NPC Natural Product Communications

2017
Vol. 12
No. 4
527 - 532

Isoflavone Composition, Total Phenolic Content and Antioxidant Capacity of Soybeans with Colored Seed Coat

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Received: July 15th, 2016; Accepted: December 11th, 2016

The influence of soybean seed coat color and genotype on antioxidant capacity, phenolic content and isoflavone profile was investigated. Isoflavone content and composition of 21 seed samples - yellow, green, brown, black and rarely mentioned ocher and reddish, were determined by high-performance liquid chromatographic analysis. Antioxidant capacity and total phenolic content (TPC) were evaluated spectrophotometrically. Significant correlation between total isoflavone content and total genisteins was established in all colored groups. Total isoflavone content was in the range from 2.11 mg/g in a green wrinkled sample to 5.24 mg/g in yellow seed. It was found that black genotypes had the highest TPC and antioxidant capacity, which were significantly different (p<0.05) from other colored soybeans. The obtained interconnections among analyzed isoflavones can be used as a model for estimation of their specific content.

Keywords: Soybean, Isoflavones, Total phenolic content, Radical-scavenging activity.

Isoflavones, so called phytoestrogens, are compounds with estrogen-like activity that are widely distributed in the seeds and other parts of many plant species of the *Leguminosae* family [1a]. Soybeans are unique among the legumes because they present a concentrated source of isoflavones used in human nutrition [1b].

These compounds are currently receiving much attention because of their potential benefit to human health due to the purported role in preventing and treating cancer, osteoporosis, menopausal symptoms, cardiovascular diseases and other human chronic ailments [1c,2]. It has been reported that soy is an exellent dietary source of natural antioxidants, which is mainly related to its high total phenolic content [3a]. Due to the assumed beneficial effects, use of products and supplements containing soy isoflavones has significantly increased. Soybeans and soy products are the most important sources of phytoestrogens in human diet. Soy contains daidzein, genistein and glycitein aglycons which can form three glucoside forms, a β-glucoside, a 6''-*O*-malonyl-glucoside and a 6''-*O*-acetyl-glucoside [3b].

It has been shown that different factors influence the content and composition of isoflavones in plant sources. There are numerous studies about the influence of genotype, location and crop year or season on isoflavone distribution in soybeans [4,5].Temperature is one of the factors that can influence isoflavone concentration. In a study of Tsukamoto *et al*. [6a] isoflavone content was significantly lower in seeds that developed in higher temperatures than in seeds exposed to low temperatures. The effect of the crop season was investigated in Taiwan, where it was observed that isoflavone contents were significantly higher in the fall crops than in the spring crops, which was mainly related to difference in the ambient temperatures [4]. In an Ohio study, it was confirmed that total isoflavone content was influenced by planting location and cultivar. The only environmental variable that correlated with total isoflavones was rainfall during seed drill [5].

Although environmental factors can have an important effect on isoflavone content, it is considered that the potential for this trait is mainly under genetic control. Hoeck *et al*. [6b] evaluated importance of genotype, year, location, and their combinations on isoflavone content in soybean cultivars. Despite the significant genotype/environment interactions, the performance of two genotypes with the highest and the lowest mean total isoflavone concentration was quite consistent among 16 different environments. These results also suggest that genotype is an important factor which potentially determines total isoflavone content. Some results indicate that content and composition of isoflavones can be derived from parent genotype to its hybrids [6c].

Soybeans can have various seed coat colors, such as yellow, green, brown or black. Commercial soybean cultivars in major soybean growing regions are predominantly yellow. Color pigmentation is due to anthocyanins, chlorophyll, and different combinations of breakdown products of these pigments [6d,6e]. Because of their phenolic structures, anthocyanins, which give the seed coat a dark color, exert a strong radical-scavenging activity and thereby behave as natural antioxidants [7]. It is less known about the influence of seed color on isoflavone content. Recent papers which investigated antioxidant activity, phenolic content or isoflavone concentration in soybeans, analyzed mainly yellow [5,8a-9] or, to a lesser extent, green and black seeds [10-12]. To our knowledge there are only few studies that investigated the effect of seed color variations on isoflavone content and profile in soybeans, and especially rare are those that included reddish and ocher seeds.

