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Characterization and Comparison of Antioxidant Properties and Bioactive Components of Virginia Soybeans

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Nine Virginia soybeans grown in a single location were evaluated and compared for their antioxidant properties and isoflavone profiles. The total phenolic content (TPC) in the soybean extracts was significantly different among different genotypes. The V01-4937, V03-1144, and MFS-511 soybeans had the highest TPC values of 3.89, 3.63, and 3.53 mg of gallic acid equiv/g of seeds, respectively. The isoflavone composition was also different among the different soybean varieties. Malonylgenistin was the major isoflavone in all soybean seeds, accounting for 75−83% of the total measured isoflavones. The V01-4937 variety had the highest total isoflavones and malonylgenistin content followed by the V03-5794. The antioxidant activities of the soybean extracts were also significantly different. V01-4937 and Teejay showed the strongest ORAC values, which were 70% higher than that of the V00-3493 soybean, which had the lowest ORAC value (115.7 μmol of Trolox equiv/g of seeds). However, their ORAC values were correlated with neither TPC nor total isoflavone content. The MFS-511, V01-4937, and Teejay soybeans had the highest DPPH radical scavenging activities of 4.94, 4.78, and 4.64 μmol of Trolox equiv/g of seeds. Overall, the V01-4937 soybean stood out among the tested Virginia soybeans with regard to having the highest TPC, ORAC value, and isoflavone content as well as the second highest DPPH scavenging activity.

KEYWORDS: Soybean antioxidant; isoflavones; TPC; ORAC

INTRODUCTION

Human consumption of soybean and soy-based products has been soaring recently, with a growth in sales from $300 million to $3.9 billion from 1992 to 2006 (*1*). This trend has been in line with the increasing volume of research linking soybean consumption with lower serum total and LDL cholesterol in humans (*2*) and the reduced risk of certain types of cancers, particularly prostate and breast cancer (*3, 4*). The diverse potential health benefits of soybean consumption have prompted scientists to further investigate specific bioactive ingredients in the soybean. Soybean isoflavones have become one of the most investigated food functional ingredients with a wide variety of beneficial activities being revealed in in vitro and clinical studies. Along with isoflavones, soybean antioxidants have also been receiving increased attention.

Soybean and soy products contain significant amounts of isoflavones known as aglycones as genistein, glycitein, and daidzein and their glycosides β-glucosides, 6″-*O*-acetyl-β-glucosides, and 6″-*O*-acetyl-β-glucosides. It has been postulated that the purported health benefits of soy products are in part due to isoflavone estrogenic activity or antioxidant activity (*5*). The structural similarities of soy isoflavones to estrogens make these bioactive compounds a unique group of phytoestrogens that may protect against hormone-dependent cancers and produce immune effects by modulating the activity of estrogen (*6*). Furthermore, as a group of natural flavonoids, soy isoflavones have also shown significant antioxidant activities by inhibiting lipid oxidation (*7*), scavenging free radicals, and promoting the expression of antioxidative enzymes (*8*). In addition to isoflavones, soybeans contain a number of other natural antioxidants such as caffeic acid, chlorogenic acid, and ferulic acid, tannins, and proanthocyanidins (*9*). Soy antioxidant extracts were shown to reduce low-density lipoprotein (LDL) oxidation and exert oxygen radical absorbance capacity (ORAC),

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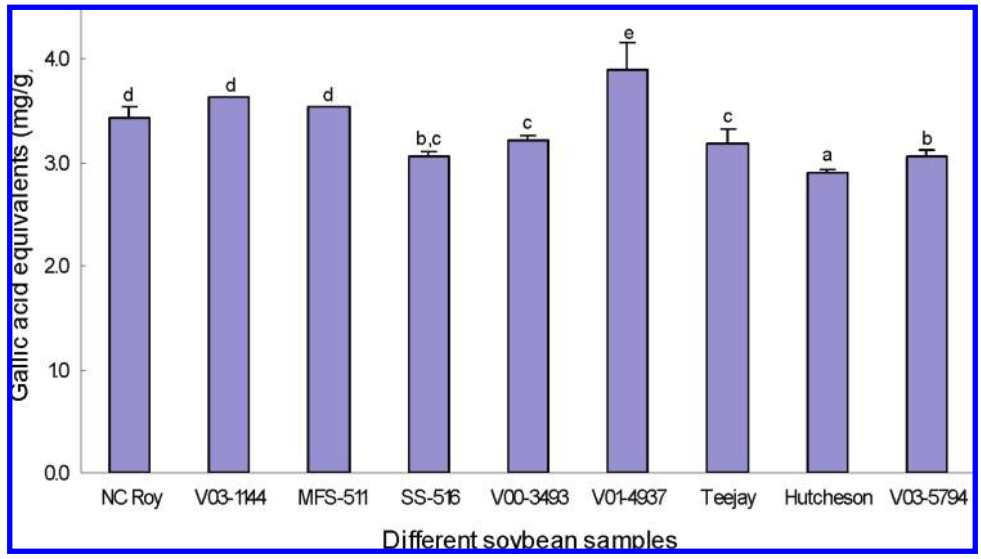

