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Antioxidant Capacity of Seed Coat, Dehulled Bean, and Whole Black Soybeans in Relation to Their Distributions of Total Phenolics, Phenolic Acids, Anthocyanins, and Isoflavones

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Black soybeans have been used as an excellent dietary source for disease prevention and health promotion in China for hundreds of years. However, information about the distribution of health-promoting phenolic compositions in different physical parts of black soybean and the contribution of phenolic compositions to overall antioxidant capacity is limited. To elucidate the distribution of phenolic composition and their contribution to antioxidant activities in black soybean, the total and individual phenolic profiles, and antioxidant capacities of seed coat, dehulled and whole black soybean were systematically investigated. The seed coat exhibited much higher total phenolic indexes and antioxidant activities than whole and dehulled black soybean. Dehulled black soybean possessed similar levels of total phenolic content, total flavonoid content, 2-diphenyl-1-picryhydrazyl (DPPH) radical scavenging activity, ferric reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC) activities as compared to whole yellow soybean. Cyanidin-3-glucoside, petunidin-3-glucoside, and peonidin-3-glucoside were detected in the seed coat but not in dehulled black soybean and yellow soybean. Among benzoic acid detected, caffeic and chlorogenic acid were the predominant phenolic acids. Whole black soybean and dehulled black soybean exhibited similar isoflavone contents in 7-*O*-β-glucosides and malonylglucosides of daidzein and genistein. The seed coat possessed significantly ($p < 0.05$) lower 7-*O*-β-glucosides and malonylglucosides of daidzein and genistein, acetylglycitin, and total isoflavones than whole and dehulled black soybean. The contribution of phenolics in the seed coat to the antioxidant activity of black soybean parts depends on the assay methods. When measured with the DPPH and FRAP methods, the seed coat contributed 90% of the total antioxidant capacity of black soybean. However, when measured with the ORAC method, the seed coat and dehulled portion contributed approximately equally the total antioxidant capacity of black soybeans. The information generated from this study on the distribution and content of their active components is useful for the effective use of black soybeans as an ingredient for promoting health.

KEYWORDS: Black soybean; seed coat; dehulled bean; phenolic acids; isoflavones; anthocyanins; antioxidants; DPPH; FRAP; ORAC; HPLC

INTRODUCTION

Black soybean (*Glycine max*), a market class of soybeans with a black seed coat, has been widely utilized as a health food and herbal material in Oriental medicine for hundreds of years. Its beneficial effects first appeared in Ben-Cao-Gang-Mu (*1*), an ancient Chinese botanical encyclopedia written in the early 16th century, which describes black soybeans as capable of increasing both blood circulation and water passage, counteracting toxic effects, relieving kidney disease, and also having antiaging effects.

Recent advances in antioxidant research show that black soybeans possess a strong inhibitory effect against in vitro low-density lipoprotein oxidation (*2, 3*) and a stronger 2-diphenyl-1-picryhydrazyl (DPPH) radical scavenging activity, ferric reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC) than yellow soybeans (*4*−*6*). The antioxidant effects of black soybeans are related to their phenolic pigments in the seed coats (*2, 4*). In vitro anticancer research shows that polysaccharide components from black soybean may inhibit proliferation and induce differentiation in human leu-

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kemic U937 cells (*7*). Anthocyanins isolated from black soybean seed coats display a strong apoptosis induction effect and growth inhibitory effects against human leukemia Molt 4B cells (*8*). In vitro antifungal and antiviral research show that an antifungal protein (glysojanin) from black soybean exhibits potent antifungal activity (*9*). The hot water extracts of black soybean possess significant antiviral activity against adenovirus, coxsackievirus, and influenza virus (*10*). Animal experiments show that intake of seed coat extract of black soybean effectively enhances memory and learning ability in rats (*11*). Anthocyanin extracts from black soybean have antiobesity effects, which can reverse the effect of a high-fat diet on body weight, adipose tissue weight, and serum lipid contents (*12*). The latest clinical case control study showed that a high consumption of cooked yellow and black soybeans had an association with reduced breast cancer in Korean women (*13*). In addition, cooked and fermented black soybean products exhibit potent physiological activities, such as radical scavenging activity (*14*) and apoptosis induction activity (*15*). These researches indicate that black soybean may serve as an excellent dietary source for disease prevention and health promotion.

Cotyledons, seed coats, and embryonic axes (germ) represent, on average, 89, 10, and 1%, respectively, of the total seed weight (*16*). The cotyledon contains the main reserve substances, basically proteins, fats, and carbohydrates. The seed coat, which acts as a protective barrier for the cotyledon, has the highest concentration of phenolic compounds (*16*). Several major anthocyanins (cyanidin 3-*O*-β-D-glucoside, delphinidin-3-*O*-β-D-glucoside, and petunidin 3-*O*-β-D-glucoside) have been isolated and identified from the seed coat of black soybeans (*8, 17, 18*). The black soybeans with black seed coats and green cotyledons are widely utilized as a health food and herbal material in China mainland, Taiwan island, Korea peninsula, and Japan. However, the major phenolic compositions, including isoflavones, phenolic acids, anthocyanins, and their distributions in the seed coat and dehulled bean of the green cotyledon soybean cultivar, as related to their contributions to antioxidant activity, have not been reported. Therefore, the aims of this study were to elucidate the types of phenolic compounds, to quantify their contents in the seed coat and dehulled bean, and further to investigate their contribution to overall antioxidant activity of a green cotyledon type black soybean. A yellow lipoxygease-free soybean was used for comparison.

MATERIALS AND METHODS

Chemicals. All authentic phenolic acids, (+)-catechin, high-performance liquid chromatography (HPLC)-grade trifluoroacetic acid (TFA), DPPH$^{\bullet}$, fluorescein disodium, Folin−Ciocalteu reagent, 2,4,6-tri-(2-pyridyl)-*s*-triazine, and 6-hydroxy-2,5,7,8-tetramethlchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Nine isoflavone standards were purchased from LC Laboratories (Woburn, MA). A mixture of six unimolar anthocyanin standards was purchased from Polyphenols Laboratories (Sandnes, Norway). 2,2′-Azobis (2-amidino-propane) dihydrochloride was purchased from Wako Chemicals USA (Richmond, VA).

Soybean Materials. Dry matured black soybeans (with black seed coats and green cotyledons) harvested in 2006 were obtained from Sinner Brothers & Bresnahan (Casselton, ND). The IA2032, a yellow lipoxygenase-null soybean, was obtained from Stonebridge Ltd. (Cedar Falls, IA). Broken seeds, damaged seeds, and foreign materials were removed from the samples. The moisture content was determined by drying the sample in an air oven at 110 °C until a constant weight was obtained (*19*). Phenolic contents and antioxidant activities were expressed on a dry weight basis.

