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Research article

Effects of ethanol-water extraction on antioxidants from Riceberry (*Oryza sativa* L.) bran

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Abstract

Importance of the work: Solvent-to-substrate ratio and extraction time are the most effective for antioxidant extraction.

Objectives: The study aimed to investigate the non-acid and acid solvents, ethanol concentration (0–70%) and extraction time (30–180 min) on the extraction of antioxidants from Riceberry (*Oryza sativa* L.) bran.

Materials & Methods: The total phenolic content (TPC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and ferric reducing antioxidant power (FRAP) were used to determine the antioxidant activity of the Riceberry bran**.** High performance liquid chromatography analysis was applied to investigate the profiles of the phenolic compounds and anthocyanins and to determine the contents of each compound.

Results: The optimum extraction conditions for Riceberry bran were 50% ethanol and an extraction time of 60 min based on the mean (± SD) TPC (17.18 ± 0.62 mg gallic acid equivalents/g dry weight (DW)), DPPH radical scavenging activity (46.68 ± 3.35 mg 3,5-di-tert-4-butylhydroxytoluene/g DW) and FRAP (1.17 ± 0.05 mol Fe(II)/g DW). The Riceberry bran contained three major phenolic compounds: ferulic acid (FA), vanillic acid (VA) and gallic acid (GA) and two main anthocyanins: cyanidin-3-O-glucoside (C3G) and peonidin-3-O-glucoside (P3G). Since FA and C3G were predominant in Riceberry bran, the optimum conditions for extraction of the phenolic compounds and anthocyanins followed the conditions of FA and C3G, respectively. The optimum conditions for extraction of FA, VA and GA were 0% ethanol and an extraction time of 180 min producing 79.79 ± 2.57 µg FA/g DW, 29.67 ± 0.53 µg VA/g DW and 49.95 ± 0.73 µg GA/g DW, respectively, whereas the optimum conditions for C3G and P3G were 0.1% HCl in 70% ethanol for 180 min (producing 356.86 ± 0.04 µg C3G/g DW and 49.42 ± 1.92 µg P3G/g DW, respectively).

Main finding: The results of this study identified the optimum extraction conditions to obtain high TPC values and antioxidant activity as well as high anthocyanin contents (C3G and P3G) and environmental-friendly extraction for FA, VA and GA.

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Introduction

Rice (*Oryza sativa* L.) is a major crop in Thailand and in many countries in Asia such as China, Japan, Korea and India with about 85% of rice generally consumed in milled form as white rice (Deng et al., 2013). However, the consumption of pigmented rice has increased by mixing it with white rice before cooking or using it as a natural food colorant in bread, ice cream, beverages and snacks and desserts (Tananuwong and Tewaruth, 2010) as well as in functional foods (Deng et al., 2013). Pigmented rice with a red, black or purple pigment contains an abundant amount of nutrients such as amino acids, essential oils, tannins, flavones, sterols and gamma-oryzanols, with the anthocyanins being the major component most responsible for bioactivity in pigmented rice (Chen et al., 2012; Deng et al., 2013; Tananuwong and Tewaruth, 2010) which is located mainly in the pericarp and aleurone layers. Anthocyanins have antioxidant, antimutagenic, anti-inflammatory and anticancer properties (Bowen-Forbes et al., 2010). Riceberry, a dark purple rice, is derived from a hybrid of the Chao Hom Nin rice variety (an antioxidant-rich rice) and Khao Dawk Mali 105 (an aromatic rice) and was developed by the Rice Research Center, Kasetsart University, Kamphaeng Saen campus, Nakhon Pathom, Thailand. It has been claimed that Riceberry bran has a high content of phenolic acid (ferulic acid) and anthocyanins (cyanidin-3-glucoside and peonidin-3-glucoside) (Leardkamolkarn et al., 2011; Prangthip et al., 2013) that are beneficial for improving hyperglycemia and hyperlipidemia and reducing inflammation (Prangthip et al., 2013).

Extraction of antioxidants from pigmented rice can be achieved using solvent extraction, a technique commonly used for the separation of biological components (Deng et al., 2013). Methanol, acetone and ethanol are commonly used as the major solvents; however, due to lower toxicity and cost effectiveness, the binary solvent of ethanol and water has great potential as a solvent (Escribano-Bailón et al., 2004). The extractability of the phenolics and anthocyanins is influenced by many factors including the compound's solubility, polarity, solvent concentration and the temperature, pH, extraction time used and chemical structure (Spigno and De Faveri, 2009; Tananuwong and Tewaruth, 2010). The optimum amount of extraction solvent is the critical factor for the extraction of the maximum amount of biological compounds (Pedro et al., 2015).