Figure 1. Total phenolic content (TPC) of Virginia soybean samples. Results are expressed as milligrams of gallic acid equivalents (GAE) per gram of soybean seed (mean ± SD, $n = 3$). Bars marked by the same letter are not significantly different ($P < 0.05$).

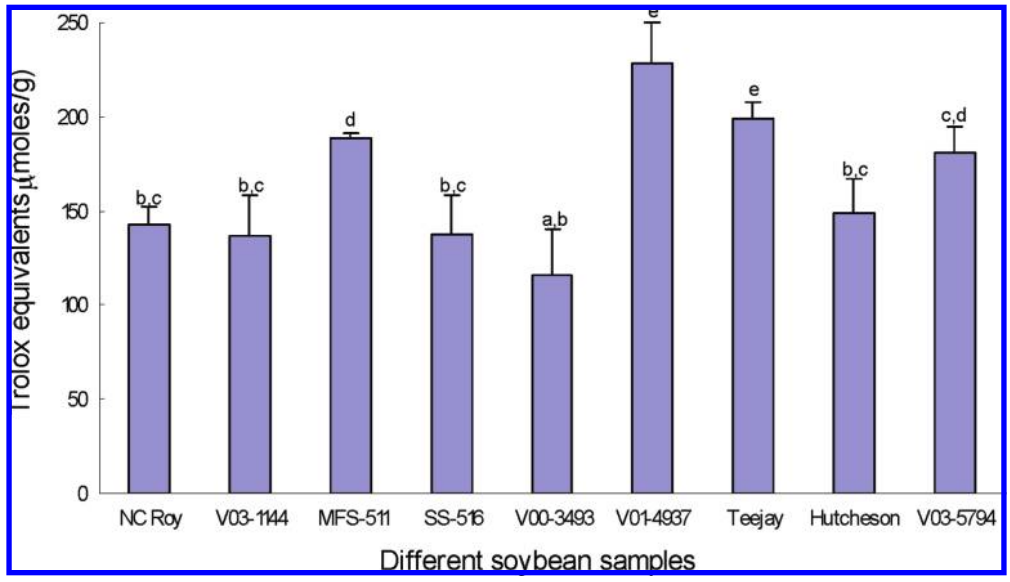

Figure 2. Oxygen radical absorbing capacity (ORAC) of Virginia soybean samples. Results are expressed as micromoles of Trolox equivalents per gram of soybean seed (mean ± SD, $n = 3$). Bars marked by the same letter are not significantly different ($P < 0.05$).

ferric reducing antioxidant power (FRAP), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity (*10*).

It was known that both soybean isoflavones and antioxidant properties can be significantly affected by their genotypes (*11, 12*). For instance, 14 Brazilian soybeans had >3 times variation in their isoflavone content and more than twice the difference in their total phenolics content (*13*). These findings suggested the possibility of identifying and developing premium soybean varieties rich in isoflavones and natural antioxidants for human consumption with enhanced health benefits. Soybeans are Virginia's largest row crop with production of 15 million bushels in 2005, which contributed over $85 million to the local agricultural economy. However, little is known about the isoflavone composition and other beneficial components in Virginia soybeans or their associated antioxidant properties. The present study was undertaken to characterize the isoflavone and phenolic content of nine soybean varieties grown in Virginia and to provide information related to their antioxidant characteristics. This project is part of our continual effort toward the development of Virginia soybean cultivars with increased levels of isoflavones and/or natural antioxidants, which may potentially benefit Virginia soybean growers and the local agricultural economy.

