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ARTICLE

Antioxidant Properties, Phytochemical Composition, and Antiproliferative Activity of Maryland-Grown Soybeans with Colored Seed Coats

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This study characterized and compared 18 colored seed coat soybeans for the isoflavone, total phenolic, and cyanidin-3-glucoside (Cy-3-glc) contents of their flour extracts and the fatty acid composition and carotenoid and α-tocopherol contents of their oils. Antioxidant assays also assessed activity of the flour extracts against peroxyl, hydroxyl, and $ABTS^+$ radicals. Black seed coat soybeans had the highest TPC, ORAC, HOSC, and $ABTS^+$ radical scavenging values, in addition to the highest isoflavone content, and were the only color to contain Cy-3-glc. Five soybeans (two black and one each brown, yellow, and green) were selected to test their effects on HT-29 human colorectal cancer cell growth. The effects of the hydrolyzed and unhydrolyzed extracts were compared to an aglycone isoflavone standard mixture of the same total molar concentration as the highest soybean concentration of 15 mg of seed flour equiv/mL of treatment media. All high doses of hydrolyzed soybean treatments except the green genotype significantly reduced cell number compared to control at 3 h of treatment time, whereas the high dose of isoflavone standard treatment took 72 h to show a significant reduction ($P < 0.05$).

KEYWORDS: Antioxidant; isoflavone; soybean; HT-29; tocopherol; carotenoid; free radical; antiproliferation

INTRODUCTION

Consumption of soy foods has been recognized to lower the risk of aging-associated diseases, including cardiovascular disease and cancer, among others (*1*−*3*). These health benefits have often been studied in relation to a particular soy component. Isoflavones, a class of flavonoids found almost exclusively in legumes and most prominently in soy, have been studied heavily in this regard. There are three aglycone isoflavones—genistein, daidzein, and glycitein. Each aglycone has three derivatives based on the placement of sugar constituents—β-, malonyl-, and acetyl-glucoside. The aglycone forms are absorbed more rapidly by intestinal cells due to their less polar structure. Additionally, enzymes present in the intestine are capable of cleaving the glucosides to their aglycones, thus providing for better absorption. Isoflavones' antioxidant abilities are well-known, and their biological activity has been demonstrated in a great number of studies—they inhibit cancer cell growth in vitro, prevent tumor development in animal models, inhibit LDL oxidation, are capable of binding to estrogen receptors, and inhibit bone resorption by osteoclasts, among a list of other activities (*2*, *3*). These results suggest that isoflavones may be at least in part responsible for the health benefits associated with soy food consumption mentioned above.

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Interestingly, a few studies have indicated that soybeans with black, brown, green, and yellow seed coats might differ in their antioxidant properties, flavonoid levels, total phenolic contents, and proanthocyanidins (*4*−*6*), indicating that this may alter their ability to affect health. Soybean extracts (*4*, *7*) and peptides (*8*) have both been shown to reduce the oxidation of LDL cholesterol in vitro (*4*, *7*) or in rats (*8*), but black soybeans have been shown to have greater inhibitory effect against lipid peroxidation in human LDL than yellow ones (*4*). Additionally, in vitro studies have shown that the isoflavone genistein and anthocyanins are independently capable of inhibiting the growth of cancer cells through various mechanisms (*2*, *9*). Because black soybeans are the only color reported to contain anthocyanins (*10*, *11*) and only brown and black soybeans contain proanthocyanins, this may result in a differing level or type of bioactivity among soybeans with different seed coat colors. Also, these data suggest the possibility of developing novel soybean lines with a selected seed coat color to be used as bioactive ingredients in functional foods targeting different health problems.

The farm value for soybeans has varied greatly in recent years and is highly dependent on factors well beyond the control of farmers, including international demand, weather conditions, and market supply size (*12*). If a high-demand, consistent retail outlet can be established for a novel soybean line because of its special health properties, small farms growing it may be better able to withstand the uncertain market and retain more consistent profitability. As part of our continuous effort to enhance the

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quality and value of Maryland-grown soybeans, this study aimed to characterize the antioxidant activity and phytochemical composition of Maryland-grown soybeans with various seed coat colors. Furthermore, this study sought to compare the phytochemical composition of extracts of these soybeans with their bioactivity in HT-29 human colorectal cancer cells, as compared to the activity of treatments of pure isoflavones.

MATERIALS AND METHODS

Materials and Chemicals. Whole soybeans of brown, green, yellow, and black seed coat colors from the 2007 growing season grown at a single location in Maryland were obtained from Dr. William Kenworthy of the Department of Plant Science and Landscape Architecture, University of Maryland (College Park, MD). In Maryland, 2007 was a drought season, which should be considered in interpreting results due to the well-accepted link between growing conditions and levels of various phytochemicals, including isoflavones and tocopherols.

ABTS chromophore diammonium salt was manufactured by Calbiochem (San Diego, CA). Iron (III) chloride, fluorescein (FL), biotech grade DMSO, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$), genistein, daidzein, cyanidin, lutein, β-carotene, β-cryptoxanthin, zeaxanthin, and α-, δ-, and γ-tocopherols were purchased from Sigma-Aldrich (St. Louis, MO). 2,2′-Azinobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). Thirty percent ACS grade H_2O_2 was purchased from Fisher Scientific (Fair Lawn, NJ). Glycitein was obtained from Indofine Chemical Co. (Hillsborough, NJ). Cyanidin-3-glucoside was purchased from Polyphenols Laboratory AS (Norway). Ultrapure water was manufactured by Cayman Chemical Co. (Ann Arbor, MI) and was used for all experiments. An ATP-Lite 1step Luminescence Assay System was obtained from Perkin-Elmer (Waltham, MA). All cell culture media components were purchased from Invitrogen (Carlsbad, CA). All other chemicals and solvents were of the highest commercial grade and used without further purification.

Seed Coat Color. A monolayer of whole, undamaged soybeans was placed in a clean glass sample container and analyzed for Hunter color values L^*, a^*, and b^* using a HunterLab ColorFlex (Reston, VA) according to the manufacturer's directions. Color value was obtained using a D65/10° (daylight 65 illuminant/10° observer) setting. Triplicate measurements were taken, with seeds gently shaken between readings.

Oil Extraction. Whole soybeans were ground in a standard household coffee grinder to a 20 mesh particle size. Five grams of ground soybeans was extracted at room temperature in 10 mL of petroleum ether (BP 35−60 °C) a total of four times. The petroleum ether was evaporated in a nitrogen evaporator to a constant weight. The oils were stored at ambient temperature under nitrogen in the dark until testing.

Antioxidant Extraction. The soy flour remaining after oil extraction was then extracted with 50% acetone at a ratio of 1 g/10 mL. The solvent was chosen according to the results of Xu and Chang (*13*) and to coincide with our previous work on soybeans (*14*). The tubes were stirred to disturb the unwetted portion at the bottom, blown off with nitrogen, vortexed three times for 15 s each, and allowed to extract overnight in the dark. The soy flour−solvent mixtures were then sonicated for 15 min, and the extracts were filtered through 0.45 μm syringe filters. The extracts were held under nitrogen in the dark until testing.

Fatty Acid Composition. Fatty acid methyl esters (FAME) were prepared from the oils by saponification followed by methylation according to a previously described laboratory protocol and subjected to gas chromatography (GC) analysis (*15*). A Shimadzu GC-2010 with an FID, an AOC-20i injector, and an AOC-20S autosampler (Shimadzu, Columbia, MD) was used for fatty acid analysis. The carrier gas was helium at a total flow rate of 12.2 mL/min through a fused silica capillary column SP-2380 (30 m × 0.25 mm with a 0.25 μm film thickness) from Supelco (Bellefonte, PA). Injection volume was 1 μL at a split ratio of 10/1. Oven temperature was initially 136 °C, increased by 6 °C/min to 184 °C, at which it was held for 3 min, and then increased again by 6 °C/min to a final temperature of 226 °C. Individual fatty acids were identified through comparison of GC retention times with those of fatty acid methyl ester external standards. Quantification was based on the area under each fatty acid peak as compared to the total area of all fatty acid peaks.

Carotenoid and α-Tocopherol Contents. Oil samples and standards were dissolved in hexane and analyzed via normal phase liquid chromatography−atmospheric pressure chemical ionization−tandem mass spectrometry (NP-LC-APCI-MS/MS) according to the method of Hao et al. (*16*). Briefly, a Zorbax RX-SIL column, 2.1 mm i.d. × 150 mm, 5 μm particle size (Agilent Technologies, Palo Alto, CA), was used at ambient temperature. Separation was achieved through gradient elution with a flow of 0.5 mL/min, with hexane for solvent A and 1% isopropanol in EtOAc for solvent B. A 5 min linear gradient from 1 to 10% solvent B was followed by a 15 min linear gradient from 10 to 50% B. The column was allowed to re-equilibrate at initial conditions for 10 min prior to injection of the next sample. The injection volume was 5 μL. Quantification was done using total ion counts compared to that of external standards.

