# Antioxidative Compounds in the Extracts of Black Rice Brans

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Recently we found that extracts from ancient rice brans, especially those from black rice bran, possess strong scavenging activities for reactive oxygen species (ROS). In this study, we examined the origin of the ROS-scavenging activities in the black rice bran extracts, and identified candidate scavengers such as cyanidin-3-glucoside (Cy-3-glu) and cyanidin. Although ferulic acid is known to be an antioxidative component of bran in currently available common white rice varieties, it was not found in the black rice bran extracts. The ROS-scavenging activities of Cy-3-glu and cyanidin, which were identified in this study, were examined using the ESR-spin trap method and in terms of protective activity against effects of ultraviolet (UVB) irradiation on an epidermal cell line (HaCaT cell). These anthocyanin compounds were found to possess both strong ROS-scavenging activities and to suppress cell-damaging effects of UVB, indicating that both Cy-3-glu and cyanidin are the active components involved in the antioxidative activity of black rice bran extracts.

**Key words** ——— black rice bran extract, cyanidin-3-glucoside, cyanidin, antioxidative activity, LC/MS, HaCaT cell

## INTRODUCTION

Smoking, drinking alcohol[1,2] and abnormal generation of reactive oxygen species (ROS) under conditions such as pathophysiological disorders and ultraviolet light exposure have long been known to induce oxidative stress. This involves lipid oxidation[3] and DNA damage[4] in living systems including skin tissue, which in turn causes cell damage. ROS and their related peroxides are also known to promote skin aging, producing wrinkles and pigmental spots, weaken immunity, and induce cancers and life-style related diseases.

Living systems possess defenses against ROS, which minimize their influence through the actions of antioxidative enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase. However, these enzyme activities tend to decline with age, due to increased psychological stresses and imbalances in eating styles. Therefore, substances possessing antioxidative activity have been widely sought.[5,6] We have also investigated materials with potential antioxidative properties, focusing on the ancient rice brans. Rice bran contains fat-soluble antioxidative components such as ferulic acid and $\gamma$-oryzanol[7,8] which are widely used in food and cosmetics. We examined antioxidative properties of white rice bran extracts with regard to SOD-like activity and ROS-scavenging activity.[9] Present-day rice bran is obtained from rice varieties produced by breeding (Koshihikari, being the most popular), and has a light yellow color, unlike the original variety. However, colored rice varieties are assumed to include several varieties similar to the black and red rice strains believed to have been brought in from China more than two thousand years ago.[10] These colored rice varieties are the only likely survivors of ancient rice and have been bred little, evolving due to the power of adaptability of the plants alone. Red rice has a reddish brown color and black rice is blackish purple. The cause of this difference in color tone has been reported to be genetic.[11] Colored plants (for example, red grape, blueberry, tomato, green tea *etc*.) are known to contain high levels of antioxidative compounds such as polyphenols. Ancient rice varieties are likely to be strongly antioxidative, because they have external

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deep colors due to the presence of polyphenolic compounds, and are vigorous in their germination. Because these original plants possess defense mechanisms against external stresses, bran extracts of ancient rice have been focused on as potential materials for daily use.

Recently we have found that ROS-scavenging activity estimated by the ESR-spin trap method and SOD-like activity determined by the nitro-blue tetrazolium (NBT) method in ancient rice bran extracts (black and red rices) were significantly stronger than those of present-day rice (Koshihikari). We also confirmed that extracts of both ancient and present-day rice brans varied in scavenging activity according to their harvest sites.[9] Furthermore, it was suggested that the active components in the black rice bran extracts were identical with anthocyanin compounds, which showed absorption maxima at approximately 530 nm.[12] Thus, in order to identify these active components, comparison with authentic several anthocyanin compounds was carried out. In the present study, we examined the active compounds in ancient rice bran extracts, in particular, black rice extracts, with regard to antioxidative properties, and found that cyanidin-3-glucoside (Cy-3-glu) and cyanidin contributed to the antioxidative properties of the extracts. On the basis of the results, black rice bran extracts are strongly indicated to be useful as antioxidative substances for use in cosmetics and health food.

## MATERIALS AND METHODS

**Analysis of the Active Compounds in the Black Rice Bran Extract ——**

*Rice*: Black rice (*Oryza sativa* L.) produced at Hiraka-gun, Akita Prefecture in Japan in 2001, was used.

*Chemicals*: Trifluoroacetic acid (TFA), methanol, HPLC-grade acetonitrile, 99.5% EtOH, formic acid, hematoporphyrin dihydrochloride (HP), hydrogen peroxide ($H_2O_2$), iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), xanthine oxidase (XOD), xanthine and L(+)-ascorbic acid were purchased from Wako Pure Chemicals (Osaka, Japan). Methanol-D4 for NMR ($CD_3OD$) was from Merck (Darmstadt, Germany). Trifluoroacetic acid-d ($CF_3COOD$) was from Cambridge Isotope Laboratories (Andover, MA, U.S.A.). Diethylenetriamine-N, N′, N″, N‴, N⁗-pentaacetic acid (DTPA) was from Dojindo (Kumamoto, Japan). 5, 5-Dimethyl-1-pyrroline-N-oxide (DMPO) was from Labotec Ltd. (Tokyo, Japan). 6-Hydroxypurine (hypoxanthine, HPX), methemoglobin (MetHb) and 2,2,6,6-tetramethyl-4-piperidone hydrochloride (TMPD) were purchased from Sigma-Aldrich, Inc. (St. Louis, U.S.A.). *tert*-Butyl hydroperoxide (*t*-BuOOH) was obtained from Katayama Chemical (Osaka, Japan). fetal bovine serum (FBS, Australia), Dulbecco's modified eagle's medium (DMEM), low glucose (1000 mg/l) and Hanks (+) were purchased from Nikken Biomedical Laboratory (Kyoto, Japan). Neutral red solution, neutral red assay fixatives (0.1% $CaCl_2$, 0.5% HCHO) and neutral red assay dissolution solution (1% $CH_3COOH$, 50% EtOH) were purchased from Kurabo Industries (Tokyo, Japan). Cy-3-glu chloride (authentic sample A), cyanidin chloride (authentic sample B), pelargonidin chloride (authentic sample C), delphinidin chloride (authentic sample D), keracyanin chloride (authentic sample E) and ferulic acid were purchased from Extrasynthese (Genay, France). Procyanidin $B_1$ (authentic sample F) was from Asahi Breweries (Tokyo, Japan). *tetra*-Methyl silane (TMS) was from Isotec Inc. (Illinois, U.S.A.). The buffers used were 0.5, 0.1 M and 20 mM phosphate buffer (pH 7.4). All other chemicals used were of commercially available analytical reagent grade.

*Preparation of Black Rice Bran Extract*: Black rice bran was obtained by milling the rice hulls (Rice mill SMD-100G, MK Seiko, Osaka, Japan). Black rice bran extract was prepared by extraction with 50% EtOH as follows: to 100 g of black rice bran, 1 l of 50% EtOH was added. The solution was refluxed at 80°C for 3 hr, cooled, left to stand at 10°C for 7 days to remove the sediment for immersion, and then filtered through a quantitative filter paper (No. 5B, 4 $\mu$m, Advantec, Tokyo, Japan). The filtrate was concentrated by rotary evaporation under reduced pressure at 40–45°C and freeze-dried until use.

*Preparation of Folin-Dennis Reagent*: A 100 g of sodium tungstate (VI) dihydrate, 20 g phosphomolybdic acid n-hydrate and 50 ml of $H_3PO_4$ were added to 700 ml of purified water and dissolved, then refluxed for 2 hr. After cooling, the volume was adjusted to 1 l. For making a saturated $Na_2CO_3$ solution, 35 g $Na_2CO_3$ was added to 100 ml of purified water and dissolved at 70–80°C. The solution was left to stand overnight at room temperature, the resulting precipitate was removed and the supernatant was used.

**Methodology for Fractionation of the Active Compounds** ——— Extracts (10 g) were fractionated by column chromatography using HP-20 columns (4.0 cm i.d. × 50 cm length), filled with 100 g Daiyaion HP-20 resinoid (Mitsubishi Chemical, Tokyo, Japan). The resinoid in the column was washed first with water (2 l), then with MeOH (2 l). The solvents used for gradient elution were $H_2O$, 50% EtOH, 60% EtOH, 80% EtOH and 99.5% EtOH in that order. Each fraction was concentrated and then freeze-dried. Following a report on more efficient extraction of polyphenols,[13] extractions were also carried out under acidic conditions. The extracts (10 g) were applied to an HP-20 column (4.0 cm i.d. × 50 cm length, 100 g Daiyaion HP-20 resinoid). The solvents used for gradient manipulations were $H_2O$, HCOOH/MeOH/$H_2O$ (1/99/100) and MeOH in this order. Each effluent fraction was collected, concentrated, and then freeze-dried.

**Determination of Total Polyphenols by the Folin-Dennis Method** ——— Total polyphenol contents in samples were measured using the Folin-Dennis method.[14]

A solution containing 3.2 ml of purified water, 200 $\mu$l of a sample solution and 200 $\mu$l of Folin-Dennis reagent was added to a test tube and stirred, and 400 $\mu$l of a saturated $Na_2CO_3$ solution then added. After allowing the sample to stand for 30 min, a UV-visible recording spectrophotometer UV-260 (Shimadzu Co., Kyoto, Japan) was used to measure the absorbance at 700 nm. Gallic acid was used as a reference standard, and the total polyphenol concentration was calculated as an equivalent concentration of gallic acid using a working curve (correlation coefficient: r = 0.995).