MATERIALS AND METHODS

Materials. The nine soybean varieties used in this experiment were grown in Warsaw, VA, by a soybean breeding project at Virginia Polytechnic Institute and State University and harvested in 2006. MFS-511, V00-3493, and V01-4397 are small-seeded varieties that could be used for food grade breeding. SS-516 is a large-seeded food grade variety. Teejay and Hutcheson are varieties that have been used for more conventional soybean uses such as meal and oil. They are not considered to be of food grade. Folin−Ciocalteu reagent, fluorescein (*14*), 2,2′-bipyridyl, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and phenolic acid standards were purchased from Sigma-Aldrich (St. Louis, MO), and 2,2′-azobis(2-aminopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). Isoflavone standards (daidzin, genistin, malonylgenistin, daidzein, glycitein, and genistein) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were of analytical or HPLC grade.

Sample Preparation. Five grams of each soybean sample was ground to a fine powder using a micromill and extracted with 50 mL of 50% acetone for 15 h by shaking at ambient temperature (*15*). The extracts were filtered with a 1.5 μm filter paper and kept in the dark until analyses. The soybean extracts were then subjected to a variety of antioxidant evaluations. To determine the isoflavone composition, the milled soybean seeds were extracted by the mixture of 0.1 N hydrochloric acid, acetonitrile, and distilled water (2:7:3, v/v/v) (*16*). After the extraction, the solution was centrifuged, and the supernatant was filtered and collected. The filtration was then evaporated under nitrogen gas. The residue was reconstituted in 1 mL of methanol and filtered with a 0.45 μm disk filter prior to HPLC analysis.

Total Phenolic Content (TPC). The TPC of soybean extracts were determined using Folin−Ciocalteu reagent with gallic acid as phenolic standard (*17*). In brief, the appropriate dilutions of extracts were mixed with Folin−Ciocalteu reagent and 20% sodium carbonate (Na_2CO_3) at ambient temperature. After reaction incubation for 2 h, the blue color developed in each assay mixture and the absorbance was recorded at 760 nm (Thermo Electron Corp., Genesys 10-UV scanning, Madison, WI). The TPC value of the soybean extracts was expressed in micrograms of gallic acid equivalent (GAE) per gram of soybean (mg/g).

Oxygen Radical Absorbance Activity (ORAC). The $ORAC_{FL}$ assay was conducted to measure the peroxyl radical scavenging activity of soybean samples with Trolox as an antioxidant standard according to the method reported previously (*18*). In brief, a fluorescein stock solution (100 μM) in phosphate buffer (75 mM, pH 7.4) was prepared and kept at 4 °C in the dark. A fresh working fluorescein solution (100 nM) was prepared daily by diluting the stock solution in phosphate buffer. Two hundred microliters of the working fluorescein solution was added to each 40 μL of sample or Trolox standard (a water-soluble analogue of vitamin E) prepared in the phosphate buffer (20, 40, 80,100, and 200 μM) in a black 96-well plate and incubated for 20 min at 37 °C. The assay was initiated by adding the peroxyl radical generator prepared in the phosphate buffer. Specifically, 35 μL of 0.36 M AAPH was added, and the fluorescence was measured (λ_{ex} = 485 nm and λ_{em} = 535 nm) every minute using a Victor^3 multilabel plate reader (Perkin-Elmer, Turku, Finland) maintained at 37 °C until the reading had declined to <5% of the initial reading. Standards and samples were run in triplicate. Results for ORAC were determined by using a regression equation relating Trolox concentrations and the net area under the kinetic fluorescein decay curve. The $ORAC_{FL}$ value of each soybean extract was expressed in micromoles of Trolox equivalents per gram of sample (μmol/g).

DPPH$^\bullet$ Scavenging Activity. Antioxidant activity of soybean samples was tested using the radical DPPH assay. This high-throughput assay, based on the reduction of the free radical DPPH$^\bullet$, was carried out using a Victor^3 multilabel plate reader (Perkin-Elmer). To begin, the reaction mixture contained 100 μL of antioxidant soybean extracts and 100 μL of 0.208 mM DPPH$^\bullet$ solution. The absorption at 515 nm was determined immediately when the reaction was initiated by gentle shaking. Each plate was read once every minute for 1.5 h. The relative DPPH$^\bullet$ scavenging capacities were expressed as micromoles of Trolox equivalents (TE) per gram of sample (μmol/g).

