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Evaluation of Isoflavone Aglycon and Glycoside Distribution in Soy Plants and Soybeans by Fast Column High-Performance Liquid Chromatography Coupled with a Diode-Array Detector

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An ultrafast HPLC/UV−vis DAD method working at 254 nm was applied for the determination of isoflavone aglycons and glycosides (genistin, genistein, daidzein, daidzin, glycitin, glycitein, ononin, formononetin, sissotrin, and biochanin A) in roots, stems, leaves, and soy pods of soy plants and in soybeans of five varieties (Korada, Quito, Rita, OAC Erin, and OAC Vison). An Atlantis dC18 ultrafast RP chromatographic column (20 mm × 2.1 mm, 3 μm particle size) was applied for separation of the isoflavone aglycons and glycosides. A flow rate of the mobile phase (0.1% (v/v) acetic acid, pH 3.75—solvent A and methanol—solvent B) was 0.35 mL min^{-1}, and the column temperature was 36 °C. A linear gradient profile from 13 up to 22% B (v/v) from zero to 2.5 min, up to 30% B to 3.21 min, up to 35% B to 4 min, up to 40% B to 4.5 min, up to 50% B to 5.14 min, and followed by negative gradient up to 13% B to 7.71 min was used. The absolute limits of detection per sample injection (5 μL) were the highest for biochanin A (166.2 fmol) and the lowest for genistin (17.0 fmol), respectively. An accelerated solvent extraction (ASE) in combination with sonication was applied for isolation of biologically active compounds. A solid-phase extraction procedure was used to purify the extracts in the case of analysis of soy plants parts. The recoveries of 96−106% were obtained for the different concentrations of the isoflavone aglycons and glycosides and the different matrixes (overall RSDs 2−9%). The highest isoflavone concentrations were found in roots (12.5 μg g^{-1} dry weight), while the amounts were about 3−1100 μg g^{-1} fresh weight in different varieties of soybeans.

KEYWORDS: Isoflavones; liquid chromatography; UV−vis DAD; accelerated solvent extraction; ultrasonic extraction; solid-phase extraction; soy plants; roots; leaves; stems; pods

INTRODUCTION

The use of plants for large-scale production of biologically active compounds (i.e., phenylpropanoids, quinones, and alkaloids), such as pharmaceuticals, foodstuffs, and cosmetics is of interest from scientific and economic points of view (*1*). The bioactive compounds derived from the phenylpropanoid pathways—which include flavonoids, isoflavones, lignins, coumarins, and many small phenolic substances—have a multiplicity of functions in structural support, pigmentation, defense, and signaling of plants (*2*−*4*). Even phytoestrogen preventive effects to certain diseases have been discovered (*5*−*7*).

Isoflavones are biologically active metabolites that accumulate in soybeans during development. Amounts of isoflavones fluctuate in different plant species and, of course, in different parts of individual plants (*5*−*11*). The highest amounts are generally found in *Fabacae* family plants (including soy plants) that are widely used to human and animal feeding (*12*). The amount of isoflavones present in soybean is variable, depending on genetic and environmental factors (*2*). That is why we studied the changes in the isoflavone content in five varieties of soybeans (Korada, Quito, Rita, OAC Erin, and OAC Vison) and in different parts of two kinds of soy plants varying in the procedure and duration of cultivation.

Analysis of isoflavones in biological samples is complicated due to low efficiency of chromatographic separation and time-consuming sample preparation (*8*, *13*−*19*). A high-performance liquid chromatography (HPLC) coupled with an electrochemical,

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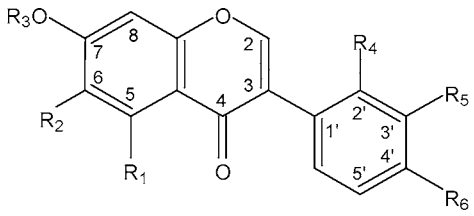

Name of isoflavone	R_1	R_2	R_3	R_4	R_5	R_6
daidzin	H	H	glc	H	H	OH
glycitin	H	OCH_3	glc	H	H	OH
genistin	OH	H	glc	H	H	OH
ononin (formononetin-7-O-β-D-glucoside)	H	H	glc	H	H	OCH_3
sissotrin (biochanin A-7-O-β-D-glucoside)	OH	H	glc	H	H	OCH_3
daidzein	H	H	H	H	H	OH
glycitein	H	OCH_3	H	H	H	OH
genistein	OH	H	H	H	H	OH
formononetin	H	H	H	H	H	OCH_3
biochanin A	OH	H	H	H	H	OCH_3

Figure 1. Chemical formulas of daidzin, genistin, glycitin, ononin, sissotrin, daidzein, genistein, glycitein, formononetin, and biochanin A.

