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Contributed Paper

Chemical Compositions and Metabolite Profiling of Rice Varieties from Chiang Rai Province, Thailand

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ABSTRACT

The objectives of this study were to analyze and compare chemical compositions and identify the metabolite profiles of Thai traditional rice cultivars. Fifteen rice varieties were grown under conventional agronomic practices. A metabolite profiling approach based on gas chromatography-mass spectrometry (GC/MS) was used to investigate metabolites in Thai traditional rice varieties. The similarity and difference of metabolite data were analyzed by principal component analysis. Statistical analysis of the amylose content showed that the rice verities could be classified into two groups: low and high amylose groups. The higher anthocyanin content was observed in the black- colored rice varieties. In addition, total 44 metabolites including organic acids, sugars and sugar-alcohols, unsaturated and saturated fatty acids were identified. The first and second principal components explained 90.18% of the total variation. The findings obtained from this study should provide the potential role of metabolite profiling in the study and improve rice production and quality.

Keywords: rice (*Oryza sativa* L.), grain quality, chemical compositions, metabolomics, principal component analysis (PCA)

1. INTRODUCTION

Rice (*Oryza sativa* L.) is one of the extensively grown agricultural products and the most important staple crop for human consumption in many countries. Total production of rice paddy was 741.48 MT in 2014 [1]. In addition, Thailand has been recognized as one of the main rice producing and exporting countries. The bulk chemical compositions including carbohydrates, proteins, dietary fibers and micronutrients influence cooking, sensory and nutritional qualities of cereal products [2]. Recently, metabolomics have been studied for the comprehensive profiling of plants, natural products, food and nutrition researches [3]. At present, there is no single analytical method or combination of analytical methods including GC-MS, LC-MS or NMR which is

able to identify all metabolites within a biological system. Gas chromatography-mass spectrometry (GC-MS) is a method for quantitative and qualitative detection of metabolites with high thermal stability and volatility. The method is highly sensitive, specific and allows for high reproducibility [4]. In addition, metabolite profiling is the measurement of a broad range of metabolites within a single extract [5]. Due to the large quantity of metabolites obtained from measurements, multivariate analysis including principal component analysis (PCA) is employed. Principal component analysis is a leading technique for treating complex metabolite data. Recently, metabolite profiling has been used to identify a range of compounds of wheat [6] and rice [7, 8]. However, metabolomics analysis of native rice in Thailand is limited to volatile aroma metabolites and components, for example, carbohydrates and proteins [2, 9].

Therefore, the objectives of this study were to analyze and compare chemical compositions and identify the metabolite profiles of Thai traditional rice cultivars.

2. MATERIALS AND METHODS

2.1 Materials

Grains of fifteen rice (*Oryza sativa* L.) cultivars grown in Chiang Rai province, Thailand were studied. Rice samples grown in the wet season (July-November) of 2015 were donated by the Rice Research Center, Chiang Rai. Colored and traditional varieties including *Kheiyw-ngu, Hemy-nong, Khao kheiyw dum Khao kum* and *Luem pua* were purchased from local farmers in different districts (*Amphoe*) (Table 1 and Figure 1). Mixed two hundreds grams of paddy obtained from three represented batches based on different rice fields were dehulled by using a laboratory scale rice dehuller (Natrawee Technology Co., Chachoengsao). Fifty grams of unpolished rice were ground to pass 0.5 mm mesh using a laboratory rice miller (McGrill type, USA) and stored at -40 °C until further analysis.

Table 1. Rice varieties grown in Chiang Rai province.