HPLC Analysis of Isoflavone Composition. Isoflavone profile in the soybean extracts was performed on an Agilent 1200 quaternary HPLC system (Agilent Technologies, Palo Alto, CA) equipped with a photodiode array detector. The isoflavone standards were separated on a Phenomenex Luna C18 column (250 mm × 4.6 mm, particle size = 5 μm) using a linear gradient elution program with a mobile phase containing solvent A (0.1% glacial acetic acid in H_2O) and solvent B (0.1% glacial acetic acid in acetonitrile) (*19*). The solvent gradient was linearly programmed from 15 to 35% solvent B in 50 min with a flow rate of 1.0 mL/min. Identification of isoflavones in each soybean sample was accomplished by comparing the retention time and absorption spectra of peaks in the extracts to those of the standard compounds. Quantification of individual isoflavone was conducted using total area under each peak with external standards.

Statistical Analysis. Data were reported as mean ± SD for triplicate determinations. The mean values within each test were compared by a two-sample *t* test. Data are presented as mean ± standard deviation (SD). Significance of variety differences was determined by analysis of variance. Difference was considered to be statistically significant when the *P* value was <0.05. A two-tailed Pearson's correlation test was conducted to determine the correlations among means.

RESULTS AND DISCUSSION

Total Phenolic Content. Natural phenolic compounds have been receiving increased attention due largely to their notable antioxidant activities. The unique structures make the phenolic compounds inherently excellent electron or hydrogen donors, which enable them to readily stabilize some reactive oxygen species (ROS) (*20*). In fact, phenolic compounds have been shown to effectively inhibit lipid oxidation of LDL, liposome, and food model systems by interacting with transitional metals and free radicals such as peroxyl, hydroxyl, and superoxide radicals (*21, 22*).

TPC of the extracts from selected soybeans is presented in **Figure 1**. The tested soybeans had a TPC range of 2.9−3.9 mg of gallic acid equivalents (GAE)/g of fresh weight, with most falling in the range of 3.2−3.6 mg of GAE/g. The TPC values were significantly different among different soybean varieties. In particular, the V01-4937 soybean had the highest TPC of 3.9 mg of GAE/g, whereas Hutcheson was the lowest with 2.9 mg of GAE/g. This distinct difference may be attributable to their genetic variation as all of the soybean samples were collected from a single growing location, which minimized environmental influence. Among the tested Virginia soybeans, the Hutcheson, V03-5794, and SS-516 varieties had the lowest TPC values, which ranged between 2.9 and 3.0 mg of GAE/g. V01-4937 had an exceptionally higher TPC than other eight varieties, indicating that this specific soybean may have unique genetic characteristics in favor of producing phenolic compounds. Overall, the TPC range of the tested Virginia soybeans was comparable to previously reported values determined in the seeds of 20 soybean hybrids (2.7−4.9 mg/g) (*9*) and in 6 yellow soybean seeds (3.0−4.5 mg/g) (*23*). Lin et al. also reported that black soybeans had significantly higher total phenolic contents than yellow soybeans did (*23*).

Oxygen Radical Absorbance Activity. ORAC measures the ability of the soybean extracts to scavenge peroxyl radicals generated in an aqueous solution. The ORAC values of the selected soybeans are expressed as micromoles of Trolox equivalents (TE) per gram (**Figure 2**). Different soybeans showed significantly different ORAC values which varied from 115.7 to 228.6 μmol of TE /g. The V01-4937 soybean had the highest value at 228.6 μmol of TE/g, the only soybean variety in our study with >200 μmol of TE/g. This exceptionally high ORAC value of V01-4937 may be associated with its highest TPC among the tested varieties. However, there was no significant correlation between the ORAC and TPC of the soybeans in our experiments. The V00-3493 soybean had the lowest ORAC value (115.7 μmol of TE/g), less than half that of V01-4937. Other soybeans with significantly lower ORAC values were the V03-1144, SS-516, NC Roy, and Hutcheson varieties at 136.7, 137.3, 142.5, and 149.0 μmol of TE/g, respectively. The difference of ORAC values strongly suggests that soybean varieties may significantly affect their antioxidant activities against peroxyl radicals. Current reports on the ORAC data of soybean seeds are scarce. Xu and Chang recently reported a range of 40.81−86.84 μmol of TE/g in the various extracts of a yellow soybean (*10*). However, our ORAC results for the Virginia soybeans were considerably higher; this may be due to the different sample preparations as well as the effect of soybean varieties and growing environment.