Separation, Weight Percentage Calculation, and Grinding of Seed Coats and Dehulled Beans. For the purpose of calculation of mass distribution, a small portion (20 g in duplicate) of whole black soybean was separated into seed coats and dehullled beans by mortar and hands. The separated parts were weighed, and the percentages of dehulled beans and seed coats were calculated. For the purpose of chemical and antioxidant assays, a large portion (around 1 kg) of whole black soybean was coarsely ground by a Strawb Grinding Mill (model 4E, Strawb Corporation, Philadelphia, PA) and then divided into the seed coats and dehulled beans by sieve and hands. The separated seed coats and dehulled beans, as well as a portion of whole soybean, were finely ground to powder with a U/D Cyclone Sample Mill (model 3010-030, UDY Corp., Fort Collins, CO) and passed through a 60 mesh sieve. The ground samples were stored at −20 °C until analysis.

Extraction of Total Phenolics from Seed Coats, Dehulled and Whole Soybeans. The extraction procedures in our recent report were followed (*5*) to provide extracts for total phenolic indexes and antioxidant evaluation. Briefly, the sample flours (0.5 g in triplicate) were extracted twice each with 5 mL of acetone/water/acetic acid (70:29.5:0.5, v/v/v). The combined extract was stored at 4 °C in the dark for use. The reason for selecting this specific solvent system was based on our recent study (*5*) that this solvent system gave the best yields of phenolic contents and antioxidant activities. All extractions for total phenolics and further analyses of total phenolic substances and antioxidant activities were performed in triplicate.

Determination of Total Phenolic Content (TPC). The TPC was determined by a Folin−Ciocalteu assay (*20*) with slight modifications (*5*) using gallic acid (GA) as the standard. The absorbance was measured at 765 nm against reaction reagents as a blank. The TPC was expressed as GA equivalents (mg GAE/g sample) through the calibration curve of GA. The linearity range of the calibration curve was 50−1000 μg/mL ($r = 0.99$).

Determination of Total Flavonoid Content (TFC). The TFC was determined using a colorimetric method described previously (*21*). The absorbance was measured at 510 nm using an UV−visible spectrophotometer (UV 160, Shimadzu, Japan). The results were expressed as micrograms of (+)-catechin equivalents (mg CAE/g sample) using the calibration curve of (+)-catechin. The linearity range of the calibration curve was 10−1000 μg/mL ($r = 0.99$).

Determination of Condensed Tannin Content (CTC). The analysis of CTC was carried out according to the method of Broadhurst and Jones (*22*) and was slightly modified in our laboratory (*5*). The absorption was measured at 500 nm against methanol as a blank. The amount of condensed tannin was expressed as mg of (+)-catechin equivalents (mg CAE/g sample) using the calibration curve of (+)-catechin. The linearity range of the calibration curve was 50−1000 μg/mL ($r = 0.99$).

Determination of Monomeric Anthocyanin Content (MAC). The MAC was determined using a pH differential method (*23*) without modification. A Shimadzu UV 160 double beam spectrophotometer was used for measuring the absorbance at 700 and 520 nm. The MAC content was expressed as cyanidin-3-glucoside equivalents (CyE) in mg/g, using an extinction coefficient of 26900 L cm^{-1} mol^{-1} and a molecular weight of 449.2 g mol^{-1}.

HPLC Analysis of Phenolic Acid Content. *Extraction of Free Phenolic Acids.* The extraction of free phenolic acids was performed according to a previous report (*24*) with slight modifications. Briefly, the flours of whole beans, dehulled beans, and seed coats (0.5 g in duplicate) were extracted twice each with 5 mL of methanol/water/acidic acid/butylated hydroxytoluene (85:15:0.5: 0.2, v/v/v/w) by shaking samples at 300 rpm at room temperature on an orbital shaker for 4 h. The two extracts were combined and filtered through Whatman #1 paper and concentrated at 45 °C under vacuum to remove solvents. The extract was dissolved in 2.5 mL of 25% methanol. The methanol solution was filtered through a 0.2 μm PVDF syringe filter (National Scientific Co., Duluth, GA) and analyzed for free phenolic acid contents by HPLC.

HPLC Analysis of Phenolic Acids. The quantitative analysis of free phenolic acids was performed by HPLC according to Robbins and Bean (*25*) with slight modifications as follows: (i) The column temperature was increased from 25 to 40 °C to get better reproducibility and resolution, (ii) a Waters Associates (Milford, MA) chromatography system equipped with a model 720 system controller, model 6000A

solvent delivery system, model 7125 loading sample injector, and model 418 LC UV detector set at 270 nm was used; and (iii) instead of C_{18} Luna column, a Zorbax Stablebond Analytical SB-C_{18} column (4.6 mm × 250 mm, 5 μm, Agilent Technologies, Rising Sun, MD) was used. Elution was performed according to previous description (*24*) without modification.

Identification and Quantification of Phenolic Acids. A stock solution (1 mg/mL) of all individual compounds was prepared and then diluted. The diluted working solutions (100 μg/mL) were subjected to HPLC. A spiking method and external standard method were used to identify HPLC peaks by comparing peak area increases and retention times. In addition, to confirm the identities of compound peaks through their UV spectrum information, individual phenolic acids and phenolic acid mixtures as well as several typical samples were selected to perform analysis on another HPLC (HP 1090, Hewlett-Packard, Waldbronn, Germany), which was equipped with a UV-PDA detector. All identified phenolic acids were quantified with external standards by using HPLC analysis as previously described (*25*).

HPLC Analysis of Anthocyanin Content. *HPLC Analysis of Anthocyanins.* The free phenolic acid extracts were also used for anthocyanin analysis. The analysis was performed on an HP 1090 series HPLC (Hewlett-Packard, Waldbronn, Germany), using a YMC Pack ODS-AM column (4.6 mm × 250 mm, S-50 μm, 120A). HPLC conditions (*26*) were as follows: solvent A, 0.1% TFA/H_2O; solvent B, CH_3CN/H_2O/TFA (50:50:0.1, v/v/v); linear gradient, initial percentage of B (15%) to 60 min (40%); column temperature, 40 °C; and flow rate, 0.5 mL/min. The filter detector was set at 540 nm.

Identification and Quantification of Anthocyanin. The identifications and peak assignments of anthocyanins were primarily based on comparison of their retention times with those of standards, a blueberry reference sample, and literatures (*8, 17, 18, 27*). The stock solution of anthocyanins mixture was prepared by dissolving standards mixture (unimolar mixture of 3-*O*-β-glucosides of delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin) in methanol to give a concentration of 1.0 mg/mL. A portion of the stock solution was then diluted using methanol to the following series of dilutions: 1 in 5, 10, 20, 40, 80, and 160. Standard curves of anthocyanins were plotted peak area against concentrations by duplicate injection of the six series diluted working solutions of standard mixture.