Many papers have been published regarding pigmented rice solvent extraction using ethanol as the solvent (Tananuwong and Tewaruth, 2010; Posuwan et al., 2013; Pedro et al., 2015). However, it is not clear which solvent-to-substrate ratio and extraction time are the most effective for antioxidant extraction. In addition, there is limited published information that has investigated Riceberry bran. Therefore, the current research aimed to investigate the effect of the ethanol concentration and extraction time to maximize the phenolic and anthocyanin contents as well as the antioxidant activity of Riceberry bran.

Materials and Methods

Materials

Riceberry (*Oryza sativa* L.) was purchased from Thanya-osod Company (Nakhon Pathom, Thailand) and polished using a rice polisher machine (model K-1, Ngek Seng Huat, Thailand) to produce bran. The initial moisture content was approximate 9.6%. All bran samples were immediately passed through a sieve (no. 60) to obtain a homogenous size. The bran was packed in aluminum foil bags, sealed and kept in a freezer at -20.0°C until use. Bran samples were prepared in duplicate.

Reagents

All chemicals and solvents were commercial grade. Ethyl alcohol and methyl alcohol were purchased from Macron Chemicals (Phillipsburg, NJ, USA). Hydrochloric acid was purchased from Merck (Darmstadt, Germany). Sodium carbonate, iron (III) chloride, potassium chloride, sodium acetate and iron (II) sulphate were purchased from Ajax Finechem (Taren Point, NSW, Australia). 2,4,6-Tris(2-pyridyl)-triazine (TPTZ) was purchased from Fluka Analyticals (Seelze, Germany). Formic acid was purchased from Fisher Scientific (Somerset, NJ, USA). Acetonitrile was purchased from RCI Labscan (Bangkok, Thailand). Folin-Ciocalteu phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3,5-di-tert-4-butylhydroxytoluene (BHT), cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Extraction

The extraction was slightly modified from the method of Chen et al. (2012). A 0.4 g sample of Riceberry bran was mixed with 10.0 mL of different concentrations of ethanol (0%, 30%, 50% or 70%) in an amber glass bottle using a magnetic stirrer

at room temperature (25°C). The time treatments varied (30 min, 60 min, 120 min or 180 min). The extracted solution was collected using centrifugation at 2,500×*g* at room temperature and passed through Whatman no.1 filter paper. The obtained filtrate samples were kept in amber glass bottles and stored at -20.0°C for future analysis. Extraction was done in duplicate.

Ethanol alone and ethanol acidified (extraction solvent types) using 0.1% HCl at different concentrations (0%, 30%, 50% and 70% ethanol) were chosen as non-acid and acid extraction solvents, respectively. The non-acid extraction solvents were used to study their effect on the total phenolic content (TPC), DPPH and ferric reducing antioxidant power (FRAP) while non-acid and acid extraction solvents were used to analyze the phenolic compounds and anthocyanins from Riceberry bran. The sample containing anthocyanins was commonly treated with acid solvent to prevent the degradation of anthocyanin and to maintain its stability in the extraction process (Escribano-Bailón et al., 2004; Pedro et al., 2015).

The optimum extraction condition was based on the ethanol concentration and extraction time providing the maximum value with the shortest extraction time.

Determination of total phenolic content

The TPC was determined using the Folin-Ciocalteau method reported by Singleton et al. (1999) with minor modification. A sample of 500.0 µL of Folin-Ciocalteu reagent was mixed with 250.0 µL of Riceberry bran extracts and 6.0 mL of distillated water. The mixture was shaken and then mixed with 2.0 mL of sodium bicarbonate (15% weight per volume), the volume was adjusted to 10.0 mL using distillated water and incubated for 2 hr at room temperature. The absorbance was measured using an ultraviolet (UV)-spectrophotometer at 750 nm. The results were expressed as milligrams gallic acid equivalents per gram of Riceberry bran dry weight (DW).

Determination of 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

The method was slightly modified from Brand-Williams et al. (1995). An aliquot of 2.0 mL of each Riceberry bran extract was vigorously mixed with 2.0 mL of freshly prepared 25 parts per million solution of DPPH radical in methanol. The mixture was incubated in the dark at room temperature for 30 min. The absorbance was measured using a UV-spectrophotometer at 517 nm. The results were expressed as milligrams BHT equivalents per gram of Riceberry bran DW.

Determination of ferric reducing antioxidant power

The FRAP was determined using slight modification of the method of Benzie and Strain (1999). A sample of 200 µL of each Riceberry bran extract was mixed with 1.3 mL freshly prepared FRAP reagent (0.3 M acetate buffer (pH 3.6) to10 mM TPTZ in 40 mM HCl:20 mM $FeCl_3$ at a ratio of 10:1:1 (volume per volume per volume)) and incubated for 30 min at 37°C. The absorbance was measured using a spectrophotometer at 595 nm. FRAP values were expressed as moles of Fe (II) equivalent per gram of Riceberry bran DW.

