



**Full Length Article**

## Simultaneous Quantification of Anthocyanins and Phenolic Acids in Pigmented Rice (*Oryza sativa*) using UPLC-PDA/ESI-Q-TOF

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### Abstract

Anthocyanins and phenolic acids are the major antioxidants in purple rice. The method conditions were specified and not covered for both anthocyanins and phenolic acids. The objective of this study was to adopt the UPLC coupled with ESI-MS method for determination of the major anthocyanins and phenolic acids under the same condition in purple, red and white rice varieties. A UPLC-PDA/ESI-Q-TOF method was optimized with a high linearity for all analyzed compounds with regression coefficients greater than 0.99. Repeatability was good with the relative standard deviation values generally less than 5.5%. The limit of detection and quantification ranged from 1.9–36.6 and 5.0–123.1  $\mu\text{g kg}^{-1}$ , respectively. Spike recoveries were between 81–116%. Mass spectroscopy was used to confirm identity in the positive ion mode for all analyzed compounds. Cyanidin-3-glucoside and ferulic acid were the most abundant anthocyanins and phenolic acids, respectively. Bound ferulic acid was higher in purple rice than in red and white rice flours. This developed procedure can be used to facilitate the rapid screening of rice seed banks for anthocyanins and phenolic acids of interest for rice pre-breeding. © 2019 Friends Science Publishers

**Keywords:** Pigmented rice; UPLC-MS; Anthocyanin; Phenolic acid

### Introduction

Pigmented rice contains a wide range of phenolic compounds including anthocyanins and phenolic acids (Huang and Lai, 2016) as well as derivatives of anthocyanidins (without glucoside). In general, cyanidin-3-glucoside and peonidin-3-glucoside are the most abundant anthocyanins in purple rice, *p*-coumaric acid and ferulic acid are the main phenolic acids (Zaupá *et al.*, 2015; Hu *et al.*, 2017). Anthocyanins and phenolic acids have been extensively quantified in rice mainly by high-performance liquid chromatography (HPLC) (Shao *et al.*, 2014; Hao *et al.*, 2015). For example, two anthocyanins were observed in black rice but anthocyanidin was not observed (Kim *et al.*, 2014) and twelve phenolic acids were identified (Chatthongpisut *et al.*, 2015; Gong *et al.*, 2017). Recently, the use of the high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) to analyse the profiles of anthocyanins and phenolic acids offers a number of advantages (Wang *et al.*, 2015; Zhang *et al.*, 2015), however the use of ultra-performance liquid chromatography-quadrupole-time-of-flight -mass spectrometry (UPLC-Q-TOF-MS) for anthocyanins and phenolic acids analysis has been successfully for many

advantages, including fast separation, high resolution with high sensitivity, and accurate mass measuring (Li *et al.*, 2010). With the more advanced UPLC-PAD-ESI-MS is still evolving in pigmented rice (Hao *et al.*, 2015; Bae *et al.*, 2017). However, the method conditions were specified in each compound and not covered for both anthocyanins and phenolic acids. For example, the positive ion mode was used for anthocyanins, while the negative ion mode found to be more suitable for some phenolic acids (Lachowicz *et al.*, 2017; Samoticha *et al.*, 2017). Although, all phenolic acids can be detected in both positive and negative modes, some phenolic acids more sensitive to the positive than to the negative ion modes (Lin *et al.*, 2015; Kumari *et al.*, 2016). Therefore, optimization with a shorter analysis time and under the same condition is desirable. Furthermore, the first step in determination compounds from natural sources as extraction is necessary to get a high extraction efficiency, such as alkaline hydrolysis (NaOH) is commonly used in the extraction of bound phenolic acids due to extraction efficiencies being higher than under acidic conditions (HCl) (Kim *et al.*, 2006) with the time of hydrolysis and NaOH concentration being assessed (Shao *et al.*, 2014). However, no studies exist for the optimization of extraction conditions for both free and

bound phenolic acids in purple rice. In addition, the current methods have limitations for some of the commonly analyzed phenolic compounds due to risks of degradation by factors such as pH, temperature and long run times (Sharma *et al.*, 2016).