UV−vis diode-array detector (DAD) and/or mass spectrometric detectors (MS) belongs to the most widely used method for the determination of the isoflavones. A combination of highly effective chromatographic techniques with effective isolation/purification techniques is very suitable for the quantitative analysis of the compounds. Commonly used methods for isolation of the isoflavones include a simple mixing, Soxhlet extraction, or ultrasonication techniques using different organic solvents. On the other hand, several new extraction procedures, such as supercritical fluid extraction (SFE), accelerated solvent extraction (ASE), etc., have been developed (*13*) in the last several years as an alternative to the traditional procedures.

The aims of this study were (i) to optimize a combined accelerated solvent extraction/sonication procedure for the isolation of the isoflavone aglycons and glycosides (daidzin, glycitin, genistin, ononin, daidzein, glycitein, sissotrin, genistein, formononetin, and biochanin A; see **Figure 1** for chemical stuctures); (ii) to optimize a solid-phase extraction (SPE) for purification of crude extracts prior to their application on an ultrafast chromatographic column; (iii) to develop a fast and effective HPLC/UV−vis DAD chromatographic method; and (iv) to apply the optimized procedure for evaluation of the isoflavone aglycon and glycoside contents in five varieties of soybeans (Korada, Quito, Rita, OAC Erin, and OAC Vison) and different soy plant parts.

MATERIALS AND METHODS

Chemicals. HPLC-grade methanol (>99.9%; v/v) and glacial acetic acid were from Merck (Darmstadt, Germany). Isoflavone aglycons and glycosides, flavone, and all other chemicals of reagent grade purity were purchased from Sigma Aldrich (St. Louis, MO). The stock standard solutions of isoflavone aglycons and glycosides and flavone at 10 μg mL^{-1} were prepared in aqueous methanol (1:1, v/v) and stored in darkness at 4 °C. The working standard solutions were prepared daily by dilution of the stock solutions with the aqueous methanol. All solutions were filtered through a 0.45 μm Teflon membrane filter (MetaChem, Torrance, CA) prior to HPLC separations.

Instruments. An HP 1100 liquid chromatographic system (Hewlett-Packard, Waldbronn, Germany) was equipped with a vacuum degasser (G1322A), a binary pump (G1312A), an auto sampler (G1313A), a column thermostat (G1316A), and a UV−vis diode array detector (model G1315A) working at 254 nm. A flow rate of the mobile phase (0.1% (v/v) acetic acid, pH 3.75—solvent A and methanol—solvent B) was 0.35 mL min^{-1}, and the column temperature was 36 °C. A linear gradient profile from 13 to 22% B (v/v) from start to 2.5 min, up to 30% B to 3.21 min, up to 35% B to 4 min, up to 40% B to 4.5 min, up to 50% B to 5.14 min, and followed by a negative gradient up 13% B to 7.71 min was used for the separation of isoflavone aglycons and glycosides on an Atlantis dC18 reversed-phase fast chromatographic column (20 mm × 2.1 mm, 3 μm particle size, Waters Corp., Ireland). The peak identity was confirmed by comparing the retention times and the UV spectra of individual compounds (match factors greater than 995) and finally by the standard addition method. The UV−vis spectra were registered in the range of 190−400 nm (SBW 100 nm).

The pH values were measured using a WTW InoLab Level 3 pH meter. The combined pH-electrode (SenTix H) was regularly calibrated by a set of WTW buffers (all from WTW, Weilheim, Germany). Accelerated solvent extraction (ASE) procedure was performed using a PSE-one extractor (Applied Separations).