Total Phenolic Contents (TPC). The TPC of each soybean extract was measured according to a laboratory procedure described previously (*17*). The reaction mixture comprised 100 μL of 50% acetone soybean extract, 500 μL of Folin−Ciocalteu reagent, 1.5 mL of 20% sodium carbonate, and 1.5 mL of ultrapure water. Absorbance was read at 765 nm on a Thermo Spectronic (Waltham, MA) Genesys spectrophotometer after 2 h of reaction at ambient temperature. Different concentrations of gallic acid were used to create the standard curve. Reactions were conducted in triplicate, and results were reported as milligrams of gallic acid equivalents (GAE) per gram of soybean flour.

Isoflavone Composition. Fifty percent acetone soybean extracts were hydrolyzed with acid, redissolved in methanol, and filtered through a 0.45 μm syringe filter prior to being subjected to HPLC analysis. The protocol by Lee et al. (*18*) was followed with modifications. The column used was a Phenomenex (Torrance, CA) Gemini C18 column (150 mm × 4.6 mm × 5.0 μm) and was housed in an oven set to 40 °C. A binary solvent system was employed with solvent A consisting of water/acetic acid at a ratio of 99.9:0.1 (v/v) and solvent B consisting of acetonitrile/acetic acid (99.9:0.1, v/v). The gradient changed linearly from 75:25 (A:B, v/v) to 67:33 (A:B, v/v) from 0 to 20 min and then was returned to initial conditions for 5 min to re-equilibrate the column prior to the next run. The flow rate was 1 mL/min, the injection volume was 10 μL, and the detection wavelength was set to 254 nm. The isoflavones were identified and quantified via comparison to external standards.

Cyanidin-3-glucoside (Cy-3-glc) Contents. Cy-3-glc was determined because it is the predominant anthocyanin present in black soybean seed coats (*10*, *19*, *20*). An HPLC protocol by Lee and colleagues (*19*) was adapted for use on a Shimadzu Prominence UFLC system (Columbia, MD) with an autosampler, an in-line degasser, a CTO-20AC oven, and an SPD-20A UV−vis detector. A gradient flow of 1.0 mL/min was employed on a Phenomenex Gemini C18 column (150 mm × 4.6 mm × 5.0 μm) in an oven set to 25 °C. Solvent A consisted of 0.1% acetic acid in water, whereas solvent B consisted of 0.1% acetic acid in acetonitrile. The gradient changed linearly from 100:0 (A:B, v/v) to 85:15 (A:B, v/v) over 10 min and then to 75:25 (A:B, v/v) over another 10 min. Then, a 5 min wash at 10:90 (A:B, v/v) was performed, after which the column was re-equilibrated at 100:0 (A:B, v/v) for 5 min. Absorbance was read at 530 nm, and the injection volume was 20 μL of unhydrolyzed extract. Cy-3-glc was identified and quantified via comparison to peak area of an external standard.

Antioxidant Activity Assays. *ABTS$^{\bullet+}$ Scavenging Ability*. The scavenging capacity against ABTS$^{\bullet+}$ was measured according to a previously reported method (*21*). Briefly, ABTS$^{\bullet+}$ working solution was prepared by reacting 2,2-azobis(3-ethylbenzothizide-6-sulfonic acid) diammonium salt with manganese oxide (MnO_2) in solution, which was then filtered and diluted to an absorbance of 0.700 ± 0.005 at 734 nm on a Thermo Spectronic Genesys spectrophotometer. Eighty microliters of sample or standard was added to 1 mL of the working ABTS$^{\bullet+}$ solution and vortexed for 30 s, and the absorbance was read at 734 nm after 90 s of reaction. Trolox standards in 50% acetone were used to create a standard curve, and samples were diluted as necessary to fit on the curve.

Oxygen Radical Absorbance Capacity (ORAC) Assay. The ORAC assay was conducted according to a previously reported laboratory protocol (*22*) with minor modifications. Trolox standards were prepared in 50% acetone, whereas all other reagents were prepared in 75 mM

Table 1. Isoflavone Composition of Soybean Extract Treatments at Three Concentrations (Micromolar)[a]

	low dose			medium dose			high dose		
	Daid	Gen	Gly	Daid	Gen	Gly	Daid	Gen	Gly
Emerald	0.5	0.7	0.9	1.6	2.3	3.0	4.8	6.9	8.9
MD 0304 WN-46	0.4	0.4	1.2	1.2	1.5	4.0	3.6	4.5	12.0
MD 0304 WN-46-1	0.2	0.3	0.7	0.6	1.0	2.2	1.7	2.9	6.7
Peking	1.8	1.2	2.1	5.9	4.0	7.0	17.7	12.1	20.9
Pi 88788	1.2	1.4	2.5	4.1	4.5	8.5	12.2	13.6	25.4
Isoflavone mixture	1.2	1.4	2.5	4.0	4.5	8.5	12.0	13.6	25.4

[a] Daid, daidzein; Gen, genistein; Gly, glycitein.

sodium phosphate buffer (pH 7.4). The initial reaction mixture contained 225 μL of freshly made 8.16×10^{-8} M fluorescein and 30 μL of sample, standard, or blank solution. Initial reaction mixtures were pipetted into a 96-well plate and preheated at 37 °C for 20 min. Then, 25 μL of freshly made 0.36 M AAPH was added to each well. The fluorescence of the assay mixture was recorded on a Victor^3 multilabel plate reader (Perkin-Elmer, Turku, Finland) once every 2 min for 3 h at 37 °C, with λ_{Ex} = 485 nm and λ_{Em} = 535 nm. Trolox equivalents (TE) were calculated for samples on the basis of area under the curve (AUC) calculations used by Ou and others (*23*). Results are expressed as micromoles of TE per gram of defatted soybean flour.

Hydroxyl Radical Scavenging Capacity (HOSC) Estimation. The HOSC assay was conducted also using fluorescein as the fluorescent probe and a Victor^3 multilabel plate reader (Perkin-Elmer) according to a previously reported laboratory protocol (*24*). The reaction mixture contained 170 μL of 9.28×10^{-8} M fluorescein, 30 μL of sample, 40 μL of 0.1990 M H_2O_2, and 60 μL of 3.43 M $FeCl_3$. The fluorescence of the reaction mixture was recorded approximately once every 4 min for 7 h at ambient temperature, with λ_{Ex} = 485 nm and λ_{Em} = 535 nm. Standards were prepared in 50% acetone. The 9.28×10^{-8} M fluorescein was prepared fresh for each assay from stock solution and 75 mM sodium phosphate buffer (pH 7.4). Trolox equivalents were calculated for samples using the same AUC calculations as in ORAC (*24*). Results are expressed as micromoles of TE per gram of defatted soybean flour.

Antiproliferative Activity against HT-29 Cells. The antiproliferation test was adopted from Wang et al. (*25*). HT-29 human colorectal adenocarcinoma cells were cultured in a humidified incubator at 37 °C and 5% CO_2. Cell culture media consisted of McCoy's 5A media supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution.

Cells were grown to 95% confluence and then plated at 2500 cells/well in a black 96-well viewplate treated for tissue culture. After 24 h, the medium was replaced with 100 μL of control or treatment medium. Proliferation of cells was assessed using the ATP-Lite 1step Luminescence Assay System from Perkin-Elmer prior to treatment and at 3, 24, 48, 72, and 96 h after initial treatment. To take a reading, plates were allowed to equilibrate at room temperature for 30 min, and then 100 μL of reconstituted ATPlite solution was added to each well immediately prior to taking luminescence readings on a Victor^3 multiwell plate reader (Perkin-Elmer). A separate plate was used for each reading. Treatment and control media were replaced every 24 h until a reading was taken on that plate.

Treatment Media Preparation. All treatment media contained 1.2% DMSO, including the control, and were filtered through a 0.2 μm retrograde cellulose syringe filter prior to treatment of cells.

For the study of the antiproliferative activity of soybean extracts, cells were treated with hydrolyzed or unhydrolyzed soybean extracts in three doses: 1.5, 5, and 15 mg of seed flour equiv/mL of media. All treatment media, including the control, contained a final concentration of 1.2% DMSO (v/v). Isoflavone compositions of the hydrolyzed extracts were calculated for genistein, daidzein, and glycitein on the basis of HPLC data and are shown in **Table 1**. Because the unhydrolyzed extracts had not had their glucoside isoflavones hydrolyzed to their aglycone forms, it is likely their isoflavones were mainly in the glucoside form. However, all 12 isoflavone forms were not quantified in unhydrolyzed extracts. For the unhydrolyzed extracts, the values in **Table 1** therefore represent isoflavone "aglycone equivalents". For comparison purposes, a standard isoflavone treatment was tested on the cells. It included a mixture of genistein, daidzein, and glycitein (each ≥99% pure and purchased from Sigma-Aldrich or Indofine), and the concentration of isoflavones in the mixture was equivalent to that of Pi 88788, which had the highest isoflavone contents of soybean extracts tested on cells.