**Identification of Active Compounds in the Fractions of the Black Rice Bran Extract** ——— The active compounds were identified using 50% EtOH (eluate A) effluent and 1% HCOOH/MeOH (1/1) (eluate B) effluent.

*Reversed Phase Thin Layer Chromato-Graphy (TLC)-Scanning Densitometric Method*: The compounds in the extracts were first detected by thin layer chromatography (TLC), using an RP-18$WF_{254S}$ plate (Merck). The extracts (10 mg/ml) and authentic samples (1 mg/ml) were dissolved in a mixed solvent composed of MeOH/0.1% TFA (1/1), and TLC of these solutions was carried out using $CH_3CN$/4% TFA (1/2) as a developing solvent. The TLC conditions were as follows: spot position, 1 cm; development length, 8 cm; spot size, 2 $\mu$l. In addition, the visible absorption spectrum and the absorbance maximum at 400–800 nm of the spot on the TLC plate were measured using a MCPD-1000 (Photal Otsuka Electronics, Tokyo, Japan) linked to a scanning densitometric method.[15,16]

*HPLC and LC/MS Methods*: HPLC analyses were carried out on the fractions (portions of the eluates A and B) obtained and the authentic samples (3 types), and each retention time (RT value) was determined.

The extracts (the fractions: 10 mg/ml) and the authentic samples (0.5 mg/ml) were dissolved in MeOH/0.1% TFA (1/1), and filtered through a membrane filter (Titan, PTFE membrane, 0.45 $\mu$m, Tomsic, Tokyo, Japan). HPLC conditions: experiments were carried out using a Shimadzu C-R6A Chromatopac system equipped with a Shimadzu SCL-6B System Controller and a Shimadzu SPD-10AU UV-VIS Detector (all from Shimadzu). The following operating conditions were used[17,18] with a Wakosill ODS column, $\phi$ 4.6 mm × 250 mm (Wako Pure Chemicals): solvent: (A) 0.4% HCOOH (v/v), and (B) 20% $CH_3CN$/0.4% HCOOH (1/1) (v/v); gradient: % B, initial, 40%; 35 min, 100%; 40 min, 100%; 50 min, 40% (run time 50 min), flow rate: 0.7 ml/min, detection wavelength: 520 nm, column temperature: 30°C, sample temperature: ambient, and injection volume: 20 $\mu$l.

The extracts from eluate A and ferulic acid were dissolved in 50% EtOH (v/v), and then filtered through a membrane filter (Titan, PTFE membrane, 0.45 $\mu$m, Tomsic). HPLC conditions: experiments were carried out using a DG660 HPLC degassing unit (GL Science, Tokyo, Japan) equipped with a chromatocorder 21 (System Instrument, Tokyo, Japan), a PU610-1X HPLC pump, a CO630/630C HPLC column oven (GL Science, Tokyo, Japan) and a UV620 HPLC UV-visible spectrometer (all from GL Science, Tokyo, Japan). The column used was a TSKgel ODS-80Ts, $\phi$ 4.6 mm × 7.5 cm (Toso, Kyoto, Japan). The solvent used was $CH_3CH_2COOH$/$CH_3CN$/$H_2O$ (1/49/228) (v/v). The flow rate was 1.0 ml/min (run time 20 min), detection wavelength was 302 nm, column temperature was 30°C, sample temperature was ambient, and injection volume was 20 $\mu$l.[19,20]

On the basis of the results of the HPLC analysis, after the extracts (portions of the eluates A and B) were dissolved in MeOH/0.1% TFA (1/1), the solutions (30 mg sample/ml and 1.0 mg authentic sample/ml) were filtered through a membrane filter (Titan, PTFE membrane, 0.45 $\mu$m, Tomsic). LC/MS conditions: LC/MS experiments were carried out on

an LCQ Advantage (Thermo-Finnigan, Arizona, U.S.A.) equipped with a HP-1100 series (Agilent Technologies, Waldbronn, Germany). Experiments were carried out under the following conditions[21–23]: column: Shiseido Capcell Pak C18 MG ($\phi$ 3.0 mm × 150 mm) (Shiseido Fine Chemicals, Tokyo, Japan); solvent: (C) 0.2% HCOOH/$H_2O$ (v/v) and (D) 20% $CH_3CN$/0.2% HCOOH (1/1) (v/v); gradient: % D: initial, 5%; 20 min, 20%; 25 min, 100%; 28 min, 100%; 30 min, 5%; 32 min, 5% (run time 32 min); flow rate: 0.4 ml/min; injection: 20 $\mu$l; column temperature: 30°C; detection: 520 nm. The followings were the MS parameters: ionization mode: electrospray ionization (ESI) positive; scan range: 200–1200 amu; scan rate: 1 scan/sec.

**Method of Cy-3-glu Isolation from the Extract (Eluate A)** —— The fraction eluted with eluate A (500 mg) was applied to an octa decil silicate (ODS) column (2.0 cm i.d. × 25 cm length, with 15 g ODS for silica gel chromatography) (Fuji Silysia Chemicals, Tokyo, Japan). After washing with MeOH (100 ml), 0.1% TFA, MeOH/0.1% TFA (30/70) and MeOH were used for fractionation.[24] The yields of samples obtained by fractionation (Fr. 1, 2, 3 and 4) were 170, 53, 11 and 190 mg, respectively. Fr. 3 (11 mg) was purified using an LH-20 column (2.4 cm i.d. × 50 cm length, containing 50 g Sehpadex LH-20 resinoid; Amersham Biosciences Corp. Piscataway, NJ, U.S.A.) and washing with MeOH (200 ml), before elution with HCOOH/$H_2O$ (5/95), MeOH/$H_2O$/HCOOH (20/75/5) and MeOH, in this order. Effluents obtained by these fractionation steps were concentrated and then freeze-dried. The yields were Fr. 3-1: none, Fr. 3-2: 1 mg, Fr. 3-3: 10 mg. Fr. 3-2 was proposed to be Cy-3-glu on the basis of TLC results, which were identical to those of an authentic sample.

*Structural Analysis of the Isolated Compounds*:

**LC/MS Method**: The effluent (Fr. 3-2 fraction) and an authentic sample were dissolved in a MeOH/0.1% TFA (1/1) solution at 0.5 mg/ml and the LC/MS analysis was carried out under the above-mentioned conditions, following membrane filtration (Titan, PTFE Membrane, 0.45 $\mu$m, Tomsic).

**$^1$H-NMR Method**: $^1$H-NMR analysis of the compounds purified from the extracts was carried out by comparison with an authentic sample (1.0 mg/ml) to elucidate the structure.[25–27] A JEOL AL-400 NMR spectrometer (JEOL, Tokyo, Japan) was used and NMR sample tubes (Wilmad Glass, New Tersey, U.S.A.) contained the solute in $CD_3OD/CF_3COOD$ (10/1) with TMS (Isotec Inc.) as an internal standard.

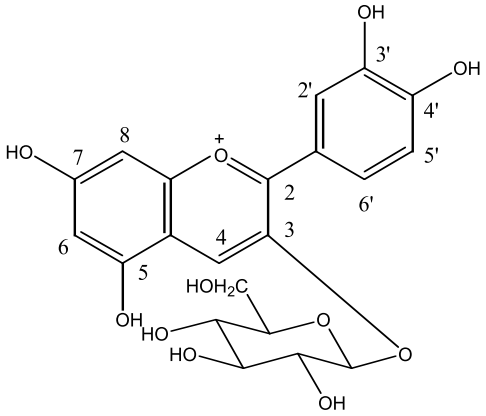


**Fig. 1.** Cyanidin-3-O-glucopyranoside

$^1$H-NMR analysis of the purified product of the extracts and the authentic sample A were carried out to elucidate the structure.

$^1$H-NMR data from the authentic sample A: $^1$H-NMR (400 MHz) in $CD_3OD/CF_3COOD$ (10/1) at 25°C, $\delta$ppm from TMS: 8.88 (1H, *s*, H-4), 8.22 (1H, *dd*, *J* = 2.2, 8.5 Hz, H-6′), 8.00 (1H, *d*, *J* = 2.2 Hz, H-2′), 7.02 (1H, *d*, *J* = 8.0 Hz, H-5′), 6.90 (1H, *d*, *J* = 2.0 Hz, H-8), 6.70 (1H, *d*, *J* = 2.0 Hz, H-6), 5.33 (1H, *d*, *J* = 7.8 Hz, Glc H-1), 3.14–3.77 (6H, m, GlcH2-6).

$^1$H-NMR data from the product purified from the extracts: $^1$H-NMR (400 MHz) in $CD_3OD/CF_3COOD$ (10/1) at 25°C, $\delta$ppm from TMS: 8.94 (1H, *s*, H-4), 8.16 (1H, *dd*, *J* = 2.2, 8.5 Hz, H-6′), 7.97 (1H, *br.s*, H-2′), 6.93 (1H, *d*, *J* = 8.5 Hz, H-5′), 6.80 (1H, *d*, *J* = 2.0 Hz, H-6), 6.57 (1H, *d*, *J* = 2.0 Hz, H-8), 5.19 (1H, *d*, *J* = 7.8 Hz, Glc H-1), 3.15–3.85 (6H, m, GlcH2-6) (Fig. 1).