HPLC Analysis of Isoflavone Content. *Extraction of Isoflavones.* Isoflavones were extracted by modified previous methods (*28, 29*). Briefly, the black soybean flours (1.0 g ± 0.01 in duplicate) were accurately weighed into a set of 15 mL screw-top VWR centrifuge tubes. Five milliliters of acetonitrile, 4.5 mL of distilled water, 0.25 mL of internal standard THB (0.1 mg/mL) (*28*), and 0.25 mL of internal standard 6-hydroxyflavone (0.1 mg/mL) were added to each tube. The 6-hydroxyflavone was used as an internal standard for the first time in our laboratory. The previous extraction procedures (*29*) were followed. The residues in the flask were dissolved in 5 mL of 80% methanol and kept in a freezer (−20 °C) for less than 12 h before analysis.

HPLC Analysis of Isoflavones. The quantitative analysis of isoflavones was performed by HPLC according to ref *29* with a slight modification by using two internal standards. The same Waters Associates chromatography system (Milford, MA) as used for phenolic acid analysis was used for quantitative analysis of isoflavones, and a UV detector at 262 nm was used. The same column and HPLC conditions as previously described were used (*29*).

Identification and Quantification of Isoflavones. Three aglycones, three 7-*O*-β-glucosides, two 6″-*O*-acetylglucosides (acetyldaidzin, acetylgenistin), and one 6″-*O*-malonylglucoside (malonylgenistin) of isoflavones were commercially available and used directly to identify the sample peaks by comparing their retention times and HPLC profiles to standard mixture. The peak identifications for noncommercially available isoflavones were confirmed by comparing the chromatograms of several identical samples performed by a well-established isoflavone analytical laboratory (Dr. Pat Murphy, Iowa State University, Ames, Iowa).

The quantification of isoflavones was performed by calibrating the peak area obtained from HPLC analyses. The contents of three aglycones, three 7-*O*-β-glucosides, two 6″-*O*-acetylglucosides (acetyldaidzin and acetylgenistin), and one 6″-*O*-malonylglucoside (malonylgenistin) in the samples were directly quantified through their external−internal calibration curves. Calibration curves were obtained for each of nine external standards by plotting response factors (RFs) of each standard against the concentration. The RFs are the ratios of peak area of external to internal standards. For the other isoflavones without commercial standards, concentrations were calculated from the standard curves that were adjusted appropriately from the standard curves of respective form of isoflavones based on the differences in molecular weights and molar extinction coefficients of the compounds (*28, 29*). Isoflavone concentrations were expressed as μg isoflavone per g of dry sample.

Radical DPPH Scavenging Activity. The DPPH free radical scavenging capacity of total phenolic extracts was evaluated according to the method of Chen and Ho (*30*) with slight modifications (*5*). The absorbance of the sample (A_{sample}) was measured using a spectrophotometer (UV 160, Shimadzu, Japan) at 517 nm against an ethanol blank. A negative control ($A_{control}$) was taken after adding DPPH solution to 0.2 mL of the respective extraction solvent. The percent of DPPH discoloration of the sample was calculated according to the following equation: % discoloration $= [1 - (A_{sample}/A_{control})] \times 100$. The free radical scavenging activity of extracts was expressed as micromoles of Trolox equivalent per gram of sample (μmole TE/g) using the calibration curve of Trolox. The linearity range of the calibration curve was 20−1000 μM ($r = 0.99$).

FRAP Assay. The FRAP was performed as previously described by Benzie and Strain (*31*). The FRAP value was expressed as millimoles of Fe^{2+} equivalent (FE) per 100 g of sample using the calibration curve of Fe^{2+}. The linearity range of the calibration curve was 0.1−1.0 mM ($r = 0.99$).

ORAC Assay. The ORAC was performed according to Prior et al. (*32*) with slight modifications (*5*). A BMG Fluostar Optima Microplate Reader (BMG Labtech GmbH, Offenburg, Germany), which was equipped with an incubator and wavelength-adjustable fluorescence filters, was used to monitor for the reaction. The ORAC value was expressed as micromoles of Trolox equivalent per gram of sample (μmole TE/g).

Statistical Analysis. The data were expressed as means ± standard deviations. Statistical analysis was performed using 2005 SAS (Version 9.1, SAS institute Inc., Cary, NC). Duncan's multiple range test was used to determine the differences between group means. Significant levels were defined as probabilities of 0.05 or less. A Pearson correlation test was conducted to determine the correlation between variables.

RESULTS AND DISCUSSION

Total Phenolic Compositions of Black Soybeans. The TPCs, TFCs, CTCs, and MACs of seed coats, dedulled beans, and whole bean samples from black soybean, as well as whole yellow soybean (used for comparison purposes), are presented in **Figure 1**. In terms of separated parts, significant differences ($p < 0.05$) in TPC, TFC, CTC, and MAC values were found among different parts of black soybean. The seed coats possessed significantly ($p < 0.05$) much higher TPC, TFC, CTC, and MAC values (in separated parts) than whole beans and dehulled beans, while whole beans possessed significantly ($p < 0.05$) higher TPC, TFC, CTC, and MAC values than dehulled beans. In addition, dehulled beans exhibited similar TPC and TFC values to that of whole yellow soybeans. CTC and MAC were not detectable in whole yellow soybeans. The MAC was not detectable in dehulled black soybeans, indicating that anthocyanins resided completely in the seed coats.

The phenolic content was also calculated based on their mass distribution. The weight percentage calculation showed that the seed coat occupied 10.3 ± 0.3% of total weight, and dehulled bean occupied 89.7 ± 0.3% of total weight. Therefore, phenolic percentage contents of seed coats and dehulled beans in whole beans were calculated based on a weight ratio (90:10, dehulled bean to seed coat in the whole bean). Whole beans possessed significantly ($p < 0.05$) higher TPC, TFC, and CTC values than