In this study, the UPLC coupled with ESI-MS method was adopted as it required low amounts of sample and solvent, and was time saving, thus facilitating rapid analysis. A single analytical UPLC-PDA/ESI-Q-TOF method for anthocyanins and phenolic acids is purposed. A further goal of the optimized method to determine the major anthocyanins and phenolic acids in the flour of four rice varieties will be investigated.

## Materials and Methods

### Chemicals and Standards

HPLC grade methanol (MeOH, purity >95%) and formic acid (HCOOH, purity >98%) were used for chromatography and purchased from Sigma-Aldrich (St. Louis, MO, USA). Cyanidin chloride (Cy), peonidin chloride (Pn), cyanidin-3-glucoside (C3G), peonidin-3-glucoside (P3G) were purchased from Extrasynthese (Genay, France). Ferulic acid (FA) and *p*-coumaric acid (*p*-Cou) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All standards used were of 95–99% purity. Ethyl acetate (EtOAc, purity >99%), hydrochloric acid (HCl, 32%, w/w) and other chemical reagents were commercially available and of analytical grade. Double deionized (DDI) water was used for chromatography and sample extraction.

Stock solutions were prepared weekly in acidified MeOH at a concentration of 1 mg mL<sup>-1</sup>. After confirming compound performance individually, two stock solutions were then prepared daily, the first containing C3G, P3G, *p*-Cou and FA, and the second containing Cy and Pn. The two stock solutions were mixed just prior to injection and used within 48 h.

### Rice Material

Four photo-period sensitive rice varieties were used in this study with two traditional improved purple rice varieties were grown in the research field of Chiang Mai University - Kum Doi Saket (KDK) and CMU125; and two popular commercial Thai varieties Hawm Mali Deng (HMLD) and Khao Dawk Mali105 (KDML105) with red and white pericarps, respectively. Seed of KDK and CMU125 were grown in a paddy field in the Chiang Mai University field station, Chiang Mai, Thailand (18 °47' N, 98 °57' E), in the wet season (August – December, 2016). Two seedlings at approximately 30-days old were transplanted at 0.25 × 0.25 m spacing between hills. Urea was applied for growth, 60 kg N ha<sup>-1</sup> at maximum tillering and repeated at flowering, following the recommendation for farmers provided by the Rice Department of Thailand. Seed of HMLD and

KDML105 were obtained from a commercial supplier (All Rice Surin Shop, Surin province, Thailand) from the current year's harvest.

Grains of all varieties were harvested by manual threshing and then air-dried until the moisture content fell below 14%. The paddy rice was kept in zip-lock plastic bags and stored in the freezer at -25°C in the dark condition for 4 months until used. The rice was machine de-husked (Model P-1 from Ngek Huat Co., Ltd., Thailand) to produce brown rice. Then, 100 g samples of the brown rice were mechanically ground for 60 sec in a hammer mill (Scientific Technical Supplies D-6072 Dreieich, West Germany). The ground samples were placed back in zip-lock plastic bags and stored in the freezer with similar condition as above for 5 months until analysis.

### UPLC-PDA/ESI-MS Method

UPLC analysis was performed on a Waters Acquity UPLC equipped with a photodiode-array detector (PDA) and a Zorbax Eclipse C18 column (2.1 × 100 mm, 3.5 μm particle size) to separate the compounds. The injection volume was optimized to 3 μL and the sample temperature was 4°C. The gradient elution mobile phases were 0.1% (v/v) formic acid in water (mobile phase A) and 0.1% (v/v) formic acid in methanol (mobile phase B).

The identity of all compounds was confirmed by MS using an Agilent Q-TOF Micromass equipped with an orthogonal electrospray ionization source operating in positive and negative ion modes with a scanning mass range of 100–1000 m z<sup>-1</sup>. The same column and mobile phases were optimized as for UPLC. ESI-MS parameters were set as follows: capillary voltage 2.6 kV; sampling cone voltage 45 V; cone voltage 5.0 V; source temperature 120; desolation temperature 350°C; cone gas flow rate 0 L h<sup>-1</sup> and desolation nitrogen gas flow rate 300 L h<sup>-1</sup>.