**Antioxidative Activities of the Active Compounds in the Extracts** —— ROS scavenging activity of the identified compounds was estimated using an ESR method. Inhibitory effects on cell injury under ultraviolet irradiation (UVB) were examined using an epidermal cell line (HaCaT cell).

*ROS Scavenging Activities as Evaluated by the ESR-Spin Trap Method*: Polyphenolic compounds such as anthocyanins are well-known to have strong antioxidative activities. Thus, we examined the ROS-scavenging activities of the anthocyanins (Cy-3-glu and cyanidin) identified from the extracts using the ESR-spin trap method. The centrifuged extracts (crude sample, elution with eluate A and eluate B) and the anthocyanins (authentic sample A and authentic sample B) were used.

By using an FR-30 ESR spectrometer (JOEL, Tokyo, Japan) and a high-sensitivity quartz cell (Labotec Ltd.), ROS scavenging activity was measured by the spin trap method, employing DMPO as a spin trap agent.[28–30] The signal intensity due to the DMPO-ROS spin adduct was expressed as the relative intensity ratio against the signal intensity due to Mn (II). ROS scavenging activity of the compounds are expressed as the $IC_{50}$ value, which defines a concentration a compound at which a 50% inhibition rate is achieved compared to the control. The formula (1) to determine the inhibition rate was calculated as follows:

Inhibition rate (%) = (intensity relative to Mn (II) in the sample solution)/(intensity relative to Mn (II) in the control) × 100 – (1).

The measurement conditions for the ESR-spin trap method were as follows: magnetic field, 377 ± 7.5 mT; frequency of magnetic field modulation, 100 kHz; modulation amplification width, 0.1 mT; time constant, 0.1 sec; output power, 4 mW; and sweeping time, 2 min.

**Singlet Oxygen ($^1O_2$) Generation System (HP-UV System)**: To a 2-ml polycarbonate tube were added 50 $\mu$l of 0.25 mM HP, and 50 $\mu$l of 200 mM TMPD, 20 $\mu$l of 50% $CH_3CN$, and 80 $\mu$l of water (total volume 200 $\mu$l). Immediately after addition of the last reagent, the solution was vortexed and transferred to the ESR cell, which was then fixed to the cavity of the ESR spectrometer for measurement. ESR measurements were initiated after ultraviolet (UVA) irradiation (650 mW/cm$^2$) (Supercure-203S-UV•Lightsource) (SAN-EI Electric, Osaka, Japan) for 30 sec.[31,32]

**Superoxide Anion Radical ($\cdot O_2^-$) Generation System (HPX-XOD System)**: To a 2-ml polycarbonate tube were added 10 $\mu$l of 0.25 M DMPO, and 25 $\mu$l of 1 mM DTPA, 30 $\mu$l of 2.8 mM HPX, 40 $\mu$l of 0.5 units/ml XOD, 75 $\mu$l of water, and 20 $\mu$l of 50% EtOH (total volume 200 $\mu$l).[33,34] After the reaction was started by addition of XOD, it was transferred to the ESR cell and ESR measurement was then started after 30 sec.

**Hydroxyl Radical (•OH) Generation System (Fenton System)**: In a 2-ml polycarbonate tube 25 $\mu$l of 0.1 mM $FeSO_4 \cdot 7H_2O$ were added to a solution containing 50 $\mu$l of 0.1 mM DTPA, 10 $\mu$l of 0.25 M DMPO, 55 $\mu$l of 0.5 M phosphate buffer (pH 7.4), 10 $\mu$l of 50% $CH_3CN$, and 50 $\mu$l of 0.1 mM $H_2O_2$ (total volume 200 $\mu$l).[35,36] The reaction was started after addition of the last reagent and the ESR spectrum was measured after 30 sec as described above.

**Tert-Butylperoxyl Radical (*t*-BuOO•) Generation System (MetHb-*t*-BuOOH System)**: To a 2-ml polycarbonate tube were added 50 $\mu$l of 1 mg/ml MetHb, 50 $\mu$l of 0.1 mM DTPA, 10 $\mu$l of 2.5% DMPO, 30 $\mu$l of water, 10 $\mu$l of 50% $CH_3CN$, and 50 $\mu$l of 100 mM (*t*-BuOOH) (total volume 200 $\mu$l). The reaction was started by addition of the last reagent, and the ESR spectrum was measured after 30 sec as described above.[37]

*Evaluation of UVB Damage in HaCaT Cells*: The extracts, the authentic sample A and the authentic sample B of identified active compounds were evaluated by their abilities to suppress HaCaT human epidermal cell injury due to UVB, according to the neutral red (NR) method.[38,39]

HaCaT cells (Cosmos Technical Center Ltd., Tokyo, Japan) were maintained at 37°C, under 5% $CO_2$ (TE-HER CPseries $CO_2$ Incubator CPD-170, Hirasawa Works, Tokyo, Japan) in a culture medium containing DMEM supplemented with glutamine (0.1 mM) and FBS (5%, v/v) as previously reported.[40]

HaCaT cells were seeded onto a 96-well microplate (Apogent, Roskilde, Denmark) at a density of $4 \times 10^4$ per well and precultured under 5% $CO_2$ at 37°C for 24 hr. The medium was then replaced with medium containing various concentrations of sample. Each sample was dissolved in 0.1 ml of Hanks (+). The control groups were added to 0.1 ml of Hanks (+). Samples were then irradiated with UVB through a UVB filter at a dose of 25 mJ/cm$^2$, using a Supercure-203S (SAN-EI Electric). The cells were washed with Hanks (+), and then cultured in sample-free 5% DMEM for 24 hr. Following addition of the neutral red solution, cells were again cultured for 2 hr. UVB-irradiated HaCaT cell damage was expressed as survival rate, estimated by the neutral red method as described previously.[41,42] Using a microplate reader (TECAN, Spectra Fluor Plus., Wako Pure Chemicals), the absorbance at 540–660 nm was measured.

## RESULTS

### Analysis of the Active Compounds in the Extracts

*Total Polyphenol Content in the Fractions*: The polyphenol content was high in the fractions derived from eluates A and B (Table 1).

*Identification of Compounds in the Fractions of the Extracts*:

**Table 1.** Total Polyphenol Contents in the Various Extract Effluents

| Material | Fraction | Polyphenol contents (%) |
|---|---|---|
| Extract | Crude | 17.58 ± 1.46** |
| | $H_2O$ | 5.08 ± 0.56** |
| | 50% EtOH (eluate A) | 34.91 ± 1.16 |
| | 60% EtOH | 25.78 ± 1.33* |
| | 80% EtOH | 9.58 ± 1.02** |
| | 99.5% EtOH | 6.04 ± 0.86** |
| | 1% HCOOH | 5.54 ± 0.43** |
| | 1% HCOOH : MeOH = 1 : 1 (FM) (eluate B) | 35.92 ± 1.52 |
| | MeOH | 31.70 ± 2.22 |

Values are expressed as the means ± S.D. ($n$ = 3). Statistical significance was evaluated by the Student's $t$-test. *$p < 0.05$, **$p < 0.01$ *vs.* FM Total polyphenol content was determined by the Forin-Denis method.

**Table 2.** Characteristics of Extracts and Authentic Samples

| Material | Fraction | Rf | Color of spot | $\lambda_{max}$ (nm)[a)] |
|---|---|---|---|---|
| Extract | Crude | 0.564 | Pale red | 520 |
| | | 0.688 | Brown | |
| | Eluate A | 0.569 | Pale red | 521 |
| | | 0.678 | Brown | |
| | Eluate B | 0.565 | Pale red | 522 |
| | | 0.685 | Brown | |
| Authentic samples | A | 0.571 | Red | 523 |
| | B | 0.331 | Red | 532 |
| | C | 0.207 | Vermilion | 514 |
| | D | 0.391 | Violet | 540 |
| | E | 0.608 | Red blue | 525 |
| | F | 0.738 | Pale flesh | None |
| Eluate A: Authentic sample A = 1 : 1 | | 0.573 | Red | 522 |
| Eluate B: Authentic sample B = 1 : 1 | | 0.575 | Red | 523 |

*a*) λmax was obtained from a scanning densitometry visible absorption spectrum.

**Reversed Phase TLC-Scanning Densitometry**: The extracts appeared as brightly reddish colored spots on TLC. An authentic samples of anthocyanins also exhibited clearly red colored spots, but authentic samples of tannins did not show clear spots. The Rf values for the extract (eluate A) and a (1 : 1) mixed solution of the extract (eluate B) and authentic sample A on TLC coincided to be Rf: 0.573, and 0.575, respectively. The absorption spectrum of each spot was measured at 400–800 nm by scanning densitometry. The spectra for the spots of the authentic sample (authentic sample A, Rf: 0.571), and the extracts (crude extract, Rf: 0.564; eluate A, Rf: 0.569; eluate B, Rf: 0.565) were observed to have an absorption maximum approximately 530 nm, close to those of anthocyanins.[12)] It was suggested that brown

**Table 3.** HPLC Analytical Results Detected at 520 nm for the Extracts and Authentic Samples, Due to Anthocyanins

| Material | Fraction | RT (min) |
|---|---|---|
| Extracts | Eluate A | 15.056 (Main peak) |
| | Eluate B | 15.142 |
| | | 28.085 (Main peak) |
| Authentic samples | A | 14.867 |
| | B | 27.307 |
| | E | 12.785 |

spot, no UV-absorbance, was due to some carbohydrate derivatives such as sugars in the black rice bran extract or unknown substances expect for anthocyanin. These results indicated that the anthocyanin in the extracts was Cy-3-glu (Table 2).