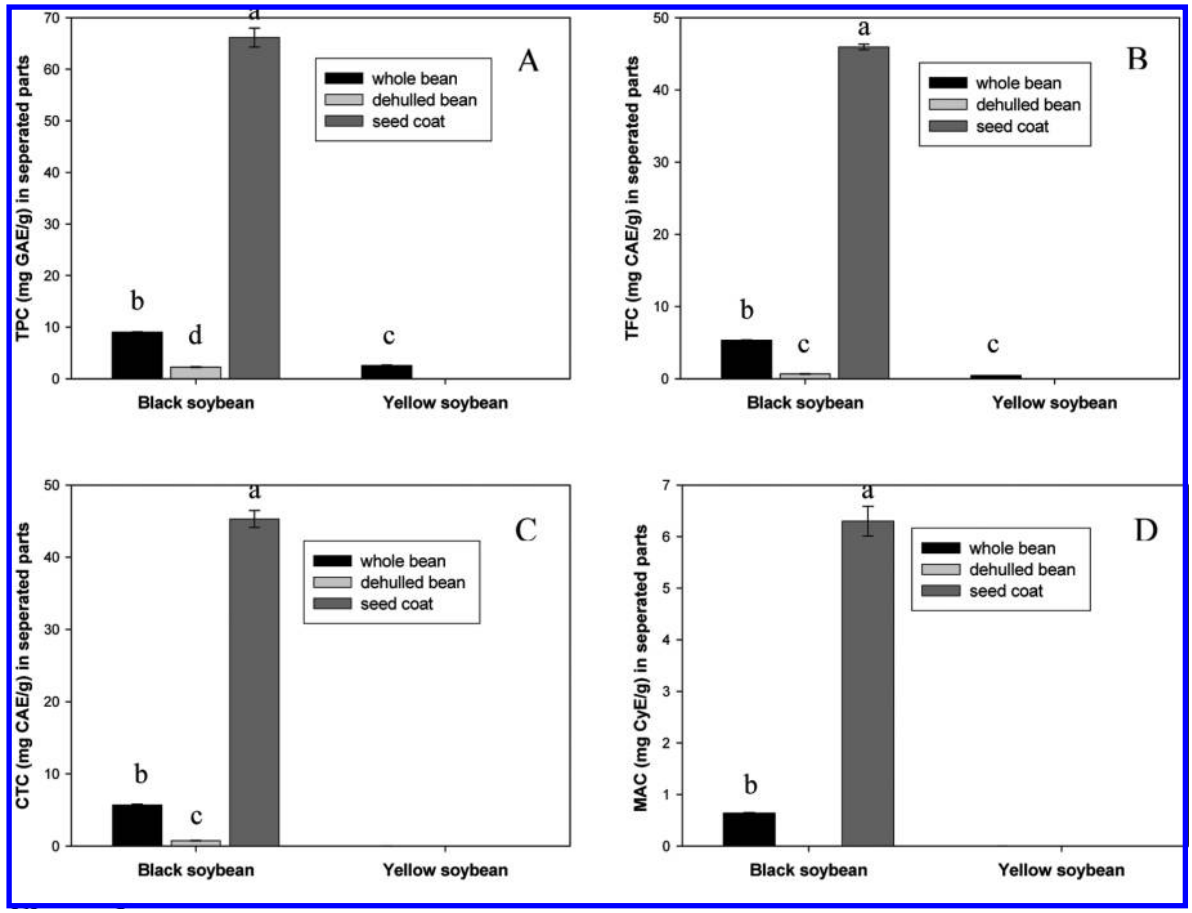

Figure 1. Phenolic contents in different physical parts of black soybean. (**A**) TPC, (**B**) TFC, (**C**) CTC, and (**D**) MAC. Data are expressed as means ± standard deviations ($n = 3$) on a dry weight basis. Values marked above the bar with different letters are significantly different ($p < 0.05$).

seed coats and dehulled beans (contents in whole beans), while seed coats possessed significantly ($p < 0.05$) higher TPC, TFC, and CTC values than dehulled beans.

The TPC value (9.0 GAE/g) of the whole black soybean was within the range of that in the four black soybeans grown in Taiwan, which was approximately 5−9 mg GAE/g (*33*) but higher than that (6.2 GAE/g) of our previous report (*5*). The differences between current results and previous reports may be attributed to the differences in the sources of the samples (harvested in different years; the current sample was subjected to analyses without long storage times). It is difficult to compare our data about TPCs in seed coats of black soybeans to that reported by Takahashi et al. (*2*), since they were expressed in a different units using (+)-catechin as equivalents. However, the reports exhibited the same trend. Namely, black seed coats possessed significantly higher TPCs than whole black soybeans, dehulled beans, and yellow soybeans.

Antioxidant Capacities of Black Soybeans. In terms of individual separated parts, significant differences ($p < 0.05$) in DPPH, FRAP, and ORAC values were found among different parts of black soybeans (**Figure 2A**,**C**,**E**). The seed coats possessed significantly ($p < 0.05$) much higher DPPH, FRAP, and ORAC values (in separated parts) than whole beans and dehulled beans, while whole beans possessed significantly ($p < 0.05$) higher DPPH, FRAP, and ORAC values than the dehulled beans. These findings confirmed a previous report (*14*) regarding the radical scavenging activity of separated parts from soybean, in which the DPPH radical scavenging activity was ranked in the order seed coat > whole bean > dehulled bean. Dehulled beans exhibited similar levels of antioxidant activities to whole yellow soybeans in DPPH, FRAP, and ORAC values.

On the basis of the mass ratios of the antioxidant activities in the fractions from 1 g of whole beans, whole beans possessed significantly ($p < 0.05$) higher DPPH, FRAP, and ORAC values than seed coats and dehulled beans (distribution in whole bean), while seed coats possessed significantly ($p < 0.05$) higher DPPH and FRAP values than dehulled beans. There were no significant differences in ORAC values between dehulled beans and seed coats. However, the ORAC value of the yellow IA2032 soybean was higher than that of the dehulled black soybean.

The antioxidant capacities in DPPH (17.9 μmol TE/g), FRAP (13.1 mmol Fe^{2+} equivalents/100 g), and ORAC (120.4 μmol TE/g) of whole black soybeans were similar to those of our earlier communication (*6*), which were 18.4, 9.4, and 131.3, respectively. It is difficult to compare our DPPH radical scavenging activities in the seed coats of black soybeans to the DPPH values of Takahashi et al. (*2*) and Kim et al. (*14*), since radical scavenging activities were expressed differently in these reports. However, both current and previous reports exhibited the same trend regarding DPPH radical scavenging activities, namely, black seed coats possessed significantly higher activities than whole black soybeans, dehulled beans, and yellow soybeans. The ORAC and FRAP values of black soybeans had not been reported by these authors.