High performance liquid chromatography analysis of phenolic compounds and anthocyanins

The phenolic compounds and anthocyanins were identified using a high performance liquid chromatography (HPLC) system (Waters; Milford, MA, USA) consisting of a Waters in-line degasser, a Waters 600 pump and controller, a Waters 2707 auto sampler and a Waters 2998 photodiode array detector. Data signals were acquired and processed on a personal computer using the Empower 2 chromatography data software (Waters; Milford, MA, USA). Analytical separation of phenolic compounds and anthocyanins was carried out using a Symmetry C18 250 mm × 4.6 mm, 5 µm column (Waters; Milford, MA, USA).

This method was slightly modified from Pothinuch et al. (2017). An amount of 20 µL of each sample was analyzed under gradient conditions using acetonitrile (A) and 1.0% formic acid in water (B). The solvent flow rate was 1.0 mL/min and the chromatogram was recorded. The solvent gradient was programmed as: 5% A at 0 min, increasing to 10% B for 5 min, 10 min 15% A, 30 min 25% B, 40 min 50% A, decreasing thereafter to 5% A for the next 5 min and equilibrating before the next injection. The phenolic compounds and anthocyanins in samples were identified by comparing their relative retention times and UV spectra with external standard compounds. Phenolic compounds were detected at 260 nm for VA, 270 nm for GA and 320 nm for FA. Anthocyanins were detected at 520 nm for cyanidin-3-O-glucoside (C3G) and peonidin-3-O-glucoside (P3G). The coefficient of variation for sample replicates was consistently below 10%.

Statistical analysis

The experimental design was performed as a 2×4×4 factorial in a randomized complete block design. Experiments

were done in duplicate. At least two repeat measurements were carried out for each replication. Analysis of variance was calculated using the Minitab version 16.0 statistical software (Minitab Inc.; State College, PA, USA) and Tukey's test was used to evaluate the difference between means at $p < 0.05$. Values were recorded as the mean ± SD.

Results and Discussion

Effect of ethanol concentration

Table 1 shows that the ethanol concentration and extraction time had significant effects on the TPC and DPPH radical scavenging capacities and on the FRAP values of Riceberry bran extraction. Among the different concentrations, 50% ethanol produced the highest content of TPC (16.63–17.73 mg gallic acid equivalents (GAE)/g DW), followed by 30% (15.54–16.39 mg GAE/g DW) and 70% (13.38–15.06 mg GAE/g DW) while the lowest content was produced in 100% water (8.56–10.05 mg GAE/g DW) (Table 2). The DPPH and FRAP values were in descending order for 50%, 30%, 70% and 0% ethanol ($p < 0.05$) which was consistent with the results for the TPC. The results suggested that a binary ethanol-and-water solvent had greater effectiveness in extracting TPC and antioxidants compared to pure water. Since the TPC and antioxidants in 30% ethanol were higher than from using 70%, it might be predicted that the TPC of Riceberry bran contains a higher proportion of hydrophilic compounds. Water is more polar than ethanol; therefore, hydrophilic compounds would be easily soluble when the polarity of the extraction solvent increased. The results were related to the compatibility between bioactive compounds and the extraction solvent. According to the "like dissolves like" principle, different ethanol concentrations would have different polarities which affected solubility and the diffusion rate (Bi et al., 2009; Thoo et al., 2010). Thoo et al. (2010) reported that the TPC of mengkudu rose increased when the ethanol concentration increased and dropped above a maximum value of 40% ethanol. Similarly, a 25–50% ethanol concentration produced the greatest TPC from longan fruit pericarp and then decreased when the ethanol concentration was higher than 75% (Prasad et al., 2009). On the other hand, Spigno et al. (2007) observed that the TPC of grape marc increased using a water content of 10–30%, remained constant using a water content of 30–40% and decreased for a water content greater than 50%. Therefore, the selection of solvent concentration is important. Considering the TPC, DPPH and FRAP values, the optimal ethanol concentration was 50%.

Effect of extraction time

The effect of extraction time might be governed by the equilibrium content of the phenolic compounds, where an excessive extraction time might reduce the TPC (Spigno et al., 2007). The TPC using 50% ethanol increased when the extraction time increased from 30 min to 60 min and remained constant from 60 m in to 180 min. The TPC in 70%, 30% and 0% ethanol increased with increasing extraction time up to 120 min and was stable with any further increase in time. The DPPH value in 50% ethanol had the same trend as the TPC in 50% ethanol. The DPPH increased from 38.91 mg BHT/g DW to 46.68 and 47.38 mg BHT/g DW with extraction times of 30 min and 60 min, respectively, and no increase was observed from 60 min to 180 min. The maximum DPPH was observed at 120

Table 1 Results (*P*-values) of the analysis of variance of the effects of extraction solvent types, ethanol concentrations, extraction times and their interactions on the extraction of total phenolic content (TPC), DPPH radical scavenging activities (DPPH), ferric reducing antioxidant power (FRAP), ferulic acid (FA), vanillic acid (VA), gallic acid (GA), cyanidin-3-O-glucoside (C3G) and peonidin-3-O-glucoside (P3G) contents

