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Determination of Phytoestrogen Composition in Soybean Cultivars in Serbia

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The growing clinical interest and use of soybean-based food products and extracts to increase dietary phytoestrogen intake have led to medical interest in the precise determination of the phytoestrogen composition of soybean products. We have analyzed the composition of these compounds in 20 domestic and introduced varieties of genetically non-modified soybean genotypes grown under the same agroecological conditions. The isoflavone content of soybeans cultivated in this region of Serbia has not been previously reported. The assays were performed, after extraction with methanol-water (8:2, v/v), by C_{18} reversed phase high-performance liquid chromatography coupled with photodiode array detection. The total phytoestrogen concentration was found to be between 2.24 and 3.79 mg g^{-1} dry bean weight. The total concentration of daidzein and its derivatives ranged from 0.96 to 1.82 mg g^{-1}, total glyciteines from 0.34-0.53 mg g^{-1}, and all genistein derivatives from 0.86-1.67 mg g^{-1} dry bean weight. Given the high biological potency of phytoestrogens and their metabolic conversion products, our data suggest that phytoestrogen content screening could be a useful tool in the selection of soybean genotypes with improved health promoting characteristics.

Keywords: phytoestrogens, soybean, HPLC analysis.

Phytoestrogens are plant-derived phenolic compounds with estrogenic activity, belonging to the class of isoflavones. Soybeans and soy products are the richest sources of these compounds in the human diet. The health-promoting activity associated with soy consumption is partly attributed to the presence of phytoestrogens [1]. Soybeans are particularly rich sources of phytoestrogens such as daidzein and genistein. Intakes of these substances are markedly higher among the Asian population than among the Western population, largely resulting from soy consumption patterns. It is widely perceived that exposure to phytoestrogens is beneficial, as the rates of breast cancer and other hormone-dependent conditions are lower in Asian than in Western countries [2]. Recently, soybean constituents with biological activity, such as anticarcinogens, antioxidants, antihemolytics, antifungal substances, and tyrosine protein kinase inhibitors, have attracted attention as well [3].

Soybean phytoestrogens are usually found in glycosidic form, such as malonyl and acetyl glycosides. It has been shown that malonylated isoflavone glycosides are major phytoestrogens in soybean seed [4]. Phytoestrogens have similar structures to both endogenous and synthetic estrogens and show "estrogen like" activities. The anticancer function of soybean phytoestrogens was shown to be associated with genistein, which inhibits protein tyrosine kinase and DNA topoisomerase and binds weakly to estrogen receptors [5].

Twelve different phytoestrogens have been detected in soybeans. Daidzein, genistein and glycitein are the aglycons, which exist usually as a β-glucoside, a 6''-*O*-malonyl-glucoside and a 6''-*O*-acetyl-glycoside [6]. Literature evidence suggests that the biological activity of soy phytoestrogens does not depend upon the glycoside form. For their absorption and activity in the organism, hydrolysis of the glycosides is necessary [7,8]. Thus, the

bioavailability of the soy phytoestrogens is not significantly different when they are consumed as either aglycone or glucoside [9]. However, the activity of the phytoestrogens depends on the type of aglycon, as genistein has a higher biological activity than daidzein and glycitein [10]. The content of phytoestrogens in soybean was found to be about 0.1-0.4% of dry weight, but the amount and composition varied. Wang and Murphy reported total phytoestrogen contents from 1176 to 3309 μg/g across several years, and from 1176 to 1749 μg/g from sites within the same year for single soybean cultivars [11].

Medical interest in soy-based food is driven by a growing number of studies indicating that the phytoestrogen content of ingested soybeans can modify the pathogenesis of some hormone-dependent and hormone-independent diseases [12-14]. The phytoestrogen content of soybean is thus of international interest because of the increasing global reliance on soybean varieties for animal and human consumption, and also because of the newly recognized physiological and pharmacological properties of phytoestrogens [5,15]. Thus, many over-the-counter products of soy isoflavones are available in pharmacies as remedies for hot flushes and post-menopause related problems [16].

Due to the widespread use of soy products, knowing the amount and kind of phytoestrogens in them is of increasing importance. Many of these products are derived from phytoestrogen-rich soy sources, yet their potency is unknown. Dietetic supplements based on soybean extracts have been adopted as a natural alternative to hormone replacement therapy in menopause due to high amounts of isoflavones [17].