### Method Validation

The specificity of the method was evaluated by determining the anthocyanins, anthocyanidins and phenolic acids in various concentrations (30, 20, 10, 2, 1 and 0 μg mL<sup>-1</sup>) by ultra-performance liquid chromatography-quadrupole-time of flight-mass spectrometry (UPLC-PDA/ESI-Q-TOF). Anthocyanins compounds and free phenolic acids were extracted based on a published method (Chatthongpisut *et al.*, 2015) but with the following changes to improve the procedure. Flour samples (0.5 g) were extracted with 6 mL of acidified MeOH (15 mL of 1.0 N HCl in 85 mL of MeOH) on a mechanical shaker for 1 h. The extract solution was centrifuged at 4,000 × g for 10 min at room temperature and then filtered through 0.22 μm Nylon membrane before analysis. After removal of anthocyanins and free phenolic acids, the bound phenolic acids were extracted using a hydrolysis protocol optimized from methods developed in our laboratory (Hao *et al.*, 2015; Gong *et al.*, 2017). Rice

flour was hydrolyzed with 10 mL of 4 M NaOH at 25°C with continuous shaking for 2 h, and 3.6 mL concentrated HCl was added to adjust the pH to 2.5. The mix was extracted with EtOAc (3 × 10 mL), centrifuged, and the bound phenolic fraction was collected and evaporated under N<sub>2</sub> gas at 35°C. The residue was taken up in 2 mL acidified MeOH. All samples were analyzed within 24 h from extraction.

Linearity was determined based on the calculation of the RSD (%) using the standard deviation of peak area values with three replicates. Accuracy was evaluated based on spiking at 120, 240 and 360 mg kg<sup>-1</sup> using three replicates each by carrying out the extraction of the sample blank (white rice extract) and the recovery of the compound was determined. Precision was determined after intraday runs at the lowest concentration of spiking samples at 10 µg mL<sup>-1</sup> for 10 injections and the data reported as RSD percent with relative standard deviation. The detection of limit (LOD) and limit of quantification (LOQ) were determined (n = 10) for each compound and calculated using calibration curves as follows: 3SD S<sup>-1</sup> for LOD and 10SD S<sup>-1</sup> for LOQ, where SD = standard deviation of the lowest concentration standard; and S = the slope (sensitivity) of the corresponding calibration curve.

### Statistical Analysis

Standard stability, compounds and rice varieties in this study were evaluated using analysis of variance (ANOVA) (Statistix version 9.0) followed by the *post hoc* LSD test at *p* < 0.05 to compare means with significant differences. Data are expressed as mean ± standard deviation of three replications.

## Results

### Optimization of the Extraction Procedure

The UPLC method reduced the time and cost of analysis, and was optimized for anthocyanins and free phenolic acids determination by UPLC under the same condition as most of the phenolic acids in rice occurs in the bound form, the extraction conditions for this component were optimized (10 mL of 4 M NaOH for 2 h gave complete digestion). The efficiency of the extraction step was optimized using EtOAc and the results showed that three extraction cycles with 10 mL EtOAc gave good separation of the organic layer after solution mixing. Using acidified MeOH to increase extraction efficiency, it is essential to assess extraction recovery through spike analysis. The residue samples (0.5 g) were spiked (n = 3) with 120, 240 and 360 µg g<sup>-1</sup> and the average extraction recoveries were 103% for *p*-coumaric acid and 113% for ferulic acid with average RSD of 8.2% and 6.3%, respectively. Therefore, this extraction method was accepted and used for bound phenolic acid analysis.

The extract solvent was first evaluated between 100% methanol and acidified MeOH (15 mL of 1.0 N HCl in 85 mL of MeOH) for determination of anthocyanins,

anthocyanidin and phenolic acids. The solvent affected the stability of standard compounds. When anthocyanidins were diluted in 100% MeOH, the anthocyanidins (cyanidin and peonidin) could not be detected. By contrast, acidified MeOH resulted in greater stability of anthocyanins and anthocyanidin. Therefore, acidified MeOH was selected for the dilution of calibration standards and extracted samples. In addition, the storage time did not affect the stability of these compounds within 48 h after mixing. Analysis of variance (ANOVA) indicated that both evaluated factors (standard concentration and time after preparation) (n = 3) had no significant effect on the changes in signal intensity of UPLC.