	TPC*	DPPH*	FRAP*	FA	VA	GA	C3G	P3G
Main effect								
Solvent types (x_1)	-	-	-	0.00	0.00	0.000	0.000	0.000
Ethanol conc. (x_2)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Extraction time (x_3)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Interactions								
x_1*x_2	0.002	0.001	0.000	0.000	0.000	0.000	0.000	0.000
x_1*x_3	-	-	-	0.407	0.000	0.000	0.000	0.001
x_2*x_3	-	-	-	0.021	0.003	0.000	0.000	0.003
$x_1*x_2*x_3$	-	-	-	0.006	0.051	0.001	0.000	0.257

*Only non-acid ethanol was used for extraction

Table 2 Total phenolic content, DPPH radical scavenging activities and ferric reducing antioxidant power of Riceberry bran extracted using non-acid extraction solvent and different ethanol concentrations at different extraction times

Ethanol (%)	Extraction time (min)	TPC (mg GAE/g DW)	DPPH (mg BHT/g DW)	FRAP (mol Fe(II)/g DW)
70	30	13.38±0.65^{h}	33.66±1.41^{e}	0.81±0.04^{f}
	60	14.23±0.71^{g}	38.09±0.82^{d}	0.99±0.03^{e}
	120	15.06±0.44^{ef}	41.47±1.01^{bc}	1.15±0.01^{c}
	180	14.48±0.88^{fg}	40.73±0.62^{cd}	1.03±0.02^{de}
50	30	16.63±0.46^{bc}	38.91±2.11^{cd}	1.15±0.11^{c}
	60	17.18±0.62^{ab}	46.68±3.35^{a}	1.17±0.05^{c}
	120	17.73±0.74^{a}	47.38±0.43^{a}	1.40±0.05^{b}
	180	17.25±0.58^{ab}	47.13±0.92^{a}	1.58±0.06^{a}
30	30	15.54±0.58^{de}	38.14±0.80^{d}	1.10±0.07^{cd}
	60	15.62±0.41^{de}	39.42±1.01^{cd}	1.01±0.04^{cde}
	120	16.39±0.17^{c}	44.41±0.90^{ab}	1.36±0.03^{b}
	180	15.90±0.60^{cd}	41.34±1.64^{bc}	1.32±0.04^{b}
0	30	8.91±0.50^{j}	24.51±0.39^{h}	0.65±0.01^{g}
	60	8.56±0.37^{j}	26.49±0.49^{gh}	0.74±0.03^{fg}
	120	10.04±0.25^{i}	29.63±0.42^{f}	0.82±0.02^{f}
	180	10.05±0.32^{i}	28.52±1.24^{fg}	0.80±0.03^{f}

DPPH = 2,2-diphenyl-1-picrylhydrazyl; GAE = gallic acid equivalents; DW = dry weight; BHT = 3,5-di-tert-4-butylhydroxytoluene.
Mean values ± SD in the same column superscripted by different lowercase letters are significantly ($p < 0.05$) different.

min for ethanol contents of 70% (41.47 mg BHT/g DW), 30% (44.41 mg BHT/g DW) and 0% (29.63 mg BHT/g DW). FRAP increased in 50% ethanol from 30 min to 180 min (1.15–1.58 mol Fe(II)/g DW) while the increase occurred from 30 min to 120 min in 70% (0.81–1.15 mol Fe(II)/g DW), 30% (1.10–1.36 mol Fe(II)/g DW) and 0% ethanol (0.65–0.82 mol Fe(II)/g DW).

The different polarities of the solvent extractions meant that the mass transfer rate was different (Prasad et al., 2009). In fact, the plant cell wall is nonpolar in character so that ethanol having a polarity lower than water could be efficient at degrading the cell wall of Riceberry bran, resulting in the release of phenolic compounds from the cell (Lapornik et al., 2005). In addition, the different extraction times for each extraction solvent could be attributed to interaction between: 1) food and phenolic compounds, 2) the structure of the phenolic compounds and the solubility between the phenolic compounds and 3) extraction solvents and the degree of polymerization of phenolic compounds (Silva et al., 2007; Thoo et al., 2010).

There are weak relationships between the TPC, DPPH radical scavenging activities and FRAP. Although phenolic compounds were responsible for the major antioxidants in the rice bran extracts, the scavenging activities were not dependent only on the TPC. The TPC can be detected based on phenols or phenol-plus-metal chelators plus reducing agents. Pigmented rice bran has not only phenolic compounds, but also contains carotenoid, minerals, tocopherols and gamma-oryzanol (Tananuwong and Tewaruth, 2010; Ryan, 2011). There are other groups of antioxidant compounds that could scavenge DPPH radicals (Prior et al., 2005; Tananuwong and Tewaruth, 2010; Ryan, 2011). DPPH assay investigates the antioxidants based on hydrogen atoms and electron transfer mechanisms; conversely, FRAP assay evaluates only a single electron (Prior et al., 2005; Deepa et al., 2006). Furthermore, the chemical structures of antioxidants could act in the assay in different ways so that individual antioxidants could be expressed by using multiple mechanisms in one system (Heim et al., 2002).