Seed coats and dehulled beans exhibited similar degrees of contribution in ORAC to the overall antioxidant activities of whole black soybeans. These are different from their contributions to overall antioxidant of whole black soybeans in DPPH and FRAP values. The differences may be attributed to the differences of antioxidant mechanisms of the former one from the latter two assay methods: ORAC reaction involves a hydrogen atom transfer mechanism, whereas DPPH

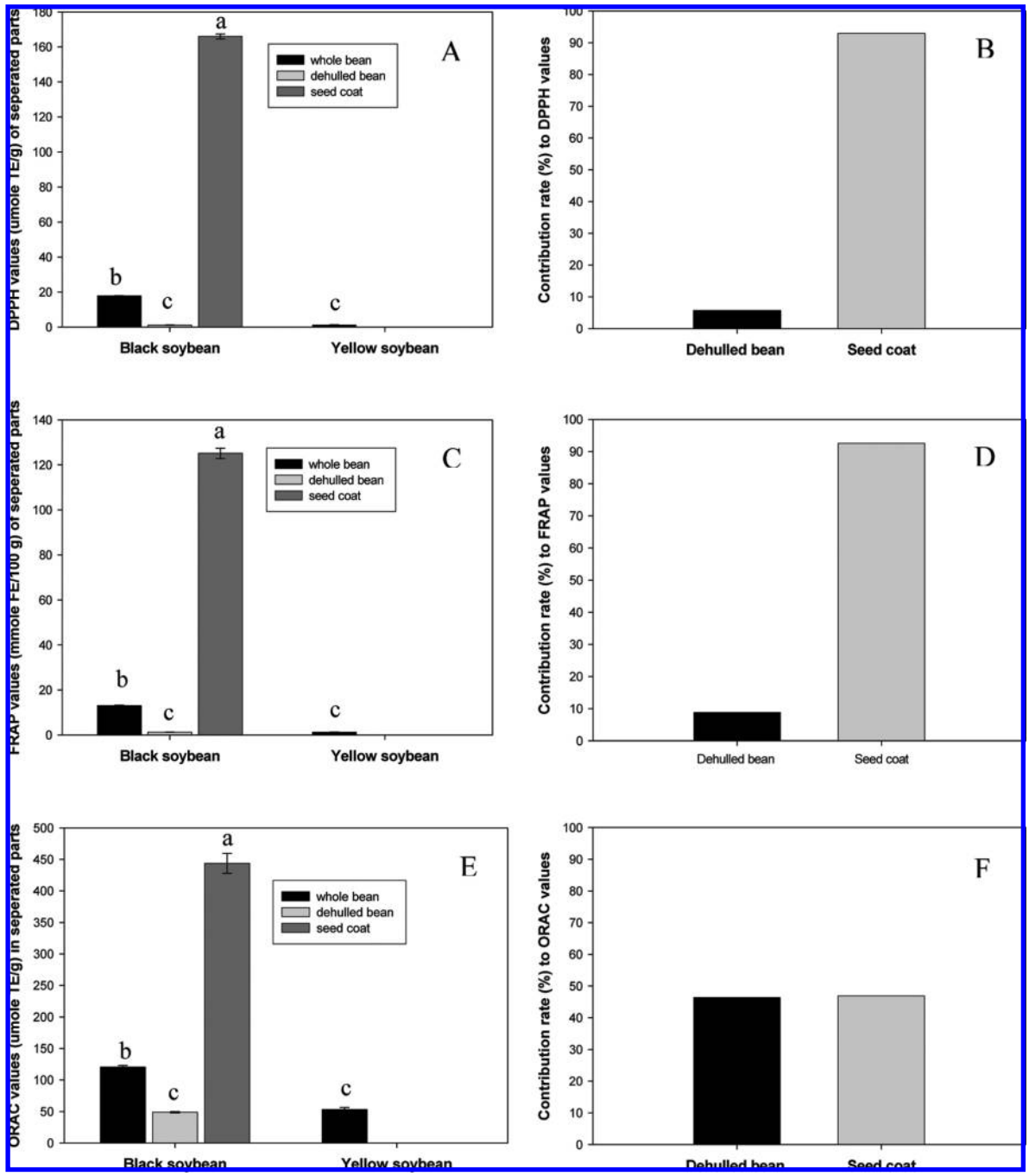

Figure 2. Antioxidant capacities of different physical parts from black soybean and contributions of different parts to overall antioxidant capacities of whole black soybean. (**A**, **B**) DPPH free radical scavenging activity, (**C**, **D**) FRAP, and (**E**, **F**) ORAC. Data are expressed as means ± standard deviations ($n = 3$) on a dry weight basis. Values marked above the bar with different letters are significantly different ($p < 0.05$).

and FRAP involve the same mechanism of single electron transfer (*32*). Functional groups in predominant phenolic compounds (such as isoflavones) in dehulled beans may be more active in transferring hydrogen atoms but may not be active in transferring single electrons. In other words, isoflavone components can contribute more ORAC values than DPPH and FRAP values of soybeans. Although there is no literature to explain the antioxidative mechanisms of isoflavones in soy foods, the findings from our previous report (*6*) at least supported these hypotheses, in which yellow soybeans possessed similar DPPH and FRAP values to yellow peas, but yellow soybeans exhibited significantly higher ORAC values than yellow peas, due to the particular components—such as isoflavones in soybeans (but not in yellow peas) contributed to the additional ORAC values.

Results from the antioxidant assay exhibited a consistent trend with those of total phenolic assays. The correlation analyses between total phenolics and antioxidant activities of all samples were performed. There were significant ($r = 0.99$, $p < 0.0001$) linear correlations between variables of total phenolics, between variables of antioxidant activities, and between total phenolics and antioxidant activities. These correlation results indicated that all antioxidant assay methods were well-correlated; meanwhile, they indicated that total phenolics were well-correlated with overall antioxidant activities of black soybean.

To better understand the distribution of phenolic compounds in different physical parts of black soybean and their contribution to the overall antioxidant, further research as below was performed to analyze individual phenolic compounds in different separated parts of black soybeans using HPLC.

Table 1. Distribution of Anthocyanins Content (μg/g) in Black Soybean[a]

	cyanidin-3-glucose	petunidin-3-glucose	peonidin-3-glucose
yellow soybean			
whole bean	ND[c]	ND	ND
black soybean			
whole bean	86.34 ± 0.11	ND	ND
dehulled bean	ND	ND	ND
seed coat (in separated parts)	851.6 ± 12.89	9.36 ± 0.05	89.85 ± 0.69
seed coat (in whole bean)[b]	85.20 ± 1.29	0.94 ± 0.01	8.99 ± 0.07

[a] Data are expressed as means ± standard deviations ($n = 2$) on a dry weight basis. [b] Calculation is based on weight ratio (90:10) of dehulled bean and seed coat. [c] ND, not detectable.

Anthocyanin Compositions in Black Soybeans. The anthocyanin contents of the whole and separated parts of black soybeans are presented in **Table 1**. Only one anthocyanin (cyanidin-3-glucoside) was detectable in whole black soybeans, while three anthocyanins (cyanidin-3-glucoside, petunidin-3-glucoside, and peonidin-3-glucoside) were detected in seed coats of black soybeans; the dominant components were cyanidin-3-glucoside and peonidin-3-glucoside. However, anthocyanins were not detected in whole yellow soybeans and dehulled black soybeans. These findings are in accordance with the literatures (*8, 14, 17, 18*), in which cyanidin-3-glucoside was the major anthocyanin in black soybeans.

