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Relationship of Radical Scavenging Activities and Anthocyanin Contents in the 12 Colored Rice Varieties in Korea

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Total polyphenolic, protein, lipid, and anthocyanin contents in grains of 12 Korean colored rice varieties were evaluated for their 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl) (DPPH) and 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium (ABTS) radical- scavenging activities. Three anthocyanins, cyanidin-3-*O*-glucoside (Cy-3-G), petunidin-3-*O*-glucoside (Pt-3-G), and peonidin-3-*O*-glucoside (Pn-3-G), were also characterized by ultra performance liquid chromatography. Among the rice varieties, 'Heugjinju', 'Heugseol', and 'Sintoheugmi' contained high total phenolic contents compared to other varieties. Anthocyanin content was highest in 'Heugjinju'; no anthocyanins were detected in 'Hongjinju' and 'Jeogjinju'. Highest antioxidant activity was observed in 'Heugjinju' and 'Jeogjinju' exhibited the lowest activity. To determine the correlation of total polyphenolic and anthocyanin contents to antioxidant activity, linear regression analysis was carried out. The results showed total polyphenolic content was strongly correlated with antioxidant activity, suggesting total phenolic content as key factor in antioxidant activity of colored rice. 'Heugjinju', 'Heugseol', and 'Sintoheugmi' could have important nutritional value.

Key words: anthocyanin, antioxidant activity, colored rice, total polyphenolic content, ultra performance liquid chromatography

Rice (*Oryza sativa* L.) is the principle cereal food for the improvement of human health in Asia and is a staple food for nearly half of the world population [Friedman, 1996; Gregorio *et al*., 1999; Chung *et al*., 2001]. There are many different kinds of rice, including white, brown, and colored rice varieties [Chung and Woo, 2001]. In Korea, consumption of colored rice varieties such as black and red rice is rapidly growing at present due to their health beneficial effects. Anthocyanins, a group of flavonoids that are red, blue or purple in color depending on pH, are reported to be the primary pigments in these rice varieties [Abdel-Aal and Hucl, 1999; 2003; Mazza and Gao 2004; Moreno *et al*., 2005]. Moreover, these components have been recognized as health-promoting functional food ingredients due to their antioxidant [Satue-Gracia *et al*., 1997; Nam *et al*., 2006; Philpott *et al*., 2006], anticancer [Kamei *et al*., 1995; Hyun and Chung, 2004; Zhao *et al*., 2004], hypoglycemic [Tsuda *et al*., 2003], and anti-inflammatory effects [Tsuda *et al*., 2002]. For these reasons, many researchers and food industries have become increasingly interested in anthocyanin rich materials for the production of supplements with preventative and therapeutic uses. The world production of anthocyanins is estimated at 10,000 tons from grapes alone, and the average daily intake is estimated as 215 mg in summer and 180 mg in winter [Kim *et al.*, 2008]. These numbers reflect the importance of anthocyanins in the human diet as well as understanding their compositional characteristics and

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functionality in food and nutrition [Du *et al*., 2008; Lee and Choung, 2011]. Although many reports have emphasized the biological activities of anthocyanins, only few have been published on the compositional components of colored rice varieties, including anthocyanin [Ryu *et al*., 2000; Lee, 2010]. Furthermore, total polyphenolic and anthocyanin contents, and antioxidant activities of various colored rice varieties have not been extensively studied.

In the present study, three anthocyanins from twelve Korean colored rice varieties were identified. Total polyphenolic, protein, lipid and anthocyanin contents of various Korean colored rice varieties were determined by the method of Soxhlet and ultra performance liquid chromatography. The effects of total polyphenolic and anthocyanin contents on antioxidant activity were also evaluated.