### Optimization of the UPLC-PDA/ESI-MS method

A sensitive and selective UPLC method for the simultaneous analysis of anthocyanins, anthocyanidin and phenolic acids was developed. Separation conditions were first assayed using UPLC with standard solutions at concentrations within 0–30 µg mL<sup>-1</sup>. The preliminary gradient elution program of the mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in methanol) were as follows: 0–2.5 min, 10% B; 2.5–4 min, 10–15% B; 4–6.50 min, 15–20% B; 6.5–9 min, 20–25% B; 9–12.5 min, 25–30% B; 12.5–15 min, 30–35% B; 15–17.5 min 35–50% B, 17.5–20 min, 50–60% B; 20–22.5 min, 60–95% B; 22.5–23 min, 95–10% B at a flow rate of 0.5 mL min<sup>-1</sup>. The column temperature was set at 30°C and the auto-sampler was conditioned at 4°C with an injection volume of 3 µL. The best separation was obtained with a total run time of 25 min to separate all compounds under the same condition. In addition, 500 nm, 310 nm and 320 nm were selected for anthocyanins compounds, *p*-coumaric acid and ferulic acid, respectively as monitoring wavelengths according to absorption maxima and the retention time (Table 1).

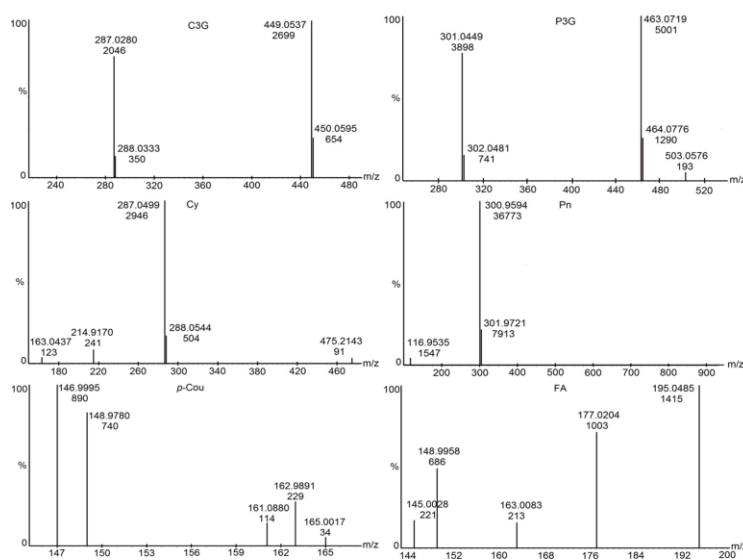
### Identification of Anthocyanins and Phenolic Acids

To facilitate establishing an accurate qualitative method, the MS profile for each compound was analyzed (Table 1 and Fig. 1). The compounds were identified according to their fragmentation data using ESI-MS. Anthocyanin compounds had high sensitivity in the positive ESI mode. Cyanidin-3-glucoside and peonidin-3-glucoside were molecular ion [M+H]<sup>+</sup> at *m/z*<sup>-1</sup> 449.0537 and 463.0719, respectively and a principal fragment ion at *m/z*<sup>-1</sup> 287.0280 (cyanidin) and 301.0449 (peonidin). Two phenolic acids were also confirmed by positive and negative ESI modes and the result indicated that the higher signal sensitivities of phenolic acids were obtained in the positive ESI mode, while poor absorptivity was observed in the negative ESI mode. Our results showed that the electrospray ionization source operated in the positive ESI mode was suitable for phenolic acids. Therefore, the positive mode was selected for identification of all compounds. In the MS full scan spectrum, the analysis showed a protonated molecular ion [M+H]<sup>+</sup> at *m/z*<sup>-1</sup> 165.0017 acid and principal fragment ion at

**Table 1:** Standard compounds detected by UPLC-PDA/ESI-MS using positive ionization mode

Compound	RT <sup>a</sup> (min)	$\lambda_{\text{max}}$ (nm)	RT <sup>b</sup> (min)
Cyanidin-3-glucoside	7.78	500	7.87
Peonidin-3-glucoside	9.44	500	9.50
Cyanidin	12.98	500	13.13
Peonidin	15.82	500	16.03
<i>p</i> -Coumaric acid	8.45	310	8.54
Ferulic acid	9.77	320	9.95