*HPLC and LC/MS*: HPLC was performed for the extracts (eluate A and eluate B) and the authentic samples (3 types) to determine the retention time in min (RT value). Values for the fraction from eluate A and the authentic sample A were in agreement, with RT of 15.1 and 14.9 respectively. Furthermore, the HPLC peak of the fraction from eluate A overlapped with that of the (1 : 1) mixed solution of the extracts and authentic sample A, and were also consistent with the RT value of 15.2, suggesting that the active compound was Cy-3-glu. Two main peaks were detected in the extracts obtained from the eluate B. The RT values for these peaks (15.1, 28.1) agreed with those of authentic sample A (14.9) and authentic sample B (27.3), respectively. The HPLC peak of the fraction from eluate B overlapped with that of a (1 : 1) mixed solution of authentic samples A and B, and agreed with the RT values (15.4 and 27.6), suggesting that the active compounds were Cy-3-glu and cyanidin respectively (Table 3).

In addition, a shown in Fig. 2, ferulic acid was not observed in the extracts from eluate A.

TLC and HPLC analyses indicated that the active compounds in the fractions obtained from eluates A and B were Cy-3-glu and the two compounds Cy-3-glu and cyanidin, respectively. Then, LC/MS analysis was further performed to identify the compounds.

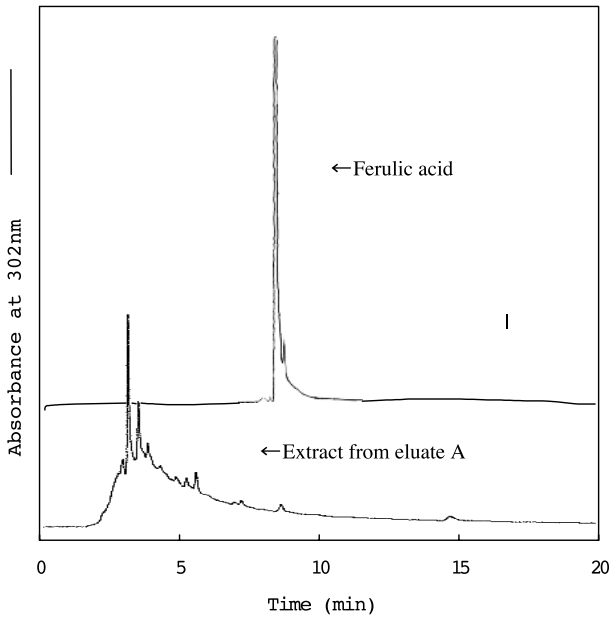


**Fig. 2.** HPLC Profiles of the Extract from Eluate A and Ferulic Acid

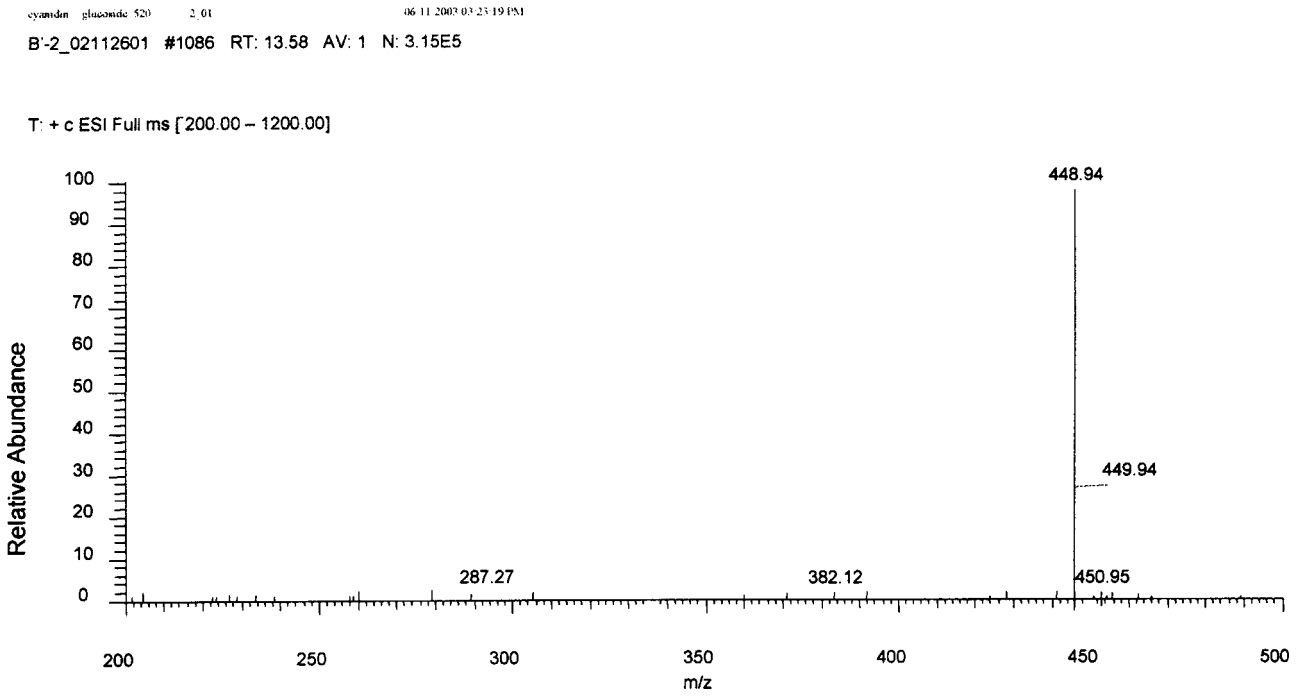


**Fig. 3.** ESI Positive MS Spectrum of Peak with RT 13.58 from LC/MS Analysis of the Extracts (eluate A)

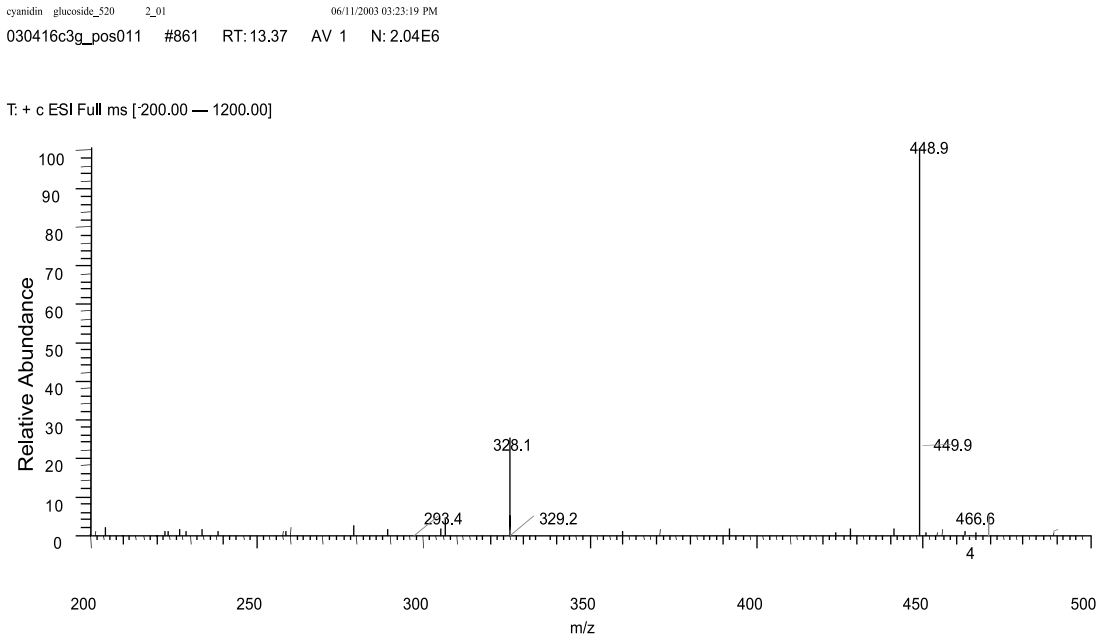


**Fig. 4.** ESI Positive MS Spectrum of Peak with RT 13.37 from LC/MS Analysis of the Purified Product from the Extracts

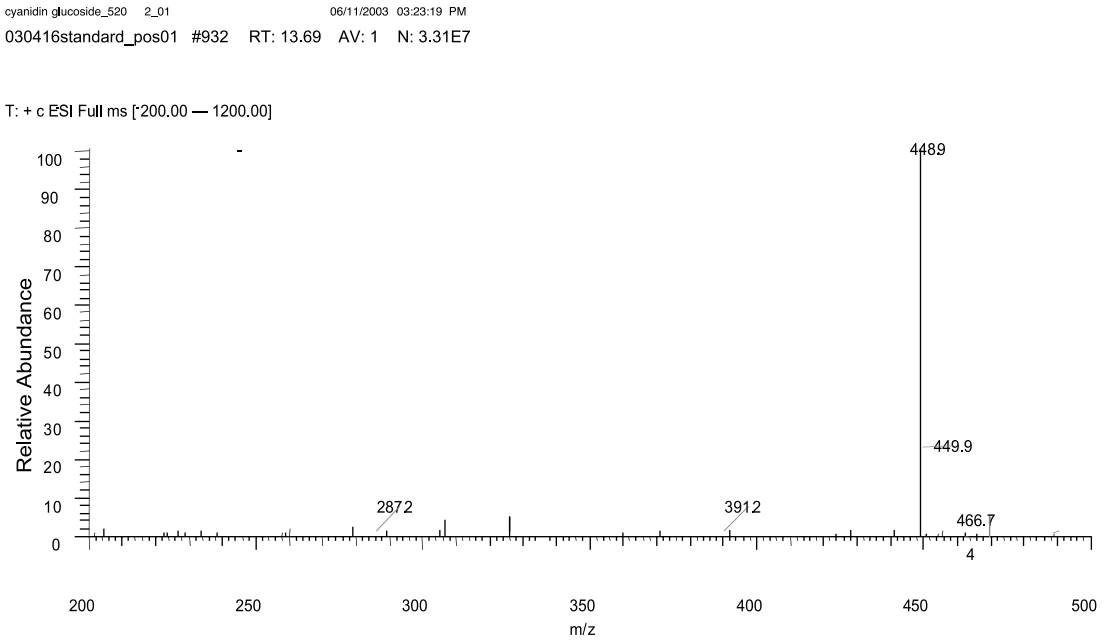