Materials and Methods

Plant materials. The grains of 12 Korean colored rice varieties, 'Heugnam', 'Sintoheugmi', 'Hongjinju', 'Jeogjinju', 'Heugjinju', 'Boseogheugchal', 'Sinnongheugchal', 'Sinmyungheugchal', 'Josaengheugchal', 'Heuggwang', 'Heughyang', and 'Heugseol' were collected on October 5, 2009 at the experimental field of the Department of Functional Crop, National Institute of Crop Science, Rural Development Administration (RDA), Milyang, Korea. After harvesting, the grains of each variety were dried, hulled with a milling machine, and stored at −40°C until analysis.

Reagents. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), butylated hydroxyl-anisole (BHA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), chlorogenic acid, sodium persulfate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, and trifluoroacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Cyanidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, and petunidin-3-*O*-glucoside were purchased from Extrasynthese Co (Genay, France). Ultra Performance Liquid Chromatograph (UPLC)-grade water and methanol were obtained from EMD Chemicals (Darmstadt, Germany).

Determination of total polyphenolic contents. Total polyphenol contents of the colored rice varieties were measured by the Folin-Ciocalteau colorimetric method [Choi *et al*., 2006]. Dried samples were extracted with 70% methanol at 37°C for 12 h. The extract (0.1 mL) was mixed with 0.2 mL of Folin-Ciocalteau's phenol reagent, followed by addition of 3 mL of 5% Na_2CO_3. The absorbance of the mixture at 765 nm was recorded using a spectrophotometer (Versa max, Molecular Devices Co., Sunnyvale, CA) after 2 h of incubation at 30°C. Total polyphenolic content was expressed in chlorogenic acid equivalents (mg CGA/100 g extract) [Kweon *et al*., 2001].

Protein and lipid analyses The protein contents of the samples were measured according to the Kjeldahl procedure using a Kjeltec Auto analyzer [Kim *et al*., 2006]. The lipid contents were measured by the Soxhlet method using the Buchi B-811 extraction system [Juliano, 1985]. Two grams of pulverized seeds was added to 200 mL of *n*-hexane in an extraction thimble, followed by boiling for 2 h at 110°C. After cooling to room temperature, the extracted oil was weighed. Total lipid contents were determined on a dry matter basis of the colored rice grains.

Measurement of fatty acids The fatty acid composition was determined by the gas chromatography-flame ionization detector) (GC-FID) analysis system [Dhakal *et al*., 2009]. For oil extraction, each sample (0.5 g) was placed in a test tube, and 10 mL hexane was mixed. The samples were placed in a shaking incubator (100 rpm) at 50°C for 2 days. The supernatant was transferred into another test tube and evaporated. Subsequently, the extracted oil (0.15 mL) from each sample was placed in a vial, and 5 mL of methylation solution [H_2SO_4 : methanol (MeOH) : Toluene = 1 mL : 20 mL : 10 mL] was added. The vial was heated in a water bath (100°C) for 1 h and then allowed to cool to room temperature. Then, 5 mL of distilled water was added to the vial and shaken thoroughly. The mixture was separated into two layers, after which the upper layer was removed by Pasteur pipette and dried using anhydrous Na_2SO_4 for 10 min. Then, 1 mL of the sample solution was directly injected into GC using an automatic sampler (Agilent 7683B, Santa Clara, CA). An Agilent 7890A gas chromatograph equipped with a FID and HP-FFAT capillary column was used. The oven temperature was raised from 150 to 230°C at a constant rate of 2.5°C per min. The injector and detector temperature were kept at 250 and 230°C, respectively. The carrier gas was nitrogen at a flow rate of 1 mL/min, and the split ratio at the injector port was 50 : 1.