The aim of this study was to determine the influence of soybean seed coat color (yellow, green, ocher, reddish, brown and black) on isoflavone content and composition, as well as on antioxidant potential and phenolic content. The correlation between the obtained parameters was analyzed in order to point out their significant interconnection. This investigation can be of importance for the selection of the raw material for further processing into food

Table 1: Isoflavone content (mg/g of dried soybean seed), average total phenolic content (TPC) and antioxidant activity (inhibitory concentration - IC_{50}) of different colored soybeans – average values according to seed coat color

seed color	total daidzein	total glycitein	total genistein	total isoflavones	TPC mg GAE/g	IC_{50} mg/mL
yellow N=1	3.26±0.001^{a}	0.31±0.002^{a}	1.65±0.001^{a}	5.24±0.002^{a}	2.05±0.01^{ab}	1.70±0.22^{ab}
green N=6	1.39±0.24^{d}	0.31±0.05^{a}	0.72±0.10^{c}	2.42±0.28^{c}	1.91±0.05^{b}	1.86±0.11^{a}
ocher N=3	1.45±0.13^{cd}	0.30±0.04^{a}	0.90±0.20^{bc}	2.65±0.29^{bc}	1.99±0.05^{b}	1.67±0.24^{a}
reddish N=2	2.15±0.51^{b}	0.31±0.02^{a}	0.99±0.17^{b}	3.45±0.71^{b}	1.99±0.04^{b}	1.77±0.13^{a}
brown N=4	1.87±0.46^{bc}	0.31±0.09^{a}	0.85±0.20^{bc}	3.03±0.63^{b}	1.93±0.10^{b}	1.27±0.20^{b}
black N=5	1.64±0.25^{bcd}	0.29±0.07^{a}	0.83±0.12^{bc}	2.76±0.31^{bc}	2.28±0.22^{a}	0.90±0.18^{c}

Values given are mean ± standard deviation of triplicate samples; values superscripted with different letters in the same column are significantly (p<0.05) different from each other; N - number of samples.

ingredients, for functional food or dietary supplements which, due to favorable characteristics, may enhance the nutritional health benefits of these products.

Results for average total isoflavone contents, as the sum of total daidzeins, genisteins and glyciteins, calculated as aglycone equivalents, are presented in Table 1. Comparing average values of total isoflavone content between colored soybeans (except the yellow control sample), it was found that the highest average value was for the reddish seeds and the lowest for the green seeds. Average total isoflavone contents decreased in the following order: yellow (5.24 mg/g) >reddish (3.45 mg/g) >brown (3.03 mg/g) >black (2.76 mg/g) >ocher (2.65 mg/g) >green (2.42 mg/g) (Table 1).

Study of 30 soybean samples (2 black and 28 yellow cultivars) from the Minnesota region showed that total isoflavone content of black seeds varied from 1.58-1.81 mg/g and of yellow seeds 1.18-2.86 mg/g [10].Values of total isoflavones in black seeds obtained in our investigation (2.33-3.15 mg/g) are higher compared with this study. It should be pointed out that the selected yellow genotype analyzed in our study was used as a control sample, but its high isoflavone content obtained is not usual for this type of seeds. Previous results show that total isoflavone content in yellow soybeans can vary from 1.18 to 4.59 mg/g [6c,8b-10] or from 0.46 to 2.1 mg/g [5] and can go even up to 9.49 mg/g [13a].

Distribution of total individual isoflavone forms was relatively consistent among the different seed groups. The yellow sample had the highest percentage of total daidzeins (62.5%) and the lowest percentage of total glyciteins (5.9%) (Figure 1). The highest average percentage of total genisteins (34%) was observed in ocher soybeans, and the highest percentage of total glyciten content (12.9%) in green seeds (Figure 1). Generally, daidzein forms were dominant in all analyzed samples (Figure 1). Total daidzein content on average was the highest in brown seeds (1.87 mg/g) and the lowest in green seeds (1.39 mg/g) (Table 1). Total glycitein content was on average the lowest in all samples and it was not significantly different (p<0.05) between the different colored soybeans. Total genistein content was on average the highest in reddish seeds (0.99 mg/g) and the lowest in green seeds (0.72 mg/g) (Table 1). It was noticed that the profiles regarding the presence of the type of isoflavones are on average similar in all analyzed seed colors. Total daidzeins were followed by total genisteins, and total glyciteins, which was consistent with some other findings [10].

Lee *et al*. [11b] showed that the sum of β-glucosides and malonyl glucosides represented 90% or more of the total isoflavones, which is also confirmed in our study. In all our colored groups this sum was higher than 90%. The only consistent difference they observed, between 268 samples of soybeans of five different colors, was the percentage of aglycones, where brown seeds had a greater proportion of aglycone forms, due to the highest concentration of glycitein compared with the other seed colors. This was not confirmed in our analysis, because all groups had a similar content of aglycone forms (1.15-1.83%).