**Fig. 5.** ESI Positive MS Spectrum of Peak with RT 13.69 from LC/MS Analysis of the Authentic Sample A: Cy-3-glu Chloride

## Measurements in the Fractionated Extracts

*The Extracts Obtained from Eluate A*: Since MS analysis of a peak confirmed by UV spectrum showed an *m/z* of 449 ($M^+$), the anthocyanin in the extracts obtained from eluate A was determined to be Cy-3-glu (Figs. 3–12). In addition, Cy-3-glu was isolated from the extracts for the elucidation of its structure.

*The Extracts Obtained from Eluate B*: Similarly, MS analysis of two peaks (RT of 14.5 and 20.9) was carried out. The two peaks were confirmed by UV spectrum occurred at *m/z* values of 449 ($M^+$) and 287 ($M^+$). The analytical results for authentic sample A [MS peak of *m/z* 449 ($M^+$) at RT 14.8] and the authentic sample B [MS peak of *m/z* 287 ($M^+$) at RT 20.9] indicated that two anthocyanins, Cy-3-glu and

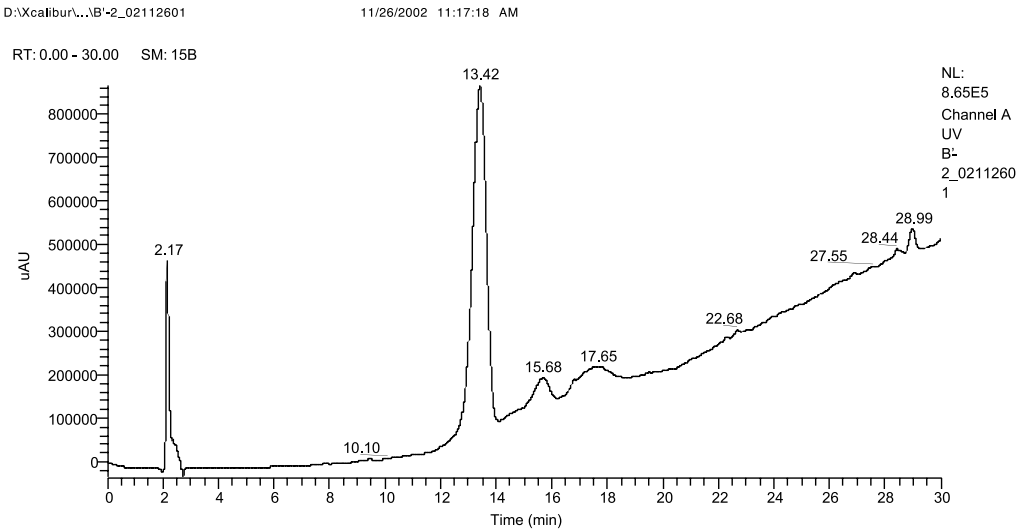


**Fig. 6.** LC (detection at 520 nm) of the Extracts (eluate A)

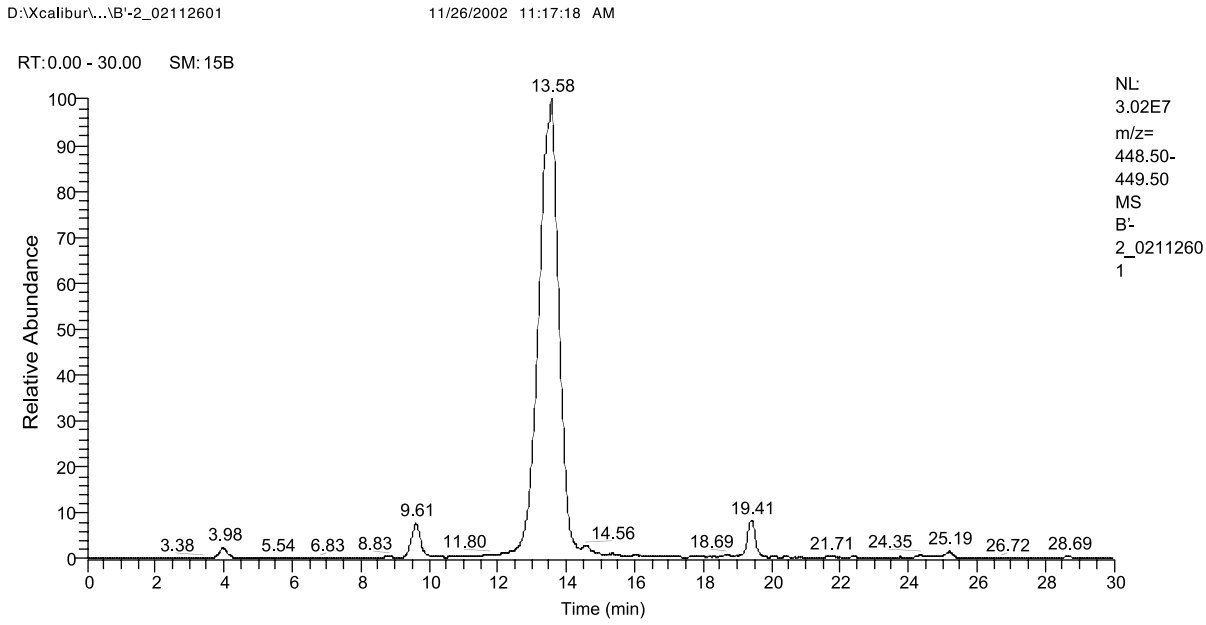


**Fig. 7.** LC (*m*/*z*: 448.5–449.5) of the Extracts (eluate A)

cyanidin, (RT values of 14.4 and 21.2 respectively) were present in the extracts (Figs. 4–16).

*Purification of the Compound in the Extracts*: LC/MS analyses of the authentic sample and the product purified from the extracts were carried out. According to the MS analysis by RT (purified product; RT = 13.4, authentic sample; RT = 13.7), both samples were recognized to have an identical peak at *m*/*z* 449 ($M^+$) and had comparable RT values. From these results, the anthocyanin in the extracts (eluate A) was identical with the authentic sample A (Figs. 4–12).

The $^1$H-NMR of the authentic sample A agreed with the literature data.[43–45] In addition, in regard to the sample purified from the extracts, this substance was identical with the cyanidin-3-O-glucoside, as evidenced by the observed anomeric proton and olefin proton of the glucose moiety. These analytical results were supported by *m*/*z* 449 ($M^+$), and 287 ($M^+$), corresponding to cyanidin.