Table 2. Isoflavone Composition of Isoflavone Treatment Medium Concentrations (Micromolar)[a]

	Daid	Gen	Gly	Cy
control	0	0	0	0
Daid only	12.0	0	0	0
Gen only	0	13.6	0	0
Gly only	0	0	25.4	0
Daid + Gen	12.0	13.6	0	0
Daid + Gly	12.0	0	25.4	0
Gen + Gly	0	13.6	25.4	0
Daid + Gen + Gly	12.0	13.6	25.4	0
Pi 88788 mimic	12.0	13.6	25.4	7.0
Peking mimic	17.7	12.1	20.9	17.7

[a] Daid, daidzein; Gen, genistein; Gly, glycitein; Cy, cyanidin.

Table 3. HunterLab Color Values of Soybeans[a]

	L^*	a^*	b^*
Black			
MD 06-5437-1	19.6 ± 0.3	0.2 ± 0.2	1.0 ± 0.2
MD 06-5440-2	16.5 ± 0.44	0.1 ± 0.2	−0.5 ± 0.1
Peking	22.7 ± 0.3	0.0 ± 0.0	0.3 ± 0.0
Pi 88788	22.3 ± 0.2	0.0 ± 0.1	0.0 ± 0.1
Pi 90763	19.3 ± 0.6	0.0 ± 0.1	−0.6 ± 0.1
Brown			
MD 0304 WN46-1	33.9 ± 0.2	11.6 ± 0.2	14.5 ± 0.1
MD 0304 WN46-2	33.3 ± 0.4	11.4 ± 0.3	14.4 ± 0.6
MD 0304 WN46-3	33.6 ± 0.5	11.4 ± 0.3	13.9 ± 0.6
MD 0304 WN46-4	32.7 ± 1.0	10.9 ± 0.6	13.5 ± 0.7
Green			
Emerald	49.6 ± 1.5	−3.4 ± 0.2	24.6 ± 0.6
Verde	51.4 ± 1.1	−3.4 ± 0.5	24.9 ± 0.3
Yellow			
MD 0304 WN-46	61.1 ± 0.7	7.9 ± 0.4	30.8 ± 0.5
MD 05-6073	63.7 ± 0.5	6.7 ± 0.1	29.3 ± 0.2
MD 05-6077	62.4 ± 0.4	6.9 ± 0.3	30.6 ± 1.1
MD 05-6079	62.4 ± 0.5	6.5 ± 0.2	29.7 ± 0.4
MD 06-5433-1	60.7 ± 1.5	7.2 ± 0.2	34.6 ± 1.8
MD 06-5433-3	60.7 ± 1.5	6.6 ± 0.1	30.7 ± 0.5
MD 06-5445-5	58.3 ± 1.3	6.0 ± 0.1	30.9 ± 1.0

[a] Values based on triplicate readings. Mean ± standard deviation shown. (n = 3).

For the study of the antiproliferative activity of isoflavones, cells were treated with the aglycone isoflavones (genistein, daidzein, and glycitein) individually and in all possible combinations at three doses each. Again, the final concentration of DMSO in the treatment media was 1.2%. Doses were chosen to be concentrations equivalent to the high dose of soybean Pi 88788. Actual doses and combinations can be seen in **Table 2**.

Another study was conducted to test the antiproliferative effect of an isoflavone and cyanidin mixture that mimic the concentration of both isoflavones and cyanidin in Peking and Pi 88788 extracts. The isoflavone and cyanidin concentrations of these two soybeans were recreated with standard compounds, with final treatment media again containing 1.2% DMSO, and antiproliferative effects were again compared to a control with 1.2% DMSO. Actual concentrations are shown in **Table 2**.

Statistical Analysis. Tests were conducted in triplicate with data reported as mean ± standard deviation. Differences in means were detected using one-way ANOVA and Tukey's test. For those instances when average values were calculated for a seed coat color group,

Table 4. Fatty Acid Composition of Soybean Oil by Seed Coat Color (Grams per 100 g of Oil)[a]

	16:0	18:0	total SFA	18:1	18:2	18:3	total PUFA
				Black			
MD 06-5437-1	11.27i ± 0.05	3.91d ± 0.03	15.18k ± 0.03	21.26c ± 0.07	52.35ij ± 0.12	8.48k ± 0.06	60.94j ± 0.17
MD 06-5440-2	11.44j ± 0.05	4.05e ± 0.01	15.49m ± 0.05	22.99d ± 0.04	50.63 g ± 0.05	7.99i ± 0.01	58.66 h ± 0.04
Peking	11.21i ± 0.08	3.46b ± 0.05	14.67i ± 0.04	17.13a ± 0.04	55.14p ± 0.12	10.78p ± 0.07	66.03o ± 0.18
Pi 88788	11.41j ± 0.02	3.48b ± 0.03	14.89j ± 0.02	18.72b ± 0.05	53.57 m ± 0.06	10.27n ± 0.03	63.89m ± 0.05
Pi 90763	11.64k ± 0.02	3.03a ± 0.03	14.67i ± 0.01	18.16b ± 0.03	53.93no ± 0.11	10.45o ± 0.02	64.49n ± 0.11
black av	11.39z ± 0.16	3.59yz ± 0.38	14.98z ± 0.33	19.65w ± 2.23	53.12z ± 1.59	9.59z ± 1.17	62.71z ± 2.73
				Brown			
MD 0304 WN46-1	5.86b ± 0.02	4.24gh ± 0.05	10.10c ± 0.05	41.48j ± 0.04	42.84bc ± 0.08	3.10a ± 0.05	46.02b ± 0.11
MD 0304 WN46-2	6.19d ± 0.02	4.11ef ± 0.03	10.30d ± 0.03	41.02j ± 0.04	43.13d ± 0.03	3.31bc ± 0.07	46.47c ± 0.05
MD 0304 WN46-3	6.05c ± 0.01	4.19fg ± 0.03	10.24cd ± 0.04	41.01j ± 0.08	42.92cd ± 0.09	3.27bc ± 0.05	46.28bc ± 0.13
MD 0304 WN46-4	6.14 cd ± 0.06	4.36h ± 0.03	10.49e ± 0.08	41.07j ± 0.10	42.63b ± 0.07	3.23b ± 0.01	45.93b ± 0.07
brown av	6.06x ± 0.13	4.22z ± 0.10	10.28y ± 0.15	41.15z ± 0.21	42.88y ± 0.20	3.23w ± 0.09	46.11x ± 0.25
				Green			
Emerald	9.58e ± 0.07	3.45b ± 0.03	13.03f ± 0.05	23.51de ± 0.11	52.53jk ± 0.09	8.33j ± 0.03	60.96j ± 0.12
Verde	10.97h ± 0.08	3.51bc ± 0.03	14.48h ± 0.08	26.08f ± 0.13	48.88f ± 0.19	7.76h ± 0.04	56.83f ± 0.16
green av	10.28y ± 0.76	3.48y ± 0.04	13.76yz ± 0.73	24.80x ± 1.41	50.71z ± 2.01	8.05y ± 0.31	58.75y ± 2.31
				Yellow			
MD 0304 WN-46	6.04c ± 0.04	3.86d ± 0.01	9.90b ± 0.04	36.03i ± 0.06	48.31e ± 0.18	3.37c ± 0.01	51.87d ± 0.18
MD 05-6073	3.54a ± 0.04	3.61c ± 0.02	7.15a ± 0.05	33.26g ± 0.02	53.68mn ± 0.05	3.76e ± 0.92	57.50g ± 0.07
MD 05-6077	3.55a ± 0.2	3.61c ± 0.02	7.16a ± 0.02	33.11g ± 0.07	53.99o ± 0.05	3.52d ± 0.01	57.56g ± 0.05
MD 05-6079	3.59a ± 0.03	3.49bc ± 0.01	7.08a ± 0.02	34.79h ± 1.11	51.36h ± 0.03	3.88f ± 0.01	55.27e ± 0.02
MD 06-5433-1	16.60m ± 0.04	6.08i ± 0.02	22.68n ± 0.04	23.86e ± 0.04	38.98a ± 0.05	5.92g ± 0.01	44.95a ± 0.04
MD 06-5433-3	10.61g ± 0.03	3.88d ± 0.11	14.50h ± 0.10	22.71d ± 0.04	52.23i ± 0.07	8.04i ± 0.03	60.34i ± 0.07
MD 06-5445-5	10.36f ± 0.02	3.48b ± 0.07	13.84g ± 0.05	21.71c ± 0.02	52.73k ± 0.04	9.07m ± 0.05	61.84k ± 0.08
yellow av	7.76xy ± 4.72	4.00yz ± 0.88	11.76yz ± 5.46	29.35y ± 5.96	50.18z ± 5.02	5.37x ± 2.24	55.55y ± 5.44

[a] Values are based on triplicate readings. Mean ± SD shown. Individual sample values in the same column marked by the same letter are not significantly different ($P \leq 0.05$). Average values in the same column marked by the same letter are not significantly different ($P \leq 0.05$). SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids.

differences between color groups were determined using one-way ANOVA with the Tukey-b post hoc option, allowing for the comparison of unequal sample sizes. Correlations among means were determined using a two-tailed Pearson correlation test. Statistics were analyzed using SPSS for Windows (version rel. 10.0.5, 1999, SPSS Inc., Chicago, IL). Statistical significance was defined at $P \leq 0.05$.