From a quantitative point of view, the cyanidin-3-glucoside content (851.5 μg/g) in the seed coats of black soybeans in our current study was lower than that (1080 μg/g) of the seed coats of black soybeans grown in Japan (*1*) and that (940−15980 μg/g) of the seed coats of 10 black soybeans grown in Korea (*18*). The differences between current results and previous reports may be attributed to the differences in the sources of the soybean materials, which are affected by cultivars and the environment of cultivation. Comparing the anthocyanin contents of seed coats to that in the whole beans, the content (85.2 μg/g) of cyanidin-3-glucoside of the seed coats was close to the content (86.3 μg/g) of cyanidin-3-glucoside of the whole beans, while cyanidin-3-glucoside was not detectable in the dehulled beans. These results indicated that anthocyanins existed in the seed coats, not in the dehulled parts of black soybeans.

Phenolic Acid Compositions in Black Soybeans. The free phenolic acid contents of the seed coats, dedulled beans, and whole bean samples from black soybeans are presented in **Table 2**. Four benzoic type phenolic acids (gallic, *p*-hydroxybenzoic, syringic acid, and vanillin) and nine cinnamic type phenolic acids (caffeic, chlorogenic, *p*-coumaric, *m*-coumaric, ferullic, sinapic, *o*-coumaric, *trans*-cinnamic acid, and syringaldehyde) were detected in the dehulled beans. Among these compounds, GA, caffeic acid, chlorogenic acid, sinapic acid, and *trans*-cinnamic acid were the predominant phenolic acids in the dehulled bean. Three benzoic type phenolic acids (protocatechuic, 2,3,4-trihydroxybenzoic, and vanillic acid) and six cinnamic type phenolic acids (caffeic, chlorogenic, *m*-coumaric, ferullic, *o*-coumaric, and *trans*-cinnamic acid) were detected in the seed coats of black soybeans. Caffeic acid, chlorogenic acid, and *trans*-cinnamic acid were predominant phenolic acids in the seed coat. Our results were consistent with the previous report that dehulled parts (embryo + cotyledon) of black soybeans contained gallic, vanillic, caffeic, chlorogenic, *p*-coumaric, ferullic, *o*-coumaric, and *trans*-cinnamic acid and seed coats of black soybeans contained vanillic, caffeic, chlorogenic, *m*-coumaric, ferullic, *o*-coumaric, and *trans*-cinnamic acid (*14*).

In terms of the separated parts, significant differences ($p < 0.05$) in individual phenolic acids were found among the different parts of black soybeans. The seed coats possessed significantly ($p < 0.05$) higher contents of protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, *m*-coumaric acid, and *o*-coumaric acid (in separated parts) than whole beans and dehulled beans, while dehulled beans possessed significantly ($p < 0.05$) higher contents of GA, *p*-hydroxybenzoic acid, vanillin, chlorogenic acid, *p*-coumaric acid, sinapic acid, *trans*-cinnamic acid, and total phenolic acids than seed coats. However, several minor phenolic acids were detected in dehulled beans, such as *p*-hydroxybenzoic acid, vanillin, and sinapic acid, and seed coats, such as ptotocatechuic acid and 2,3,4-trihydroxybenzoic acid, in our current investigation but were not found in a previous report (*14*). Whole black soybeans contained comparable but a little bit lower contents in terms of major phenolic acids, such as caffeic, chlorogenic, and *trans*-cinnamic acids, and total phenolic acids as compared to the whole yellow soybeans (**Table 2**).

In terms of the mass distributions of phenolic contents in the seed coats and dehulled beans from the whole beans, the whole beans possessed significantly ($p < 0.05$) higher contents of individual phenolic acids, subtotal benzoics, subtotal cinnamics, and total phenolic acids than those of seed coats and dehulled beans (contents in whole beans). Literature values on phenolic acid compositions in black soybeans are very limited. Only one work was done on black soybeans grown in Korea (*14*). Five varieties of black soybeans were involved in this investigation. However, from a quantitative point of view, the contents of detected phenolic acid in dehulled beans and seed coats from our current investigation were much higher than the respective phenolic acids in that report (*14*). The differences may be attributed to the differences in the sources of the materials as well as the differences of extraction and quantification methods.

Isoflavone Compositions in Black Soybeans. The isoflavone contents of the seed coats, dedulled beans, and whole bean samples from black soybeans are presented in **Table 3**. Eleven of the total 12 isoflavone compounds (except for glycitein) were detected in both dehulled beans and seed coats. Most isoflavones existed as β-glucosides; the highest proportion at more than 66.5% of the total was 6″-*O*-malonyl-β-glucosides, followed by 7-*O*-β-glucosides at 24.5%, whereas 6″-*O*-acetyl-β-glucosides and aglycones occurred in only very small proportions. Regarding separated parts, significant differences ($p < 0.05$) in individual isoflavone content were found among different parts of black soybeans. Dehulled beans possessed significantly ($p < 0.05$) higher contents (in separated parts) of individual isoflavones (except for acetyldaidzin), subtotal isoflavones, and total isoflavones than those of seed coats, while individual and total isoflavone contents in dehulled bean were close to those of whole black soybeans. These results indicate that most isoflavones exist in the dehulled parts (cotyledon and embryo).

In terms of the mass distributions of phenolic contents in the seed coats and dehulled beans from the whole beans, the dehulled beans possessed significantly ($p < 0.05$) higher contents of individual isoflavones, subtotal isoflavones, and total isoflavones than those of the seed coats (contents in whole beans), whereas individual and total isoflavones contents in dehulled beans were a little low or close to those of whole beans.

Chemical profiles of isoflavones in selected black soybeans grown in Taiwan (*33*) and Korea (*34*) have been investigated. From a quantitative point of view, the total isoflavone contents of whole black beans (960.1 μg/g) and dehulled beans (893.8 μg/g) in the current study were in the range of whole beans (317.3−1321.2 μg/g) and dehulled beans (81.0−951.0 μg/g) of

