[(Korean J. Crop Sci.) 43(3) : 179~183(1998)]

# Quantitative Analysis and Varietal Difference of Cyanidin 3-glucoside in Pigmented Rice

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## ABSTRACT

**The cyanidin 3-glucoside (C3G) extracted from pigmented rice seeds in 0.5% TFA (Trifluoro acetic acid) −95% ethanol was separated by High Performance Liquid Chromatography (HPLC). A HPLC system using a Develosil ODS-5 column and 0.1% TFA-$H_2O$~0.1% TFA-$CH_3CN$ gradient elution was selected for separation and quantitative determination of C3G.**

**Regression equation obtained for the standard content of C3G pigment was as Y=21.95293*X-14.726771 (r=0.99**). Using this method, 326 domestic and introduced collections were evaluated for the C3G content. The Korean bred cultivar 'Heugjinjubyeo', showed highest C3G content (552 mg/100g seed) among the tested cultivars. Among the pigmented rice cultivars ten cultivars were selected for containing a high content of C3G. The content of C3G per 100g seeds was in high order as follows : Heugjinjubyeo (552mg) >Cheng Chang (321mg)>Kilimgeugmi (240mg)>PI1609-79-2 (224mg)>Hong Shei Lo (221mg)>Heugnambyeo (191 mg)>Mitak = PI160979-1 (186mg)>Suwon425 (163mg)> Sanghaehyanghyeolla (108mg). The C3G pigment was not detected in the common white rice cultivars.**

**Key words : pigmented rice, cyanidin 3-glucoside, C3G, quantitative analysis.**

Black rice (*Oryza sativa* L.) having dark purple-colored seed is a major rice in South Asia and China. It is broadly known as an enriched rice with medical effects, and especially its purple pigment is widely used as food colorants in the processing of bread, ice creams and liquor, etc (Yoshinaga et al., 1986 ; Takahashi et al., 1987 ; Cho et al. 1996).

Pigments of rice cultivars vary greatly in distribution and intensity, and thus provide a fascinating array of topic for taxonomic and genetic studies. Various pigmented rices can be characterized by the anthocyanin and nonanthocyanin pigments, which are found in the bran or hull. Development of pigmented rice seeds by genetic engineering in the early 1970's began a surge in world production of various kinds of rice seeds (Chang et al., 1991).

Anthocyanin has only recently begun to be regarded as a biologically active substance, as well as colorants. For example, anti-inflammatory activities (Vlaskovska et al., 1990), redox potentials (Gabor, 1988), and antioxidative activity (Drenska et al., 1989 ; Costantion et al., 1992 ; Meunier et al., 1989 ; Igarashi et al., 1989) have been studied.

Recently, the antioxidative activity of human low-density lipoprotein caused by the phenolic substances in red wine (at pH 7.4) was also reported by Frankel et al (1983). Anthocyanins are now being isolated from the residue of pressed grapes used in red wine manufacturing to make colorant for foods and pharmaceuticals (Furtsov et al., 1989).

There is considerable interest in the development of food colorants from natural sources to replace synthetic food colorants. In particular, the role of anthocyanins as food coloring agents became very important since they are universally associated with attractive, colorful, and flavorful fruits (Francis, 1989), Tsuda et al., (1994) reported that cyanidin 3-glucoside from red bean, fruits, and vegetables showed strong antioxidative activity. Therefore, the importance of natural antioxidants has greatly increased.

As anthocyanins are very reactive compounds, and easily degraded or condensed to polymeric pigments, a rapid and efficient method is necessary for isolation and purification of anthocyanin pigment (Spagna et al., 1992). Recently, the column chromatography using several synthetic resins was developed for isolation and purification of anthocyanin of food and plants (Shi et al., 1992).

Since cyanidin 3-glucoside, which is abundant in the pigmented rice, have been known to exert diverse physiological functions in addition to their strong antioxidant activity, antioxidative active in food have been extensively investigated in rice (Osawa et al., 1985 and 1990 ; Tsuda et al., 1994).

The purpose of this work was to isolate, and characterize the anthocyanin pigments of newly bred black rice using a rapid and efficient method and varietal differences of C3G content were also studied.