No.	Variety	Source	Type	Symbol
1.	*RD15*	*Amphoe Phan*	Polished	R1
2.	*Khao dawk mali 105*	*Amphoe Phan*	Polished	R2
3.	*Phitsanulok 2*	*Amphoe Phan*	Polished	R3
4.	*Sew Mae Chan*	*Amphoe Mae Chan*	Polished	R4
5.	*Sanpathong 1*	*Amphoe Mae Chan*	Polished	R5
6.	*Kheiyw-ngu*	*Amphoe Mae Chan*	Polished	R6
7.	*Hemy-nong*	*Amphoe Wieng Pa Pao*	Polished	R7
8.	*Rice berry*	*Amphoe Wieng Chai*	Black	R8
9.	*Hom nil*	*Amphoe Muang*	Black	R9
10.	*Deang mun poo*	*Amphoe Wieng Pa Pao*	Red	R10
11.	*Hom deang*	*Amphoe Wieng Chai*	Red	R11
12.	*Khao kheiyw dum*	*Amphoe Wieng Pa Pao*	Black	R12
13.	*Khao kum*	*Amphoe Mae Suay*	Black	R13
14.	*Luem pua*	*Amphoe Muang*	Black	R14
15.	Japanese rice	*Amphoe Wieng Pa Pao*	Polished	R15

Figure 1. Representative of rice samples: polished rice (A); Japanese rice (B); *Rice berry* (C); *Hom nil* (D); *Khao kheiyw dum* (E); *Khao kum* (F); *Luem pua* (G); *Deang mun poo* (H) and *Hom deang* (I).

2.2 Chemicals

All chemicals were of analytical grade. Total starch assay kit (AA/AMG) was obtained from Megazyme International Ireland, Wicklow, Ireland.

2.3 Moisture Content

Moisture content was determined by following the method of AOAC method 940.56 [10].

2.4 Amylose Content

Amylose content was determined by using the method of Juliano [11] with slight modifications. A hundred milligrams of milled rice powder was mixed with 1 mL of 95% ethanol and 9 mL of 1N sodium chloride solution. To obtain gelatinized starch, the mixture was incubated in a warm water bath at 95°C for 10 min. Consequently, starch solution was cooled down to room temperature. The starch solution (5 µL) was transferred to a hundred mL volumetric flask and then 1 mL of 1N acetic acid and 2 mL of 1% iodine solution was added prior to adjusting the volume to 100 mL with distilled water. The absorbance of the solution was measured at 620 nm by using UV-visible spectrophotometer (Biochrom-Libra S22, Biochrom Ltd.,UK) after 20 min of reaction at room temperature. Starch contents were obtained by comparing the absorbance of the standard curve prepared from potato amylose and expressed as the percentage of amylose.

2.5 Starch Content

The determination of starch content was done by using total starch assay kit (AA/AMG), Megazyme International Ireland.

2.6 Extraction of Anthocyanins

Colored rice varieties were chosen for further studies to investigate the anthocyanin content. Colored rice flour (1 g) was extracted at room temperature with 10 mL of 0.1 M HCl/methanol [85:15, (v/v)] for 30 min on a shaking machine and then separated by

centrifugation. The supernatants were collected and kept in the dark and cold (4°C) place prior to analysis.

2.7 Total Anthocyanin Content

The determination of total anthocyanin content was performed by using the pH difference method with slight modifications [12]. The rice extract (1 mL) was diluted with 9 mL buffer solution including potassium chloride buffer (pH 1.0) and sodium acetate buffer (pH 4.5). The diluted solutions were incubated in the dark for 15 min. The absorbance values of diluted solutions were measured at 510 nm and 700 nm by using a UV-visible spectrophotometer (Biochrom-Libra S22, Biochrom Ltd.,UK). The content of anthocyanin was calculated by using the following Equations (1) and (2), respectively.

$$A = (A_{510} - A_{700})\ \text{pH 1.0} - (A_{510} - A_{700})\ \text{pH 4.5} \quad \text{Equation (1)}$$

$$\text{Total anthocyanin content (mg/L)} = \frac{(A \times MW \times DF \times 1000)}{(\varepsilon \times 1)} \quad \text{Equation (2)}$$

Where MW is molecular weight; DF is dilution factor; ε is molar absorptivity; MW = 449.2; l = 1 and ε = 26,900

2.8 GC/MS Based Approach Metabolomics

Metabolite profiles of rice grains were determined by using GC/MS based approach metabolomics [4] with slight modifications.