<sup>a</sup> and <sup>b</sup> indicate retention time obtained by PDA and ESI, respectively



**Fig. 1:** MS of cyanidin-3-glucoside (C3G), peonidin-3-glucoside (P3G), cyanidin (Cy), peonidin (Pn), *p*-coumaric acid (*p*-Cou) and ferulic acid (FA)

$m/z$  146.9995, 148.9780, 162.9691 and 161.0880 for *p*-coumaric. Ferulic acid produced a protonated  $[M+H]^+$  at  $m/z$  195.0485 with the principal fragments ion at  $m/z$  177.0204 and a minor ion at  $m/z$  148.9958, 145.0028 and 163.0083.

### Method Validation

Calibration curves were plotted using six different concentrations of mixed standards within the range of 0–30  $\mu\text{g mL}^{-1}$ . The linearity was estimated by calibration curves from three replicates of a standard mixture with concentrations of 0, 1, 2, 10, 20 and 30  $\mu\text{g mL}^{-1}$ . The PDA detector responses of all compounds were linear with regression coefficients ranging from 0.9993 to 0.9998. Moreover, from the slope of the calibration curves with the PDA detector, *p*-coumaric acid had the highest sensitivity, whereas the lowest sensitivity was obtained for peonidin-3-glucoside. Precision was calculated for the individual compounds after intraday runs with data from a minimum of 10 injections. The repeatability values ranged from 0.5 to 5.5% of RSD. The LOD and LOQ varied from 1.50 to 36.94

$\mu\text{g kg}^{-1}$  and 5.00 to 123.14  $\mu\text{g kg}^{-1}$ , depending on the compound. Spike recovery values at 120, 240 and 360  $\mu\text{g g}^{-1}$  ranged from 89.2 to 137.1%, from 97.4 to 137.1% and from 80.7 to 128.8%, respectively, with the RSD of the compounds in the range 5.3 to 9.1% (Table 2).

### Determination of Anthocyanin Compounds, Free and Bound Phenolic Acids in Purple, Red and White Rice

The proposed method was applied to the determination of anthocyanins and phenolic acids in diverse rice varieties. Rice samples were analyzed in triplicate and injection randomized. Quality control samples were run every five samples of injection for checking the stability of compound and performance of this method. Again, excellent measurement was observed between technical replicates for all six compounds, the method was applied successfully with average RSD ranging from 0.05–4.91%, indicating that the analytical procedure was precise (Table 3). Cyanidin-3-glucoside and peonidin-3-glucoside were the predominant anthocyanins in purple rice (Table 3) and LC chromatograms are presented in Fig. 2A. Cyanidin-3-

**Table 2:** Analytical parameters obtained for the UPLC analysis and spike recovery at three spiking levels and repeatability (n = 10) of white rice sample

Compound	Slope	R <sup>2</sup>	Repeatability RSD (%)	LOD ( $\mu\text{g kg}^{-1}$ )	LOQ ( $\mu\text{g kg}^{-1}$ )	Recovery (%), spike at ( $\text{mg kg}^{-1}$ )			
						120	240	360	RSD (%)
Cyanidin-3-glucoside	653.9	0.9994	5.5	9.50	31.68	97.6	105.4	94.2	5.8
Peonidin-3-glucoside	570.9	0.9998	3.7	6.49	21.65	106.0	116.2	105.4	5.6
Cyanidin	2388.8	0.9993	1.5	1.50	5.00	93.4	97.0	81.2	9.1
Peonidin	779.1	0.9993	3.3	36.94	123.14	90.2	94.3	84.8	5.3
<i>p</i> -Coumaric acid	4218.1	0.9996	0.5	7.90	26.32	101.4	113.0	105.8	5.5
Ferulic acid	2787.0	0.9998	0.9	7.29	24.30	99.6	109.8	99.9	5.6

**Table 3:** Determination of concentration ( $\text{mg kg}^{-1}$ ) for anthocyanins and phenolic acids in the flour of four rice varieties