The results from the TPC and DPPH radical scavenging activities had to be considered together to determine the optimum conditions for FRAP. An optimum treatment of FRAP was different from that producing the optimal TPC and DPPH radical scavenging activities. FRAP in 50% ethanol for 180 min produced the maximum activity of 1.58 ± 0.06 mol Fe/g Riceberry bran against the TPC and DPPH radical scavenging activities which were produced in 50% ethanol for 60 min. However, the shortest extraction time was considered as the critical factor. Therefore, 50% ethanol with an extraction time of 60 min was suggested as the optimum conditions for antioxidant extraction from Riceberry bran.

High performance liquid chromatography analysis of phenolic compounds and anthocyanins

Phenolic compounds

The phenolic compounds of Riceberry bran were determined using HPLC-DAD based on the retention time and spectral matching in the ultraviolet region. The results showed that FA was a major phenolic compound, followed by VA and GA. FA had maximum absorption at 320 nm for a retention time of 26.7 min, VA at 260 nm for 17.3 min and GA at 260 nm for 6.0 min.

Extraction solvent types (x_1), ethanol concentrations (x_2) and extraction times (x_3); and the interaction between solvent types and ethanol concentrations ($x_{1*}x_2$) and between ethanol concentrations and extraction times ($x_{2*}x_3$) significantly affected the extraction of FA, VA and GA ($p < 0.05$) (Table 1). The interaction between the solvent types and extraction times ($x_{1*}x_3$) showed statistical significance for VA and GA only. The combination of solvent types, ethanol and extraction times ($x_{1*}x_{2*}x_3$) statistically significantly affected FA and GA. The results indicated that non-acid ethanol was more effective for FA, VA and GA extraction than acid ethanol ($p < 0.05$) (Fig. 1). In addition, 0% non-acid ethanol was the most effective for FA extraction while 50% non-acid ethanol was the most effective for VA extraction (Table 3). On the other hand, GA produced different results from FA and VA. Both 0% ethanol and 0.1% HCl in 0% ethanol were more effective for GA. The solubility of the phenolic compounds is governed by the polarity of the extraction solvents and the chemical nature of the phenolic compounds (Dai and Mumper, 2010). Focusing on the chemical structure, the number of hydroxyl and carboxyl groups is responsible for solubility (Mota et al., 2008). The solubility of FA in pure water was higher than in the ethanol-water mixture. FA is composed of an aromatic ring with one hydroxyl and one carboxy methylene group. When FA was dissolved in the non-acid ethanol, the hydroxyl and carboxyl on the methylene group were responsible for solubility. GA would have the highest solubility in water because it has three hydroxyl groups resulting in less effect in an acid solution. In contrast, VA has one hydroxyl on an aromatic ring, indicating less interaction with a high water content.

The extraction time was governed by the equilibrium content of FA, GA and VA. The increase in extraction time increased their contents. The results of the effect of extraction time indicated that 100% water content in the extraction solvent resulted in a slow equilibrium content for FA and VA, where the maximum value of FA and VA was observed at 180 min and 120 min respectively. This could be explained by mass transfer differences among extraction solvents (Lapornik et al., 2005; Dai and Mumper, 2010). Furthermore, there are many factors that could govern the equilibrium rate of the extraction, including interaction between food and phenolic compounds, the solubility between phenolic compounds and extraction solvent and the structure of the phenolic compounds (Silva et al., 2007; Thoo et al., 2010).

FA was predominant in Riceberry bran, followed by GA and VA. Therefore, the optimum conditions to obtain the highest yield should be 0% non-acid ethanol for 180 min, producing values of 79.79 ± 2.57 µg FA/g Riceberry bran DW; 29.67 ± 0.53 µg VA/g Riceberry bran DW; and 49.95 ± 0.73 µg GA/g Riceberry bran DW, respectively. FA was also the predominant phenolic compound in red and black varieties from Thailand, China and Sri Lanka (Sompong et al., 2001), black japonica rice bran and a Hong Kong type of black rice bran (Laokuldilok et al., 2011). The use of zero content ethanol produced an environmental-friendly solvent for FA GA and VA.