Plant Samples. Nature Plants. Five varieties of soybeans (Korada, Quito, Rita, OAC Erin, and OAC Vison) were obtained from the Institute of Crop Science and Breeding (Brno, Czech Republic). The soy plants (*Glycine max*, cv. Rita, from the same source) were cultivated at the experimental field of the Mendel University of Agriculture and Forestry (MZLU) in Žabčice (average year temperature, 9.2 °C and yearly rainfall, 450 mm) in 2003. During sowing of the soy kernels, a rhizobial preparation HISTICK (B.O.R., Czech Republic) was applied. The soy plants were harvested after 105 days (15 weeks) of cultivation. The harvested soy plants were divided into roots, stems, leaves, soy pods, and soybeans and finally dried at room temperature. The roots of the plants were cleaned from soil by water flooding. The height of individual soy plants varied from 70 to 85 cm at the end of cultivation. The weights of the individual soy plant parts varied between 25 and 35 g for soy pods, 50−60 g for soybeans, 5−10 g for roots, 15−25 g for stems, and 5−10 g for leaves.

Cultivated Plants. Before cultivation, the kernels of soy plants (*Glycine max*, cv. Rita) were sterilized for 30 min in 10% H_2O_2 to eliminate fungus spores and bacillus. The soy kernels were cultivated in plastic phototrays (260 mm × 220 mm × 50 mm). Glass platters (260 mm × 50 mm × 2 mm, five per tray) were situated about 1 cm over the solution level. The glass platters were covered with strips of a filter paper (250 mm × 90 mm) the longer edges of which were inserted into a cultivation solution containing water. Individual soy kernels were placed on the wet filter paper (10 pieces on each glass platter). The germination was conducted for 3 days at 25 °C in darkness of a thermostatic box (Model TER 5/1, Chirana, Brno, Czech Republic). The germinating soybeans were replaced into cultivation vessels containing water. The cultivation was run in a Versatile Environmental Test Chamber (MLR 350, Sanyo, Japan) in two cycles: (i) 14 h, 22 °C, humidity 60%, lighting 200 μE m^{-2} s^{-1} and (ii) 10 h, 18 °C, humidity 60%, darkness. The harvested soy plants were divided into roots, stems, and leaves, and the soy plant parts were dried in an oven (24 h, 105 °C; KDB, Poland).

Homogenization of Soybeans and Soy Plant Parts. The sample (2000 ± 5 mg) of dried soy plant parts (roots, stems, leaves, soy pods, and soybeans) and soybeans (Korada, Quito, Rita, OAC Erin, and OAC Vison) was spread in a mortar and homogenized by an Ika A11 basic grinder (IKA Werke GmbH and Co., Staufen, KG, Germany) to tiny particles (less than 0.1 mm).

Extraction Procedures. Combined Sonication/Accelerated Solvent Extraction (S/ASE) Procedure. A modified accelerated solvent extraction (ASE) procedure (*14*) was used for preparation of real samples for the determination of isoflavone aglycons and glycosides. It was advantageous to use 5 min sonication prior to ASE due to increasing the yield of the ASE procedure. The homogenized sample (500 ± 5 mg) was wrapped in filter paper (small envelope like format) and sprinkled with 3.0 g of SPE-ed matrix, 1.5 g of florisil (15.5% MgO, 84% SiO_2, 0.5% Na_2SO_4; pH 8.5; 60−100 μm particle size), and 3.0 g of Ottawa sand (Allentown, PA). Flavone was dosed as an internal standard on the top of the sample (100 μL, 10 μg mL^{-1}). The mixture was placed into a 10 mL stainless steel extraction cell and extracted under controlled conditions in two steps: (i) preheating period (5 min), the solvent was hexane (elution of lipophilic compounds), temperature 145 °C, pressure 140 bar, two extraction cycles (5 min),

Table 1. Validation Data for the Determination of Isoflavone Aglycons and Glycosides ($n = 5$) at 254 nm

isoflavones	t_R[a] (min)	LOD[b] (ng mL^{-1})	RSD[c] (%)	LOD[d] (fmol)	isoflavones	t_R[a] (min)	LOD[b] (ng mL^{-1})	RSD[c] (%)	LOD[d] (fmol)
daidzin	1.32	3.0	2.1	36.2	glycitein	5.77	2.0	3.8	35.1
glycitin	1.64	2. 9	2.3	32.4	sissotrin	6.32	1.5	1.2	25.7
genistin	3.14	1.5	2.9	17.0	genistein	6.57	1.1	1.6	20.9
ononin	5.15	2.5	1.3	46.1	formononetin	7.20	1.9	2.2	36.4
daidzein	5.44	1.6	1.5	30.7	biochanin A	8.06	9.4	1.9	166.2

[a] Retention times in min, total time of chromatographic run ca. 10 min as compared to the times of chromatographic analyses 20–60 min (*8*, *12–20*). [b] Limits of detection (3 S/N). [c] Relative standard deviations ($n = 5$). [d] Limits of detection per column injection (5 μL).