Table 2. Distribution of Phenolic Acid Compositions in Black Soybean[a]

		content in separated parts from black soybean (μg/g)		content in whole black soybean (μg/g)	mass distribution in 1 g of whole black soybeans (μg)[b]		content in whole yellow soybean (μg/g)
derivates type	compounds	dehulled bean	seed coat	whole bean	dehulled bean	seed coat	whole bean
benzoic acids	GA	33.37 ± 1.84 a	ND[c]	29.59 ± 1.78 a A	30.03 ± 1.66 A	ND	61.61 ± 3.08
	PA	ND	32.92 ± 1.97 a	5.39 ± 0.03 b A	ND	3.29 ± 0.19 B	ND
	TBA	ND	30.91 ± 1.06 a	3.09 ± 0.03 b A	ND	3.09 ± 0.11 A	4.69 ± 0.05
	HBA	5.26 ± 0.20 a	ND	5.83 ± 0.18 a A	4.73 ± 0.02 B	ND	ND
	VA	ND	29.37 ± 1.56 a	2.94 ± 0.03 b A	ND	2.94 ± 0.16 A	5.64 ± 0.41
	VN	7.83 ± 0.36 a	ND	7.90 ± 0.02 a A	7.05 ± 0.04 B	ND	ND
	SA	1.49 ± 0.38 b	ND	2.39 ± 0.03 a A	1.34 ± 0.04 B	ND	2.96 ± 0.18
	subtotal	53.13 ± 1.44 b	93.21 ± 4.59 a	58.14 ± 2.03 b A	47.82 ± 0.14 B	9.32 ± 0.46 C	74.90 ± 2.79
cinnamic acids	CFA	78.37 ± 1.14 b	346.3 ± 14.83 a	107.8 ± 5.99 b A	70.53 ± 0.11 B	34.63 ± 1.48 C	65.77 ± 3.43
	CLA	511.2 ± 3.22 a	129.9 ± 7.84 b	480.9 ± 37.19 a A	460.1 ± 0.32 A	12.99 ± 0.78 B	587.3 ± 12.72
	PCA + SD	9.25 ± 0.52 a	ND	9.79 ± 0.97 a A	8.32 ± 0.05 B	ND	5.58
	MCA + FA	3.50 ± 0.44 b	22.71 ± 0.68 a	5.42 ± 0.02 b A	3.15 ± 0.04 B	2.27 ± 0.07 C	ND
	SPA	38.45 ± 1.51 a	ND	37.56 ± 2.02 a A	34.61 ± 0.15 A	ND	22.33 ± 0.19
	OCA	2.84 ± 0.13 c	25.11 ± 0.58 a	5.28 ± 0.01 b A	2.56 ± 0.01 B	2.51 ± 0.06 B	2.44 ± 0.24
	TCA	210.1 ± 2.19 a	83.07 ± 5.58 b	199.6 ± 1.48 a A	189.1 ± 0.22 A	8.31 ± 0.56 B	249.1 ± 4.89
	subtotal	853.7 ± 2.99 a	607.1 ± 16.98 b	833.2 ± 41.65 a A	768.3 ± 0.30 B	60.6 ± 1.69 C	932.5 ± 20.75
	total	906.8 ± 1.55 a	700.3 ± 12.39 b	891.3 ± 43.68 a A	816.1 ± 0.16 B	70.03 ± 1.24 C	1007 ± 23.55

[a] Data are expressed as means ± standard deviations ($n = 2$) on a dry weight basis. Values marked by the same lowercase letter (for comparison of contents in separated parts) or capital letters (for comparison of contents in whole bean) within each row are not significantly different ($P < 0.05$). [b] Calculation is based on the weight ratio (90:10) of dehulled bean and seed coat. [c] ND, not detectable. Phenolic acids: PA, protocatechuic acid; TBA, 2,3,4-trihydroxybenzoic acid; HBA, *p*-hydroxybenzoic acid; VA, vanillic acid; VN, vanillin; SA, syringic acid; CFA, caffeic acid; CLA, chlorogenic acid; PCA + SD, *p*-coumaric acid + syringaldehyde; MCA + FA, *m*-coumaric acid + ferullic acid; SPA, sinapic acid; OCA, *o*-coumaric acid; and TCA, *trans*-cinnamic acid.

Table 3. Distribution of Isoflavones in Black Soybean[a]

		content in separated parts (μg/g)		content in whole black soybean (μg/g)	mass distribution in 1 g of whole black soybeans (μg)[b]		content in whole yellow soybean (μg/g)
isoflavones		dehulled bean	seed coat	whole bean	dehulled bean	seed coat	whole bean
7-*O*-β-glucosides	Din	105.3 ± 10.17 c	72.60 ± 1.98 b	122.4 ± 3.16 a A	94.78 ± 9.15 B	7.26 ± 0.19 C	260.7 ± 13.16
	Gin	254.6 ± 0.56 a	83.15 ± 5.30 b	240.7 ± 3.19 a A	229.2 ± 0.06 A	8.32 ± 0.53 B	333.3 ± 14.30
	Gly	67.08 ± 0.51 a	32.09 ± 0.23 b	62.58 ± 1.82 a A	60.37 ± 0.05 A	3.21 ± 0.02 B	58.09 ± 4.72
6″-*O*-malonylglucosides	MDin	561.7 ± 4.39 b	183.2 ± 0.96 c	656.5 ± 34.50 a A	505.5 ± 0.44 B	18.32 ± 0.09 C	712.1 ± 31.07
	MGin	341.5 ± 17.82 a	306.9 ± 7.78 b	340.6 ± 5.06 a A	307.3 ± 1.78 B	30.69 ± 0.78 C	1980 ± 20.58
	MGly	156.9 ± 0.64 a	108.4 ± 5.82 b	158.3 ± 7.24 a A	141.3 ± 0.06 B	10.84 ± 0.58 C	87.54 ± 3.02
6″-*O*-acetylglucosides	ADin	8.63 ± 0.50 c	18.36 ± 0.40 a	10.33 ± 0.94 b A	7.78 ± 0.05 B	1.84 ± 0.04 C	88.32 ± 3.21
	AGin	15.41 ± 0.18 a	8.25 ± 0.11 b	15.23 ± 0.38 a A	13.86 ± 0.02 B	0.83 ± 0.01C	32.76 ± 1.67
	AGly	101.9 ± 1.05 a	17.09 ± 0.60 c	93.24 ± 6.54 b A	91.67 ± 0.11 A	1.71 ± 0.06 B	34.86 ± 1.61
aglycones	Dein	12.89 ± 0.85 a	12.29 ± 0.84 a	12.62 ± 0.85 a A	11.60 ± 0.08 A	1.23 ± 0.08 B	14.56 ± 2.18
	Gein	26.19 ± 0.73 a	18.56 ± 0.38 b	25.14 ± 0.13 a A	23.57 ± 0.07 B	1.86 ± 0.04 C	31.12 ± 2.19
	Glein	ND[c]	ND	ND	ND	ND	ND
subtotal [d]	T-Dein	454.4 ± 7.72 a	153.9 ± 0.05 c	425.0 ± 20.02 b A	408.9 ± 0.77 B	15.34 ± 0.01C	582.9 ± 23.36
	T-Gein	366.4 ± 8.31 a	235.2 ± 0.42 b	356.2 ± 4.55 a A	329.8 ± 0.83 B	23.52 ± 0.04 C	1290 ± 18.42
	T-Glein	172.3 ± 0.59 a	88.39 ± 3.31 b	178.9 ± 8.84 a A	155.1 ± 0.06 B	8.84 ± 0.33 C	104.2 ± 3.69
total [e]	isoflavones	993.1 ± 16.62 a	477.5 ± 2.93 b	960.1 ± 33.42 a A	893.8 ± 1.66 B	47.75 ± 0.29 C	1978 ± 45.47