UPLC apparatus and measurements. Variations in anthocyanins contents of colored rice varieties were analyzed according to the method of Hosseinian *et al*. [2008] with slight modification. The colored rice (0.1 g) was extracted with 10 mL of 40% MeOH containing 1.0% (v/v) HCl for 12 h in a vortex mixer at room temperature to form the final extract, which was then centrifuged. The extracts used for UPLC analysis were passed through a 0.2-μm filter (Advantec MFS, Inc. Dublin, CA) before injection into a reverse phase Bondapak^{TM} C_{18}. Subsequently, 2 μL portions of these

solutions were injected into the UPLC system (Waters 2695 Alliance, Milford, MA). The mobile phase was water containing 0.1% trifluoroacetic acid (TFA) in H_2O (A) and 0.1% TFA in MeOH (B). The gradient conditions were as follows: 0.4 min, 15% B; 8.1 min, 20% B; 0.5 min, 30% B, and then a hold for 1 min before returning to the initial conditions. The flow rate was adjusted to 0.4 mL/min, and the detection wavelength was set at 530 nm with the temperature held constant at 30°C.

Anthocyanin Standard solution and calibration curves. One milligram each of cyanidin-3-*O*-glucoside (Cy-3-G), petunidin-3-*O*-glucoside (Pt-3-G), and peonidin-3-*O*-glucoside (Pn-3-G) were accurately weighed and dissolved in 40% MeOH containing 1.0% (v/v) HCl to obtain stock solution of 1.0 mg/mL. Calibration curves were made for each standard with at seven different concentrations (1, 2.5, 5, 10, 25, and 50 μg/mL). Mean areas (n=3) generated from the standard solutions were plotted against each concentration to establish a calibration equation.

Measurement of DPPH radical-scavenging activity. The method described by Hatano *et al*. [1988] was used to determine the DPPH radical-scavenging activities of the colored rice extracts. Absorbance of the remaining DPPH radicals was measured against a blank of pure MeOH including only DPPH radical at 517 nm using a UV-visible spectrophotometer for 30 min at room temperature. The DPPH radical-scavenging capacity was calculated as the difference in absorbance between the tested samples and expressed as the percentage of DPPH radical remaining. Inhibition of DPPH radical as a percentage (%) was calculated as follows:

$$\text{Antioxidant activity of DPPH (\%)} = (A_{blank} - A_{sample})/A_{blank} \times 100$$

Measurement of Trolox equivalent antioxidant capacity (TEAC). TEAC assay was based on the relative ability of antioxidants to scavenge the radical cation $ABTS^{\bullet+}$ in comparison to a standard (Trolox) [Re *et al*., 1999; Choi *et al*., 2005]. The radical cation was prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate. The reaction mixture was maintained for 4-8 h until the mixing was completed and the absorbance stabilized. $ABTS^{\bullet+}$ solution was diluted with ethanol, and the absorbance was read at 734 nm. For the photometric assay, 0.9 mL of $ABTS^{\bullet+}$ solution and 0.1 mL of test sample were mixed for 1 min, after which the absorbance was immediately measured at 734 nm. The antioxidant activity of each compound was calculated by determining the decrease in absorbance at different concentrations using the following equation: $E = [(A_{blank} - A_{sample})/A_{blank}] \times 100$, where A_{sample} and A_{blank} were the absorbances of the samples with and without the test samples, respectively. Antioxidant activity was expressed in TEAC values.

Statistical analysis All measurements were repeated three times and the results were expressed as the means ± SD. Results were analyzed using Sigma plot 2001 (SPSS Inc., Chicago. IL).

Results and Discussion

Determination of total polyphenolic contents. Total polyphenol contents of the 70% MeOH colored rice extracts were expressed as mg/g of CGA. A calibration curve was constructed using different concentrations of chlorogenic acid (12.5, 25, 50, 100, 200, 400, and 1,000 μg/mL) as standards [Chun and Kim, 2004]. The linear regression equation of the curve and coefficient of determination (r^2) were: $y=0.000231x+0.008562$, $r^2=0.9976$. These results show that the content of total polyphenolics was higher in the 'Heugjinju', 'Heugseol', and 'Sintoheugmi' varieties (1,338.58±94.42, 1,173.27 ±75.57, and 1,119.43±67.07 mg CGA/100 g, dry weight (DW), respectively), whereas that of 'Jeogjinju' had the lowest (221.00±36.99 mg CGA/100 g, DW) (Table 1). The total polyphenolic content of 'Heugjinju' was 6 times higher than that of the 'Jeogjinju' variety.