DPPH$^\bullet$ Scavenging Activity. Other than ORAC, which measures antioxidant activity on the basis of hydrogen transfer mechanisms, the DPPH assay involves electron transfer mecha-

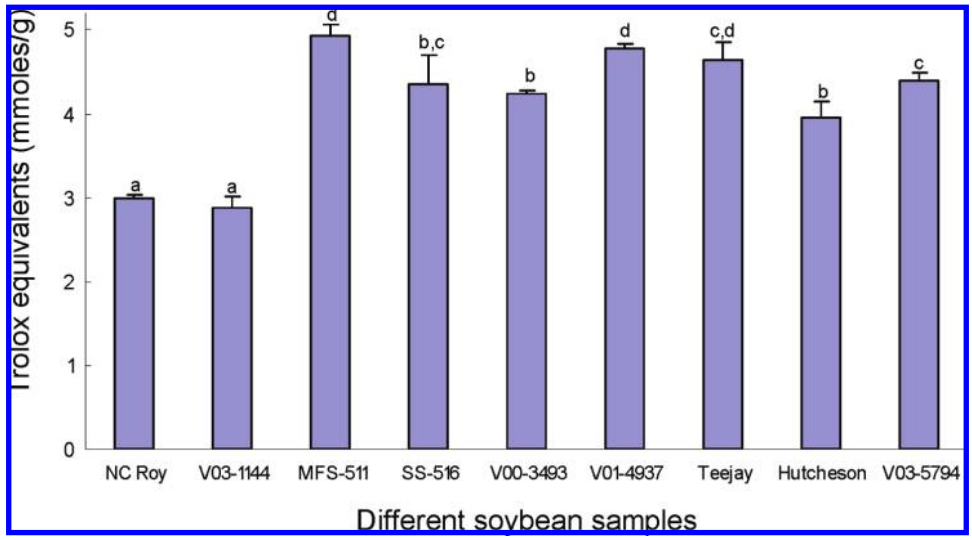

Figure 3. DPPH• radical scavenging activity of Virginia soybean samples. Results are expressed as micromoles of Trolox equivalents per gram of soybean seeds (mean ± SD, $n = 3$). Bars marked by the same letter are not significantly different ($P < 0.05$).

Table 1. Isoflaovone Composition of Virginia Soybean Samples[a]

variety	daidzin (μg/g)	genistin (μg/g)	malonylgenistin (μg/g)	daidzein (μg/g)	genistein (μg/g)	total (μg/g)	malonygenistin/total (%)
NC Roy	275.2e ± 5.5	301.1e ± 2.4	1998.7a ± 12.8	33.4d ± 0.9	27.0f ± 0.5	2635.4	75.8
V03-1144	268.5e ± 12.8	376.2g ± 9.2	2225.8c ± 88.0	32.1cd ± 0.6	29.1g ± 0.0	2931.7	75.9
MFS-511	231.9e ± 2.2	179.8a ± 0.7	2317.8d ± 0.0	42.7e ± 1.7	20.4d ± 1.4	2792.6	83.0
SS-516	212.4d ± 2.3	179.5a ± 8.6	2064.6ab ± 4.5	26.6ab ± 0.8	12.7a ± 1.6	2495.8	82.7
V00-3493	194.4c ± 1.1	198.6b ± 9.6	2244.9c ± 36.8	33.9d ± 2.1	17.8b ± 0.2	2689.6	83.5
V01-4937	251f ± 2.4	340.8f ± 2.8	2539.8e ± 94.2	43.1e ± 0.6	30.4h ± 2.9	3205.1	79.2
Teejay	176.9b ± 1.4	247.7c ± 26.5	2102.4b ± 98.7	26.5ab ± 0.2	16.6b ± 2.4	2570.1	81.8
Hutcheson	152.9a ± 6.9	269.3d ± 10.1	2093ab ± 144.739	27.5bc ± 3.5	23.6e ± 0.5	2566.3	81.6
V03-5794	275.8e ± 2.7	408.4f ± 2.5	2354.9d ± 50.1	50.2f ± 1.8	38.1i ± 4.0	3127.4	75.3

[a] Entries in a column marked by the same letter are not significantly different ($P < 0.05$).