## MATERIALS AND METHODS

### Material and reagent

Anthocyanin pigments in 326 rice cultivars including 186 white rice, 30 pigmented rice, 40 collected weedy red rice in domestic and 70 pigmented rice accessions from gene bank Rural Development Administration of were quantified by high performance liquid chromatography

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Received 29 May 1998.

(HPLC) using reversed ODS-5 column and detection at 530nm.

The rice seeds were cleaned and stored at 4℃ until use. The rice seeds were polished in sieve to obtain uniform seed fractions. Trifluoroacetic acid (TFA) were obtained from Sigma Chemical Co. (St. Louis Mo, USA). All laboratory chemicals used were of reagent grade.

### Extraction, isolation, and identification of C3G

Ground rice seeds (50g) was defatted with 400 ml of n-hexane. The residue was dried, and then anthocyanin pigments were extracted with 500 ml of 0.5% TFA in 95% ethanol overnight before being, filtered and concentrated to a small volume at temperature below 30℃ in vacum. The anthocyanin fraction of 0.1% TFA in 70% methanol were collected, and concentrated as before. These partially purified aqueous anthocyanin pigments were further isolated, and separated by preparative HPLC. Anthocyanin were identified by their retention times or standard addition. Anthocyanin were purchased from Indofine Chemical Co., Inc. (Somerville, NJ)

TLC (Thin Layer Chromatography) of crude extracts and partially purified pigments was carried out on reversed-phase RP-18, F254, (10×20 cm 0.25 mm, Merck, Darmstadt, Germany) by using two different solvent system: (A) 1st direction, i-BAW (isobutanol: water = 15 : 3 : 82 v/v), (B) 1st direction, i-BAW, AHW and 1% HCI as a solvent, respectively (Table 1).

Separation of anthocyanins was performed by a slight modification of HPLC procedure reported by Kondo et al (1985). The partially purified, aqueous anthocyanin pigments were adsorbed onto a water Sep-pak C-18 Cartridge (Milford, MA, USA), washed with water, eluted with acidified methanol, and concentrated by flushing with nitrogen pas. Furthermore, purification of the pigments was conducted with HPLC on a JASCO Twincle HPLC (Japan Spectroscopic Co. Ltd., Tokyo, Japan) using a reversed-phase Develosil ODS-10 column (20 mm × 250 mm, Nomura Chemical Co. Ltd., Seto, Japan) and an UV spetrophotometric detector (JASCO UVIDEC-100; Japan Spectroscopic, Tokyo, Japan) at 530 nm. The solvent was 3%$H_3PO_4$ in AcOH-$CH_3CN$-$H_2O$ (6.0 : 7.5 : 86.5) at a flow rate of 5.0 ml/min over 80 min.

### Quantification of anthocyanin pigment levels

HPLC analysis were performed with P-2000 pump (TSP Co.), Datajet integrator, and a model of Spectro Focus Spectrophotometric detector set at 530 nm. About 1 g of sample was accurately weighed and shaken with 20 ml of 0.5% TFA- 95% EtOH for 9 hrs at room temperature. The crude sample was separated by decantation and filterated by using C18 sep-pak SPE catridges attached to 10 ml syringes. The pigment conent of this filterate was determinated by HPLC. HPLC was performed by using a Develosil ODS-5 column (4.6×250 mm), Spectrophotometric detector with UV 530 nm and linear gradient from 0.1% TFA-$H_2O$ to 0.1% TFA- $CH_3CN$ for 30 min as elution solvent at a flow rate of 1.0 ml/min. The standard solutions of 4, 15, 40, and 60 ppm of cyanidin 3-glucoside was injected on the HPLC. Standard calibration curve was made by average value of peak area for the triplicate determinations of cyanidin 3-glucoside.

## RESULT AND DISCUSSION

### Extraction, isolation, and identification of anthocyanin from pigmented rices

The flow chart for preparation of anthocyanin pigments from rice is shown in Fig 1. The crude pigment solution was loaded onto the Amberlite XAD-7 (Organo Co. Ltd., Tokyo, Japan, 20~50 mesh) column (25 mm× 500 mm). The column was washed stepwise with glass-distilled water, MeOH-$H_2O$ (40:60) to remove sugars, amino acids, organic acids, low molecular phenols, and the polymerized dark brown pigments, and then finally eluted the bright purple-colored anthocyanin pigments with 0.1% TFA in MeOH-$H_2O$ (70:30).