2.9 Metabolite Extraction

Fifty milligrams of rice flour was extracted with chloroform (750 µL) and distilled water (1,400 mL) in a glass vial by gently mixing for 1 min. The mixture was centrifuged at 2,200 g for 15 min and the supernatant (150 µL) was transferred to a new glass vial. The vial was placed in a nitrogen evaporator for completely drying of the supernatant.

For the derivatization, the dried residue was re-dissolved in 40 µL of 20 mg/mL methoxyamine hydrochloride in pyridine. Sixty milliliters of ribitol (0.2 mg/mL) was added as internal standard before and the solution was derivatized at 37°C in a warm water bath for 2 h. Consequently, 70 µL of N-methyl-N-(trimethylilyl)-trifluoroacetamide (MSTFA) was added and incubated at 37°C for 30 min. The sample was then transferred into 1.5 mL autosampler vials with glass inserts and stored at -20°C prior to GC-MS [4].

2.10 GC-MS Analysis

Metabolite profiling by GC-MS was performed using Agilent 7890A/5975C GC-MS system (Agilent Technologies, Santa Clara, California, USA) for all analyses. One µL sample volume was injected with a split ratio of 25:1. GC separations were carried out on a HP-5MS capillary column with inner diameter 30 m × 0.25 mm and film thickness 0.25 mm. Injection temperature was 230°C and He was used as a carrier gas at a constant flow rate of 1 mL/min. The GC temperature program was held at 70°C for 5 min, then increased to 310°C at a rate of 4°C/min, and held at 310°C for 7 min, giving a total analysis time of 60 min per sample. The MS source was adjusted to 230°C and the mass range of m/z 70-700 was recorded. The mass spectra were acquired in electron impact ionization mode [4].

The peak area integration and chromatogram visualization were performed using Agilent ChemStation software (Agilent Technologies, Waldbronn, Germany). For peak identification and mass spectra evaluation, NIST Standard Reference Database version 14 (NIST 14) (National

Institute of Standards and Technology, Boulder, CO, USA) was used.

2.11 Statistical Analyses

All experiments were performed in triplicate analyses and expressed as mean ± standard deviation (SD). Statistical analysis was calculated by using SPSS statistical software (IBM Inc. NY, USA). Analysis of variance (ANOVA) was performed using a least significant difference of 5% by Duncan's multiple range tests. Multivariate statistics for comparison of metabolite concentrations were performed using principal component analysis (PCA). In addition, the PCA was performed using XLSAT statistical software (Addinsoft Inc., NY, USA)

3. RESUTLS AND DISCUSSION

3.1 Chemical Compositions

Fifteen rice cultivars were analyzed for moisture, starch and amylose contents (Table 2). The moisture contents were significantly different ($p < 0.05$) and ranged from 9.53 to 11.45%. All rice cultivars had moisture contents lower than maximum moisture content (14%) for Thai aromatic rice [13]. Starch content influences the nutritional quality of rice.

Table 2. Chemical compositions of rice varieties.