Therefore, the aim of this study was to investigate the concentration of phytoestrogens in different soybean genotypes grown under defined agroecological conditions, not previously studied. Obtained data should enable the selection of soybean genotypes highly rich in biologically active compounds that could further be processed into either functional food or pharmaceutical raw material.

The phytoestrogen distribution of twenty soybean samples was studied. All twelve described isoflavones were successfully separated and identified using the applied HPLC conditions. The variance of results was lower than 5% for isoflavones in each soybean cultivar (n=4). The highest total phytoestrogen content was obtained for soybean genotype 2 (3.79 mg/g of dried seeds), while the lowest was in genotype 3 (2.24 mg/g of dried seeds) (Table 1). These results showed that the isoflavone content of the analyzed samples is higher than in the recently performed study on 30 soybean samples from the USA [7]. In that investigation, the total isoflavone content ranged between 1.18-2.86 mg/g of dried seeds. Also, the average total isoflavone value (2.98 mg/g) of all twenty soybean samples analyzed in our study is considerably higher than some values reported earlier, such as for 30 cultivars from Minnesota (1.86 mg/g), 210 South Dakota cultivars (average value 1.98 mg/g) [18] and 18 Korean (average value 1.45 mg/g) (based on dry weight) [11].

Table 1: Total daidzein, glycitein, genistein and isoflavone contents of analyzed cultivars (mg/g dry plant material).

No.	Cultivar	TDZ	TGLY	TGE	TI
1	LN92-7369	1.41	0.41	1.35	3.17
2	1581/99	1.66	0.46	1.67	3.79
3	1511	1.00	0.38	0.86	2.24
4	1499/99	0.96	0.51	0.93	2.40
5	lori	0.98	0.53	0.86	2.38
6	linda	1.54	0.50	1.13	3.16
7	balkan	1.82	0.49	1.21	3.52
8	BL-8	1.56	0.50	1.54	3.60
9	alisa	1.03	0.34	1.11	2.48
10	tara	1.05	0.50	0.96	2.52
11	meli	1.60	0.53	1.49	3.62
12	sava	1.50	0.42	1.11	3.03
13	venera	1.61	0.42	1.37	3.40
14	morava	1.60	0.43	1.17	3.20
15	LN92-7369 x 1581/99	1.41	0.44	1.26	3.11
16	1499/99 x 1581/99	1.28	0.48	1.18	2.94
17	1499/99 x 1511	1.07	0.37	1.08	2.51
18	lori x LN92-7369	1.06	0.38	0.93	2.38
19	linda x LN92-7369	1.55	0.43	1.31	3.29
20	balkan x BL-8	1.27	0.34	1.14	2.74
average		1.35	0.44	1.18	2.98

Abbreviations: No-sample number; TDZ-total daidzein content; TGLY-total glycitein content; TGE-total genistein content.

Data analysis by the ANOVA test shows statistically significant ($p<0.05$) differences between the analyzed cultivars considering the total isoflavone content, as well as for the total amount of genistein, glycitein and daizdein. Figure 1 (obtained using the SPSS 10.0 program) shows the isoflavone contents of the analyzed cultivars with regard to three parameters: total isoflavone content (x-axis), total genistein content (y-axis) and total daidzein content (z-axis).

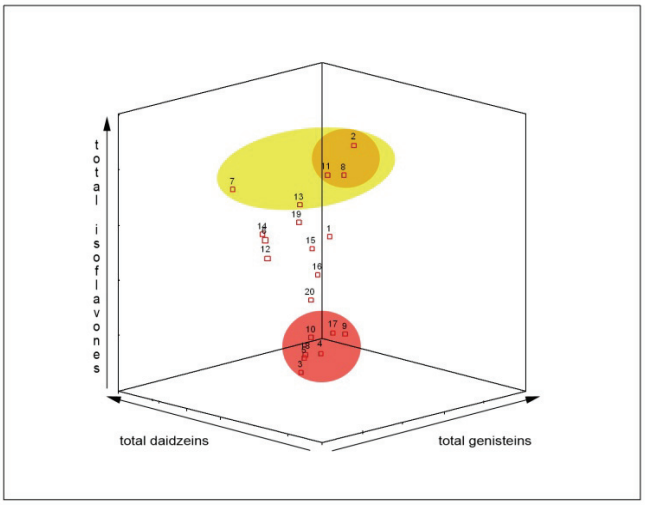

Figure 2: Distribution of isoflavone content in analyzed cultivars, regarding total isoflavones, total genisteins and total daidzeins.