Comparison of protein and lipid contents. Protein and lipid contents in 12 different Korean colored rice varieties are shown in Table 1. The protein contents were determined using a Tecator Kjeltec Auto Analyzer. 'Heugseol' showed the highest concentration of proteins (9.26±0.09%), whereas 'Sinnongheugchal' displayed the lowest (6.98±0.05%). The lipid content was determined by the Soxhlet method. 'Heugseol' variety contained the highest lipid content (3.32±0.06%), whereas that of 'Sintoheugmi' had the lowest (2.48±0.03%). The above results showed protein and lipid contents were not significantly different among the Korean colored rice grains. Thus, protein and lipids are not important components in the development of colored rice with improved quality in terms of the breeding aspect.

Analysis of fatty acid composition. Lipid content is an important quality in some plants due to the many biological activities of unsaturated fatty acids, such as oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids. In particular, they can reduce cholesterol levels in human blood [Wang and Oram, 2002] and enhance long-term potentiation and depression [Honore *et al*., 1994]. The fatty acid contents of 12 Korean colored rice varieties grains are shown in Table 2. The majority of the colored rice varieties showed elevated fatty acid contents. In the case of whole grains, palmitic acid (C16:0) ranged from

Table 1. Contents of total polyphenol, protein, and lipid from the grains of 12 Korean colored rice varieties

Rice varieties	Content of total polyphenol[1)] (mg CGA/100g, DW)	Content of protein and lipid[1)] (%, DW)	
		Protein	Lipid
Heugnam	295.24±22.20	7.87±0.05	2.58±0.11
Sintoheugmi	1,119.43±67.07	7.84±0.19	2.48±0.03
Hongjinju	536.28±70.09	7.97±0.05	2.92±0.00
Jeogjinju	221.00±36.99	8.02±0.00	2.70±0.11
Heugjinju	1,338.58±94.42	9.14±0.05	2.58±0.06
Boseogheugchal	720.44±85.08	8.51±0.19	2.76±0.02
Sinnongheugchal	831.58±69.64	6.98±0.05	3.31±0.13
Sinmyungheugchal	536.29±26.92	8.23±0.29	2.51±0.07
Josaengheugchal	994.94±46.59	7.56±0.05	2.78±0.06
Heuggwang	434.84±50.31	8.76±0.14	2.55±0.01
Heughyang	575.68±82.30	7.46±0.00	2.69±0.00
Heugseol	1,173.27±75.57	9.26±0.09	3.32±0.06

[1)]Values indicate the means ± SD of three replications

Table 2. Comparison of fatty acid composition in grains of 12 Korean colored rice varieties

Rice varieties	Fatty acid content[1)] (%, DW)						
	Palmitic acid ($C_{16:0}$)	Stearic acid ($C_{18:0}$)	Oleic acid ($C_{18:1}$)	Linoleic acid ($C_{18:2}$)	Linolenic acid ($C_{18:3}$)	SF*	USF**
Heugnam	16.64±0.83	2.25±0.08	43.82±2.19	35.90±1.84	1.38±0.00	19.73±0.99	85.14±5.11
Sintoheugmi	15.66±0.69	2.23±0.09	45.34±2.58	35.61±1.78	1.17±0.06	18.58±1.03	86.47±2.59
Hongjinju	18.39±0.92	2.46±0.14	42.52±2.13	35.33±1.95	1.30±0.02	21.77±1.09	83.23±4.16
Jeogjinju	15.09±0.58	2.41±0.12	48.25±2.84	33.23±1.66	1.03±0.05	18.08±0.48	87.00±4.35
Heugjinju	18.81±0.94	2.59±0.11	38.98±1.95	38.31±2.08	1.30±0.07	22.35±1.12	82.62±4.96
Boseogheugchal	18.50±1.05	2.45±0.12	42.66±2.56	35.09±1.75	1.30±0.06	22.00±1.35	83.36±4.17
Sinnongheugchal	19.20±0.96	2.58±0.15	43.38±2.17	33.60±1.26	1.23±0.03	22.75±1.14	81.64±2.45
Sinmyungheugchal	19.37±1.12	2.93±0.15	40.93±2.45	35.43±1.77	1.33±0.07	23.43±0.85	81.91±4.10
Josaengheugchal	19.52±0.98	2.51±0.07	46.18±2.31	30.70±0.97	1.09±0.00	23.00±1.15	81.25±2.44
Heuggwang	16.18±0.91	2.51±0.13	44.03±2.15	36.10±1.80	1.18±0.06	19.60±1.23	85.27±5.12
Heughyang	16.52±0.83	2.38±0.07	45.58±2.28	34.24±1.57	1.28±0.04	19.73±0.99	84.95±4.25
Heugseol	17.91±1.03	2.54±0.13	39.40±1.58	38.78±1.94	1.37±0.07	21.48±0.99	83.07±4.98