Symbol	Variety	Chemical compositions		
		Moisture content (%)	Starch content (% DM)	Amylose content (% DM)
R1	*RD15*	11.34 ± 0.11 a	78.26 ± 2.17 cd	19.17 ± 0.59 d
R2	*Khao Dawk Mali* 105	10.94 ± 0.15 b	79.52 ± 1.26 b	16.38 ± 0.38 e
R3	*Phitsanulok* 2	9.61 ± 0.11 hg	77.25 ± 1.01 cdf	26.63 ± 0.76 a
R4	*Saew Mae Chan*	9.78 ± 0.04 fg	84.09 ± 0.48 a	6.82 ± 0.38 i
R5	*Sanpathong 1*	9.77 ± 0.05 fg	83.65 ± 1.44 a	5.01 ± 0.0 k
R6	*Kheiyw-ngu*	11.13 ± 0.08 b	83.41 ± 2.03 a	6.17 ± 0.09 ij
R7	*Hemy-nong*	9.86 ± 0.05 f	80.02 ± 3.17 bc	5.79 ± 0.09 jk
R8	*Rice Berry*	10.99 ± 0.08 bc	75.95 ± 0.33 fe	13.47 ± 0.08 g
R9	*Hom Nil*	10.55 ± 0.08 d	81.90 ± 2.53 b	17.25 ± 1.00 e
R10	*Deang Mun Poo*	11.43 ± 0.06 a	82.17 ± 0.37 b	20.39 ± 0.52 c
R11	*Hom Deang*	9.65 ± 0.04 hg	81.84 ± 1.78 b	18.72 ± 0.68 d
R12	*Khieiyw Dum*	10.69 ± 0.10 d	74.54 ± 2.22 e	4.81 ± 0.80 k
R13	*Khoa Kum*	10.23 ± 0.10 e	77.42 ± 2.15 cdf	8.51 ± 0.57 h
R14	*Luem Pua*	9.53 ± 0.24 h	79.34 ± 0.24 bc	7.91 ± 0.42 h
R15	Japanese rice	11.45 ± 0.07a	80.15 ± 1.45 bc	24.28 ± 0.75 b

Mean values within the same column followed by different letters are significantly different at $p < 0.05$.

In this study, the starch content varied between 74.54 to 84.09%. Starch consists of two of molecules: amylose and amylopectin. Generally, starch in plant has 20-25% amylose and 75-80% amylopectin by weight.

According to the amylose content in rice, rice cultivars can be classified into five groups including waxy (1-2%), very low (2-9%), low (10-20%), intermediate (20-25%) and high (25-33%) [14]. Therefore, in this study,

very low amylose type rice consists of R5, R6, R7, R12, R13 and R14. Rice samples including R1, R2, R4, R8, R9, R10 and R11 are classified as low amylose type. In addition, high amylose content in R3 and R15 samples was 26.63 and 24.28%, respectively. Cooking quality of rice is affected by the amylose content. Low amylose type rice (10-20%) are moist and sticky after cooking while high amylose type rice (>25%) are dry and fluffy but become hard upon cooling due to retrogradation of the amylose molecules [2].

In addition, colored rice grains including red rice (R10 and R11) and black rice (R8, R9, R12, R13 and R14) samples were analyzed for their total anthocyanin content. The total anthocyanin content ranged from 1.91-11.04 and 16.25-77.37 mg/100 g DM in red and black rice varieties, respectively (Figure 2). However, both contents are lower than those in colored rice varieties in the previous study. The highest anthocyanin contents have been found in black rice, with an average of 327.6 mg/100 g, and that of red rice 9.4 mg/100 g [12]. This discrepancy might come from the differences in rice varieties, agronomic practices and analytical methods. In rice, the black-colored rice grain contains mainly cyanidin 3-glucoside and peonidin 3-glucoside and their contents ranging from 19.4-140.8 mg/100 g DM and 11.1-12.8 mg/100 g DM, respectively [2].

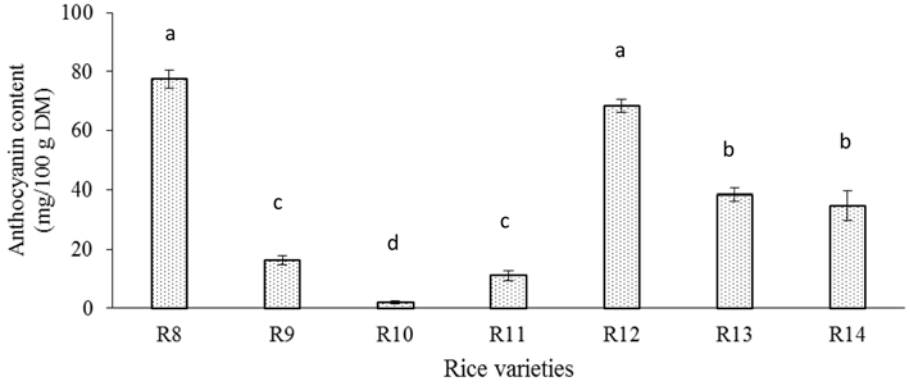

Figure 2. Total anthocyanin content in some rice varieties. Mean values with different letters on the same bars are significantly different at $p < 0.05$