Two main distinctive groups of cultivars could be recognized; these are marked in red and yellow. From all analyzed cultivars, samples with the highest content of total isoflavones have been placed in the yellow graph area. It can be noticed that samples 2, 8 and 11, placed in the orange subgroup, are distinguished from the other samples by containing also the highest amount of genisteins. This might be important considering that daidzein and glycitein forms have less estrogenic activity than genistein forms [7].

The cultivars with the lowest total isoflavone content are in the red area of the diagram. At the same time, all the samples in that area have the lowest total genistein content. Therefore, by looking at the graph, we can clearly distinguish soybeans with different potential for biological activity, and thus the best cultivars for development for pharmaceutical and/or food supplements. The best isoflavone composition was found in cultivars no 2, 11 and 8, taking into consideration the content of total isoflavones (1.63; 1.51; 1.46 mg/g, respectively) and the contribution of total genisteins in the total isoflavone content of 44, 41 and 43%, respectively.

The isoflavone contents of 20 analyzed soybean cultivars are shown in Table 2. The two last rows in the table represent the minimal and maximal standard deviation for the 4 different analyses of the same sample. Considering the average contents of all analyzed samples, the most abundant components were malonyl daidzin (1.53 mg/g, dry weight) and malonyl genistin (1.53 mg/g, dry weight), while the concentration of acetyl genistin was the lowest (0.01 mg/g, dry weight). This observation is in agreement with previously reported results [1,11,19,20,]. Daidzin and genistin are present in considerable amounts in all soybean samples (average values are 0.70 and 0.56 mg/g, dry weight, respectively).

In the analyzed soybeans, isoflavones were mostly present in the form of glycosides. The average content of isoflavones in the free aglycon forms (daidzein, glycitein and genistein) is only 0.92% of the total isoflavone content. Isoflavones are dominantly present as malonyl glucosides. On average, regarding all the twenty analyzed samples, malonyl glucosides (malonyl daidzin, malonyl glycitin and malonyl genistin) represented 67% of the total isoflavone content. The average sum of glucoside derivatives (daidzin, glycitin and genistin) formed 27% of total isoflavones, and the acetylglucosides (acetyl daidzin, acetyl glycitin and acetyl genistin) existed only in small proportions; these data are consistent with the literature reports [3,11,19].

In Figure 2, the distribution of total daidzeins, total glyciteins and total genisteins is shown, in relation to the total isoflavone content. Daidzeins varied between 41-52% of the total, genisteins from 34-45%, and glyciteins from 12-22%. The average amount of total daidzeins in the analyzed cultivars was the highest (45.6%), followed by total genisteins (39.3%), while total glyciteins were present in the least amount in all analyzed samples (15.0%). The ratios of total daidzin, total glycitin and total genistin contents compared with total isoflavone content are similar in all 20 analyzed cultivars. These results are in agreement with the previous observations that soybeans and soy food usually contain similar amounts of genistein and daidzein forms and a much lower amount of glycitein forms [11, 20].The results related to the antioxidant activity of the same samples used in this study are previously reported [21]. By comparison of these results with those obtained from our analysis we can conclude that there was no correlation between the content of total isoflavones and antioxidant activity in analyzed soybean seeds ($p>0.05$). Soy isoflavones seemed to be less effective antioxidants compared with other compounds present in soybean [22]. This may be due to dependence of antioxidant activitiy on isoflavone structure. For example, the glucose linkage to the aglycone reduced the antioxidant activities of isoflavones by approximately 50-100 times [23].

Table 2: Isoflavone content of analyzed cultivars (mg/g dry plant material).