RESULTS AND DISCUSSION

Seed Coat Color. In the typical yellow soybean, chlorophyll disappears as seeds mature and dry in the field, allowing the remaining flavonoid pigments' color to show. Some soybeans do not lose their chlorophyll as they dry and remain green at maturation, whereas others have additional pigments, including anthocyanins and proanthocyanins, that impart deep black and brown seed coat colors. It is these pigments that are believed to contribute at least in part to the antioxidant capacity differences discussed later.

Seed coat color values are shown in **Table 3**. The L^* reading is an indicator of blackness of the sample, with closer to zero being more black. Appropriately, the black seed coat L^* values are in the vicinity of 20, whereas the others are higher: brown around 33, green around 50, and yellow around 60. Negative a^* readings indicate a green color, whereas positive a^* readings indicate a red color. As expected, the green seed coats have the most negative a^* value, and the brown have the highest positive a^* value. Finally, b^* represents the yellow to blue continuum, with positive values indicating yellow and negative values indicating blue. The yellow seed coats have the highest positive b^*, whereas the black seed coats have the lowest value.

Fatty Acid Composition. As shown in **Table 4**, oils from soybeans with different seed coat colors might differ significantly in their fatty acid compositions ($P \leq 0.05$). This is relevant due to the considerable efforts dedicated to altering the fatty acid composition of soybeans oils for specific uses: low-linolenic varieties have been sought due to their improved oxidative stability, whereas other varieties (including low-saturated or high-linolenic) have been sought for their health-promoting attributes.

The black genotypes had the lowest 18:1 contents and the highest average 16:0 level, along with relatively high levels of total saturated fatty acids (SFA) around 15% of total fatty acids and on average the highest 18:2 and 18:3 contents (**Table 4**). The brown genotypes had the highest 18:1 content, ranging from 41.0 to 41.5% of total fatty acids in their oils, along with around 10% SFA and low 18:3 contents at 3%, with higher 18:1 and 18:2 to compensate. The two green genotypes had SFA at 13 and 14.5% and an average 18:3 content of 8%. This 18:3 level is comparable to that of 8.0−10.5% detected in the soybeans with black coats (**Table 4**). Finally, the most variation in fatty acid composition was seen among the yellow genotypes, with SFA ranging from 7 to 23%, 18:1 from 22 to 35%, 18:2 from 39 to 54%, and 18:3 from 3 to 9%. In summary, black-coated soybeans may serve as a dietary source for n-3 fatty acid, whereas soybeans with brown coats may be candidates for future breeding efforts to develop high-oleic and low-linolenic and saturated fatty acids lines.

Carotenoid and α-Tocopherol Contents. Lutein, β-carotene, cryptoxanthin, and zeaxanthin contents of the soybean oils are summarized in **Table 5**. Lutein was the primary carotenoid compound present in the soybeans regardless of seed coat color, and zeaxanthin was also detected in most of the tested soybean oils. The greatest lutein content was 907 μg/g in the oil of MD 05-6079 yellow soybean, and MD 05-6077 yellow soybean oil had

Table 5. Carotenoid and α-Tocopherol Content of Soybeans by Seed Coat Color[a]

	μg/g of oil					
	α-tocopherol	lutein	β-carotene	cryptoxanthin	zeaxanthin	total carotenoids (μmol/g of oil)
Black						
MD 06-5437-1	526.6hi ± 39.6	171.8b ± 3.7	30.4a ± 3.3	6.9f ± 0.6	95.3e ± 2.1	0.539fg ± 0.011
MD 06-5440-2	1083.8m ± 97.0	191.8b ± 4.4	ND	ND	60.0d ± 3.2	0.443ef ± 0.013
Peking	144.1ab ± 5.3	270.7d ± 16.4	ND	ND	62.5d ± 3.6	0.586g ± 0.034
Pi 88788	744.1jk ± 75.7	375.9e ± 8.2	ND	1.9cd ± 0.4	119.0f ± 1.4	0.874h ± 0.013
Pi 90763	360.8fg ± 17.3	510.3f ± 17.7	49.1b ± 1.8	6.4f ± 0.4	217.4g ± 10.4	1.382i ± 0.037
Brown						
MD 0304 WN46-1	282.7def ± 6.5	201.4bc ± 8.6	ND	ND	45.0c ± 1.7	0.433e ± 0.013
MD 0304 WN46-2	262.1cdef ± 6.5	391.9e ± 12.8	ND	3.1e ± 0.4	89.7e ± 2.5	0.852h ± 0.024
MD 0304 WN46-3	308.9ef ± 1.7	64.8a ± 2.7	ND	ND	10.6a ± 0.9	0.133b ± 0.005
MD 0304 WN46-4	240.7bcde ± 10.5	178.4b ± 9.2	ND	ND	37.9c ± 1.4	0.380de ± 0.016
Green						
Emerald	250.0cde ± 6.7	ND	ND	ND	ND	0.000a ± 0.000
Verde	431.3gh ± 26.7	82.4a ± 6.7	ND	ND	14.4a ± 0.9	0.170b ± 0.013
Yellow						
MD 0304 WN-46	332.7efg ± 15.8	226.9bcd ± 17.0	ND	0.5ab ± 0.3	27.0b ± 1.0	0.447ef ± 0.032
MD 05-6073	161.8abc ± 6.3	80.4a ± 4.7	ND	ND	ND	0.141b ± 0.008
MD 05-6077	109.9a ± 7.5	ND	ND	2.9de ± 0.4	ND	0.005a ± 0.001
MD 05-6079	201.3abcd ± 14.4	906.5g ± 64.0	ND	1.3bc ± 0.2	14.4a ± 1.1	1.621j ± 0.111
MD 06-5433-1	618.7ij ± 23.2	66.9a ± 5.9	36.4a ± 3.4	ND	19.3ab ± 1.2	0.219bc ± 0.015
MD 06-5433-3	677.8jk ± 24.7	99.5a ± 3.7	ND	2.3de ± 0.3	60.6d ± 1.4	0.286cd ± 0.009
MD 06-5445-5	121.0a ± 8.4	256.9cd ± 6.9	ND	2.4de ± 0.2	86.8e ± 2.7	0.608g ± 0.016

[a] Values based on triplicate readings. Mean ± SD shown. Values in the same column marked by the same letter are not significantly different ($P \leq 0.05$). ND, not detected.

no detectable lutein or zeaxanthin. The highest zeaxanthin content was 217 μg/g in the oil of Pi 90763 black soybean oil, and black soybean oils contained, on average, higher zeaxanthin than soybeans with other color coats (**Table 5**). The oils of Pi 90763 and MD 06-5437-1 black soybean lines had all four carotenoid compounds, but the oil of MD 05-6079 yellow soybean line had the highest total carotenoid content of 1.62 μmol/g of oil, followed by 1.38 μmol/g of oil detected in the oil of Pi 90763 soybeans with black seed coats (**Table 5**). The average total carotenoid content was about 0.76, 0.48, 0.45, and 0.09 μmol/g oil for soybeans with black, yellow, brown, and green coats, whereas the yellow genotypes contained the widest variation in carotenoid content from 0.005 to 1.621 μmol/g of oil. It was also noted that the oil of Emerald green soybean contained no detectable carotenoids, and the other green-coated soybean cultivar (Verde) contained < 0.2 μmol of total carotenoids/g in its oil. However, two samples are not enough to determine whether a significant trend exists.

The oils from soybeans with different color coats were also evaluated for their α-tocopherol contents (**Table 5**). All oils contained α-tocopherol, with the low value from the yellow MD 05-6077 at 109.9 μg/g of oil and the highest value from the black MD 06-5440-2 at 1083.8 μg/g of oil. No specific trend was apparent between α-tocopherol content and seed coat color, although the average levels of α-tocopherol were 572, 341, 318, and 274 μg/g of oil for black, green, yellow, and brown soybean genotypes. Soybean oil with higher carotenoid content did not necessarily contain higher α-tocopherol. These data indicated that soybeans with different coat colors generally contain comparable levels of carotenoids and α-tocopherol, with higher possibility to find a black soybean line or cultivar rich in these beneficial components.

Some carotenoids (including β-carotene and cryptoxanthin) are forms of provitamin A, although the soybeans in the present study were low in these components. The predominant carotenoid detected was lutein, and consumption of lutein has protective effects against a variety of ocular diseases. Tocopherols are members of the vitamin E family. Both carotenoids and tocopherols are lipophilic antioxidants and may have protective effects against oxidative damage for oils during storage and cellular components after ingestion/absorption.