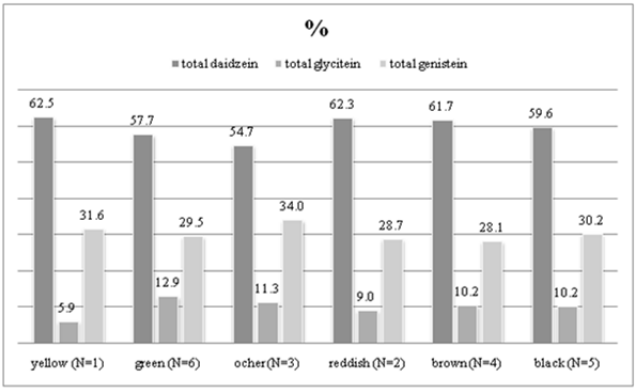

Figure 1: The average percentage (%) of total daidzein, total glycitein and total genistein compared with total isoflavones regarding seed coat color; N - number of samples

Some recent studies examined the same interdependence between soybean seed coat color and isoflavone concentration. Lee *et al*. [11b] analyzed soybeans categorized into five groups according to their seed coat color (black, brown, green, mottled and yellow). It was concluded that seed coat and cotyledon color differences are not strongly associated with differences in isoflavone concentration. Similar results, concerning dependence of isoflavone content with seed coat color, were obtained in a study of Kumar *et al*. [11a], where isoflavone levels were not significantly different between yellow, black and green soybean seeds. Our research confirmed that there is no distinctive and significant difference between isoflavone content in different colored seeds, which corroborates previously reported results, including also reddish and ocher seeds, which were not analyzed in previous studies.

Total isoflavone content in the analyzed samples was in the range 2.11-5.24 mg/g dried seed. The highest content was found in yellow seed and the lowest in one type of green seed (green wrinkled) (Table 2). Regarding colored samples (with the exception of yellow seed) reddish 2 genotype showed the highest total isoflavone content (4.07 mg/g). In the study of Xu and Chang [10] total isoflavone content ranged from 1.18 to 2.86 mg/g in 30 investigated cultivars from North Dakota (28 yellow and 2 black). Yang and Chung [13b] reported that the range of isoflavone contents of 60 soybean cultivars varied from 1.54 to 6.47 mg/g, and their results were the most similar to those obtained in our study. Lee *et al*. [3b] determined the range of 1.88 to 9.48 mg/g in the average of 15 yellow samples, indicating that Korean varieties have higher isoflavone contents than those from other regions. Total isoflavone, daidzein and genistein contents of the control yellow sample, being much higher, were significantly different (p<0.05) from the colored genotypes. Among the colored seeds, reddish 2 genotype had the highest total daidzein content (2.59 mg/g), while the highest total genistein was observed in brown 3 (1.10 mg/g). For total glycitein content, brown shine sample had the highest (0.42 mg/g) and significantly different (p<0.05) content from other genotypes (Table 2).

Table 2: Total daidzein, glycitein and genistein content and their sum – total isoflavones (mg/g dried seed), total phenolic content (TPC) and antioxidant capacity (inhibitory concentration - IC_{50}) in different genotypes of colored soybean seeds.