3.2 GC-MS Metabolite Profiling

The total ion current (TIC) GC-MS chromatograms of the representative four different rice grains are shown in Figure 3. The majority peaks with different relative intensities were observed in all samples.

Table 3 shows the identified metabolites obtained from rice varieties. Total 44 metabolites were extracted and identified including organic acids, amino acids, sugars, sugar-alcohols, saturated and unsaturated fatty acids. Sucrose, 8TMS were observed in all rice grain samples. Major unsaturated fatty acids present were the 18-carbon triglycerides of oleic, linoleic and linoelaidic acids, which have one, two and two (*trans*) double bonds, respectively. Saturated fatty acids such as myristic (C14), palmitic (C16) and stearic acids (C18) were also detected. In addition, the unsaturated fatty acids in milled rice might contribute to rice fragrance due to low-odor threshold volatile compounds which can be derived from the oxidation of unsaturated fatty acids [15]. The compositions of these metabolites in rice grain samples vary from cultivar to cultivar.

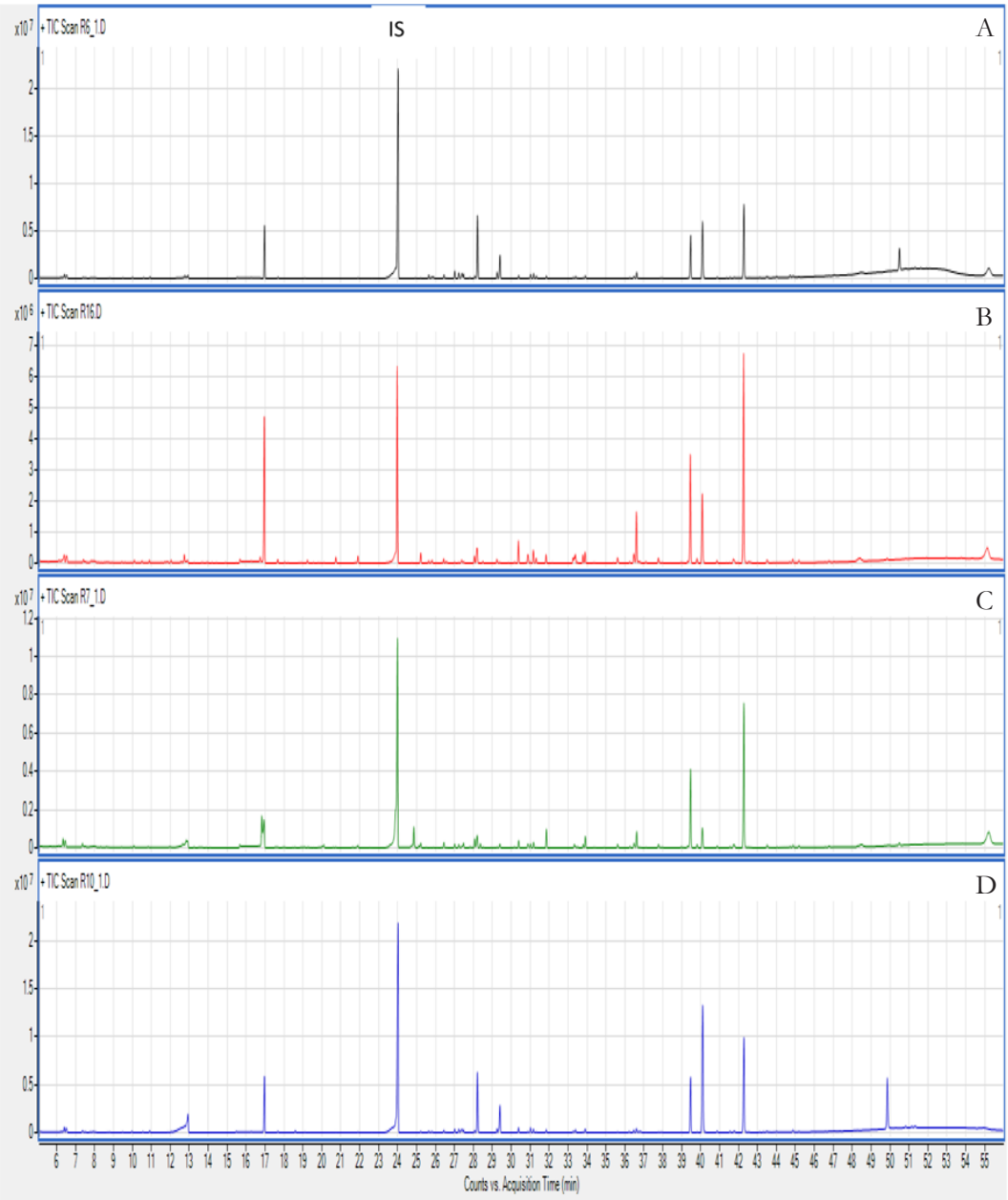

Figure 3. Representative of total ion current (TIC) of some rice samples: R1 sample (A); R5 sample (B); R10 sample (C) and R13 sample (D); IS = internal standard**.**

Table 3. Metabolites of rice grains.

No.	Retention time (min)	Metabolites	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15
1	6.393	1,3-Propanediol, 2TMS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	6.510	Lactic acid, 2TMS	-	+	-	-	-	-	-	+	-	+	-	+	-	+	-