Sample	DE	DI	mDI	aDI	GY	GYI	mGYI	aGYI	GE	GI	mGI	aGI
1	0.02	0.58	1.76	0.27	0.03	0.15	0.47	0.07	0.07	0.47	1.82	0.01
2	0.02	0.69	2.00	0.37	0.04	0.15	0.54	0.09	0.02	0.66	2.32	tr
3	0.02	0.45	1.16	0.21	0.03	0.13	0.42	0.07	0.01	0.35	1.18	tr
4	tr	0.42	1.13	0.24	0.03	0.20	0.62	0.07	0.01	0.36	1.29	tr
5	tr	0.45	1.17	0.21	0.02	0.29	0.57	0.06	0.01	0.35	1.18	tr
6	tr	0.71	1.91	0.25	0.03	0.19	0.59	0.07	0.00	0.48	1.55	tr
7	0.02	0.88	2.20	0.27	0.02	0.15	0.61	0.09	0.01	0.53	1.62	tr
8	tr	0.77	1.78	0.35	0.03	0.20	0.57	0.09	0.01	0.72	2.02	tr
9	tr	0.53	1.15	0.23	0.02	0.13	0.38	0.08	0.01	0.50	1.47	tr
10	tr	0.53	1.21	0.22	0.02	0.23	0.58	0.06	0.00	0.44	1.26	tr
11	tr	0.82	1.83	0.31	0.03	0.19	0.65	0.07	0.01	0.69	1.96	tr
12	0.01	0.83	1.68	0.24	0.03	0.15	0.51	0.06	0.01	0.55	1.41	tr
13	0.03	0.85	1.81	0.28	0.02	0.15	0.50	0.07	0.01	0.71	1.69	tr
14	0.02	0.87	1.78	0.27	0.03	0.16	0.52	0.06	0.01	0.59	1.47	tr
15	0.01	0.76	1.55	0.28	0.03	0.21	0.50	0.04	0.01	0.63	1.59	tr
16	tr	0.71	1.39	0.27	0.02	0.22	0.57	0.04	0.01	0.63	1.44	tr
17	tr	0.62	1.13	0.22	0.02	0.15	0.42	0.05	0.01	0.61	1.29	tr
18	tr	0.63	1.14	0.19	0.03	0.21	0.42	0.02	0.01	0.52	1.12	tr
19	0.01	0.91	1.68	0.26	0.03	0.21	0.47	0.04	0.01	0.71	1.58	tr
20	0.02	0.80	1.26	0.23	0.02	0.16	0.36	0.05	0.01	0.71	1.32	tr
Av.	0.02	0.69	1.53	0.26	0.03	0.18	0.51	0.06	0.01	0.57	1.53	0.01
Min StD x10^{-5}	4.0	0.6	5.5	0.9	0.9	0.1	0.13	2.9	1.5	2.4	0.9	35.2
Max StD x10^{-5}	83.5	218.5	268.8	65.8	42.0	127.4	310.7	104.6	56.0	105.8	163.1	35.2

Abbreviations: DE, daidzein; DI, daidzin; mDI, malonyl daidzin, aDI, acetyl daidzin; GY, glycitiein; GYI, glycitin; mGYI malonyl glycitin, aGYI acetyl glycitin; GE genistein; GI, genistin; mGI, malonyl genistin; aGI acetyl genistin; TI, total isoflavone; tr, trace amount; av, average value; Min StD, Minimal Standard Deviation for 4 injections of the same sample; Max StD, Maximal Standard Deviation for 4 analyses of the same sample.

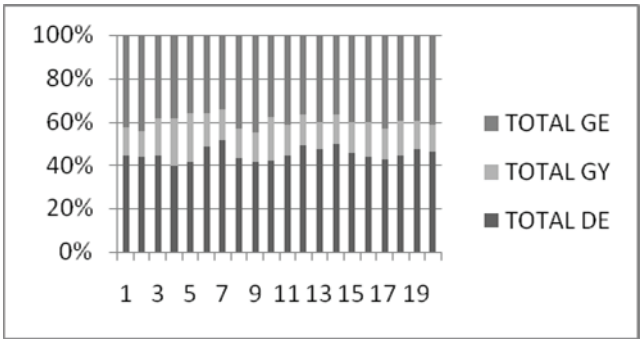

Figure 2: Total daidzein, total glycitein and total genistein distribution in 20 soybean cultivars

Experiments on antioxidant activity of isoflavone aglycones *in vivo* showed significantly lower excretion of urinary secondary lipid oxidation products, a measure of lipid peroxidation *in vivo* [24]. The low percentage of isoflavones in aglycone form (less than 1% in analyzed samples) may cause the relatively low antioxidant activities of soybean extracts. The prospective comparison of the antioxidant activity before and after hydrolysis of glycosides in soybean extracts should reveal more detailed information related to their effectiveness.