genotype	total daidzein	total glycitein	total genistein	total isoflavones	TPC mg GAE/g	IC_{50} mg/mL
yellow	3.26±0.001^{a}	0.31±0.002^{cde}	1.65±0.001^{a}	5.24±0.002^{a}	2.05±0.01^{ab}	1.70±0.22^{bcdefg}
green 1	1.74±0.001^{gh}	0.21±0.0001^{gh}	0.72±0.03^{ef}	2.67±0.03^{efg}	1.90±0.005^{ab}	1.91±0.04^{ab}
green 2	1.55±0.01^{ijk}	0.32±0.005^{cde}	0.76±0.001^{def}	2.63±0.008^{efgh}	1.86±0.06^{ab}	2.03±0.07^{a}
green 3	1.53±0.003^{k}	0.31±0.006^{cde}	0.87±0.003^{bcdef}	2.71±0.01^{efg}	1.96±0.008^{ab}	1.79±0.05^{abcde}
green 4	1.25±0.007^{p}	0.31±0.003^{cde}	0.70±0.0008^{ef}	2.26±0.009^{ij}	1.91±0.018^{ab}	1.86±0.006^{abcd}
green 5	1.19±0.0003^{q}	0.29±0.008^{e}	0.64±0.002^{f}	2.12±0.006^{j}	1.94±0.11^{ab}	1.84±0.05^{abcde}
green wrinkled	1.09±0.003^{r}	0.39±0.002^{b}	0.63±0.18^{f}	2.11±0.18^{j}	1.91±0.02^{ab}	1.73±0.11^{abcde}
ocher opaque	1.56±0.0007^{j}	0.31±0.0006^{de}	1.09±0.32^{bcd}	2.96±0.32^{de}	2.02±0.01^{ab}	1.53±0.17^{defg}
ocher shine	1.29±0.02^{o}	0.25±0.004^{f}	0.84±0.01^{bcdef}	2.38±0.005^{ghij}	1.94±0.02^{ab}	1.52±0.06^{efg}
ocher grey	1.50±0.01^{l}	0.33±0.03^{cd}	0.77±0.02^{cdef}	2.60±0.001^{fgh}	2.01±0.08^{ab}	1.95±0.14^{ab}
reddish 1	1.71±0.002^{h}	0.29±0.0003^{e}	0.84±0.008^{bcdef}	2.84±0.006^{def}	1.97±0.007^{ab}	1.66±0.07^{bcdef}
reddish 2	2.59±0.002^{b}	0.34±0.002^{c}	1.14±0.01^{b}	4.07±0.02^{b}	2.01±0.05^{ab}	1.87±0.05^{abc}
brown 1	2.31±0.003^{c}	0.25±0.001^{f}	0.94±0.007^{bcdef}	3.49±0.002^{c}	1.97±0.03^{ab}	1.07±0.001^{hij}
brown 2	1.75±0.006^{g}	0.20±0.0003^{gh}	0.75±0.0003^{ef}	2.70±0.006^{efg}	1.90±0.005^{ab}	1.35±0.02^{fgh}
brown 3	2.21±0.008^{d}	0.37±0.003^{b}	1.10±0.01^{bc}	3.68±0.03^{c}	2.03±0.0002^{ab}	1.12±0.002^{hi}
brown shine	1.22±0.002^{q}	0.42±0.004^{a}	0.60±0.001^{f}	2.24±0.003^{ij}	1.79±0.08^{b}	1.54±0.06^{cdefg}
black1	1.85±0.005^{f}	0.19±0.002^{h}	0.76±0.01^{def}	2.82±0.02^{ef}	2.17±0.07^{ab}	0.99±0.003^{ijk}
black2	1.33±0.002^{n}	0.31±0.0001^{de}	0.69±0.01^{ef}	2.33±0.01^{hij}	2.33±0.32^{ab}	1.14±0.09^{ghi}
black 3	1.66±0.002^{i}	0.38±0.0005^{b}	0.92±0.01^{bcdef}	2.95±0.008^{de}	2.39±0.28^{a}	0.89±0.21^{ijk}
black 4	1.94±0.01^{e}	0.22±0.0005^{fg}	0.99±0.002^{bcde}	3.15±0.01^{d}	2.14±0.02^{ab}	0.69±0.01^{k}
black 5	1.43±0.001^{m}	0.34±0.003^{c}	0.78±0.02^{cdef}	2.55±0.02^{fghi}	2.37±0.39^{a}	0.78±0.002^{jk}

Values given are mean ± standard deviation of triplicate samples; values superscripted with different letters in the same column are significantly (p<0.05) different from each other

Observing different seed colors, the concentration of malonyl daidzin, the most abundant compound in all samples, was on average the highest in reddish genotypes (2.22 mg/g). Reddish soybeans also had the highest average contents of malonyl glycitin (0.23 mg/g) and malonyl genistin (0.91 mg/g).

It has been shown that, in general, the total content of daidzeins, genisteins and glyciteins is inconsistent and varies according to genotype and environmental factors, but usually the concentration of glyciteins is the lowest [9]. The results obtained in our analysis showed that glycitein forms are the least present in all soybean samples (on average 0.30 mg/g). The highest average content was determined in total daidzeins (1.71 mg/g), while average content of total genisteins was 0.86 mg/g.

Statistical analysis showed that, in general, the examined soybean genotypes had significantly different (p<0.05) total daidzein contents. Only green 1 genotype was not significantly different from reddish 1 and brown 2, and green 2 was similar to green 3 and ocher opaque, according to total daidzein content (Table 2).

Results of isoflavone analysis obtained for colored soybean seeds showed that all isoflavone forms were successfully separated and 11 of 12 isoflavones, generally present in soybeans, were detected in samples investigated in this study. Only acetyl genistin was not present in any of the analyzed seeds, while daidzein was present in 12 and genistein in 14 of 21 samples. All the other 9 isoflavones were detected in all samples (Table 3).