*SF: saturated fatty acid, **USF: unsaturated fatty acid, [1)]Values indicate the means ± SD of three replications

15.09~19.52%; stearic acid (C18:0) 2.23~2.93%; oleic acid (C18:1) 38.98~48.25%; linoleic acid (C18:2) 30.70~38.78%, and linolenic acid (C18:3) 1.03~1.38%. The fatty acid compositions among the colored rice varieties showed only slight variation (Table 2). Thus, the fatty acid composition and protein and lipid contents do not need to be considered in the selection of colored rice grains of high quality.

Determination of anthocyanin contents. The anthocyanins Cy-3-G, Pt-3-G, and Pn-3-G were measured by quantitative analysis using UPLC, and identified by comparing their physical and spectroscopic data with those of an authentic standard. A calibration curve was constructed using different concentrations of the three anthocyanins (1, 2.5, 5, 10, 25, and 50 μg/mL) as standards. The concentrations of anthocyanins Cy-3-G, Pt-3-G, and Pn-3-G were determined on the basis of the peak areas in the chromatogram as follows: Cy-3-G, y = 24,409.1x+8,046.3, r^2=0.9999, Pt-3-G, y=40,904.9x+15,635.6, r^2=0.9998, and Pn-3-G, y=12,713.7x−416.8, r^2=0.9997. The crude colored rice extracts were directly analyzed by UPLC chromatography. Significant differences in anthocyanin content among the varieties were observed (Table 3). The Cy-3-G, Pt-3-G, and Pn-3-G contents of the 12 colored rice varieties ranged from 0 to 113.63±0.13, from 0 to 0.10±0.02, and from 0 to 11.99±0.22 mg/100 g of DW, respectively. The average Cy-3-G content was 2,474 and 10 times higher than the average Pt-3-G and Pn-3-G contents, respectively. The highest anthocyanin content was observed in 'Heugjinju'

Table 3. Contents of anthocyanin in grains of 12 Korean colored rice varieties

Rice varieties	Content of anthocyanin[1] (mg/100 g, DW)			
	Cy-3-G (1)	Pt-3-G (2)	Pn-3-G (3)	**Total anthocyanin**
Heugnam	8.86±0.12	ND	1.35±0.20	**10.21±0.11**
Sintoheugmi	103.94±0.20	0.08±0.01	11.99±0.22	**116.01±0.19**
Hongjinju	tr	ND	ND	-
Jeogjinju	ND	ND	ND	-
Heugjinju	113.63±0.13	0.04±0.01	8.98±0.34	**122.65±0.24**
Boseogheugchal	24.53±0.07		3.85±0.29	**28.38±0.36**
Sinnongheugchal	63.80±0.49	tr	6.45±0.14	**70.26±0.62**
Sinmyungheugchal	15.43±0.06		1.87±0.22	**17.30±0.26**
Josaengheugchal	78.19±0.06	tr	6.17±0.24	**84.36±0.19**
Heuggwang	20.48±0.16		1.86±0.15	**22.34±0.26**
Heughyang	20.45±0.10		4.57±0.25	**25.02±0.17**
Heugseol	101.48±0.13	0.10±0.02	10.69±0.15	**112.28±0.29**