Compound	Variety				RSD (%)
	KDK	CMU125	HMLD	KDML	
Anthocyanins					
Cyanidin-3-glucoside	496.3 $\pm$ 73.7Ab	1998.4 $\pm$ 30.4Aa	nd	nd	4.91
Peonidin-3-glucoside	475.3 $\pm$ 11.3Ab	648.4 $\pm$ 18.2Ba	nd	nd	1.02
Cyanidin	7.5 $\pm$ 3.6Bb	16.1 $\pm$ 0.8Ca	nd	nd	1.63
Peonidin	0.3 $\pm$ 0.16Cb	1.8 $\pm$ 0.5Da	nd	nd	nd
Free phenolic acids					
<i>p</i> -Coumaric acid	2.6 $\pm$ 0.2Bb	6.7 $\pm$ 0.3Ba	0.8 $\pm$ 0.1Bc	1.0 $\pm$ 0.1Bc	7.47
Ferulic acid	10.5 $\pm$ 0.5Ab	12.9 $\pm$ 1.4Aa	4.7 $\pm$ 0.1Ac	5.0 $\pm$ 0.2Ac	0.05
Bound phenolic acids					
<i>p</i> -Coumaric acid	21.5 $\pm$ 2.1Bc	16.7 $\pm$ 2.9Bd	74.6 $\pm$ 2.08Ba	67.4 $\pm$ 3.1Bb	8.57
Ferulic acid	412.4 $\pm$ 22.4Aa	399.3 $\pm$ 21.0Aa	292.0 $\pm$ 4.7Ac	324.3 $\pm$ 4.8Ab	3.44

Values are expressed as mean  $\pm$  SD (n = 3). Different upper and lower letters indicate a significant difference between column and row ( $p < 0.05$ ). "nd" indicates not detected (the concentration is less than LOD)

glucoside (1998.4  $\text{mg kg}^{-1}$ ) was the most abundant compound in CMU125, followed by peonidin-3-glucoside (648.4  $\text{mg kg}^{-1}$ ).

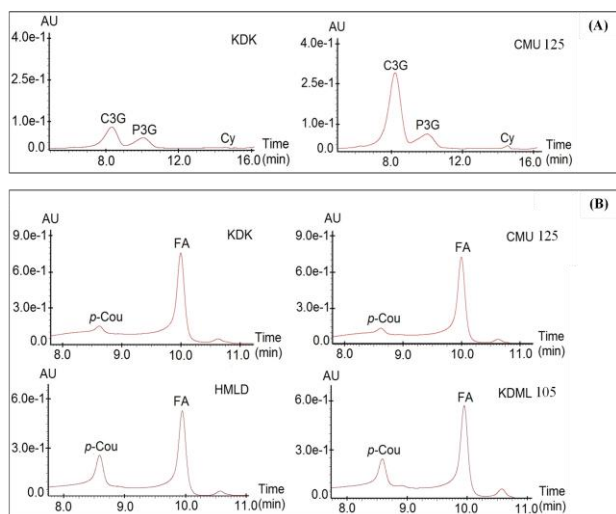
The concentrations of cyanidin-3-glucoside (496.3  $\text{mg kg}^{-1}$ ) and peonidin-3-glucoside (475.3  $\text{mg kg}^{-1}$ ) were similar in KDK. Individual anthocyanins varied among varieties, with CMU125 having higher cyanidin-3-glucoside, peonidin-3-glucoside and cyanidin than KDK, about 3, 0.3 and 1 times, respectively. Cyanidin and peonidin were in higher concentration in CMU125 (16.1 and 1.8  $\text{mg kg}^{-1}$ , respectively) than in KDK (7.5 and 0.3  $\text{mg kg}^{-1}$ , respectively). Overall, the concentration of anthocyanins was much higher than anthocyanidin in the purple rice flour, by about 122 and 65 times in CMU125 and KDK, respectively.