Anthocyanins

Solvent types (x_1), ethanol concentrations (x_2) and extraction times (x_3); and the interaction between solvent types and ethanol concentrations ($x_{1*}x_2$), between ethanol concentrations and extraction times ($x_{2*}x_3$) and between solvent types and extraction times ($x_{1*}x_3$) significantly affected the extraction of C3G and P3G ($p < 0.05$) (Table 1). The interaction of solvent types, ethanol and extraction times ($x_{1*}x_{2*}x_3$) significantly affected C3G only. The effect of extraction solvents on anthocyanins is shown in Table 3. C3G and P3G were the major anthocyanins found in Riceberry bran at 520 nm for retention times of 17.3 min and 19.5 min, respectively (data not shown). The HPLC profiles for the Riceberry bran were similar to Japanese black rice, where C3G (85%) was the first peak and P3G (15%) was the second (Yawadio et al., 2007). The levels of C3G and P3G increased as the ethanol content increased ($p < 0.05$) for both non-acid and acid ethanol, as follows: 70% > 50% > 30% > 0% (Fig. 1). Acid ethanol produced a higher level ($p < 0.05$) of C3G extraction than non-acid ethanol for the same extraction times: acid 70% ethanol > 70% ethanol; acid 50% ethanol > 50% ethanol and similarly for the other concentrations. The maximum amount of C3G was produced using 0.1% HCl in 70% ethanol (Table 3). The P3G content was not significantly different between the mixtures of 70% ethanol and 0.1% HCl in 70% ethanol and this was higher than for the other

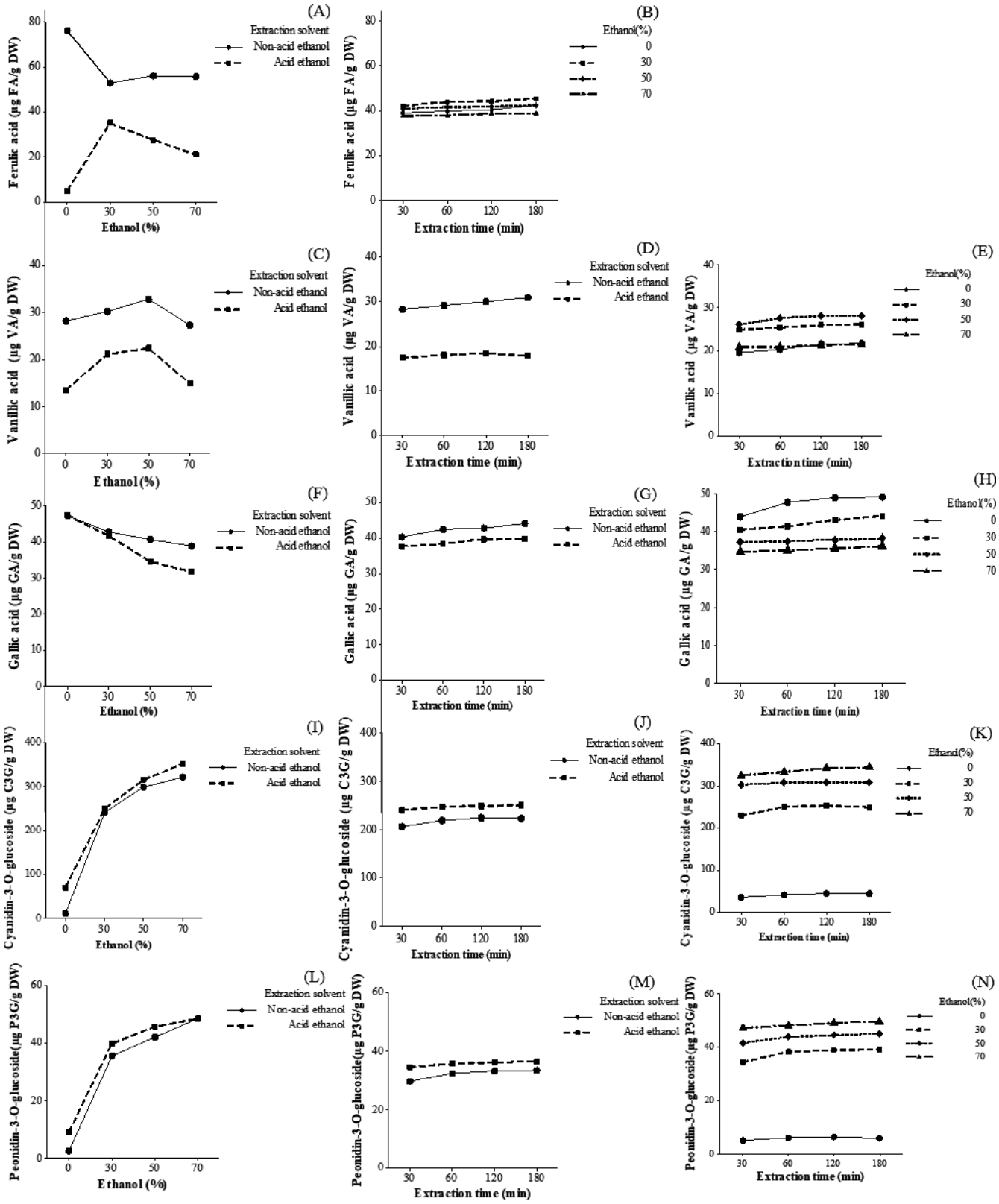

Fig. 1 Profile plots showing interaction between solvent types and concentrations (A, C, F, I and L), solvent types and extraction times (D, G, J and M) and solvent concentrations and extraction times (B, E, H, K and N) on quantity of ferulic acid (A and B), vanillic acid (C, D and E), gallic acid (F, G and H), cyanidin-3-O-glucoside (I, J and K) and peonidin-3-O-glucoside (L, M and N). (Only significant interactions are shown.)