[1]Values indicate the means ± SD of three replications; tr, trace; ND, not detected

(Cy-3-G: 113.63±0.13, Pt-3-G: 0.04±0.01, Pn-3-G: 8.98±0.34, and total anthocyanins 122.65±0.24 mg/100 g), whereas 'Hongjinju' and 'Jeogjinju' did not contain any anthocyanins (Table 3).

Radical-scavenging activity of colored rice extract. DPPH and ABTS radical-scavenging assays are both commonly used to measure the total antioxidant status of a biological specimen due to their reproducibility and ease of quality control. Therefore, to measure the antioxidant activities of the colored rice extracts, UV/Vis spectrophotometry was used to detect DPPH and ABTS radicals. The DPPH and ABTS radical-scavenging activities (IC_{50}) of the 12 colored rice varieties ranged from >1,000 to 246.94±11.95 μg/mL, and from >200 to 19.96±1.03 μg/mL, respectively. The highest antioxidant activity was observed in 'Heugjinju' (IC_{50}, DPPH: 246.94±11.95 and ABTS: 19.96±1.03 μg/mL) and the lowest was found in 'Jeogjinju' (not inhibited at DPPH: >1,000 and ABTS: >200 μg/mL) (Table 4). To determine the correlation among total polyphenolic, total anthocyanin content, and antioxidant activity, linear regression analysis was carried out. The results show that total polyphenolic content was correlated strongly with antioxidant activity; the correlation coefficients (r^2) were 0.8512 (DPPH) and 0.7839 (ABTS) (Figs. 1 and 2).

Table 4. Radical scavenging activities in grains of 12 Korean colored rice varieties

Rice varieties	Radical-scavenging activity[1] (IC_{50}[2], μg/mL)	
	DPPH	ABTS
Heugnam	961.41±52.31	97.62±5.26
Sintoheugmi	287.39±13.26	25.78±2.03
Hongjinju	614.15±35.45	38.81±1.86
Jeogjinju	>1,000	>200
Heugjinju	246.94±11.95	19.96±1.03
Boseogheugchal	583.31±32.45	65.48±2.85
Sinnongheugchal	483.36±27.85	44.44±2.34
Sinmyungheugchal	634.52±30.29	74.44±3.89
Josaengheugchal	457.90±21.67	39.38±2.08
Heuggwang	767.30±40.26	81.79±4.25
Heughyang	497.06±29.56	58.83±3.04
Heugseol	381.33±20.57	31.08±1.87
BHA/Trolox	6.02±0.39 (BHA)	3.38±0.56 (Trolox)

[1]Values indicate the means ± SD of three replications; [2]Concentration at which 50% inhibition is achieved.

In the present study, the total polyphenolic, protein, lipid, and anthocyanin contents of grains from 12 Korean colored rice varieties were compared. Anthocyanin and polyphenol contents showed significant differences, whereas protein and lipid contents exhibited minor differences. Moreover, evaluation of the relationship between the above contents and DPPH and ABTS radical-scavenging activities showed that total polyphenolic content was correlated strongly with antioxidant activity. 'Heugjinju', 'Sintoheugmi', and 'Heugseol' exhibited higher total polyphenolic contents (1,338.58±94.42, 1,173.27±75.57, and 1,119.43±67.07 μg CGA/100 g, DW, respectively) and showed stronger antioxidant activities (DPPH IC_{50}: 246.94±11.95, 287.39±13.26, and 381.33±20.57; ABTS IC_{50}: 19.96±1.03, 25.78±2.03, and 31.08±1.87 μg/mL, respectively). Overall, 'Heugjinju', 'Sintoheugmi', and 'Heugseol' could be very important nutritional food sources with possible health-related benefits for humans.