Regarding individual isoflavone profile, in all analyzed samples malonyl daidzin was the isoflavone present in the highest concentration, although its content varied among different genotypes (Table 3). Except for this compound, a high concentration in samples was observed mainly for daidzin and in some cases malonyl genistin. Generally, malonyl forms are the most abundant forms of all isoflavones [8b,9], which is in accordance with the results obtained in this study; it was the case in 18 of 21 analyzed samples. The exceptions were observed in ocher opaque, brown 2 and black 4, where glycitin showed a higher content than malonyl glycitin (Table 3). Usually malonyl genistin and malonyl daidzin are the dominant isoflavones in raw soybeans. Yellow soybean showed the highest content of daidzin, acetyl daidzin, malonyl daidzin, glycitein, genistin and malonyl genistin (Table 3, in bold). Accordingly, this sample, used as a control compared with colored seeds, had the highest total isoflavone content of all genotypes (Table 2).

The ocher opaque sample had the highest concentration of daidzein, brown shine the highest content of glycitein and malonylglycitin, green 3 the highest concentration of acetyl glycitin, and reddish 2 genotype the highest concentration of genistein (Table 3, in bold). In general, of all detected compounds, the least present were genistein and daidzein (on average 0.011 mg/g and 0.028 mg/g respectively), while the average content of the third aglycone, glycitein, was also low (0.042 mg/g). These data are in accordance with previously published results [6c,8b].

Among other isoflavone types, β-glucosides (daidzin, glycitin, genistin) showed on average a high content in different colored soybeans, as demonstrated earlier [8b]. This β-glucoside form is in second place regarding the distribution of the total content in analyzed soybeans. As mentioned above for the malonyl form, daidzin also was the most present β-glucoside in all genotypes. On the other hand, in the study performed by Correa *et al.* [14], the major isoflavones present in black soybeans were daidzin and genistin and their corresponding acetylated forms, but this result is explained by the decomposition of heat labile malonyl forms during the roasting process.

Acetyl isoflavones were detected in low amounts compared with the above mentioned malonyl and β-glucoside forms. On average, acetyl glycitin was present in lower content (0.065 mg/g) than acetyl daidzin (0.29 mg/g), while acetyl genistin was under the detection limit in all samples.

Generally, the percentage of aglycones was the lowest, which is usually determined for unprocessed soybean seeds [13a,15]. It is well known that, of 12 known isoflavone forms, the most common in unprocessed soybean seed are malonyl and β-glucoside forms [13a,15], which was also confirmed in our investigation.

All obtained data were subjected to statistical analysis in order to point out significant correlations, presented in Table 4. It was observed, in all groups, that there is a significant correlation (p<0.01) between total isoflavone content and total genisteins. Genistein forms, after ingestion, convert to the aglycone genistein,

Table 3: Individual isoflavone content (mg/g of dried seed) in different genotypes of colored soybean.

genotype	DE	DI	ADI	MDI	GY	GYI	AGYI	MGYI	GE	GI	AGI	MGI
yellow	0.03	**2.02**	**0.62**	**3.27**	**0.08**	0.15	0.07	0.18	0.01	**1.13**	TR	**1.53**
green 1	TR	0.99	0.31	1.90	0.04	0.11	0.07	0.12	TR	0.55	TR	0.72
green 2	0.07	0.94	0.29	1.48	0.03	0.21	0.05	0.24	TR	0.62	TR	0.71
green 3	TR	0.83	0.35	1.63	0.04	0.17	**0.08**	0.22	TR	0.65	TR	0.89
green 4	0.07	0.68	0.29	1.19	0.03	0.18	0.08	0.22	0.01	0.55	TR	0.65
green 5	0.04	0.70	0.25	1.17	0.03	0.18	0.07	0.20	0.01	0.51	TR	0.58
green wrinkled	0.04	0.66	0.22	1.05	0.03	0.25	0.07	0.30	0.01	0.42	TR	0.67
ocher opaque	**0.08**	1.00	0.32	1.38	0.04	0.19	0.08	0.19	0.02	0.74	TR	1.17
ocher shine	TR	0.82	0.31	1.19	0.04	0.14	0.07	0.15	0.01	0.70	TR	0.75
ocher grey	TR	0.93	0.27	1.52	0.04	0.20	0.07	0.22	TR	0.66	TR	0.69
reddish 1	0.04	1.08	0.25	1.72	0.04	0.17	0.05	0.21	0.02	0.68	TR	0.76
reddish 2	0.05	1.59	0.34	2.72	0.05	0.19	0.07	0.25	**0.02**	0.90	TR	1.07
brown 1	0.05	1.42	0.26	2.45	0.05	0.14	0.06	0.14	0.02	0.73	TR	0.90
brown 2	0.04	1.13	0.23	1.77	0.04	0.11	0.05	0.11	0.01	0.61	TR	0.67
brown 3	0.05	1.33	0.34	2.30	0.05	0.21	0.08	0.25	0.01	0.85	TR	1.06
brown shine	TR	0.73	0.19	1.31	0.04	**0.26**	0.06	**0.33**	TR	0.47	TR	0.59
black 1	TR	1.17	0.26	1.96	0.04	0.10	0.05	0.10	TR	0.60	TR	0.73
black 2	TR	0.85	0.18	1.40	0.04	0.20	0.05	0.21	TR	0.55	TR	0.66
black 3	TR	1.04	0.27	1.72	0.04	0.24	0.06	0.26	0.01	0.74	TR	0.86
black 4	0.03	1.24	0.30	1.96	0.04	0.12	0.07	0.11	0.01	0.78	TR	0.94
black 5	TR	0.95	0.23	1.42	0.04	0.22	0.05	0.23	0.01	0.64	TR	0.70