mixtures (Table 3). At the lower ethanol concentrations (50%, 30% and 0% ethanol), the acid ethanol had more influence on P3G extraction than the non-acid solvent at the same concentration because the stability of anthocyanin was higher in acidic ethanol than in non-acid ethanol. The phenomena involved an ionic nature in the anthocyanin molecule. When the ionic nature shifted, the molecular structure of anthocyanin would change as a result of degradation. The change in the ionic nature in anthocyanin depended on the pH value accounting for color and hue differences. In an acidic solution, there are four main equilibrium species of anthocyanin that present different colors: flavylium cation (pH < 2.0 exhibits red color), quinonoidal base (blue or violet), carbinol or pseudobase (colorless) and chalcone (colorless) (Escribano-Bailón et al., 2004; Cavalcanti et al., 2011). As the pH increases, a flavylium cation would change the structure to the quinoidal form. This compound was unstable and would easily change into carbinol or a pseudobase and

Table 3 Ferulic acid (FA), vanillic acid (VA), gallic acid (GA), cyanidin-3-O-glucoside (C3G) and peonidin-3-O-glucoside (P3G) contents of Riceberry bran extracted using different extraction solvent types (non-acid and acid solvents) and different ethanol concentrations at different extraction times

Extraction solvent	Ethanol (%)	Extraction time (min)	FA (µg FA/ g DW)	VA (µg VA/ g DW)	GA (µg GA/ g DW)	C3G (µg C3G/ g DW)	P3G (µg P3G/ g DW)
Non-acid ethanol	70	30	55.08±0.31^{cde}	26.97±0.37^{fg}	38.19±1.53^{k}	302.18±0.01^{m}	46.47±3.46^{bcde}
		60	55.37±1.34^{cde}	26.93±0.44^{fg}	38.69±0.64^{jk}	317.17±0.03^{h}	48.13±1.23^{abc}
		120	56.57±1.02^{c}	27.14±0.67^{fg}	39.26±1.31^{ijk}	332.98±0.07^{e}	49.64±1.34^{ab}
		180	55.84±0.55^{cde}	28.34±2.16^{ef}	39.79±1.57^{hijk}	331.67±0.03^{f}	49.83±0.49^{a}
	50	30	55.42±0.82^{cde}	30.98±0.62^{cd}	40.02±1.23^{hijk}	292.20±0.10^{p}	38.50±2.43^{hij}
		60	55.94±1.42^{cde}	32.74±0.97^{ab}	40.62±1.74^{ghij}	298.45±0.08^{o}	42.41±1.10^{fg}
		120	56.17±2.33^{cd}	33.12±1.29^{ab}	41.09±1.33^{fghi}	300.22±0.02^{n}	42.78±0.87^{fg}
		180	56.67±1.31^{c}	34.23±0.16^{a}	41.30±1.12^{efgh}	302.55±0.06^{l}	43.98±0.34^{ef}
	30	30	51.24±1.36^{f}	28.80±0.62^{e}	40.70±1.09^{ghi}	216.06±0.06^{x}	31.22±0.91^{k}
		60	53.10±0.91^{ef}	29.71±0.10^{de}	42.54±1.98^{efg}	249.08±0.05^{u}	36.37±1.50^{j}
		120	53.42±1.04^{def}	30.66±0.42^{cd}	42.79±0.79^{ef}	253.17±0.06^{q}	36.92±0.26^{j}
		180	54.22±1.63^{cde}	31.52±1.46^{bc}	45.18±1.61^{c}	247.63±0.01^{v}	37.68±0.23^{hij}
	0	30	74.01±0.66^{b}	26.32±0.44^{g}	42.62±2.43^{efg}	9.97±0.00^{ae}	2.17±0.06^{m}
		60	75.09±2.58^{b}	27.11±0.39^{fg}	48.06±1.04^{ab}	12.02±0.01^{ad}	3.00±0.12^{m}
		120	75.57±2.10^{b}	29.41±0.95^{de}	48.46±0.78^{ab}	12.45±0.00^{ac}	3.24±0.01^{m}
		180	79.79±2.57^{a}	29.67±0.53^{de}	49.95±0.73^{a}	9.63±0.00^{af}	2.32±0.10^{m}
Acid ethanol	70	30	20.40±0.84^{j}	14.69±0.60^{kl}	31.07±1.19^{n}	347.83±0.12^{d}	47.87±0.63^{abcd}
		60	20.87±0.41^{j}	14.87±0.76^{kl}	31.47±0.86^{n}	349.86±0.03^{c}	48.14±0.24^{abc}
		120	21.16±1.09^{j}	15.54±0.68^{k}	31.79±1.18^{n}	350.06±0.02^{b}	48.41±0.79^{abc}
		180	21.79±0.81^{j}	14.67±0.10^{kl}	32.58±1.26^{mn}	356.86±0.04^{a}	49.42±1.92^{ab}
	50	30	26.80±0.60^{i}	21.27±0.78^{ij}	34.30±0.61^{lm}	313.04±0.10^{k}	44.90±1.49^{def}
		60	27.39±1.26^{i}	22.62±1.01^{hi}	34.35±1.26^{lm}	316.93±0.04^{i}	45.27±0.82^{cdef}
		120	27.89±0.84^{i}	23.22±1.21^{h}	34.59±0.85^{l}	317.80±0.02^{g}	46.16±0.76^{cde}
		180	28.43±0.83^{i}	22.20±0.81^{hij}	35.15±0.71^{l}	314.76±0.01^{j}	46.23±1.03^{cde}
	30	30	33.39±2.08^{h}	20.99±0.78^{ij}	40.20±0.97^{hij}	242.61±0.02^{w}	37.47±0.41^{ij}
		60	34.94±0.71^{gh}	21.21±0.79^{ij}	40.31±2.07^{hij}	251.34±0.01^{s}	40.27±1.10^{ghi}
		120	35.09±1.24^{gh}	21.29±0.56^{ij}	43.29±1.59^{cde}	251.92±0.01^{r}	40.64±0.79^{ghi}
		180	36.92±1.05^{g}	20.81±0.60^{j}	43.13±0.41^{de}	250.69±0.00^{t}	40.65±1.57^{gh}
	0	30	4.49±0.10^{k}	12.80±0.69^{m}	45.11±1.57^{cd}	58.85±0.05^{ab}	8.00±0.31^{l}
		60	5.16±0.09^{k}	13.44±0.58^{lm}	47.44±0.48^{b}	69.93±0.05^{aa}	9.06±0.37^{l}
		120	5.30±0.16^{k}	13.79±0.57^{lm}	49.22±1.76^{ab}	75.10±0.02^{z}	9.44±0.09^{l}
		180	5.60±0.03^{k}	13.67±0.48^{lm}	48.15±1.03^{ab}	77.31±0.00^{y}	9.47±0.15^{l}