Isoflavone Composition. Isoflavone compositions of these soybeans with different seed coat colors were compared using the hydrolyzed 50% acetone extracts of the defatted soy. Genistein, daidzein, and glycitein were detected in all soybean flours, with glycitein as the predominating isoflavone in each sample (**Table 6**). The black soybeans Peking, Pi 88788, and Pi 90763 had significantly higher total isoflavones among all of the soybeans tested ($P \leq 0.05$). Soybeans with a black seed coat had an average level of total isoflavone content of 2.1 μmol/g of whole soybean, which was much higher than the 1.0, 0.8, and 0.7 μmol/g of whole bean observed in the green, brown, and yellow soybeans, respectively (**Table 6**). This observation was supported by a trend seen in two previous papers using 50% acetone as the extraction solvent, in which total flavonoid contents were 3−4-fold higher in black soybeans than in yellow (*5*, *13*). However, two other previous studies reported black soybeans to contain fewer isoflavones than yellow soybeans, although these studies used a different solvent system and detection method (acetonitrile/water and HPLC), which limits their relevance for direct comparison to this study (*10*, *26*). It is recognized that the isoflavone values of this study may be lower than ranges of values presented in other analyses, a difference believed to be mainly due to the extraction conditions.

It was also noted that soybeans with the same seed coat color may differ significantly ($P \leq 0.05$) in their total isoflavone contents and isoflavone compositions (**Table 6**). For instance, Pi 88788 and Peking black soybean samples had significantly

Table 6. Isoflavones and Cyanidin-3-glucoside Detected in Soybeans by Seed Coat Color[a]

	μmol/g of flour				
	daidzein	genistein	glycitein	total isoflavones	Cy-3-Glc (mg/g of flour)
			Black		
MD 06-5437-1	0.18bc ± 0.00	0.16ab ± 0.00	0.47def ± 0.00	0.80 cd ± 0.00	0.03a ± 0.01
MD 06-5440-2	0.30f ± 0.00	0.32c ± 0.00	0.53gh ± 0.01	1.15g ± 0.01	0.25c ± 0.02
Peking	1.18i ± 0.03	0.81e ± 0.08	1.39k ± 0.03	3.38j ± 0.12	0.57e ± 0.02
Pi 88788	0.81g ± 0.01	0.91f ± 0.01	1.69m ± 0.02	3.41j ± 0.03	0.22b ± 0.01
Pi 90763	0.94 h ± 0.01	0.78e ± 0.00	1.41k ± 0.01	3.13i ± 0.01	0.30d ± 0.02
			Brown		
MD 0304 WN46-1	0.11a ± 0.00	0.20b ± 0.01	0.45de ± 0.01	0.76bc ± 0.02	ND
MD 0304 WN46-2	0.19bcd ± 0.00	0.28c ± 0.00	0.52hi ± 0.00	0.98f ± 0.01	ND
MD 0304 WN46-3	0.19 cd ± 0.00	0.28c ± 0.01	0.48efg ± 0.01	0.95ef ± 0.02	ND
MD 0304 WN46-4	0.19 cd ± 0.00	0.27c ± 0.00	0.44cde ± 0.01	0.90ef ± 0.01	ND
			Green		
Emerald	0.32f ± 0.00	0.46d ± 0.02	0.59i ± 0.01	1.37h ± 0.01	ND
Verde	0.26e ± 0.01	0.31c ± 0.01	0.38ab ± 0.01	0.95ef ± 0.03	ND
			Yellow		
MD 0304 WN-46	0.24e ± 0.00	0.30c ± 0.00	0.80j ± 0.01	1.34h ± 0.01	ND
MD 05-6073	0.12a ± 0.01	0.13a ± 0.00	0.34a ± 0.05	0.60a ± 0.05	ND
MD 05-6077	0.12a ± 0.00	0.17ab ± 0.00	0.36ab ± 0.00	0.65ab ± 0.00	ND
MD 05-6079	0.19cd ± 0.00	0.20b ± 0.00	0.50fgh ± 0.01	0.89ef ± 0.01	ND
MD 06-5433-1	0.22d ± 0.00	0.21b ± 0.00	0.50efg ± 0.01	0.93de ± 0.01	ND
MD 06-5433-3	0.17b ± 0.00	0.17ab ± 0.00	0.41abc ± 0.01	0.75bc ± 0.00	ND
MD 06-5445-5	0.17b ± 0.00	0.20ab ± 0.00	0.42bcd ± 0.00	0.79bcd ± 0.00	ND

[a] Values based on triplicate readings. Mean ± SD shown. Values in the same column marked by the same letter are not significantly different ($P \leq 0.05$). ND, not detected.

higher total isoflavone content than the other three black soybeans ($P \leq 0.05$). Peking black soybeans had about the same amounts of glycitein and daidzein, whereas Pi 88788 black soybeans had twice as much glycitein as daidzein, although total isoflavone contents did not differ between the two (**Table 6**). Although overall trends may exist in isoflavones based on color, it cannot be assumed that any one color contains a specific level. Consequently, the genotype of soybean for use in a high-isoflavone food must still be evaluated individually and not solely by seed coat color.

These data indicated that black soybeans may serve as an excellent dietary source of isoflavones, which have been shown to slow or inhibit the development of a variety of chronic diseases (cardiovascular disease, various cancers, among others), in addition to their ability to lessen the symptoms of menopause. Because of this, black soybeans may serve as a valuable ingredient in functional foods targeting these diseases and life stage.

Cyanidin-3-Glucoside Contents. Recently, nine anthocyanins were characterized in the seed coat of black soybeans, and Cy-3-glc was shown to be the predominant anthocyanin compound in black soybean seed coats and was detected in whole black soybeans (*10*, *19*, *20*). In the present study, all soybean samples were examined for their Cy-3-glc contents, which were detected in all soybeans with black seed coats, but not in other colors. As shown in **Table 6**, Peking contained the highest amount at 0.57 mg/g of flour and was almost double the next nearest sample, Pi 90763. Although Peking contained the most Cy-3-glc of the soybeans tested, Choung and others (*20*) reported its anthocyanin content to be in the middle of a range of 10 black soybeans tested with acidified MeOH as the extraction solvent. Data in **Table 6** also suggested the huge possible variation of anthocyanins among black soybean cultivars and lines.

Soybeans with black seed coats have long been recognized by various Eastern cultures to have health beneficial properties, with attributions of anti-inflammation and antitoxic activities (*9*). In various studies, anthocyanins of black soybean seed coats were able to suppress lipid peroxidation in human low-density lipoproteins (LDL), inhibit the proliferation of HT-29 human colon cancer cells, and reduce cyclooxygenase-2 (COX-2) mRNA level and the number of aberrant crypt foci numbers in F344 rats (*7*, *9*). Black soybean anthocyanins have also been shown to mediate the damage incurred by ischemic myocardial infarction in rats (*27*) and by ultraviolet B radiation in both human and mice skin cells (*28*). Various components of black seed coat soybeans have been implicated as having antiobesity properties, including peptides (*8*, *29*) and anthocyanins (*30*). Taken together, identification and/or development of black soybean lines rich in anthocyanins may well be an approach to enhance their farm gate value through use in health beneficial functional foods.

Total Phenolic Content and Antioxidant Activities. It is prudent to perform a variety of antioxidant activity tests on samples to gain a broad perspective of the antioxidant properties of the samples. TPC, ORAC, HOSC, and ABTS radical cation scavenging capacity were chosen for this study because of their activities as discussed here: The TPC assay is used to compare the reducing power of a sample compared to a standard phenolic, in this case gallic acid. ORAC is perhaps the most commonly used antioxidant activity assay and utilizes competitive kinetics between a fluorescent probe and the sample's antioxidants to provide information on a sample's ability to scavenge peroxyl radicals through a hydrogen atom transfer. The HOSC assay is a relatively new assay compared to ORAC, but uses a similar method to estimate a sample's ability to scavenge hydroxyl radicals. ABTS radical cation scavenging capacity measures the ability of a sample to reduce the nonphysiological radical.

All soybeans sampled contained significant levels of phenolics, and the black seed coat soybeans exhibited much higher levels of TPC than soybeans of the other seed coat colors (**Figure 1**).