The actual phytoestrogen concentrations in this crop are of interest because of the growing number of professionals who may recommend increasing dietary intake of phytoestrogens to modulate lipid oxidation [25,26], retard the pathogenesis of cancer, notably breast cancer [27], and reduce the risks of coronary artery disease and osteoporosis [28,29]. There is also growing interest in the commercialization of phytoestrogen preparations and soy extracts. Introduction of selection criteria based on bioactive properties could help in developing soybean genotypes with better health promoting characteristics.

In this study twenty soybean cultivars were analyzed for isoflavone contents. The objective was to compare results between cultivars and to determine selection criteria for developing genotypes with favorable health promoting characteristics. Also, considering that the investigated soybeans were cultivated in a region not generally accepted as their ecological surroundings, the results obtained were of value for comparison with the isoflavone contents of soybeans bred in previously reported regions. The total isoflavone values of all twenty soybean samples analyzed in our study were considerably higher than some reported earlier [11,18]. Our analysis showed that, considering isoflavone distribution, some specific cultivars can be distinguished. Of the investigated samples, there was a group which exhibited a significantly higher content of isoflavones than the others. The group with the higher content

of genisteins, biologically the most active phytoestrogen, could also be clearly distinguished. It has been shown that the isoflavone content should be considered an important characteristic of soybean seeds. Phytoestrogen screening could provide valuable information regarding the capacity for biological activity of different soybean cultivars and, at the same time, could be useful for selection of cultivars for pharmaceutical purposes. Our results suggest that analyzed genotypes may be of interest to producers, plant breeders and phytopharmacists due to their elevated content of phytoestrogenes.

Experimental

Materials: The seeds of 14 soybean genotypes grown on experimental fields at Rimski Šančevi, near Novi Sad, were obtained from the Institute of Field and Vegetable Crops in Novi Sad, Serbia. Daidzein was purchased from Fluka (Buch, Switzerland), genistein from Serva (Heidelberg, Germany) and glycitein from Aldrich (Steinheim, Germany). Solvents used for sample preparation, ethanol and *n*-hexane, were of analytical grade and purchased from Centrohem (Šabac, Serbia). Methanol and acetonitrile were of HPLC grade and purchased from J.T. Baker (Deventher, Netherlands).

Sample preparation: All the samples were harvested on the same day and were stored for 2 months in a storage room, without removal of the seed coat. The moisture content of seeds, determined by heating at 105ºC to constant weight, was 5.5%. For further analysis, soybeans were ground using a coffee mill. A powdered portion (500 mg) was defatted by extraction with *n*-hexane (2 x 10 mL for 30 min), followed by centrifugation for 30 min at 3500 rpm, then extracted for 2 h with 8 mL methanol-water (8:2, v/v) and centrifuged (30 min, 3500 rpm) [29]. Prior to HPLC injection, each extract was passed through a 0.45 µm membrane filter.

HPLC analysis: An Agilent model 1100 HPLC equipped with binar pump, degaser, autosampler and diode array detector (DAD) was used to separate, identify and quantify phytoestrogens. Separation of these compounds was achieved using a 5 µm Zorbax SB C_{18} reversed phase HPLC column (150 x 4.6 mm) with a Zorbax SB C_{18} guard column. Mobile phase gradients were formed between two degassed 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. A post-time period of 20 minutes was applied. The column temperature was 25°C, the solvent flow rate 0.6 mL/min and the injection volume 10 µL. The spectra were collected between 240 and 400 nm by DAD and components in the eluate were detected at 260 nm. Phytoestrogens were identified by retention times, by comparison of UV spectra with those of standard compounds, and from literature data.

Aglycons were quantified from three five-point regression curves ($R \geq 0.9998$) obtained using the corresponding standards (daidzein, glycitein and genistein). Actual concentrations of phytoestrogens in glycoside forms were calculated from the regression curve of the corresponding aglycones, after applying corrections for differences in molecular weight between aglycones and glycosides.

Statistical analysis: Isoflavone analysis of each cultivar was repeated in quadruplicate. The data were analyzed statistically using analysis of variance (Origin 6.1); p value < 0.05 was considered significant. For the data analysis, the SSPS 10.0 program was also used.

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