3	11.871	Benzoic acid, TMS	-	-	-	+	+	-	+	-	-	+	-	-	-	-	+
4	12.899	Glycerol, 3TMS	+	+	-	+	+	-	-	+	+	+	+	+	+	+	+
5	16.475	Malic acid, 3TMS	-	-	-	-	+	-	-	-	+	+	+	-	-	-	+
6	19.099	Erythritol, 4TMS	+	-	-	-	-	-	-	-	+	-	-	-	-	+	-
7	19.131	L-5-Oxoproline, 2TMS	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
8	19.256	Benzoic acid, 4-ethoxy	-	-	-	-	+	-	-	-	-	+	-	-	-	+	+
9	20.751	Diethyl phthalate	-	+	-	+	+	+	+	-	-	-	-	-	-	+	+
	23.989	Ribitol (Internal standard)															
10	25.539	Azelaic acid, 2TMS	+	-	-	-	-	-	-	-	-	+	+	-	+	+	-
11	25.669	D-(-)-Fructofuranose, pentakis iso1	+	-	-	-	+	-	+	-	+	+	+	-	-	+	-
12	25.795	D-(-)-Fructofuranose, pentakis iso2	+	-	-	-	-	-	+	-	+	+	+	-	-	+	-
13	25.934	Protocatechoic acid, 3TMS	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-
14	26.563	Myristic acid, TMS	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+
15	26.828	D-Glucopyranose, 5TMS iso1	+	-	-	-	+	-	+	-	+	-	-	+	-	-	-
16	27.166	D-Glucopyranose, 5TMS iso2	+	-	-	-	+	-	+	+	+	+	+	-	+	+	-
17	27.776	Hexadecanoic acid, methyl ester	-	-	+	-	-	-	-	-	+	-	-	+	-	-	-
18	27.406	Galactonic acid, gamma-lactone	+	-	-	-	-	-	-	-	-	+	+	-	+	+	-
19	27.513	Talose, 5TMS derivative	+	-	-	-	-	-	-	-	-	+	+	-	+	+	-
20	27.442	Gluonic acid, gamma-lactone	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-
21	27.618	Dimethyl palmitamine	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
22	27.730	D-Mannose, 2,3,4,5,6-pentakis	-	+	-	-	-	-	-	-	+	+	+	-	-	-	+

+ = present and - = absent

Table 3. Continued.