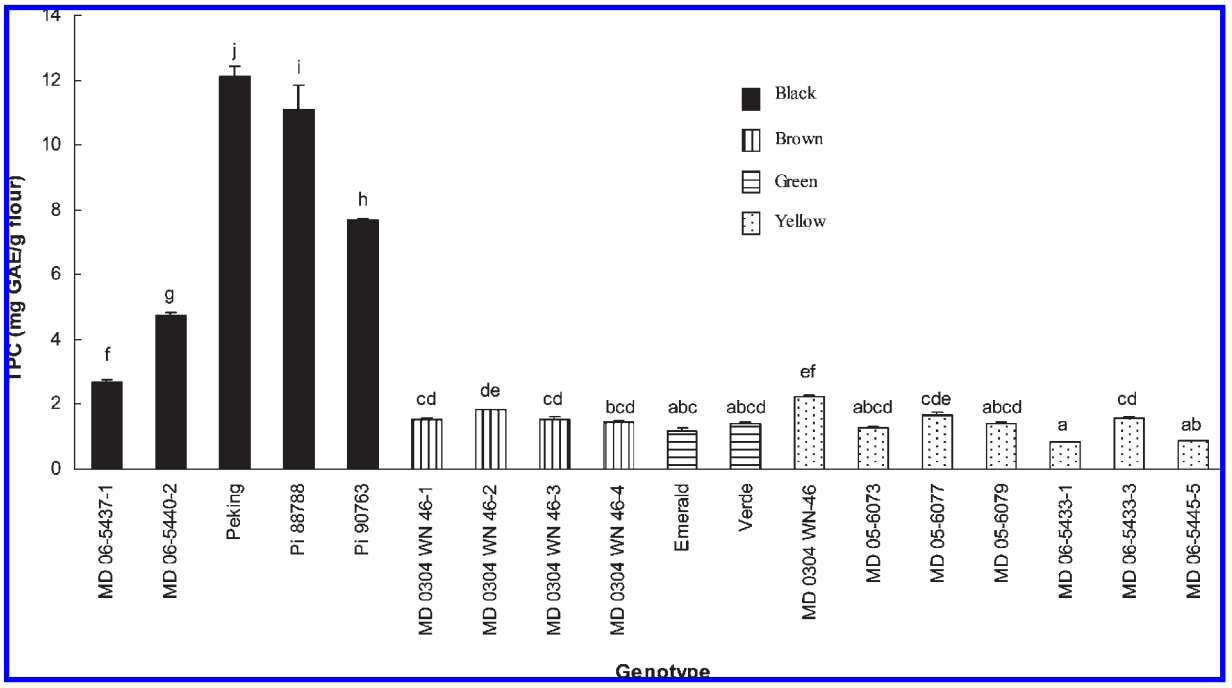

Figure 1. Total phenolic contents of colored seed coat soybean extracts. Values are based on triplicate tests and expressed as milligrams of gallic acid equivalents per gram of soybean flour. Means and SD are shown ($n = 3$). Genotypes marked by the same letter are not significantly different ($P \leq 0.05$).

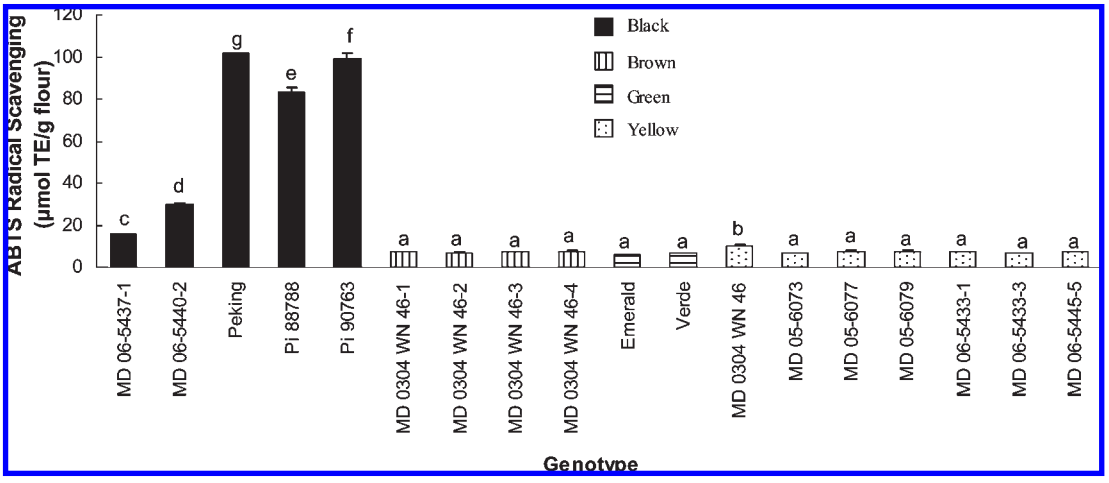

Figure 2. $ABTS^{+}$ radical scavenging capacity of colored seed coat soybean extracts. Values are based on triplicate tests and expressed as micromoles of Trolox equivalents per gram of soybean flour. Means + SD are shown ($n = 3$). Genotypes marked by the same letter are not significantly different ($P \leq 0.05$).

Peking had the highest TPC value of 12.1 mg of gallic acid equivalents (GAE)/g of flour, and MD 06-5437-1 with black seed coat had about 2.7 mg of GAE/g of flour, which was the lowest among all black soybeans. The soybeans with brown, green, and yellow seed coats had values between 0.8 and 2.2 mg of GAE/g of flour, with most values clustered between 1.2 and 1.8 mg of GAE/g of flour.

All soybeans sampled also contained scavenging capacities against peroxyl (ORAC), hydroxyl (HOSC), and $ABTS^{+}$ radicals, and black seed coat soybeans exhibited significantly higher antioxidant activities ($P \leq 0.05$) than soybeans of the other seed coat colors in all three assays (**Figures 2−4**). Peking black soybeans, which had the highest TPC, also had the highest $ABTS^{\bullet+}$ scavenging capacity of 102 μmol of TE/g of flour, whereas Pi 88788 had the greatest ORAC value of 255 μmol of TE/g of flour and a HOSC value of 238 μmol of TE/g of flour. MD 0304 WN-46 yellow soybeans had significantly stronger radical scavenging capacities against all three tested radicals than other yellow, green, or brown soybeans. The values for TPC, ORAC, HOSC, and $ABTS^{\bullet+}$ scavenging capacity were in very strong agreement—each of these four tests was significantly ($P < 0.01$), strongly ($r > 0.92$), and positively correlated to each other test.

These data agreed with previously published studies in both approximate values and the magnitude of differences seen between black soybeans and other color soybeans for both TPC and antioxidant activity, measured in other studies by ORAC, DPPH radical scavenging capacity, and FRAP assays (*5*, *10*, *13*, *26*, *31*). Limited data were available on values of $ABTS^{\bullet+}$ scavenging capacity and HOSC for soybeans. The HOSC assay was chosen in this study because it is the only radical scavenging capacity assay validated for radical purity and concentration consistency, and it is also performed under the physiological pH of 7.4 (*24*).

Soybeans with black seed coats have repeatedly been shown to have higher antioxidant contents than those with other seed coat colors in almost every antioxidant measurement that is commonly

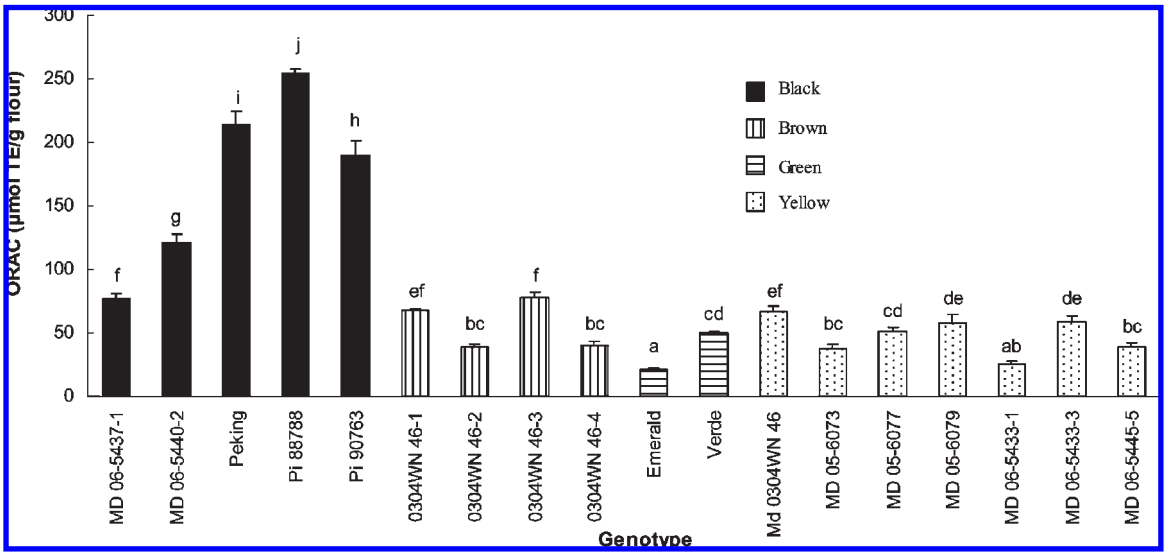

Figure 3. Oxygen radical absorbance capacity of colored seed coat soybean extracts. Values are based on triplicate tests and expressed as micromoles of Trolox equivalents per gram of soybean flour. Means + SD are shown ($n = 3$). Genotypes marked by the same letter are not significantly different ($P \leq 0.05$).

