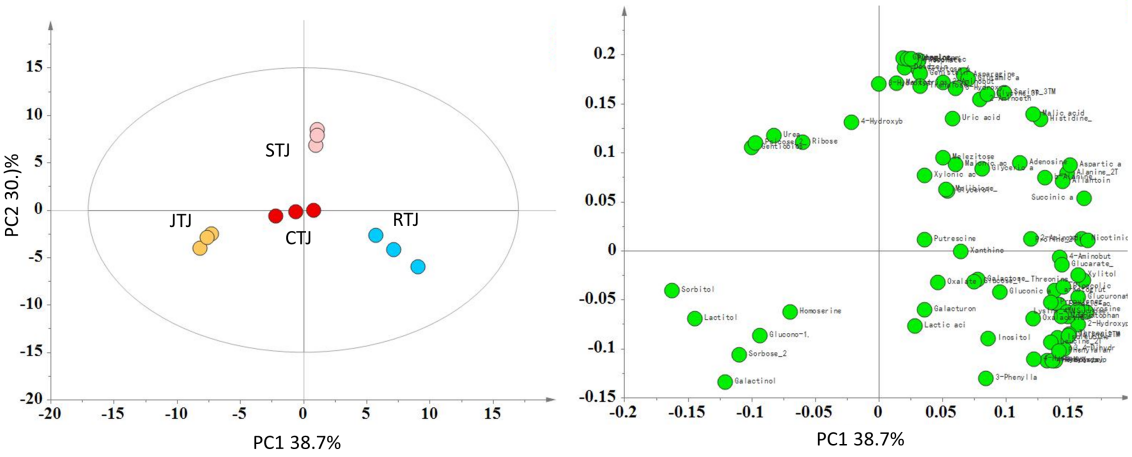
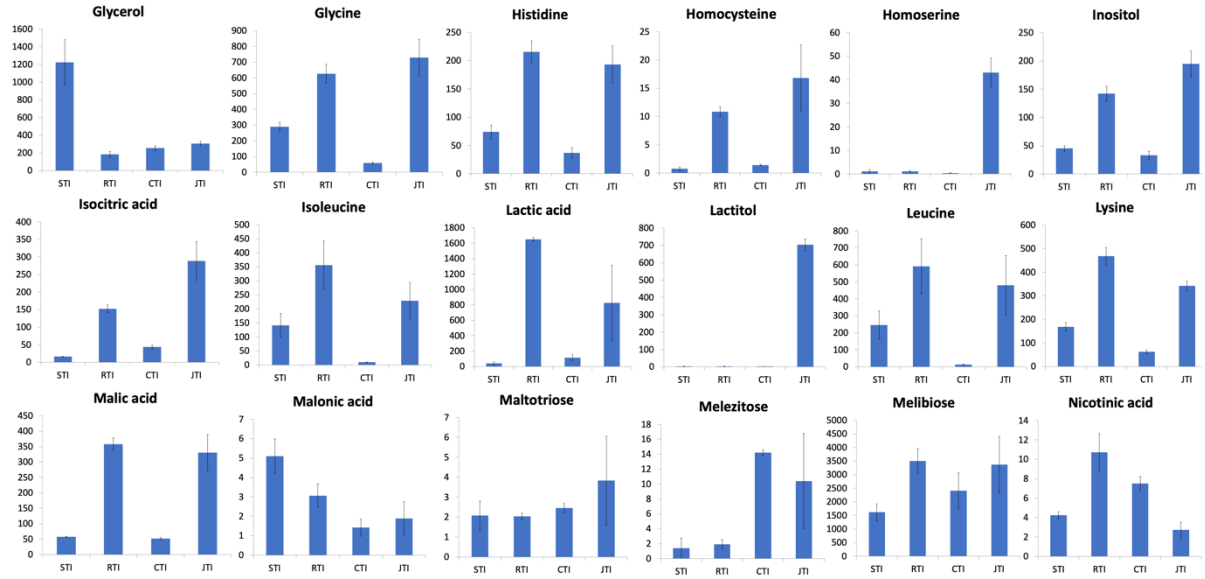
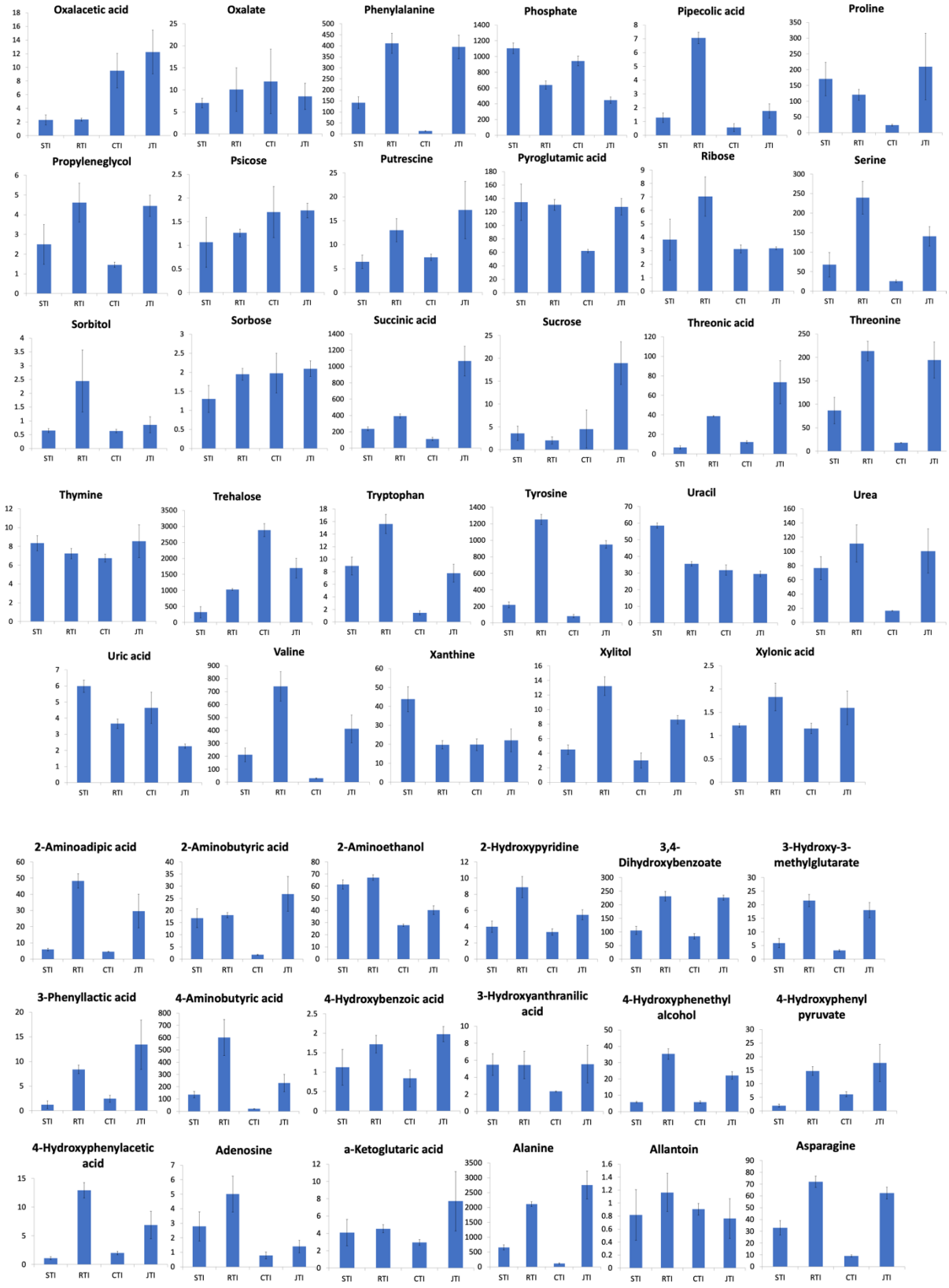


Figure S3. Principal component analysis (PCA) score plot and bar graph of legume tempe produced in Japan based on GC-MS. **A**. Score plot of legume tempe**. B.** Loading plot of legume tempe. Points indicate legume types (STJ: soybean tempe Japan; RTJ: red kidney bean tempe Japan; CTJ: Cowpea bean tempe Japan; JTJ: Jack bean tempe Japan)




Oxalacetic acid