nisms (*24*). This explains that the higher ORAC values of the samples did not necessarily suggest stronger DPPH scavenging activity. Soybean extracts and derived compounds have been shown to be effective scavengers of DPPH radicals (*5, 9*). For instance, Takahashi et al. reported that the IC_{50} values of soybean polyphenols for DPPH radicals were 39 and 34 μg/g for yellow soybean and black soybean, respectively (*25*). The DPPH results of our selected soybeans are expressed as micromoles of Trolox equivalents (TE) per gram (**Figure 3**). The highest DPPH scavenging activity was observed for MFS-511 (4.9 μmol of TE/g), followed by V01-4937 (4.8 μmol of TE/g) and Teejay (4.6 μmol of TE/g). Other soybeans were in the range of 4.0−4.4 μmol of TE/g except for two varieties, NC Roy and V03-1144, which had remarkably lower DPPH values of 2.9 and 3.0 μmol of TE/g, respectively. This range was higher than that of the yellow soybean extracts (0.6−2.0 μmol of TE/g) and significantly lower than that of the black soybean extracts (7.1−17.9 μmol of TE/g) (*10*). The DPPH scavenging activities of the soybeans were significantly corrected with neither ORAC nor TPC values even though V01-4937, MFS-511, and Teejay were the top three varieties identified in both ORAC and DPPH experiments. These results suggest that the V01-4937, MFS-511, and Teejay varieties may potentially provide enhanced health benefits as a result of their stronger antioxidant activities as compared to the other Virginia soybean varieties. Overall, further investigation into the individual soybean antioxidants and their bioactivities is warranted.

HPLC Analysis of Isoflavone Composition. Isoflavones belong to a class of plant compounds called phytoestrogens, which exhibit both estrogenic and antiestrogenic properties in both cell and animal models (*26, 27*). Isoflavones are also a group of flavonoids that showed potent antioxidant properties. The unique chemical and functional properties of isoflavones have widely stimulated research on their potential health benefits. In fact, isoflavones have been associated with the protection of a wide variety of chronic diseases and hormone-related complications such as atherosclerosis (*28*), breast cancer (*29*), osteoporosis, and menopausal symptoms (*30*). Isoflavones are being marketed as dietary supplements, and the main dietary sources of isoflavones are soybeans and soy-based products. However, there is a large variability in isoflavone concentration and profile among the soybeans depending on factors such as their genotypes and environmental conditions.

The isoflavone compositions of the selected soybeans are presented in **Table 1**. The total measured isoflavones were in the range of 2495.8−3205.1 μg/g, which is comparable to the 1563−3309 μg/g reported in 8 American and 3 Japanese soybeans (*31*) and to the 1443.1−3803.6 μg/g detected in 17 Ohio soybeans (*5*). Soybean V01-4937 was found to have the highest total isoflavone content followed by V03-5794 (3127.4 μg/g) and V03-1144 (2931.7 μg/g), whereas the lowest isoflavone content was observed from the soybeans SS-516 (2495.8 μg/g), Teejay (2570.1 μg/g), and Hutcheson (2566.3 μg/g). The isoflavones variation among 11 soybean varieties was relatively smaller than the findings of Kirakosyan et al., who reported that total isoflavones in two American varieties (Cisne and Ripley) and 3three from China varied from 425 to 6115 μg/g (*32*). Both results suggest that soybean genotypes played an important role in the total isoflavone content in the seeds. The isoflavone profile was also different among the different soybeans. Malonylgenistin was the major isoflavone determined in all of the soybean samples, representing 75−84% of the total isoflavones, followed by genistin (6−13%) and daidzin (5−10%), respectively. Daidzein and genistein were

detected in very low amounts. This finding indicated that most soy isoflavones exist in the seeds in the form of glucosides instead of aglycones (*33*). These results are in agreement with previous reports that malonylgenistin was the predominant isoflavone in soybean seeds (*11, 12, 31*). Similar to the total isoflavones, the amounts of the individual isoflavones were also found to be significantly different among the soybeans. For instance, the V01-4937 soybean contained the highest malonylgenistin content (2539.8 μg/g), which was 27 and 23% higher than those of NC Roy and SS-516, respectively. Such significant differences were also reflected in the amounts of genistin and daidzin. The malonylgenistin content in the tested Virginia soybeans was significantly higher than that of 11 soybeans grown in the state of Iowa (290−958 μg/g) (*31*), but comparable to that of 17 Ohio soybeans (1213.1−3048.4) (*5*). These differences may be attributable to soybean varietal difference, growing conditions, or even sample treatment procedures. In brief, our results suggest that the V01-4937 and V03-5794 soybeans could be recommended as potential Virginia varieties with significantly higher isoflavone content.

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