No.	Retention time (min)	Metabolites	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15
23	28.087	D-Sorbitol, 6TMS	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+
24	28.204	D-Glucitol, 6TMS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
25	29.232	D-Glucose,5TMS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
26	29.455	D-Gluconic acid, 6TMS	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+
27	29.843	Palmitoleic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
28	30.318	Palmitic acid, TMS	-	-	+	-	-	-	-	-	+	-	-	+	-	-	-
29	30.936	Oleanitrile	+	+	+	+	-	-	-	+	+	-	+	+	-	-	+
30	31.015	Myo-Inositol, 6TMS	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+
31	31.215	Linoelaidic acid	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
32	31.332	9-Octadecenoic acid (Z)	+	+	-	+	+	-	+	+	-	+	+	+	-	+	-
33	31.885	Methyl stearate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
34	33.287	Linoleic acid	+	+	-	+	+	-	+	-	+	+	+	+	-	+	+
35	33.402	Elaidic acid, TMS	+	+	-	+	+	-	-	-	+	-	-	+	+	+	+
36	33.387	Oleic Acid, (Z)-, TMS	-	-	+	-	+	-	+	+	-	-	-	-	-	-	-
37	33.916	Stearic acid, TMS	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
38	35.759	9-Octadecenamide, (Z)	+	+	-	+	+	+	+	+	-	+	-	+	+	+	+
39	36.967	1-Monopalmitin, 2TMS	-	-	-	+	-	+	-	+	-	+	-	-	-	-	+
40	36.929	Oleamide, TMS	+	+	+	+	+	-	-	-	-	+	-	-	+	+	+
41	39.118	2-Palmitoylglycerol, 2TMS	-	-	-	+	-	+	+	-	-	+	-	-	+	-	+
42	39.474	1-Monopalmitin, 2TMS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
43	40.097	Sucrose, 8TMS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
44	41.968	2-Monostearin, 2TMS	+	-	+	+	-	+	-	+	+	-	-	+	+	-	-

+ = present and - = absent

3.3 Principal Component Analysis of Rice Metabolites

Principal component analysis (PCA) is a multivariate procedure used to determine similarities and differences in the metabolites. Figures 4 and 5 show the PCA score and loading plots from identified metabolites obtained from rice GC-MS based metabolic approach. The loading is used to interpret the scores. It showed the magnitude (large/small correlation) and the manner (positive/negative correlation) which the variables (metabolites) contribute to the scores (principal components) [16]. The first two combined principal components (PC1 and PC2) explain 90.18% of the total variance. The positive value direction of PC1 exhibits a cluster of all Thai traditional rice samples (R1 to R14) while the Japanese rice sample (R15) was clearly separated and located in the negative value direction. The Japanese rice is a member of the *Japonica* family while Thai traditional rice grains belong to the *Indica* family. The differences between both rice varieties in the important rice traits of grain and cooking qualities are observed. *Japonica* rice grains are short and roundish. After cooking, cooked rice does not shatter easily. However, *Indica* rice grains are long to short and flat in shape. Cooked rice shatters more easily. Interestingly, the position of Thai improved rice cv. *Rice berry* (R8) was located close to the location of the Japanese rice (R15). *Rice berry* is a newly-registered rice variety from Thailand, originated from a cross-breeding between *Khoa Hom Nil,* the local non-glutinous purple rice and *Khoa Dawk Mali* 105, the Thai *Hom Mali* rice. For PC2, its upward positive value shows the cluster of *Saew Mae Chan* (R4) and *Kheiyw-ngu* (R6) while its negative value indicates the cluster of the majority of rice grain samples. Interestingly, both R4 and R6 samples were waxy rice and grown in *Amphoe Mae Chan*.