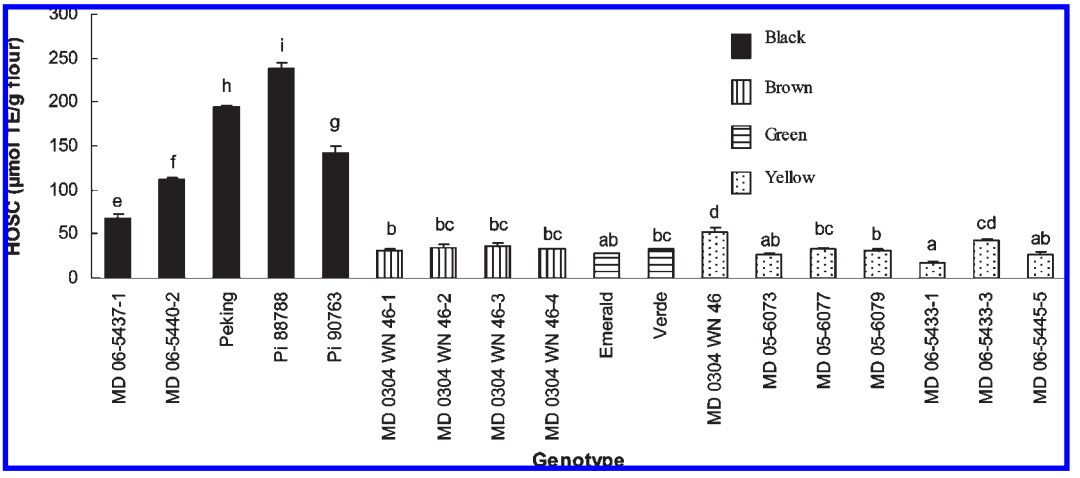

Figure 4. Hydroxyl radical scavenging capacity of colored seed coat soybean extracts. Values are based on triplicate tests and expressed as micromoles of Trolox equivalents per gram of soybean flour. Means + SD are shown ($n = 3$). Genotypes marked by the same letter are not significantly different ($P \leq 0.05$).

used: TPC, ORAC, DPPH radical scavenging capacity, and FRAP (*5*, *6*, *26*, *31*). Worthy of note, the vast majority of isoflavones have been shown to be found in the cotyledon and germ, not the seed coat (*10*), and some studies have found fewer isoflavones in black seed coat soybeans as compared to other colors (*10*, *26*). The difference in antioxidant activity arises at least in part from the presence of anthocyanins in the seed coats, which only black seed coats contain (*11*). They serve as both pigment and antioxidant. In addition, previous research has shown that brown and black seed coat soybeans contain proanthocyanins (*11*), which may contribute to the overall health properties of black and brown soybeans, although they were not measured in the present study.

Antiproliferative Activity against HT-29 Cells. In the present study, two black soybean samples (Peking and Pi 88788), one brown soybean (MD 0304 WN 46-1), one green soybean (Emerald), and one yellow soybean sample (MD 0304 WN-46) were selected because of their stronger antioxidant activities within their same seed coat color groups and investigated for their potential antiproliferative effect using HT-29 human colon cancer cells. The antiproliferative effects of five soybean extracts were compared to the control containing only solvent and to the isoflavone mixture, at three different concentrations shown in **Table 1**. The isoflavone mixture contained daidzein, glycitein, and genistein at the same levels that were present in the Pi 88788 soybean extract, which had the highest total isoflavones among all tested soybean samples.

Figure 5A shows the comparison of cell number after 48 h of treatment by unhydrolyzed extracts, whose isoflavones are therefore mostly in their original glycoside forms on the same per soybean flour weight basis. **Figure 5B** shows cell number after 48 h treatment by the hydrolyzed extracts, which are therefore in their aglycone forms. As seen in **Figure 5A**, Peking soybean extract at the high dose of 15 mg of soy flour equiv/mL is the only unhydrolyzed extract to significantly ($P \leq 0.05$) suppress HT-29 cell proliferation after 48 h of treatment, whereas the isoflavone mixture and unhydrolyzed Pi 88788 black soybean extract at the high testing dose did not produce a statistically significant reduction in cell number until 72 h of treatment time (data not shown). Among the hydrolyzed extracts, the highest treatment level (15 mg of flour equiv/mL) of black Peking and Pi 88788, yellow MD 0304 WN-46, and brown MD 0304 WN-46-1 soybeans, and the medium treatment level (5 mg of flour equiv/mL) of Peking and MD 0304 WN-46 significantly ($P \leq 0.05$) reduced cell number at 48 h of treatment

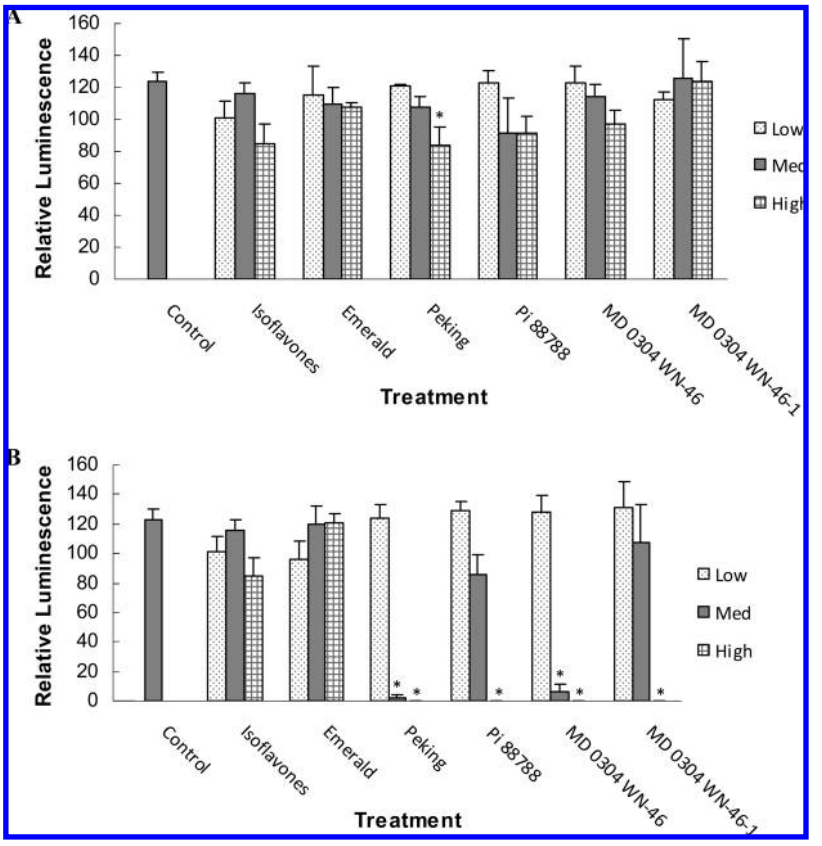

Figure 5. Antiproliferative effects of hydrolyzed and unhydrolyzed soy extracts. Data were collected at 48 h of treatment: (**A**) treated by glycoside forms of soybean extracts; (**B**) treated by aglycone forms of soybean extracts (hydrolyzed). Isoflavone treatment contains genistein, daidzein, and glycitein. Low, medium, and high doses of soybean extracts represent 1.5, 5, and 15 mg of flour equiv/mL treatment, with isoflavone concentrations of each shown in **Table 1**. The isoflavone standard was designed to mimic the concentration of isoflavones in Pi 88788. Relative luminescence is proportionate to the number of viable cells. Values are based on triplicate tests, with mean + SD shown ($n = 3$). Columns marked by an asterisk are significantly different from the control ($P \leq 0.05$).

(**Figure 5B**). Data in **Figure 5** suggest that soybeans with different seed coat color may differ in their antiproliferative components. Data in **Figure 5** also suggest that the hydrolyzed, aglycone forms of soybean extracts might be more effective in suppressing HT-29 colon cancer cell growth than their corresponding glycosides on the same per molar concentration basis. This may be partially explained by the greater cellular availability of the aglycones because of the reduced water solubility and polarity, rendering them better able to cross the cell membrane and exert effects intracellularly.

The hydrolyzed, aglycone form of the Peking, Pi 88788, MD 0304 WN-46, and MD 0304 WN 46-1 extracts and the soy isoflavone mixture suppressed HT-29 cancer cell proliferation in a dose- and time-dependent manner (**Figure 6**). At the high treatment dose, the aglycone forms of black Peking and Pi 88788, brown MD 0304 WN46-1, and yellow MD 0304 WN-46 soybean flour extracts significantly ($P \leq 0.05$) reduced cell number after 3 h of treatment (**Figure 6B**−**E**). The aglycone forms of Peking soybean extracts also reduced cell number significantly ($P \leq 0.05$) in the medium dose (5 mg of defatted flour equiv/mL) at 3 h of treatment, whereas the only other medium dose to significantly ($P \leq 0.05$) affect antiproliferative capacity was the yellow MD 0304 WN-46 after 24 h of treatment (**Figure 6B**,**E**). No treatment displayed any inhibitory effect at the low dose at any time under the experimental conditions.

The isoflavone standard mixture (**Figure 6A**) did not produce as effective a reduction in cell number as Pi 88788, whose isoflavone concentration it was designed to mimic (**Figure 6C**), nor were its effects as fast. The isoflavone standard mixture produced a statistically significant reduction in cell number by 72 h, as compared to 3 h by the actual Pi 88788 aglycone extract. Also, by 72 h the Pi 88788 aglycone extract had essentially eliminated viable cells to 0% of the vehicle, whereas the isoflavone mixture reduced cell numbers to only 60% of the vehicle.