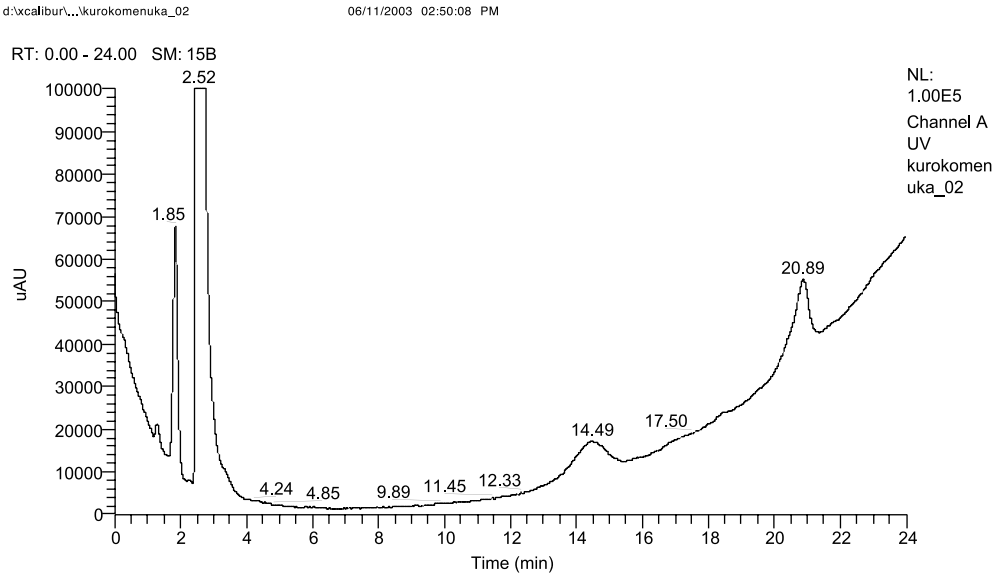


**Fig. 8.** LC (detection at 520 nm) of the Extracts (eluate B)

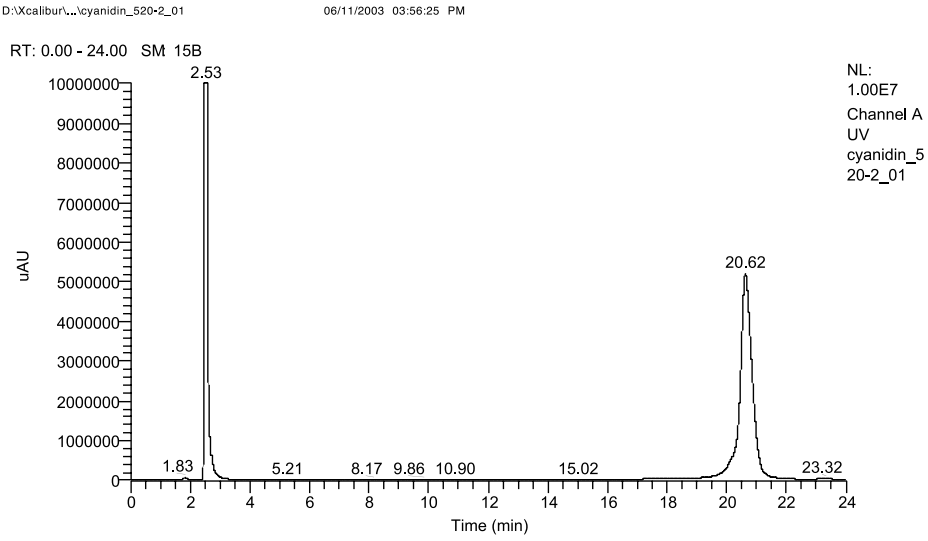


**Fig. 9.** LC Analysis of the Authentic Sample B: Cyanidin Chloride

## Antioxidative Activity of the Active Compounds

*ROS Scavenging Activity*: ROS scavenging activities of the compounds obtained (cyanidin and Cy-3-glu) and of polyphenol compounds were evaluated. ROS scavenging activities of samples obtained from the eluate B were significantly higher than that of the crude extract. It was explained that the scavenging activities of polyphenol compounds depend on the nature of the ROS present.

As shown in Table 4, the authentic samples A and B (identical to the samples obtained from the extracts) exhibited higher inhibitory activities against $\bullet O_2^-$ and *t*-BuOO• than the other anthocyanins. In particular, the extracts have been found to have higher inhibitory activities against $\bullet O_2^-$ than the other polyphenols. Regarding inhibitory activities against $^1O_2$, authentic samples C and D were high, and authentic sample B was the lowest. With respect to activity against •OH, authentic sample B showed high inhibition. It is also suggested that these activities were derived from cyanidin (Table 4), based on evidence that the scavenging activity of the eluate B against •OH was relatively higher than those of eluate A and authentic samples. Although ferulic acid,