chalcone form, if attacked by water. Considering the chemical structure of the molecule, anthocyanins become polar due to the hydroxyl group and sugar in its structure (Escribano-Bailón et al., 2004) whereas different ethanol concentrations were more selective. Both C3G and P3G could be soluble in an ethanol concentration with a less polar nature.

In the extraction of C3G, it was found that the amount of C3G increased with the extraction time ($p < 0.05$). In the extraction of P3G, the extraction time did not affect P3G in acid ethanol at the same concentration, while non-acid ethanol (30%, 50% and 70% ethanol) reached an equilibrium content of P3G at 60 min. It could be described by the different mass transfers of the extraction solvents (Lapornik et al., 2005). In addition, the interaction between food and anthocyanins, the structure of the anthocyanins, the solubility between the anthocyanins and extraction solvent also had influenced the extraction time (Silva et al., 2007; Thoo et al., 2010). Furthermore, 0%, 30% and 70% non-acid ethanol and 30% and 50% acid ethanol for 180 min reduced the amount of C3G. This result was possibly due to degradation or polymerization of anthocyanins because of the excessive extraction time (Spigno et al., 2007).

The optimum conditions for producing C3G and P3G based on the highest antioxidant activity levels were 0.1% HCl in 70% ethanol for 180 min with values of 356.83 ± 0.04 µg C3G/g Riceberry bran DW and 49.42 ± 1.92 µg P3G/g Riceberry bran DW, respectively. The results for C3G and P3G were in the same reported range for black rice bran (Shao et al., 2014).

Extraction solvents and times affected the TPC, DPPH radical scavenging activity, FRAP and the amounts of extracted phenolic compounds and anthocyanins. The optimum conditions for the extraction of antioxidants from Riceberry bran (based on the TPC, DPPH radical scavenging activity and FRAP results) were 50% ethanol for 60 min. To produce the highest yield, the optimum conditions for FA, VA and GA were 0% ethanol for 180 min, whereas the optimum conditions for C3G and P3G were 0.1% HCl in 70% ethanol for 180 min. As a result, it was concluded that these optimum extraction conditions would maximize the extraction of phenolic acid and anthocyanin contents; in addition, there should be no utilization of organic solvents to ensure the environmental-friendly extraction of FA, VA and GA.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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