90 s using pressurized nitrogen and (ii) preheating period (5 min), the solvent was 90% aqueous methanol, temperature 145 °C, pressure 140 bar, two extraction cycles (5 min), 90 s using pressurized nitrogen.

The final extracts were collected in 60 mL glass vials with Teflon coated rubber caps and centrifuged at 4000*g* (Hettich, Germany). The supernatants were evaporated to dryness in a rotary vacuum evaporator (IKA RV 05-ST) with an HB 4 water bath (both, IKA-Werke GmbH and Co., Staufen, KG, Germany). The residue was reconstituted in 1 mL of methanol and filtered through a 0.45 μm Teflon membrane filter (MetaChem, Torrance, CA) prior to injection into the HPLC system.

Solid-Phase Extraction. S/ASE extracts obtained from soy plant parts were purified by a solid-phase extraction (SPE) procedure applying a computer-controlled robotic system Aspec XL (Gilson). An HLB OASIS (Waters Corp. Milford) SPE cartridge was equilibrated by 1 mL of methanol and then 1 mL of distilled water before extraction. The final SPE procedure consisted of four steps: (i) addition of sample extracts diluted with methanol up to 3 mL; (ii) addition of 1 mL of aqueous (v/v) solution containing 5% methanol and 2% acetic acid; (iii) addition of 1 mL of aqueous (v/v) solution containing 20% methanol and 2% ammonium hydroxide; and (iv) addition of 1 mL of aqueous (v/v) solution containing 90% methanol and 2% ammonium hydroxide. The fourth fraction was evaporated to dryness in a rotary vacuum evaporator (IKA RV 05-ST) with an HB 4 water bath (both, IKA-Werke GmbH and Co., Staufen, KG, Germany). The residue was reconstituted in 1 mL of methanol and filtered through a 0.45 μm Teflon membrane filter (MetaChem, Torrance, CA) prior to injection into the HPLC system.

RESULTS AND DISCUSSION

Isoflavone HPLC/UV−Vis DAD Separation. Recently, we published an HPLC/UV−vis DAD determination of daidzin, glycitin, genistin, ononin, daidzein, glycitein, sissotrin, genistein, formononetin, and biochanin A in soy bit samples pretreated by an accelerated solvent extraction (ASE) procedure (*15*). The isoflavone aglycons and glycosides were well-separated and easily detected. Linear relationships between the peak heights and/or the peak areas and the isoflavone aglycon and glycoside concentrations ($R^2 = 0.9901$ for glycitein; $R^2 = 0.9999$ for genistein) were obtained with RSDs about 1.3−3.8%. The limits of detections (LODs for the signal-to-noise ratio S/N = 3 criterion) ranged from 9.4 to 1.1 ng mL^{-1} (166.2−17.0 fmol per injection, 5 μL) for biochanin A and genistein, respectively (see **Table 1** for basic characteristics of the modified chromatographic procedure).

Combined S/ASE Extraction Procedure. Recovery of the combined S/ASE extraction procedure (see Materials and Methods) was checked by the addition of known amounts of the isoflavone aglycons and glycosides to soybean homogenates. The recoveries of 96−106% were obtained for the different concentrations of the studied isoflavone aglycons and glycosides and the different matrixes (spikes: 1−30 μg mL^{-1}). The intra- and interday repeatabilities (RSDs 2.3−5.2 and 3.2−5.8%, respectively) of the procedure were determined using six repetitive analyses of representative samples over 5 days. Good precision was obtained for the studied isoflavone aglycons and glycosides in food samples.

Table 2. Mean Values of the Amount (in μg g^{-1} FW) of the Extracted Soybean Isoflavone Aglycons and Glycosides ($n = 6$) Using Flavone (100 μL, 10 μg mL^{-1}) as an Internal Standard

	amount of isoflavone aglycons and glycosides (in μg g^{-1} FW)[b]				
isoflavone[a]	Korada	Quito	Rita	OAC Erin	OAC Vision
daidzin	1094.0	661.0	1009.2	580.4	497.8
glycitin	55.7	118.5	144.1	103.4	742.6
genistin	1022.0	409.1	656.3	482.3	405.3
ononin	66.8	294.3	590.6	632.1	447.2
daidzein	28.9	13.4	49.2	261.2	18.0
glycitein	69.6	31.0	49.5	261.7	32.9
genistein	14.0	3.7	28.2	101.4	8.6
total[c]	2351	1531	2527	2161	2152

[a] Sissostrin, formononetin, and biochanin A not detected. [b] RSDs 2.1–9.0%, $n = 6$. [c] 1161–2743 μg g^{-1} FW was found by Wang et al. (*8*).