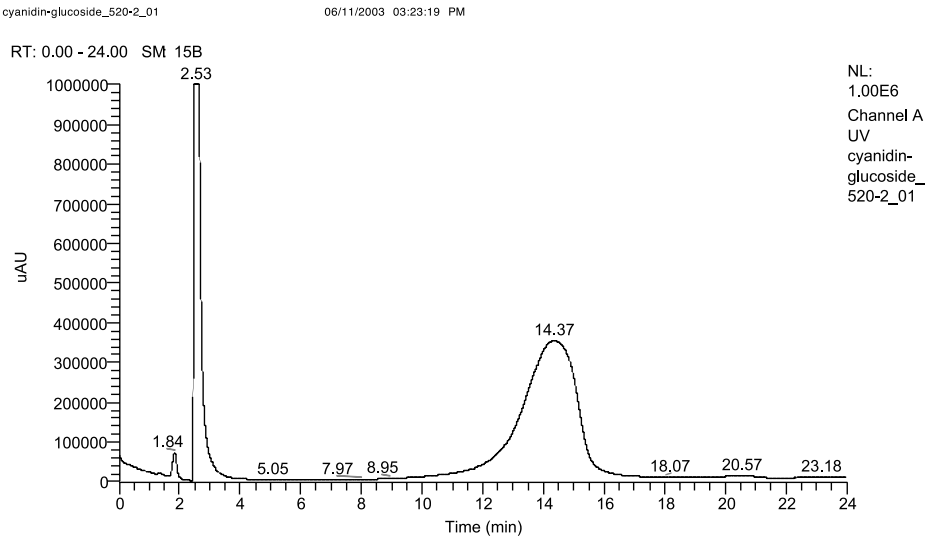


**Fig. 10.** LC Analysis of the Authentic Sample A: Cy-3-glu Chloride

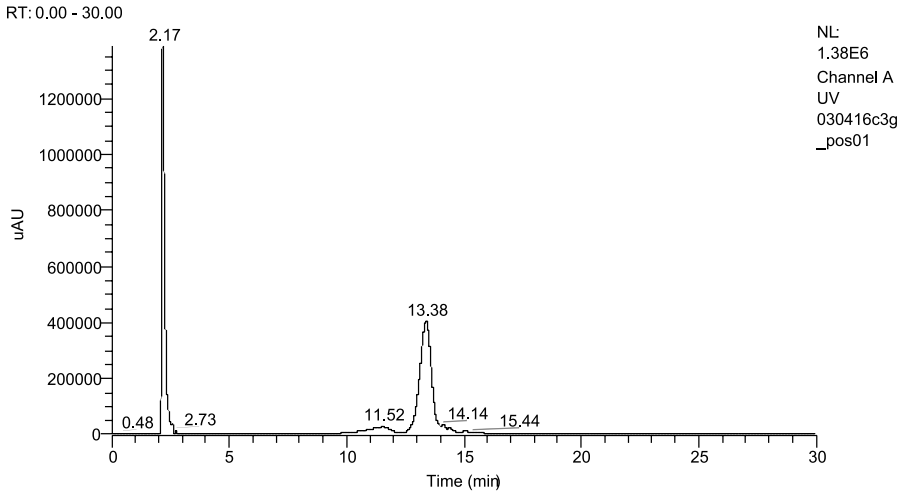


**Fig. 11.** LC Analysis of the Purified Product from the Extracts

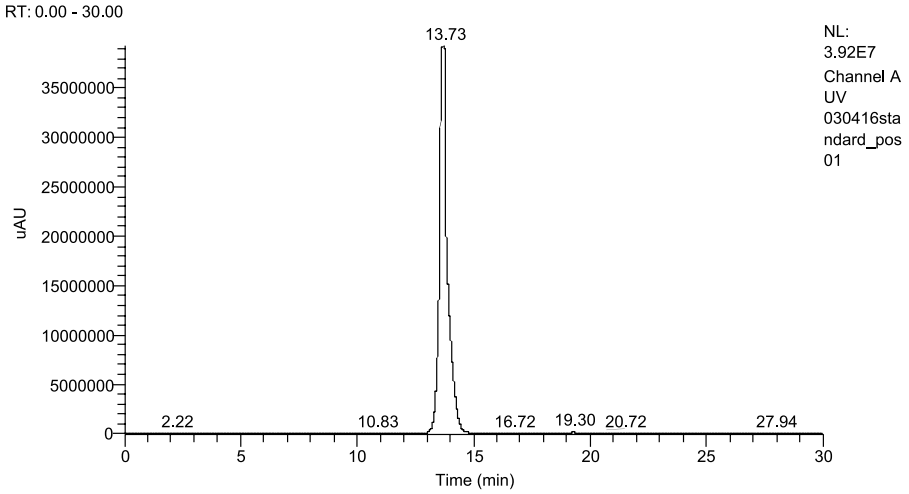


**Fig. 12.** LC Analysis of the Authentic Sample A: Cy-3-glu Chloride

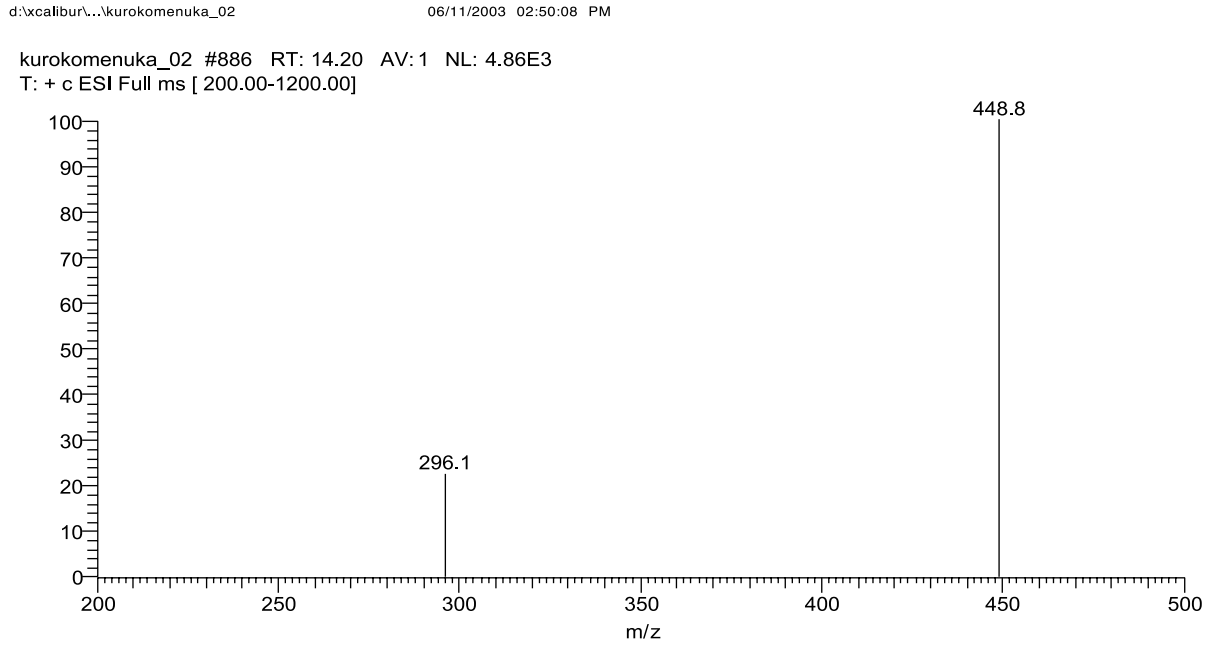


**Fig. 13.** ESI Positive MS Spectrum of Peak with RT 14.20 from LC/MS Analysis of the Extracts (eluate B)

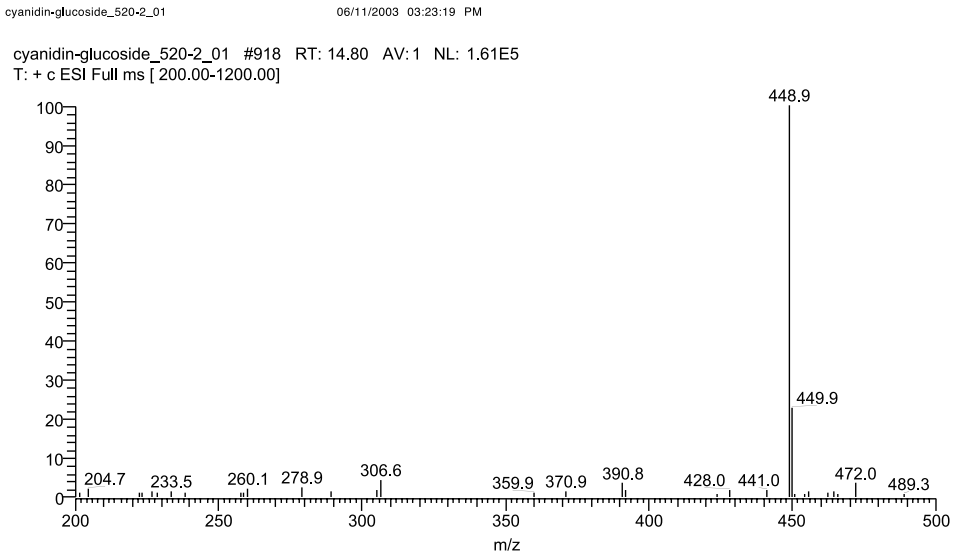


**Fig. 14.** ESI Positive MS Spectrum of Peak with RT 14.37 from LC/MS Analysis of the Authentic Sample A: Cy-3-glu Chloride

a well known compound obtained from rice bran, showed the scavenging activities against $^1O_2$ similarly to the authentic sample A (Cy-3-glu), its scavenging activities against another ROS were lower than those of the identified compounds and black rice bran extracts (Table 4).

*UVB Injury Relaxation Action in HaCaT Cells*: The suppressive effect of the compounds on UVB-mediated cell injury was evaluated. As shown in Fig. 17, all samples (extracts, authentic samples A and B) exhibited a concentration-dependent inhibitory action (Fig. 17).

## DISCUSSION

After evaluation of total polyphenol content, the UV spectrum was measured by the reversed phase TLC-scanning densitometric method. In addition, HPLC-, LC/MS- and $^1$H-NMR analyses were car-

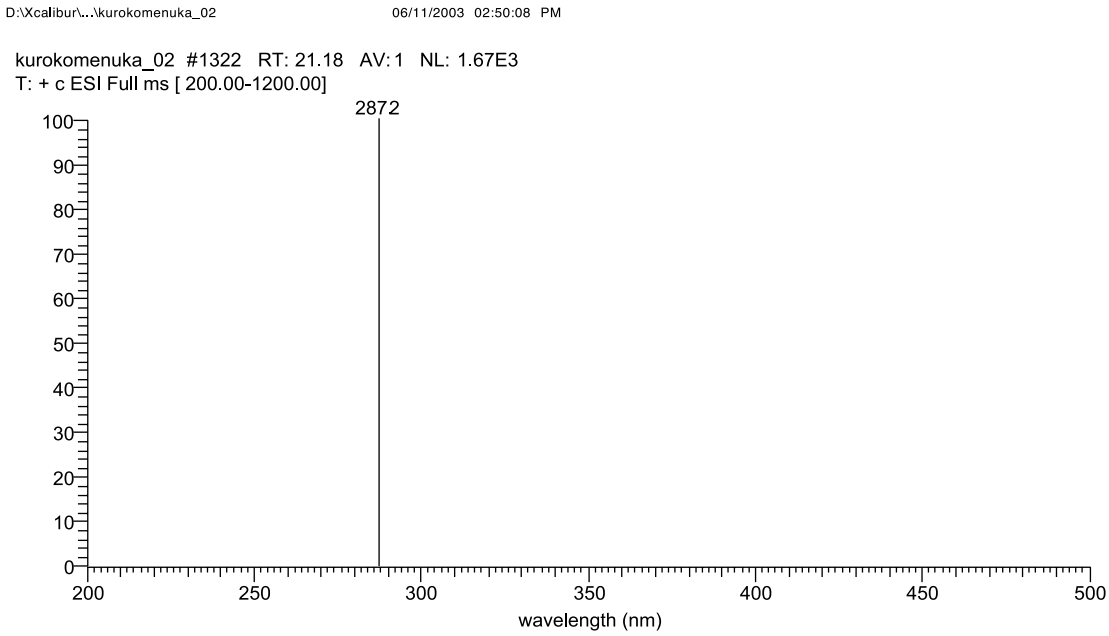


**Fig. 15.** ESI Positive MS Spectrum of Peak with RT 21.18 from LC/MS Analysis of the Extracts (eluate B)

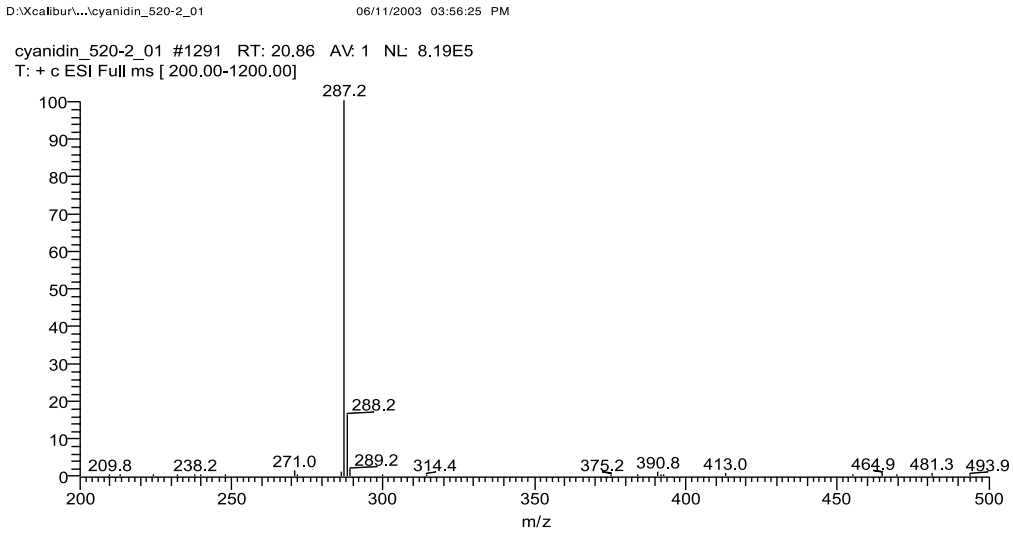


**Fig. 16.** ESI Positive MS Spectrum of Peak with RT 20.86 from LC/MS Analysis of the Authentic Sample B: Cyanidin Chloride

ried out. It has been shown that the extracts obtained from eluate A contained Cy-3-glu,[46–47] while the extracts obtained from eluate B contained Cy-3-glu and its aglycone. The results indicated that different compounds were obtained, depending on the solvent system used. This may be due to the difference in polarity of the two solvent systems. In addition, ferulic acid, an antioxidative component in rice bran, was not detected in the present extracts, suggesting that the extraction conditions in the present studies were due to the use of a high polarity solvent such as 50% EtOH. Since ferulic acid is a high lipophylic compounds, it was not detected in the black rice bran extracts under the present experimental conditions.