[a] Data are expressed as means ± standard deviations ($n = 2$) on a dry weight basis. Values marked by the same lowercase letter (for comparison of contents in separated parts) or capital letters (for comparison of contents in whole bean) within each row are not significantly different ($P < 0.05$). [b] Calculation is based on a weight ratio (90:10) of dehulled bean and seed coat. [c] ND, not detectable. [d] Subtotal, moles of four forms of isoflavones multipled by the molecular weight of their aglycone form isoflavone. [e] Total, sum of the subtotal individuals of aglycones. Din, daidzin; Gin, genistin; Gly, glycitin; MDin, malonyldaidzin; MGin, malonylgenistin; MGly, malonylglycitin, ADin, acetyldaidzin; AGin, acetylgenistin; AGly, acetylglycitin; Dein, daidzein; Gein, genistein; Glein, glycitein; T-Dein, subtotal daidzein; T-Gein, subtotal genistein; and T-Glein, subtotal glycitein.

black soybeans reported in Korea (*34*), while the total isoflavone contents of the seed coat (47.8 μg/g) were higher than that (21.6−33.8 μg/g) of seed coats from black soybeans in Korea (*34*). However, the total isoflavone contents of the whole black bean were lower than those (1795.3−2492.8 μg/g) of black soybeans reported in Taiwan (*33*). The differences may be attributed to the differences in the sources (varieties, growth conditions in habitat) of the materials as well as the differences of quantification methods.

Distribution of Phenolic Compounds and Antioxidant Activities. The percentage (%) distributions of total and individual phenolic compound in the two parts (seed coats and dehulled beans) of black soybeans are summarized in **Table 4**. In whole black soybeans, most phenolics (about 73.4% of TPC, 85.9% of TFC, 79.5% of CTC, and 100% MAC) were distributed in seed coats. A great majority of the phenolic acids resided in the dehulled beans since the dehulled beans possessed 91.6% of total phenolic acids, while seed coats possessed 7.97% of total phenolic acids in whole black soybeans. Dehulled beans possessed 93.1% of total isoflavones, while the seed coats possessed 4.97% of total isoflavones in whole black soybeans. These results verified that most isoflavones exist in dehulled parts (cotyledon and embryo), and seed coats contained considerably lower amounts of isoflavones as compared to other parts of soybeans (*34*). These results indicated that seed coats contributed to most phenolic compositions of the black soybeans, while dehulled black soybeans still retained substantial amounts of total phenolics, which were similar to that of the whole yellow soybeans. Seed coats could be a good source of anthocynins or condensed tannins, while dehulled beans could be a good source of isoflavones and phenolic acids. **Figure 2B**,**D** showed seed coats, when the mass of the part was taken into consideration, contributed most antioxidant activities in DPPH

Table 4. Percentage Distribution of Whole Bean Phenolics in Bean Parts

compounds	distribution (%) [a] in seed coat	in dehulled bean
total phenolics	73.4	22.5
total flavonoids	85.9	11.0
condensed tannins	79.5	11.8
monomeric anthocyanins	100	0
anthocyanins		
cyanidin-3-glucoside	100	0
phenolic acids		
GA	0	100
protocatechuic acid	61.0[b]	0
2,3,4-trihydroxybenzoic acid	100	0
p-hydroxybenzoic acid	0	81.1
vanillic acid	100	0
vanillin	0	89.2
syringic acid	0	56.1[b]
subtotal benzoics	16.0	82.2
caffeic acid	32.1	65.5
chlorogenic acid	2.7	95.7
p-coumaric acid	0	85.0
m-coumaric acid	41.9	58.1
sinapic acid	0	92.1
o-coumaric acid	50	50
trans-cinnamic acid	4.2	94.7
subtotal cinnamics	7.3	92.2
total phenolic acids	7.9	91.6
isoflavones		
daidzin	5.9	77.4
genistin	3.5	95.2
glycitin	5.1	94.5
malonyldaidzin	2.8	77.0
malonylgenistin	9.0	90.2
malonylglycitin	6.8	89.3
acetyldaidzin	17.8	75.3
acetylgenistin	5.4	91.0
acetylglycitin	1.8	98.3
daidzein	9.7	91.3
genistein	7.4	93.7
subtotal daidzein	3.6	96.2
subtotal genistein	6.6	92.6
subtotal glycitein	4.9	86.7
total isoflavones	4.9	93.1

[a] Calculation is based on a weight ratio (90:10) of dehulled bean and seed coat. [b] Percent contents of these compounds were not normalized to 100 due to low concentrations in samples.

values (about 92.9%) and FRAP values (about 92.6%), while seed coats and dehulled beans contributed approximately equal levels to the total ORAC activity of the whole beans (**Figure 2F**).

These above results indicated that seed coats contributed most antioxidant activities in DPPH and FRAP of black soybeans, while dehulled black soybeans still retained substantial antioxidant capacities, which were similar to that of DPPH, FRAP, and ORAC values in whole yellow soybeans. The differences in antioxidant capacity as measured by different assay methods were due to the differences in reaction mechanisms elicited by differences in antioxidant chemical compositions. The ORAC assay mechanism is based on hydrogen atom transfer, whereas DPPH and FRAP are based on single electron transfer mechanisms (*32*).

The individual phenolic compounds identified in this study included only the major phenolic compounds in black soybeans. It should be noted that other minor phenolic compounds also existed but were not identified in this study. Coumestrol (the most common coumestan) is a trace (0.5 μg/g) component in dry soybeans (*35*). Nonphenolic compounds such tocopherols may partly contribute to the overall antioxidant activities of soybeans. Tocopherols are lipophilic components and are not extracted by the solvent system used in this study. DPPH and FRAP methods can not analyze lipophilic antioxidants. Although lipophilic components can be accessed by the ORAC assay, a previous study (*36*) showed that lipophilic-ORAC values were generally lower than hydrophilic-ORAC values in beans.

Soybeans are very complex materials and could vary from sample to sample, depending on varieties, cultivation conditions, postharvest storage, and processing methods. Nonetheless, our study contributed to the understanding of the relationships between major hydrophilic phenolic compounds and antioxidant capacities of black soybeans and provided useful information for effective utilization of black soybeans as ingredients for enhancing health. Further studies may be directed to variations among black soybean varieties and how storage and processing affect their antioxidant properties.

ABBREVIATION USED

TPC, total phenolic content; TFC, total flavonoid content; CTC, condensed tannin content; MAC, monomeric anthocyanin content; DPPH, 2-diphenyl-1-picryhydrazyl radical; FRAP, ferric reducing antioxidant power; ORAC, oxygen radical absorbing capacity; GA, gallic acid.

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