DE-daidzein, DI-daidzin, ADI-acetyl daidzin, MDI-malonyldaidzin, GY-glycitein, GYI-glycitin, AGYI-acetyl glycitin, MGYI-malonylglycitin, GE-genistein, GI-genistin, AGI-acetyl genistin, MGI-malonylgenistin, TR- in traces; in bold – maximal values

which has the highest biological activity among the soy isoflavones [16].This correlation between total isoflavones and total genisteins confirmed a previous report [11a]. It can be used for the prediction of relative genistein content using only preliminary information about total isoflavone content. Regarding the biological potential of genistein, this information is relevant for usage of raw soy material for different purposes.

As expected, in the majority of analyzed groups (except ocher samples) a significant correlation (p<0.01) between total isoflavones and total daidzein content was determined. This is in accordance with the fact that daidzeins contributed the most to total isoflavones. In the darkest colored seeds (brown and black) significant negative correlation between IC_{50} values and total genistein content is observed. Some of the established correlations were noticed also by Kumar and collaborators [11a]. In their study, significant correlations were determined for total isoflavone content and daidzeins in green seeds, and total isoflavone content and genisteins in green and black seeds, which our analysis confirmed.

Analysis of 21 soybean samples showed that the average value of TPC was 2.03 mg GAE/g, and analysis of antioxidant capacity showed that the average IC_{50} value was 1.47 mg/mL.

Regarding average values of TPC according to seed coat color, it was found that black genotypes had the highest TPC (2.28±0.22 mg GAE/g), which was significantly different (p<0.05) from other colored soybeans (Table 1). In the study of Kumar *et al*. [11a] TPC varied from 0.81 to 5.89 mg GAE/g, but there was no significant difference (p<0.05) of TPC between yellow, green and black soybeans, although the average value of TPC was the highest in black seeds, which corresponds to our results. In accordance with the high TPC values, the antioxidant potential of black seeds was the highest (IC_{50} 0.90±0.18 mg/mL) and significantly different (p<0.05) from the antioxidant activity of the other colored samples (Table 1). The lowest TPC value was determined in green seeds (1.91±0.05 mg GAE/g), as well as the lowest antioxidant capacity (IC_{50} 1.86±0.11 mg/mL) (Table 1).

In the study of Furuta *et al*. [17a] it was confirmed that the DPPH scavenging activity was decreasing in the following order: soybean genotypes with black seed coats > those with red-brown seed coats> those with green or yellow seed coats, and that the activity was mainly dependent on anthocyanin content, one of the polyphenols in these genotypes. In our study black seeds also had the highest and significantly different antioxidant potential compared with other seed colors, and green colored seed had the lowest antioxidant activity, which is in accordance with earlier reported results.

Table 4: Significant correlation coefficient observed between total daidzein, total glycitein, and total genistein content, total isoflavones, TPC and IC_{50} values in soybean with different seed coat colors.

seed color	parameters	correlation coefficient (r)
green		
	total glycitein - total daidzein	-0.75**
	total isoflavones - total daidzein	0.92**
	total isoflavones - total genistein	0.81**
ocher		
	total isoflavones - total genistein	0.86**
reddish		
	total glycitein - total daidzein	0.99**
	total genistein - total daidzein	0.99**
	total isoflavones - total daidzein	0.99**
	total genistein - total glycitein	0.99**
	total isoflavones - total glycitein	0.99**
	total isoflavones - total genistein	0.99**
brown		
	total genistein - total daidzein	0.91**
	total isoflavones - total daidzein	0.97**
	TPC - total daidzein	0.90**
	IC_{50} - total daidzein	-0.99**
	total isoflavones - total genistein	0.98**
	TPC - total genistein	0.93**
	IC_{50} - total genistein	-0.91**
	TPC - total isoflavones	0.92**
	IC_{50} - total isoflavones	-0.97**
	IC_{50} - TPC	-0.92**
black		
	total glycitein - total daidzein	-0.67*
	total genistein - total daidzein	0.68*
	total isoflavones - total daidzein	0.90**
	total isoflavones - total genistein	0.92**
	IC_{50} - total genistein	-0.72*