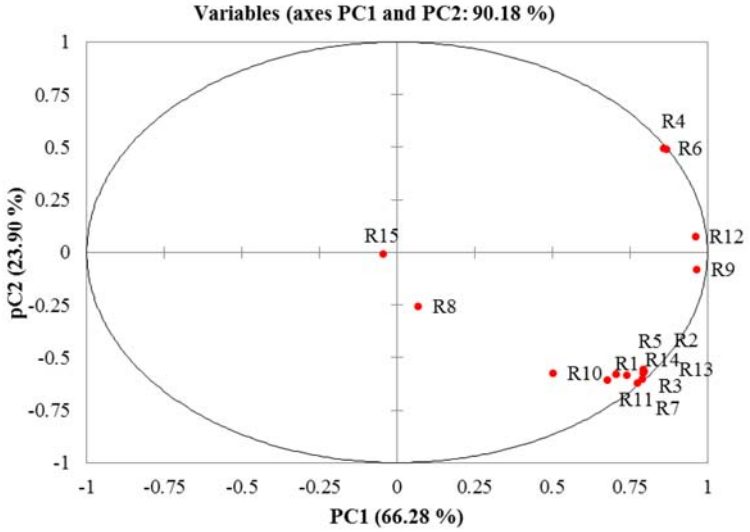

Figure 4. Score plot of rice metabolites.

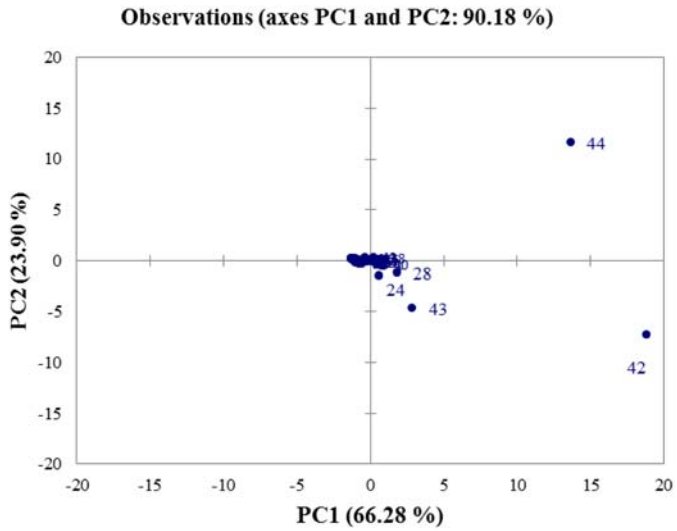

Figure 5. Loading plot of rice metabolites.

The load values indicated that PC1 is separated by 2-monostearin, 2TMS in the positive half. The PC2 axis is identified by 1-monopalmitin, 2TMS and sucrose, 8TMS. However, the majority of the metabolites were poorly distinguished. In addition, higher levels of metabolites including fatty acids, free fatty acids, organic acids and amino acids were observed in red rice and black rice when they were compared to polished rice. This finding is in agreement with the previous study [8].

The previous studies reported a small genotype influence and large effects of crop year and genotype-by-environment interaction on the metabolite compositions and quality of the wheat grain [6]. In order to improve and produce a good quality of rice grains in Chiang Rai province, future work should be studied on the relationship between more rice genotypes and agronomic areas and practices including conventional and organic applications on the metabolite compositions.

4. CONCLUSION

This study was able to indicate the key chemical compositions of high quality rice varieties. GC-MS based metabolomics was performed to analyze metabolites including organic acids, sugars, sugar alcohols, unsaturated and saturated fatty acids in rice grain samples. This can be distinguished by unsaturated fatty acid profile by using multivariate analysis via principal component analysis. Further work should be carried out to study the key compounds which contribute to the profiling of volatile metabolites.

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