

Pressurized Solvent Extraction of Genistein and Its β -Glucoside Conjugates from Soybean Flours and Soy-Based Foods¹

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Cereal Chem. 84(1):44–47

Nonsteroidal phytoestrogens, including isoflavones, are plant-derived compounds present in the human diet that are associated with reduced risks of heart disease, cancer, and possibly osteoporosis (Boersma et al 2001; Lichtenstein 2001; Ho et al 2001). In October 1999, the U.S. Food and Drug Administration approved a health claim of a reduced risk of heart disease with increased consumption of soy protein (Hayes 2001).

Soybeans are a rich source of isoflavones including genistein, diadzein, and glycitein (Fig. 1). Genistein occurs as the β -glucosidic conjugate called genistin, which is most frequently malonylated or acetylated at the 6-hydroxyl of the glucose moiety. The levels of the three derivatives of genistein and their total are highly variable in soybean cultivars as well as in soy ingredients and soy foods because of genetic and environmental effects on the bean (Eldridge and Kwolek 1983).

Isoflavones have been extracted from soybeans and soy products using 10–20 parts of a polar organic solvent (methanol, ethanol, and acetonitrile) often mixed with \approx 20–80% (v/v) water. Sometimes the water in the extraction medium is acidified, and heating, sonication, or ultrasound waves (Rostagno et al 2003) are applied during the extraction step. Solid-phase micro-extraction (SPME) has also been successfully used to extract genistein and diadzein (Mitani et al 2003). Acetonitrile and water mixtures are efficient solvents for extraction of the isoflavones (Griffith and Collison 2001). Murphy et al (2002) have shown that aqueous acetonitrile can be more effective than aqueous methanol, ethanol, and acetone for extraction of total isoflavone forms from most soy products when using a 2-hr stirring extraction.

The primary objective of this study was to extract genistein from soybeans and soy foods by low- and high-pressure extraction methods, and to compare the levels of total genistein in the extracts by HPLC. A secondary objective was to develop a rapid, automated high-pressure extraction method.

MATERIALS AND METHODS

Materials

Soybean (raw) samples 1, 2, and 4 were obtained from a local supermarket, and sample 3 was donated by David Sauer, USDA,

ARS, Grain Marketing and Production Research Center. Soybean sample 5 was an experimental line obtained from the USDA Federal Grain Inspection Service (FGIS), Kansas City, MO. The soy nut sample (intact roasted beans), soy protein isolate (finely granulated), and soy meat substitute (patty) were also obtained from local stores.

Raw soybeans and roasted soy nuts (20 g) were ground for 2 min in a Stein high-speed impact mill (Atchison, KS). The soy meat substitute was pan-fried according to package directions, allowed to air-dry overnight, and then ground as the other samples. The commercial soy protein isolate was analyzed “as is”. Genistein and genistin standards were purchased from Fluka Chemie AG (Milwaukee, WI), while 6''-*O*-acetylgenistin standard was purchased from LC Laboratories (Woburn, MA). The purity of the standards was 98% as estimated by response area in HPLC chromatograms. Fluorescein (CAS no. 2321-07-05) was purchased from Sigma (St. Louis, MO). 2,4,4'-Trihydroxydeoxybenzoin (THB, 1-[2,4 Dihydroxy-phenyl] – [3-hydroxyphenyl] – ethanone), a synthetic compound developed as an internal standard for isoflavone analysis, was donated by Patricia Murphy, Department of Food Science and Human Nutrition, Iowa State University, Ames, IA. All other chemicals were reagent-grade and were purchased from Burdick and Jackson (Muskegon, MI).

Moisture Measurement

Moisture contents were determined for all samples by Approved Method 44-15A (AACC International 2000).

Extraction at Atmospheric Pressure

The reference method for a stirring extraction of isoflavones was a modified stirring method of Barnes et al (1994). Soy material (500 mg, as-is moisture basis [mb]) was extracted with 10 mL of 80% (v/v) aq methanol by stirring 1 hr at room temperature in a 50-mL Erlenmeyer flask. The extract was then defatted with hexane (3 \times 5 mL) before HPLC analysis.