Correlations marked with * are significant at p<0.05 and with ** at p<0.01
r – Pearson's correlation coefficient

Kumar *et al*. [11a] concluded that antioxidant substances other than isoflavones contribute to higher values of free radical-scavenging activity in black soybeans. The test for free radical-scavenging activity (DPPH) showed that black soybeans had significantly higher antioxidant capacity than yellow and green samples. Their results also showed that black genotypes did not necessarily contain

higher level of total phenols than yellow or green soybean, which opens the possibility that other non-phenolic compounds can contribute to the antioxidant capacity of soybean extracts. Significant correlation (p<0.01) between total isoflavone content and antioxidant activity was observed only in brown soybean seeds (Table 4), which indicates that compounds other than isoflavones can have more potent antioxidant activity. Regression analysis for the total isoflavone content and IC_{50} values in the study of Malenčić *et al*. [17b] showed a low correlation, which is in agreement with some previous findings [5,8b,9].

Results obtained for individual TPC and antioxidant capacity of soybean seeds are presented in Table 2. Observed TPC values varied from 1.79 mg GAE/g in brown shine genotype to 2.39 mg GAE/g in black 3. The highest antioxidant capacity was detected for black 4 genotype (IC_{50} 0.69 mg/mL), while the lowest was determined in green 2 (IC_{50} 2.03 mg/mL) (Table 2). Total phenolic contents among genotypes were not significantly different (p<0.05). Green soybeans had similar antioxidant activity within the group, but IC_{50} values for other samples did not group according to seed coat color (Table 2). Ranges for TPC values for six black soybeans reported by Kumar *et al*. [11a] were 0.81-5.89 mg GAE/g, and for six green seeds 0.96-2.89 mg GAE/g. These ranges are much wider than those obtained in our research (for black samples 2.14-2.39 mg GAE/g, for green samples 1.90-1.96 mg GAE/g) (Table 2). Large variations between genotypes in the black and green soybean groups according to TPC values in the study of Kumar *et al*. [11a] result in similar average TPC in the colored groups to those obtained in our study. Results of our investigation can be used for selection of raw material with a predefined amount of biologically active compounds. The pharmaceutical industry requires raw material with high content of biologically active substances (in the case of soybeans - isoflavones and polyphenoles). For example, black seeds having similar isoflavone content as other colored seeds, but higher total phenolic content, and correspondingly antioxidant capacity, would be suitable for this purpose. It was observed that feeding with high isoflavone content could have a negative impact on livestock reproductive functions, so a low content of these compounds is needed for this use.

There are only few published investigations concerning the influence of soybean seed colors on isoflavone content. Therefore, our study was focused on the analysis of green, brown and black as well as the rarely mentioned reddish and ocher seeds. Significant correlation between total isoflavone content and total genisteins was established in all colored groups, and in the majority of analyzed groups, a significant correlation between total isoflavones and total daidzein content. Obtained liaisons between the examined characteristics can contribute to the estimation of a specific isoflavone composition. Among the colored seed, the reddish had the highest total isoflavone content, and black seeds the highest TPC and antioxidant capacity. Our investigation also confirmed that there is no distinctive connection between seed coat color and isoflavone composition. The results of our study are useful for breeding of specific soybean varieties with targeted content and composition of biologically active compounds.

Experimental

Soybean samples: Plant material was grown in experimental fields of the Institute of Field and Vegetable Crops, near Novi Sad, Serbia. Material for the analyses was derived from the single cross between commercial variety Balkan and a genotype from the Institute's soybean germplasm collection characterized by black seed coat color. The crossing resulted in a number of different seed coat colors, and color combinations in the offspring. It was possible to develop 6 groups of soybean lines based on seed coat color, and a total of 21 soybean samples were analyzed - 1 yellow (control sample), 2 reddish, 6 green, 3 ocher, 4 brown and 5 black genotypes.