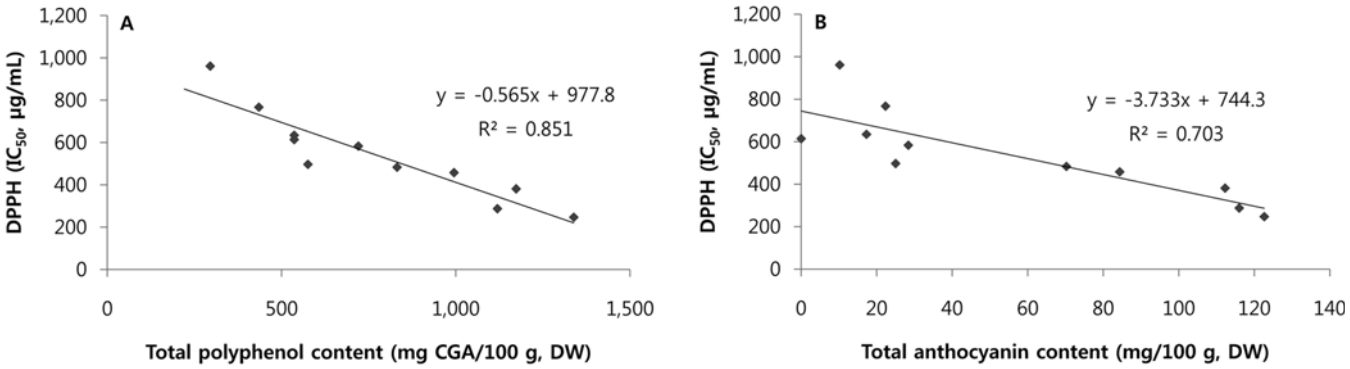

Fig. 1. Linear regression analysis of DPPH radical activity with total polyphenol (A), and total anthocyanin (B) contents in 12 Korean colored rice varieties.

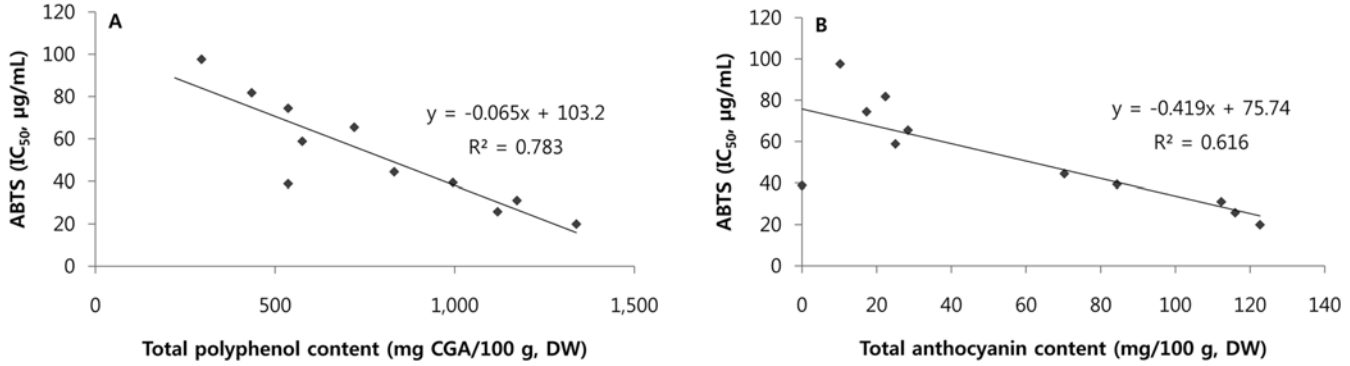

Fig. 2. Linear regression analysis of ABTS radical activity with total polyphenol (A) and total anthocyanin (B) contents in 12 Korean colored rice varieties.

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