The determined amounts of daidzin, genistin, and ononin were about 60−1100 μg g^{-1} fresh weight (FW), and the amounts of isoflavone aglycons and glycosides (daidzein, genistein, and glycitein) were about 3−270 μg g^{-1} FW. The higher variability of the isoflavone aglycon and glycoside amounts in the soybeans (**Table 2**) is caused by the differences among the varieties. The total amounts of the compounds were comparable with those found by Wang et al. (*8*) They studied 210 soybean cultivars grown in South Dakota and found out that the total isoflavone contents ranged from 1161 to 2743 μg g^{-1} FW.

SPE Purification Procedure. When the HPLC/UV−vis DAD chromatographic procedure was applied to the analyses of the extracts of the soy plant parts obtained by combined S/ASE procedures, we had found out that the determination of isoflavone aglycons and glycosides was seriously influenced by a number of undesirable compounds (**Figure 2A**). Thus, it was necessary to remove the undesirable compounds from the extracts by using a solid-phase extraction SPE prior to the HPLC/UV−vis DAD procedure (obtained chromatogram is shown in **Figure 2B**). The cleanup procedure seriously simplifies the chromatograms.

Accuracy, precision, and recovery of the analytical procedure were evaluated with the samples of plant materials spiked with 100 μL of isoflavone standards (1−30 μg mL^{-1}) and flavone (100 μg mL^{-1}). Homogenates were assayed blindly, and isoflavone aglycon and glycoside concentrations were calculated from the calibration curves. Relative standard deviations (RSD 3.1−8.2%) of the intraday assay were obtained using six homogenates. Interday precision (RSD 4.1−9.0%) was determined by analysis of six homogenates over a 5 day period.

Isoflavone Aglycon and Glycoside Determination in Soy Plant Parts. We studied the changes in the isoflavone aglycon and glycoside content in two different kinds of soy plants varying in the procedure and duration of cultivation. The first