Interestingly, the green Emerald soybean had isoflavone levels similar to or greater than those of the yellow MD 0304 WN-46 and brown MD 0304 WN-46-1 soybeans, but had no detectable antiproliferative activity at any dose at any time under the experimental conditions (**Figure 6F**), whereas both yellow MD 0304 WN-46 and brown MD 0304 WN-46-1 soybean extracts significantly ($P \leq 0.05$) suppressed HT-29 cell growth (**Figures 5B** and **6D**,**E**). In addition, the MD 0304 WN-46 soybean extract had the lowest total isoflavones, which was approximately 25% of that in the isoflavone mixture, but its aglycone form elicited significant reduction in cell number after 3 h of treatment at both the medium and high doses, whereas the isoflavone mixtures did not significantly ($P \leq 0.05$) inhibit cell proliferation until 72 h of treatment at the high dose. These data suggested that soybean lines and cultivars with the same seed coat color might differ in their antiproliferative activities and that other factors in soybeans beyond isoflavones and cyanidin may contribute to the overall antiproliferative activity of soybean flour extracts.

Finally, the isoflavone mixture was broken down into its individual components to test which may be contributing to the antiproliferative activity. Additionally, cyanidin was introduced to the mixture to mimic the concentration of the predominant anthocyanidin in addition to the isoflavones of the two black soybean extracts present after hydrolysis. Concentrations of treatment media are shown in **Table 2**. Results at 96 h are

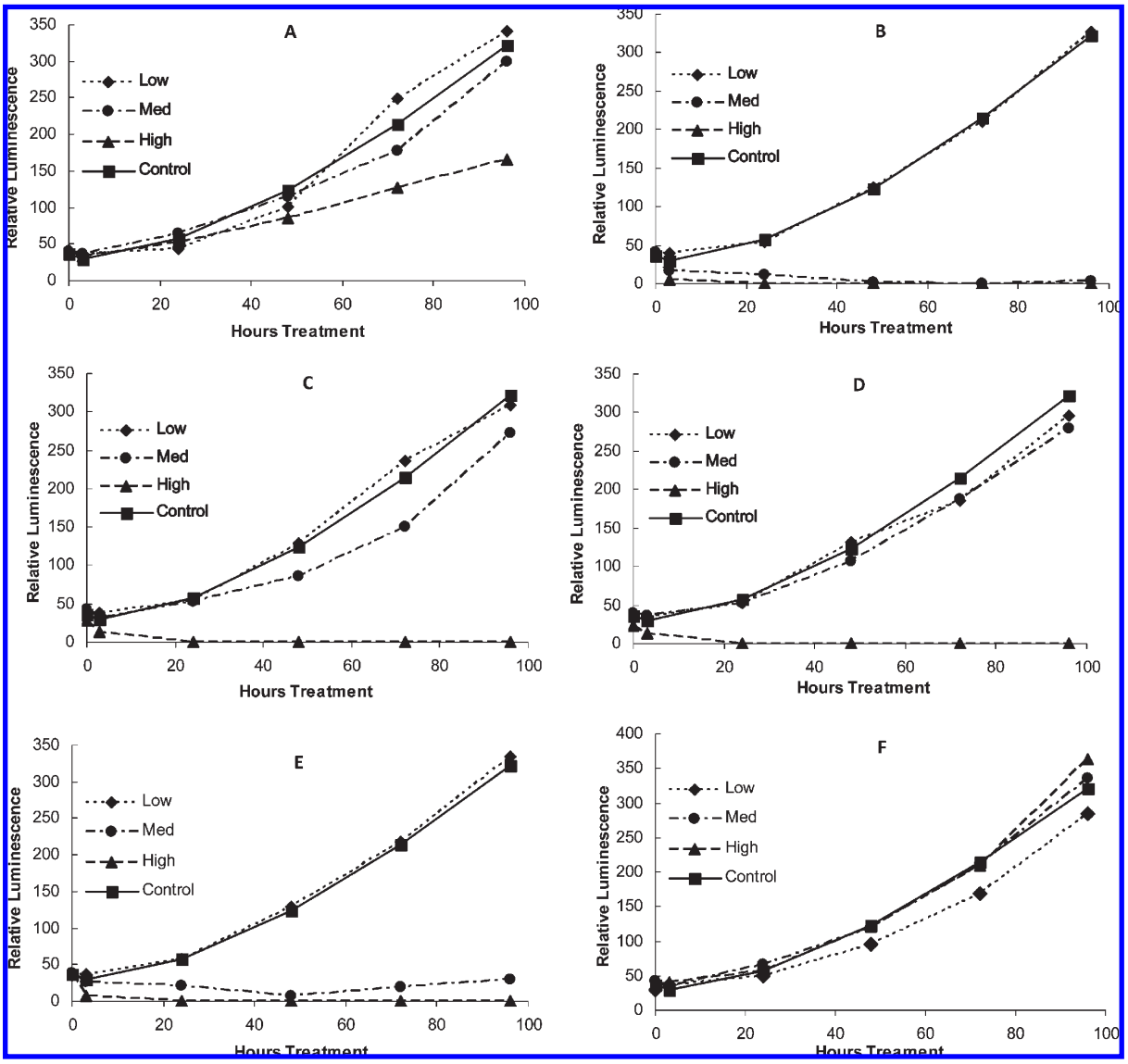

Figure 6. Time and dose effects of soy isoflavones and hydrolyzed soybean extracts on HT-29 cell proliferation:(**A**) soy isoflavones consisting of genistein, daidzein, and glycitein; (**B**) black Peking; (**C**) black Pi 88788; (**D**) brown MD 0304 WN46-1; (**E**) yellow MD 0304 WN-46; and (**F**) green Emerald extracts were hydrolyzed with base prior to cell treatment to convert all isoflavones to aglycone form. Relative luminescence is proportionate to the number of viable cells. Values are based on triplicate tests, with mean values shown ($n = 3$). Low, medium, and high doses of soybean extracts represent 1.5, 5, and 15 mg of flour equiv/mL treatment, with isoflavone concentrations of each shown in **Table 1**. The isoflavone standard was designed to mimic the concentration of isoflavones in Pi 88788.

presented in **Figure 7**. Every combination of two or three isoflavones produced significant reduction in HT-29 cell proliferation at 96 h. Glycitein was the only individual isoflavone to produce significant cell reduction in 96 h. This is likely because the glycitein concentration was approximately twice that of genistein or daidzein in the treatments (designed to mimic the concentrations seen in Pi 88788). However, it may be important to note that although all of the combination treatments were successful at inhibiting cell proliferation versus the control, they were not significantly different from each other ($P \leq 0.05$). In other words, the glycitein treatment contained less total isoflavones than any of the combination treatments, but it was similarly effective at reducing cell proliferation. A difference was seen in the time required to significantly ($P \leq 0.05$) reduce cell proliferation—the Peking and Pi 88788 mimics with isoflavones and cyanidin elicited a significant inhibition of cells by 48 h, whereas the mixture of three isoflavones and glycitein alone exhibited significant inhibition in 72 h. These data suggested that variations within a certain range of isoflavone type and dose, in combination with cyanidin, may elicit the same ultimate response by cells.

Previous work has suggested that isoflavones and anthocyanidins independently inhibit cancer cell growth in their aglycone form in in vitro studies (*32*−*34*). Taking into account the data in **Figures 5**−**7**, the present study suggests that soybeans contain antiproliferative components, although their antiproliferative activities may not be fully explained by their isoflavone content, cyanidin content, and seed coat color.

This study is fundamentally limited in its analysis of only one crop year. It is well-accepted that environmental conditions (temperature, solar radiation levels, soil composition, water availability, etc.) affect the nutritional and phytochemical composition of crops, including soybeans. For example, soy isoflavone levels have been shown to be depressed during growth at higher temperatures (*35*). For this reason, additional studies analyzing multiple crop years may serve to identify if the trends noted here hold true across a variety of growing conditions.

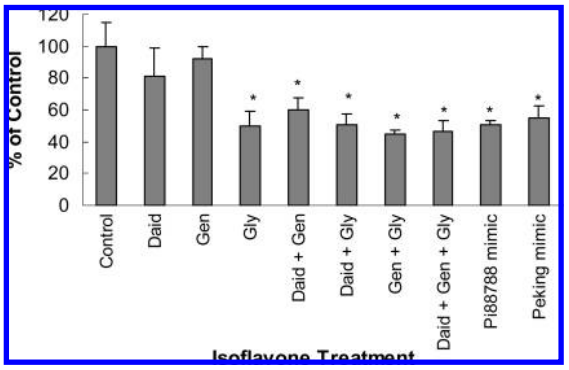

Figure 7. Effects of cell isoflavone treatments at 96 h. Daid, daidzein; Gly, glycitein; Gen, genistein. Treatment concentrations are shown in **Table 2**, with high doses shown here. All treatments, including control, contained 1.2% DMSO. Values are based on triplicate tests, with mean ± SD shown. Columns marked by an asterisk are significantly different from the control ($P \leq 0.05$).

There has been a question if the bioactivity and prevention of diseases by soybeans have been falsely attributed to any individual phytochemical or component. Data from this study suggest that whereas black soybeans may have higher levels of isoflavones, anthocyanins, and antioxidant activity, their health beneficial property as a whole may not increase proportionately with these levels. More studies are necessary comparing the effects of isolated soy components with those of the whole bean or bean part.

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