Free and bound phenolic acids were observed in this study (Table 3) and LC chromatograms are presented in Fig. 2B. The higher free phenolic acids in most varieties was ferulic acid (4.6–12.9  $\text{mg kg}^{-1}$ ) about 3.0, 0.9, 4.8 and 3.9 times for KDK, CMU125, HMLD and KDML105, respectively, compared to *p*-coumaric acid (0.8–6.7  $\text{mg kg}^{-1}$ ). The concentration of free phenolic acids in purple rice was significantly higher than in the red and white varieties. The concentration of *p*-coumaric acid was higher in CMU125 (6.7  $\text{mg kg}^{-1}$ ) than KDK (2.6  $\text{mg kg}^{-1}$ ), but levels were very low in HMLD and KDML105 (0.8–1.0  $\text{mg kg}^{-1}$ ). Ferulic acid concentrations followed the same trend: CMU125 (12.9  $\text{mg kg}^{-1}$ ) > KDK (10.5  $\text{mg kg}^{-1}$ ) > HMLD (4.7  $\text{mg kg}^{-1}$ ) > KDML105 (5.0  $\text{mg kg}^{-1}$ ). Furthermore, after alkaline hydrolysis, bound phenolic acids concentrations differed among rice varieties. Red rice (HMLD) had the

highest *p*-coumaric acid concentration (74.56  $\text{mg kg}^{-1}$ ) and purple rice (CMU125) had the lowest concentration (16.68  $\text{mg kg}^{-1}$ ), whereas purple rice (KDK) had the highest concentration of ferulic acid (412.4  $\text{mg kg}^{-1}$ ), and the lowest concentration was in red rice (HMLD) (292.0  $\text{mg kg}^{-1}$ ). Overall, our results showed that the most abundant phenolic acids in rice flour were present in bound forms, with ferulic acid being 2.9–22.9 times higher than *p*-coumaric acid.

## Discussion

The improved and simplified extraction methods are time and cost saving. For optimal extraction, acidified MeOH can be utilized to increase compound stability, which may be better than using distilled water or 80% MeOH for making the final volume (Ti *et al.*, 2014; Gong *et al.*, 2017). When anthocyanins and anthocyanidin were diluted in acidified MeOH resulted in great stability of these compounds, suggesting the anthocyanins or anthocyanidins are most stable when the positive ion or cation on the molecule is maintained under acid conditions, while it is unstable when the molecules become deprotonated and formed a negative ion or anion under alkaline condition (Castaneda-Ovando *et al.*, 2009). The acidified MeOH was used to extract anthocyanins and free phenolic acids as in previous study. The extraction and injection were separated in each determination of anthocyanins and free phenolic acids (Chatthongpisut *et al.*, 2015). Unlike published protocols, this improved method was a single extraction performed with low amounts of extracted solution (6 mL) and sample



**Fig. 2:** UPLC chromatograms of anthocyanin compounds of two purple rice varieties (A) and bound phenolic acid of four rice varieties (B)

(0.5 g) which was injected into the UPLC under the same condition. The HPLC method requires a much longer preparation time with two periods of extraction followed by the need to concentrate the solution (Kim *et al.*, 2014).

Bound phenolic acids extraction was optimized under a hydrolysis condition with good recovery. The procedure developed for rice differs from that used for some other cereals. For example, the highest bound phenolic acids content in maize was obtained when the extraction was applied at the midpoint (3 M NaOH for 90 min) (Fuentelba *et al.*, 2016). For rice, the NaOH concentration and the ratio of NaOH to rice sample were important for digestion, and differences in amylose content between varieties may also impact on digestion efficiency. Six g of whole cooked rice was digested with 60 mL 4 M NaOH (Scaglioni *et al.*, 2014) while 0.5 g rice bran flour was digested with 20 mL of 2 M NaOH (Zhang *et al.*, 2010). The efficiency of the extraction step was optimized using EtOAc with 30 mL EtOAc gave good separation of the organic layer after solution mixing, which was less than the 70 mL EtOAc used for each extraction cycle step with 2 g of rice (Shao *et al.*, 2014).