In particular, it was suggested that the Amberlite XAD resins could be more generally used because they offer a better resolution and high recovery of pigments by means of gradient elution of anthocyanins with alcohol (Shi et al 1992). However, it was found that black rice pigments were not adsorbed onto polystylene resin such as Amberlite XAD-2 and XAD-4 (Organo Co. Ltd., Tokyo, Japan) but were readily adsorbed onto acrylester resin like an Amberlite XAD-7 (Organo Co. Lte., Tokyo, Japan), thereby eluting pigments with methanol more easily.

The structure of anthocyanin pigment Cyanidin 3-glucoside has been identified as shown in Fig. 2 based on FAB-MS and NMR spectral and chemical evidence (Choi et al., 1994).

TLC analysis, on the other hand, is useful for isolation and identification of anthocyanins. As shown in Table 1, three pigment bands with Rf 0.01, 0.3, 0.41 were isolated

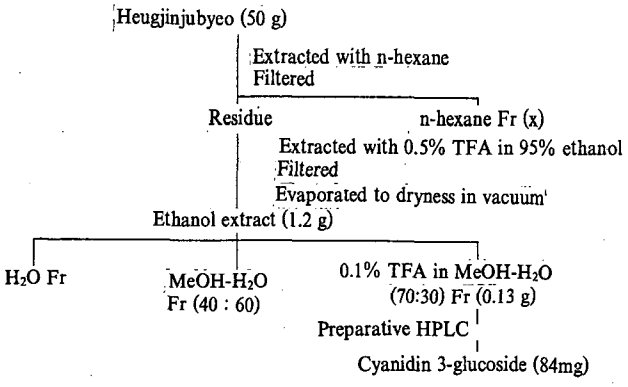


Fig. 1. Flow chart of preparation of anthocyanin pigments from pigmented rice.

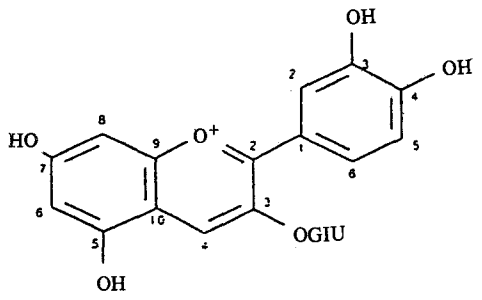


Fig. 2. Structure of rice anthocyanin pigment cyanidin 3-glucoside.

Table 1. Composition of Rf values of anthocyanins in pigmented rice cv. 'Heugjinjubyeo'.

| Anthocyanin | Rf values (×100) in | | |
|---|---|---|---|
| | i-BAW† | AHW† | 1% HCl |
| No. 1 | 30 | 22 | 5 |
| No. 2 | 41 | 30 | 11 |
| No. 3 | 1 | 21 | 15 |
| C3G‡ | 30 | 21 | 6 |

† i-BAW; isobutanol : acetic acid : water = 8 : 2 : 3 (v/v).
AHW; acetic acid : hydrochloric acid : water = 15 : 3 : 82 (v/v).
‡ C3G ; Cyanidin-3-glucoside.

from crude pigments of black rice seeds extracted with 5% TFA in 95 % ethanol. Anthocyanin was identified by their retention times or standard addition. Among them, two major band with Rf 0.3 and 0.41 could be separated on Amberlite XAD-7 column by using 0.1% TFA in 70% methanol. Band (No.1) was the most abundant anthocyanin in rice seed coats. Band (No. 3) was present in low concentration when compared with other rice pigments. Co-chromatography with authenic anthocyanin confirmed that Band (No.1) was cyanidin 3-glucoside (Table 1).