Oxalate
Phenylalanine
Phosphate
Pipecolic acid
Proline
Propyleneglycol
Psicose
Putrescine
Pyroglutamic acid
Ribose
Serine
Sorbitol
Sorbose
Succinic acid
Sucrose
Threonic acid
Threonine
Thymine
Trehalose
Tryptophan
Tyrosine
Uracil
Urea
Uric acid
Valine
Xanthine
Xylitol
Xylonic acid
2-Aminoadipic acid
2-Aminobutyric acid
2-Aminoethanol
2-Hydroxypyridine
3,4-Dihydroxybenzoate
3-Hydroxy-3-methylglutarate
3-Phenyllactic acid
4-Aminobutyric acid
4-Hydroxybenzoic acid
3-Hydroxyanthranilic acid
4-Hydroxyphenethyl alcohol
4-Hydroxyphenyl pyruvate
4-Hydroxyphenylacetic acid
Adenosine
a-Ketoglutaric acid
Alanine
Allantoin
Asparagine
STI
RTI
CTI
JTI

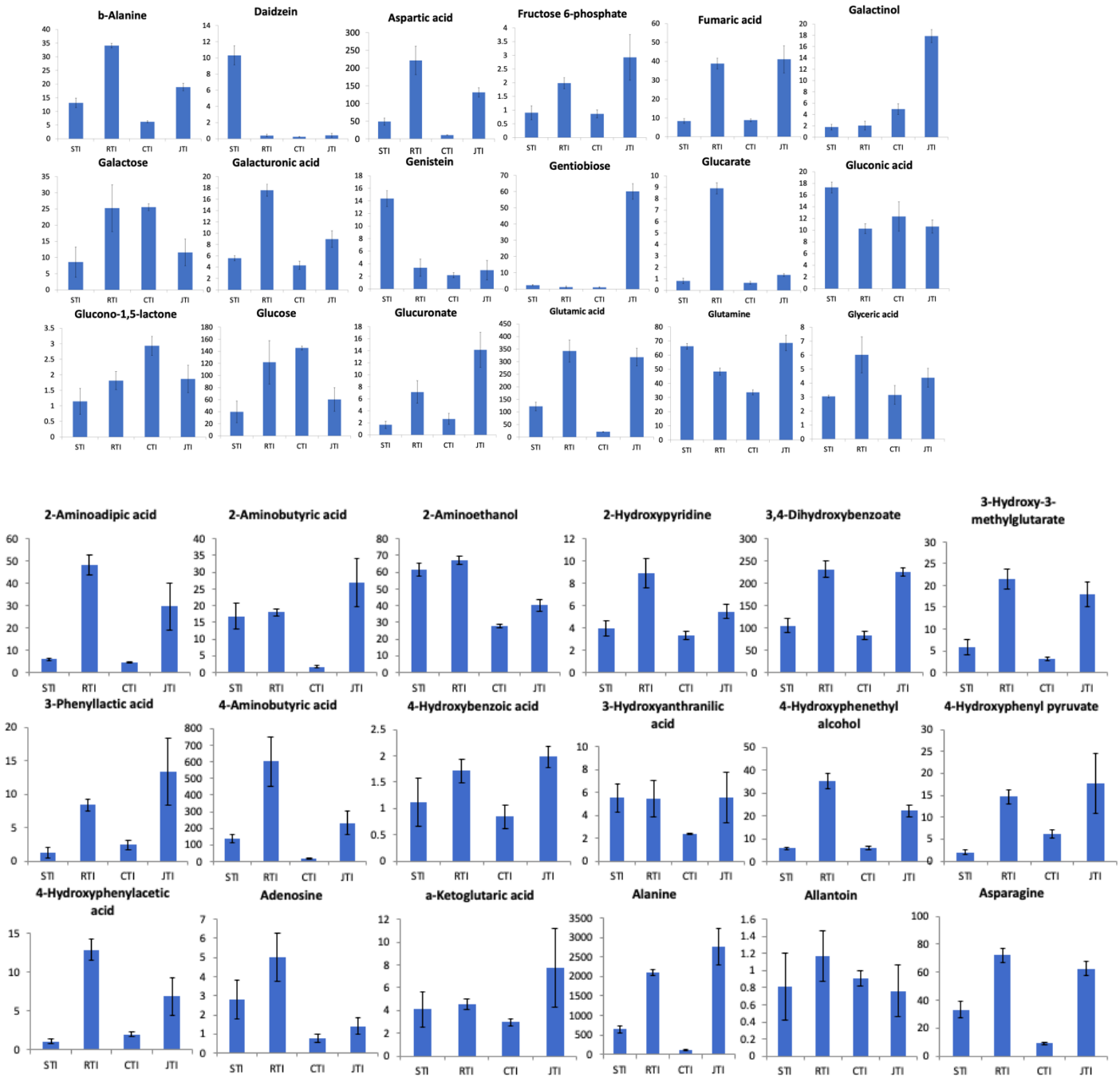


Figure S4. Bar graph of metabolites annotated in loading plot of legume tempe produced in Indonesia based on GC-MS. The vertical axis indicates relative intensity, and the horizontal axis indicates samples. The error bar shows the standard deviation from three technical replicates.