The recommended protocols allow anthocyanins and phenolic acids in pigmented rice to be determined under the same condition such as electrospray ionization and UPLC condition. In this study, the recommended protocols allow anthocyanins and phenolic acids in pigmented rice to be determined under the same condition, electrospray ionization in the positive mode was suitable for both anthocyanins and phenolic acids. In previous studies, electrospray ionization with positive ion mode was used for anthocyanins, while the negative ion mode was used for phenolic acids (Shao *et al.*, 2014; Chatthongpisut *et al.*, 2015; Zaupa *et al.*, 2015). However, the expression of different phenolic acids in both positive and negative ESI

modes has been reported (Lachowicz *et al.*, 2017). The identified compounds showed the principal fragment ion of cyanidin-3-glucoside and peonidin-3-glucoside were obtained after the loss of the glucose moiety ( $[M+H-162]^+$ ), which has been confirmed by other authors (Hao *et al.*, 2015; Zaupa *et al.*, 2015). The fragment ion of *p*-coumaric acid at  $m/z^{-1} 146.9995$  corresponded to loss of  $H_2O$   $[M+H-18]^+$  (Lin *et al.*, 2015). Ferulic acid produced a protonated  $[M+H]^+$  at  $m/z^{-1} 195.0485$  with the principal fragments ion at  $m/z^{-1} 177.0204$   $[(M+H-18)]^+$  that was produced by the loss of  $H_2O$  and consistent with previous reports (Spinola *et al.*, 2015; Zaupa *et al.*, 2015).

A UPLC method was optimized for quantification of antioxidant compounds in rice with high repeatability with the relative standard deviation values generally less than 5.5% for example, the *p*-coumaric acid and ferulic acid had a lower value (0.5 to 0.9% of RSD) compared with a previous method (2.1 to 2.6% of RSD) (Scaglioni *et al.*, 2014), indicating the acceptable precision of this method. The higher recovery was found with the recommended HPLC method from purple rice wine from 61 to 70% for anthocyanins and from 80 to 102% for phenolic acids (Wang *et al.*, 2014). The purity of analysis standard declared that all compounds were >95%, while the mean recoveries of anthocyanidin, cyanidin and peonidin were 81.2% and 84.4%, respectively. It is possible that anthocyanins (without sugar) leading to instability and easily degraded as compared to anthocyanins or phenolic acids. However, the performance of the method was within 80–120% of the acceptable range in terms of recovery according to the AOAC (AOAC, 2002).

The proposed methodology has been successfully applied in rice samples. Cyanidin-3-glucoside was the most abundant compound in purple rice, followed by peonidin-3-glucoside. The 2:1 ratio of these compounds was lower than found in China purple rice (10:1) (Hao *et al.*, 2015). However, anthocyanins in both purple varieties were at higher concentrations than in some varieties of Thai purple rice (492 and 200  $\mu\text{g g}^{-1}$  for cyanidin-3-glucoside and peonidin-3-glucoside) (Chatthongpisut *et al.*, 2015). Overall, the concentration of anthocyanin was much higher than anthocyanidin in the purple rice flour. Using HPLC, cyanidin-3-glucoside had the highest distribution approximately 80% to the total anthocyanins, while by only 1% to the cyanidin in a purple rice (Chen *et al.*, 2017). Thus, the anthocyanins in purple rice are the major compound to indicate the total anthocyanins concentration but not anthocyanidin. Purple rice had higher ferulic acid than white and red rice, and ferulic acid in purple rice was about 1.2 times higher than in China purple rice i.e., 18.57 mg 100  $\text{g}^{-1}$  (Shao *et al.*, 2014). Overall, our results showed that the most abundant phenolic acids in rice flour were present in bound forms, with ferulic acid was higher than *p*-coumaric acid, which was consistent with previous reports (Sumczynski *et al.*, 2016).

## Conclusion

This study developed and validated procedures using UPLC-MS for quantifying anthocyanins and phenolic acids compounds in rice grain. The proposed method is rapid with a run time of 25 min, sensitive (LOD = 1.5–36.94  $\mu\text{g kg}^{-1}$  and LOQ = 5.00–123.14  $\mu\text{g kg}^{-1}$ ), precise (RSD  $\leq$  5.5%) and accurate with high recovery. Furthermore, the extraction of bound phenolic acid using NaOH hydrolysis was optimized. The recommended protocols applied in this experiment allowed anthocyanins and phenolic acids in pigmented rice to be determined under the same condition and is time and cost saving.

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