Partially purified anthocyanin pigments were finally separated by preparative reversed-phase HPLC on Develosil ODS-10 column using 3% $H_3PO_4$ in AcOH-$CH_3$-CN-$H_2O$ (6.0 : 7.5 : 86.5) as a solvent. A HPLC condition using a Develosil ODS-5 column and linear gradient from 0.1% TFA-$H_2O$ to 0.1% TFA-$CH_3CN$ (30 min) was selected for separation and quantitative equation of regression for cyanidin 3-glucoside (Table 2). Regression equation of for C3G pigment contents standard obtained from pigmented rice was Y=21.952934*X-14.726771 (r=0.99**)

### Varietal difference of C3G content

The yields of anthocyanin were 1.5~2% from original rice seeds (Ryu et al., 1998). Frequency distribution of C3G content in collected pigmented rice are given in Table 3. C3G content showed a wide distribution. 124

Table 2. Retention times(tR) of anthocyanins studied, and relationship between peak area and anthocyanin contents by HPLC.

| Anthocyanin (Chloride) | tR±S.D† | Relationship (y=ax+b)‡ | | |
|---|---|---|---|---|
| | | a | b | r§ |
| C3G¶ | 11.34±.011 | 21.95293 | −14.726771 | 0.99 |

† Mean values and standard deviations of retention times for two determination.
‡ Coefficients of the regression equation y=ax+b, where x is anthocyanin concentration (ppm) and y is peak area, for concentrations ranging 4 to 60 ppm.
§ Correlation coefficients of the regression equation.
¶ See Table 1 for abbreviation.

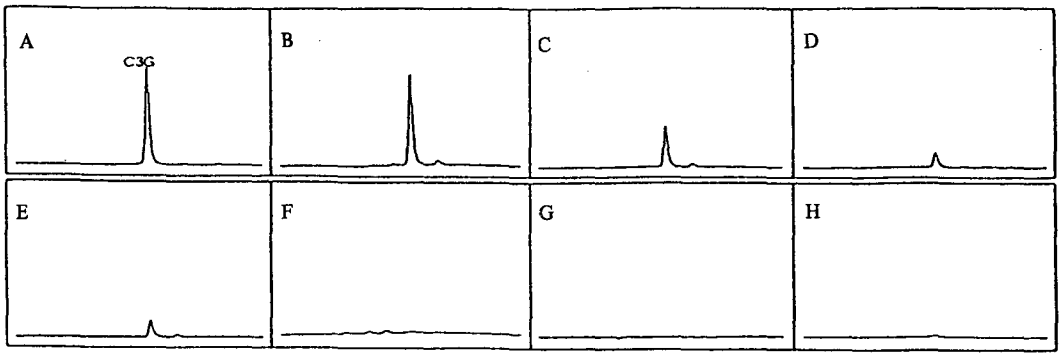


Fig. 3. Typical chromatogram of C3G standard, some pigmented rices(B~G) and a non pigmented rice(H). (A : standard, B : Heugjinjubyeo C : Suwon 425, D : Heugnambyeo, E : Sanghaehyanghyeolla, F : Jagwangbyeo, G : Goryeong red rice, H : Ilpumbyeo).

Table 3. Frequency distribution of C3G content in collection pigmented rices.

| C3G content | No. of cultivars |
|---|---|
| Below 10(mg/100g) | 124 |
| 10~50 | 4 |
| 50~100 | 2 |
| 100~200 | 5 |
| 200 over | 5 |

Table 4. Cyanidin 3-glucoside (C3G) content in pigmented rice cultivars.

| Cultivars | C3G mg/100g | Remark |
|---|---|---|
| Heugjinjubyeo | 552 | Korea |
| Suwon 425 | 163 | Korea |
| Sanghaehyanghyeolla | 108 | Korea |
| Heugnambyeo | 191 | Korea |
| Jagwangbyeo | Trace | Korea |
| Mitak | 186 | India |
| Pi 160979-1 | 186 | China |
| Hong Shei Lo | 221 | China |
| PI 160979-2 | 224 | China |
| Kilimheugmi | 240 | China |
| Cheng Chang | 321 | China |
| Hsinchu 56 | 55 | Taiwan |
| Nakabe | 56 | Japan |
| A-5 | Trace | Japan |
| A-11 | Trace | Japan |
| Kaeu N 5846 | Trace | Russia |
| Ilpumbyeo | Not detected | Korea |

cultivars showed C3G content of below 10mg/100g. 4 with 10~50mg, 2 with 50~100mg, 5 with 100~200mg, and 5 showed more than 200mg C3G content(Table 3) 10 mg/100g seed in 124 cultivars, 10~50mg in 4 cultivars, 50~100mg in 2 cultivars, 100~200mg in 5 cultivars, and over than 200mg in 5 cultivars, respectively.