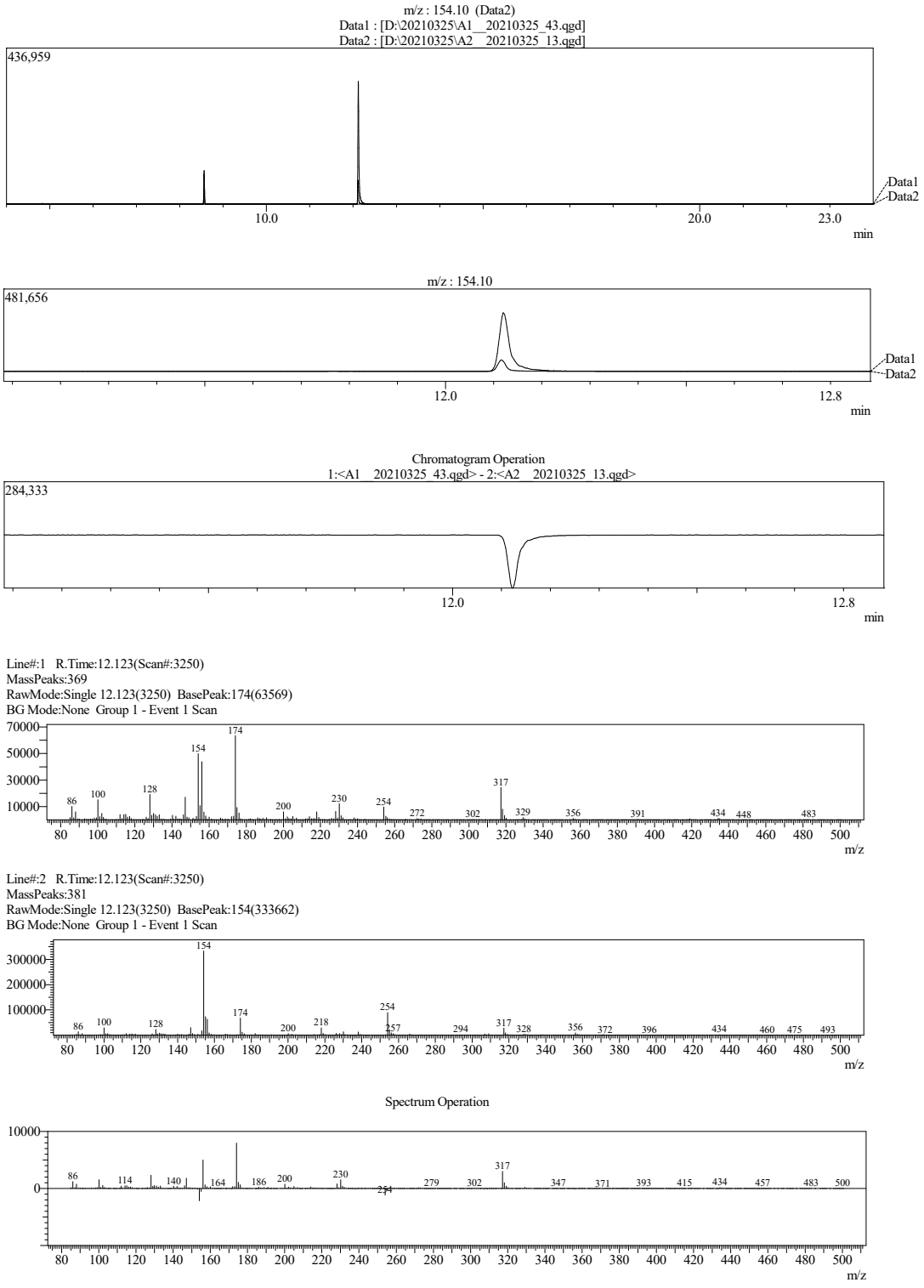


Figure S5. Chromatogram of soybean *tempe* sample with histidine standard at m/z 154 & retention time (RT) 12.1 minutes for histidine. Data 1 represent a sample without standard, and data 2 represent a sample with standard histidine.