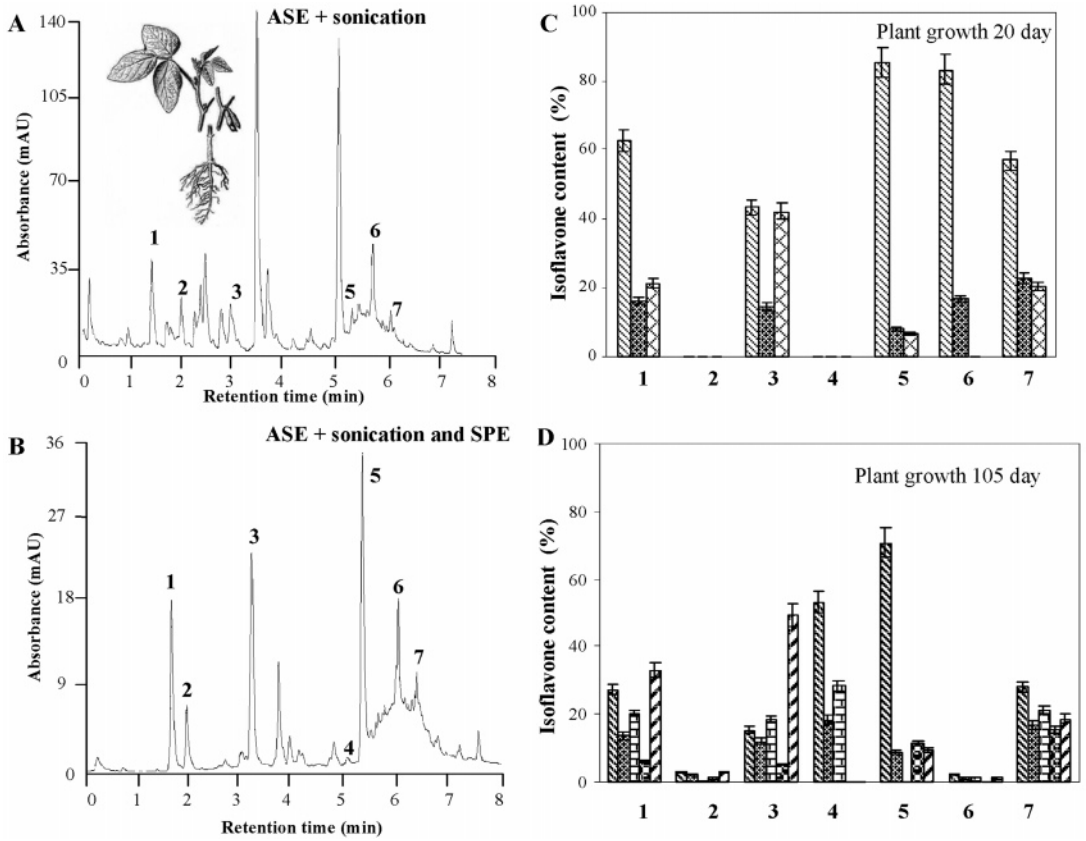

Figure 2. HPLC–UV chromatograms of isoflavone aglycon and glycoside soy plant extracts obtained by ASE + sonication procedure (**A**) without and (**B**) with a solid-phase extraction procedure. Amount of isoflavone aglycon and glycoside (columns from left to right—content in roots, stem, and leaves and roots, stem, leaves, soy pods, and soybeans, respectively) detected in soy plants cultivated in a versatile environmental test chamber under controlled conditions for 20 days (**C**) and cultivated in the field for 105 days (**D**). 100% was 12.5 μg g^{-1} DW. Description of the peaks (**A** and **B**) and the substances (**C** and **D**): 1, daidzin; 2, glycitin; 3, genistin; 4, ononin; 5, daidzein; 6, glycitein; and 7, genistein. For other details, see Materials and Methods.

group of studied soy plants was cultivated under controlled conditions for 20 days (see Materials and Methods), and the second group of studied soy plants was cultivated in the field for 105 days. We determined daidzin, genistin, daidzein, glycitein, and genistein by the optimized HPLC/UV−vis DAD method in the first group of studied plants. The highest amount of the determined isoflavone aglycons and glycosides was detected in roots (**Figure 2C**). The same results were described earlier (*9*). We determined daidzin, genistin, daidzein, glycitein, genistein, glycitin, and ononin in the second group of studied plants. Their highest amounts were detected in roots and soybeans (**Figure 2D**).

It is known that in response to infection, soy plants produce isoflavonoid phytoalexins that inhibit pathogen growth (*9*, *10*). In addition, isoflavonoids can act as inducers of nodulation genes of *Rhizobium* species in the early steps of symbiosis between soy roots and soil microorganisms (*11*). We assume that the increased concentration of studied isoflavone aglycons and glycosides in plant parts obtained in the second group of the studied plants is related to the ability of the plants to control the development and numbers of the rhizobial bacteria. On the contrary, we did not apply any rhizobial bacteria at cultivation of the first group of the studied plants. That is why it was possible to assume that the synthesis of isoflavone aglycons and glycosides proceeding in roots served for plant protection against pathogens. Moreover, we determined lower concentration of the isoflavone aglycons and glycosides in the leaves, stems, and soy pods in comparison with the roots and soybeans (**Figure 2C,D**). It is well-known that daidzein, glycitein, genistein, and 7-β-D-glukosides (daidzin, glycitin, and genistin) obviously occurred in soybeans (*14*).

In this work, optimization of rocket-powered analysis of isoflavone aglycons and glycosides (daidzin, glycitin, genistin, ononin, daidzein, glycitein, sissotrin, genistein, formononetin, and biochanin A) by fast column high-performance liquid chromatography coupled with a diode-array detector is described. In addition, the optimized method was used for the evaluation of the amounts of the isoflavone aglycons and glycosides in different varieties of soybeans and soy plant parts. The proposed procedure is faster (ca. 8 min) that most of the procedures listed in the literature (*15*−*20*) without losing its separation efficiency (up to 10 isoflavonoids can be determined as compared to four to 20 compounds in the literature) and sensitivity (tens to hundreds of fmol as compared to hundreds of fmol in the literature). It is very suitable for the control of plant growth and also for fast process control and routine analysis.

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