The C3G levels in rice seeds and its HPLC chromatogram are given in Fig. 3.

Seed (100g) C3G content with 326 cultivars ranged from trace to 552mg. The maximum C3G content was showed by Heugjinjubyeo (552mg/100g seed) and Cheng Chang (321mg/100g seed), and the minimum by Jagwangbyeo (trace) and Ilpumbyeo (trace).

The Korean bred cultivars, Heugjinjubyeo, showed the highest C3G content. In the non pigmented rice cultivars, C3G pigment was not detected (Table 4). Among the 140 pigmented rice cultivars, ten cultivars were selected for containing high amount of C3G. The content of C3G in 100g of seeds was in high order as follows; Heugjinjubyeo (552mg)>Cheng Chang (321mg)>Kilimheugmi (240mg)>PI160979-2 (224mg)>Hong Shei Lo (221mg)>Heugnambyeo (191mg)>Mitak=PI160979-1 (186mg)>Suwon 425 (163mg)>Sanghaehyanghyeolla (108mg).

The color variation of C3G in 0.5% TFA 95% ethanol extracted from pigmented rice is shown in photo 1. After mass up of 1 g of pigmented rice to 25 ml, the colors of C3G showed to variation depending on the concentration of C3G. The color variation of C3G pigment in 1 g of rice seed was in high order as follows : A>B>C>D>E>F>G. It seems that a close relationship between the extracted color and the C3G concentration in the pigmented rice exists.

In particular, cyanidin 3-glucoside, an important OARC (Oxygen Radical Absorbing Capacity) ability agent (Wang et al., 1997), was the most abundantly pres-

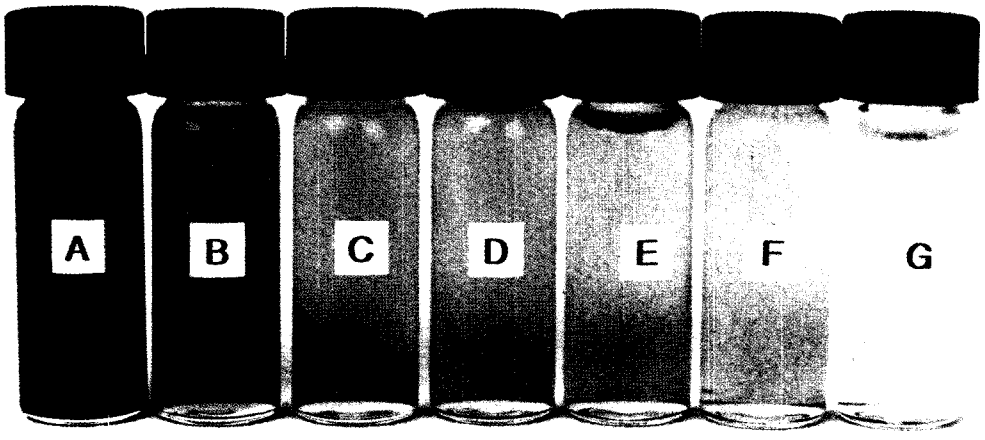


Photo. 1. Color variation of C3G in 0.5% TFA-95% ethanol extract from pigmented rice
A : Heugjinjubyeo B : Suwon 425 C : Heugnambyeo D : Sanghaehyanghyeolla E : Sanghaehyanghyeolla F : Jagwangbyeo G : Goryeong red rice

ent in Heugjinjubyeo and Cheng Chang among the samples examined. Rice cultivars "Heugjinjubyeo" may be also related to high antioxidative activity, and further studies including environment variation and genetic characteristics of these compounds are needed.

## ACKNOWLEDGEMENT

The authors wish to acknowledge the financial support of the Korea Research Foundation made in the program year of 1997

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