Sample preparation: Soybeans were ground using a coffee mill without removal of the seed coat. A powdered portion (500 mg) of soybean seed was defatted by *n*-hexane extraction (2 x 10 mL, 30 min and subsequent centrifugation, 30 min, 1780 rpm) and then extracted for 2 h with 8 mL methanol:water (4:1, v/v) and centrifuged (30 min, 1780 rpm) [18]. The obtained extracts were used for isoflavone and total phenolic content determination and antioxidant activity analysis. Prior to HPLC injection, each extract was filtered through an Agilent technologies 0.45 µm membrane (Delaware, Wilmington).

HPLC analysis of isoflavones: Isoflavone analysis was performed according to the reported method of Lee *et al.* [3b], with slight modifications. Separation, identification and quantification of isoflavones was performed on a Zorbax SB C_{18} reversed phase HPLC column (150 x 4.6 mm, 5 µm) with a Zorbax SB C_{18} guard column. The mobile phase consisted of 2 solvents. Solvent A was 1% (v/v) acetic acid in water and solvent B 100% acetonitrile. Gradient conditions were: 0-5 min 15% B; 5-44 min from 15 to 35% B; 44-45 min from 35 to 15% B, 45-50 min 15% B, with a post time period of 20 min. The column was set at 25°C, the solvent flow rate was 0.6 mL/min and injection volume 10 µL. Spectra were collected between 240 and 400 nm by DAD and components in the eluate were detected at 270 nm. Isoflavones were identified by comparing the retention times in HPLC chromatograms and UV spectral patterns with those of standard compounds and literature data [3b,18]. Isoflavone concentrations were quantified by external standard (5-point regression curves, r ≥ 0.9997) of daidzein, glycitein and genistein standard compounds. Standard solutions were made by dissolving standard compounds in a mixture of methanol:water (4:1, v/v). As only standards of phytoestrogene aglycones were used, the content of the corresponding glucoside forms was obtained by calculation. For this purpose, calibration curves of the corresponding aglycones were used and corrections for differences in molecular weight between aglycones and glucosides were applied following the pattern given by Romani *et al*. [19a]:

$$\mathbf{c(glucoside) = c(corr\ aglycone) \cdot \frac{Mr\ (glucoside)}{Mr\ (corr\ aglycone)}}$$

Contents of individual isoflavones were given after correction according to molar masses of corresponding glucosides, and when total isoflavone contents were presented, they were given as aglycone equivalents, without the mentioned correction.

Determination of total phenolic content (TPC): The content of total phenolics was quantified by the Folin-Ciocalteu method using gallic acid as a reference standard [19b]. The sample (0.1 mL) and Folin-Ciocalteu reagent (0.5 mL) were placed in a volumetric flask and mixed well. After 6 min, 0.4 mL of saturated Na_2CO_3 solution (177 g Na_2CO_3/L) was added. The absorbance at 740 nm was measured after 120 min. The results were expressed as gallic acid equivalents per g of soybean (mg GAE/g) using a gallic acid standard curve.

Free radical scavenging capacity: The radical scavenging activities of soybean genotypes were evaluated after the reaction with DPPH [19c]. For the purpose, 1 mL of a methanol DPPH solution (0.097 mM) was mixed with 20 to 400 µL of sample extract and diluted with 80% methanol to 4 mL. The reduction of the DPPH radical

was measured after 60 min at 515 nm. For every concentration of soybean extract the radical scavenging capacity (RSC) percentage was calculated using the formula: RSC=100-100*$A_{extract}/A_{blank}$, where $A_{extract}$ represents absorbance of the analyzed sample extract and A_{blank} represents absorbance of blank sample. The calibration curve RSC versus extract concentrations was plotted and inhibitory concentration (IC_{50}) in mg/mL was calculated as concentration of extract necessary to achieve RSC value of 50%.

Statistical analysis: All sample measurements were made in triplicate. The data were expressed as mean ± standard deviation and analyzed by analysis of variance (ANOVA). Tukey test was employed to draw the comparison between means and the significance was accepted at $p<0.05$. The correlations between examined parameters were determined by the Pearson's correlation coefficient (r). The significance of the Pearson's coefficient was also investigated at either $p<0.05$ or $p<0.01$. Statistical analysis was made using Statistica (version 10).

Abbreviations used: TPC – total phenolic content; DPPH - 2,2-diphenyl-1-picryl-hydrazyl radical; RSC – radical scavenging capacity; GAE – gallic acid equivalents; HPLC – high performance liquid chromatography; DAD – diode array detector.

Acknowledgments - This work is a part of a project financed by the Ministry of Science and Technological Development, Republic of Serbia, no. TR31022. Also thanks to project H2020-MSCA-RISE-2015 MEDLEM, no 690876.

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