On the other hand, the extracts contain trace metals, such as Mn and Fe at high levels,[48] that are contained in the active centers of SOD and catalase. It is possible that complexes of these metals with anthocyanin pigments in the extracts contribute to the synergistic antioxidative properties. We have already found that the antioxidative properties of rice bran extracts from a particular kind of rice change

**Table 4.** ROS Scavenging Activities of the Extracts and Authentic Samples Evaluated by the ESR-Spin Trap Method

| Samples | | Number of OH | $IC_{50}$ (mg/ml) | | | |
|---|---|---|---|---|---|---|
| | | | $\cdot O_2^-$ | ·OH | $^1O_2$ | *t*-BuOO· |
| Black rice bran | Crude | | 0.020 ± 0.002** | 0.900 ± 0.10* | 0.093 ± 0.009**,‡ | 0.140 ± 0.10 |
| (50% EtOH) | Eluate A | | 0.016 ± 0.002*,‡ | 0.660 ± 0.01** | 0.130 ± 0.002**,‡ | 0.150 ± 0.01**,‡ |
| | Eluate B (FM) | | 0.010 ± 0.001 | 0.430 ± 0.02 | 0.035 ± 0.002 | 0.043 ± 0.009 |
| Authentic | A[a)] | 4 | 0.006 ± 0.0001** | > 3.20 | 0.021 ± 0.001** | 0.019 ± 0.004* |
| samples | (molarity) | | (12.30 ± 0.20 μM) | (> 6.60 mM) | (43.30 ± 2.00 μM) | (39.20 ± 8.20 μM) |
| | B[a)] | 5 | 0.008 ± 0.0001*,‡ | 0.25 ± 0.005** | 0.053 ± 0.002**,‡ | 0.013 ± 0.001* |
| | (molarity) | | (24.70 ± 0.30 μM) | (0.77 ± 0.02 mM) | (164.20 ± 6.10 μM) | (40.20 ± 3.00 μM) |
| | C | 4 | 0.140 ± 0.02**,‡ | > 1.23 | 0.011 ± 0.0003**,‡ | 0.019 ± 0.002 |
| | (molarity) | | (0.46 ± 0.07 mM) | (> 4.01 mM) | (35.80 ± 0.90 μM) | (61.90 ± 6.50 μM) |
| | D | 6 | 0.250 ± 0.01**,‡ | > 1.46 | 0.011 ± 0.0002**,‡ | 0.013 ± 0.0002* |
| | (molarity) | | (0.74 ± 0.03 mM) | (> 4.31 mM) | (32.40 ± 0.50 μM) | (38.30 ± 0.50 μM) |
| | E | 4 | 0.020 ± 0.001*,‡ | > 3.05 | 0.044 ± 0.003† | 0.023 ± 0.007 |
| | (molarity) | | (31.60 ± 1.50 μM) | (> 4.83 mM) | (69.70 ± 4.70 μM) | (36.40 ± 11.00 μM) |
| | F | 10 | 0.230 ± 0.050** | > 1.38 | 0.020 ± 0.0002** | 0.071 ± 0.002*,‡ |
| | (molarity) | | (0.40 ± 0.09 mM) | (> 2.39 mM) | (69.70 ± 4.70 μM) | (36.40 ± 11.00 μM) |
| | Ferulic acid | 1 | > 0.44 | > 12.60 | 0.029 ± 0.001 | > 6.52 |
| | (molarity) | | (2.27 mM) | (64.9 mM) | (149.3 ± 5.1 μM) | (33.6 mM) |

Values are expressed as the means ± S.D. ($n = 3$). Statistical significance was evaluated by the Student's *t*-test.*$p < 0.05$, **$p < 0.01$ *vs.* Black (FM), †$p < 0.05$, ‡$p < 0.01$ *vs.* Authentic sample A: Active compounds identified from the extracts. Authentic sample A: Cyanidin-3-glucoside. Anthentic sample B: Cyanidin chloride. *a*) Active ingredients identified from black rice bran extract.

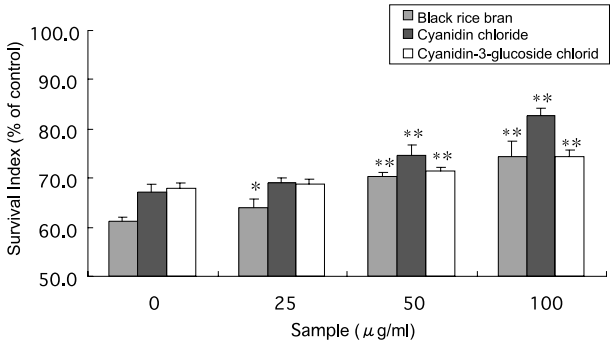


**Fig. 17.** Suppressive Action of the Compounds against UVB-Induced Injury of the HaCaT Cell

Values are expressed as the means ± S.D. ($n = 4$). Statistical significance was evaluated by the Student's *t*-test. *$p < 0.05$, **$p < 0.01$ *vs.* sample free.

in accordance with the harvest site.[9)] It is suggested that the antioxidative properties are due to not only anthocyanin polyphenols but also these trace metals contained in the rice brans. Thus, we have tried to explain the relationship between the trace metals in these molecules and their antioxidative properties. From the measurements of ROS-scavenging activities by ESR-spin trap method, the eluate B-derived effluent possessed significantly higher activities than the 50% EtOH extract (crude extract). It can be inferred that this was due to the results from differences in anthocyanin content estimated from the amounts of total polyphenols, as shown in Tables 1 and 4. The two anthocyanin compounds (cyanidin and Cy-3-glu) in the extracts showed high chemical ROS-scavenging activities. This verified that the active anthocyanin compounds showing the high antioxidative properties of the extracts were of two kinds, *i.e.* Cy-3-glu, and its aglycone. The scavenging activities against •OH radicals measured by ESR-spin trap method were also considered to be due to the cyanidin. It was explained that ROS-scavenging activities of ferulic acid, a known compound obtained from rice bran, were lower than those of two active compounds (cyanidin and Cy-3-glu). Thus, the black rice bran extracts were considered to be effective antioxidative materials when compared with ferulic acid.

The correlation between the amount of total polyphenols and ROS-scavenging activities (ESR-spin trap method) in the extracts confirmed that the greater the polyphenol content in the extracts, the higher the ROS-scavenging activities (a highly positive correlation was observed especially for $\cdot O_2^-$ and $^1O_2$). There was also a positive correlation between the amount of polyphenols and antioxidative activity as shown in Table 5.

Comparisons of ROS-scavenging activities of the polyphenol compounds obtained from the present

**Table 5.** Correlation between Polyphenol Content and ROS-Scavenging Activities in the Extracts

| | Antioxidative activities of ROS | | | |
|---|---|---|---|---|
| | $^1O_2$ | $\cdot O_2^-$ | ·OH | *t*-BuOO· |
| Polyphenol content | r = 0.831 | r = 0.895 | r = 0.469 | r = 0.469 |

r: correlation coefficient.

studies with authentic samples A, B, C, D, E and F were carried out, showing that authentic samples A and B had significantly higher activities against $\cdot O_2^-$ than others. Although both authentic samples A and E have the same cyanidin skeleton of anthocyanidin, the differences in activity appear to be due to differences in the site at which glucose binds. Activities of authentic sample A against $^1O_2$ were lower than B and D, and activities of authentic sample A against *t*-BuOO• were lower than B and D. Only authentic sample B was found to possess high activity against •OH. Thus, the activities were found to vary according to radical species. The scavenging activities seem to be due to not only the number of hydroxyl groups, but also the differences in chemical structure and steric hindrance resulting from various three-dimensional structures. This was clearly shown by the fact that the number of hydroxyl groups on the aglycone alone was not correlated with scavenging activity, as shown in Table 4, *i.e.* the number of hydroxyl groups was 5 for authentic sample B, 6 for D, 10 for F, 4 for A, 4 for E and 4 for C. In addition, HaCaT cells were used to evaluate the suppression of UVB-mediated cell damage in terms of cell survival ratio. The results showed that the extracts exhibited inhibitory action in a concentration-dependent manner. Moreover, authentic samples A and B were observed to show significantly high activity in suppressing cell damage, in a concentration-dependent manner. The extracts, in particular, were confirmed to show significantly higher ratios of cell survival than the control, even at a concentration as low as 25 $\mu$g/ml. The results indicated the possibility that not only the compounds found in these studies, but also complexes with metals and organic substances such as amino acids and the other components that contributed to an increase in antioxidative effects.

Because the extracts possess both high ROS-scavenging activities and suppress the cell damage by UVB, they are expected to show effectiveness in reducing damage occurring in the living body, including on the skin surface. These observations strongly indicate that the extract which possesses high antioxidative activities, will be useful as an anti-aging material in antioxidative cosmetics and in health food.

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