High-Pressure Extraction

Pressurized solvent extraction (PSE) was conducted on an ISCO model SFX 3560 (Lincoln, NE), which is an automated, dual-module supercritical fluid extractor/pressurized solvent extractor. The PSE method was conducted at an 80°C extraction chamber temperature, 138 bar (2,000 psi) extraction pressure, and 5 mL/min flow rate. The restrictor temperature was 40°C (Fig. 2). The soy material (500 mg, as-is) was weighed into an ISCO 20- μ m fritted extraction cartridge, and the soy sample was extracted for 1 min in 10 mL of 80% aq methanol without flow (static extraction). Then 10 mL of 80% aq methanol was forced through the extraction cartridge at a flow rate of 5 mL/min and the extracts were collected in ISCO 20-mL glass test tubes. After the extraction steps, the cartridge was flushed for 7 min with supercritical CO₂ into a second test tube. Total extraction time for PSE, including automated sample handling and pump pressurization, was 20 min. The total volume of aqueous methanol collected in both tubes was \approx 20 mL.

¹ Cooperative investigations, U.S. Department of Agriculture, Agricultural Research Service, and the Department of Grain Science and Industry, Kansas State University. Contribution No. 07-063-J from the Kansas Agricultural Experiment Station, Manhattan, KS 66506. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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The contents of the two tubes were combined, followed by extraction with hexane (3 × 5 mL) to remove lipids. The bottom phase was evaporated to dryness under reduced pressure at 40°C on a rotary evaporator (Buchi, Germany). The residue was dissolved in a mixture of 9.0 mL of 80% aq methanol and 1.0 mL of 80% aq methanol containing fluorescein standard. Isoflavone extract (1 mL) was then centrifuged at 16,000 × g for 2 min before HPLC injection (Fig. 2).

HPLC Analysis

Reversed-phase (RP) HPLC separation was conducted on a Hewlett Packard (Palo Alto, CA) 1090 dual pump chromatograph with a diode array detector set at 262 nm with a 4-nm bandwidth. The stationary phase was an HP Zorbax 4.6 × 150 mm C8 column with the particle size of the stationary phase at 5 μm. The pressure for this method was 150 bar (2,176 psi) with a flow rate of 1.5-mL/min and a runtime of 30 min plus a 10 min post-run column equilibration time. The mobile phase was a linear gradient of 0.1% trifluoroacetic acid (TFA) in water/0.1% TFA in acetonitrile. Sample (12 μL) was injected into the column with an HP auto injector. After injection, the 0.1% TFA in acetonitrile was increased at a linear rate from 0 to 50% over 30 min, and was then increased to 100% over 5 min. The amount of TFA in acetonitrile was then reduced to 0% over the last 5 min of the run to reequilibrate the column. Standard curves for genistein, genistin, and acetylgenistin were obtained using reference standards. Malonylgenistin standard was not commercially available at the time of this study and the minor isoforms of the malonyl and acetyl described by Griffiths and Collinson were not identified in this work. The molar standard curve prepared from the acetyl glucoside standard was also used to quantify the malonyl and acetyl glycosides due to the similarity of their molar extinction coefficients. The weight of aglycone genistein was calculated by multiplying the glycoside weight (x) by the ratio of the molecular weight of genistein (270) to the molecular weight of the glycoside (432 for genistin). Fluorescein was used as an internal standard to gauge retention time drift.

Recovery of 2,4,4' Trihydroxydeoxybenzoin (THB), A Model Compound for Isoflavones

Recovery of THB, A model substance for isoflavones, was tested using a multiple recovery addition scheme. THB was added to ground soy sample 1 at three levels, 56, 112, and 168 ppm. The sample was allowed to dry and then extracted. The recovery of the three levels was 98, 96, and 86%, respectively.

Statistics

All samples were extracted in triplicate except for ground soy sample 2, which was extracted in duplicate. Triplicate analysis was performed at each THB level in the recovery experiment. Analysis of variance was performed using statistical analysis (v.8.0, SAS Institute, Cary, NC) and was used to determine significant differences in the amount of total genistein (normalized to aglycone weight) extracted by PSE compared with 1 hr of stirring.

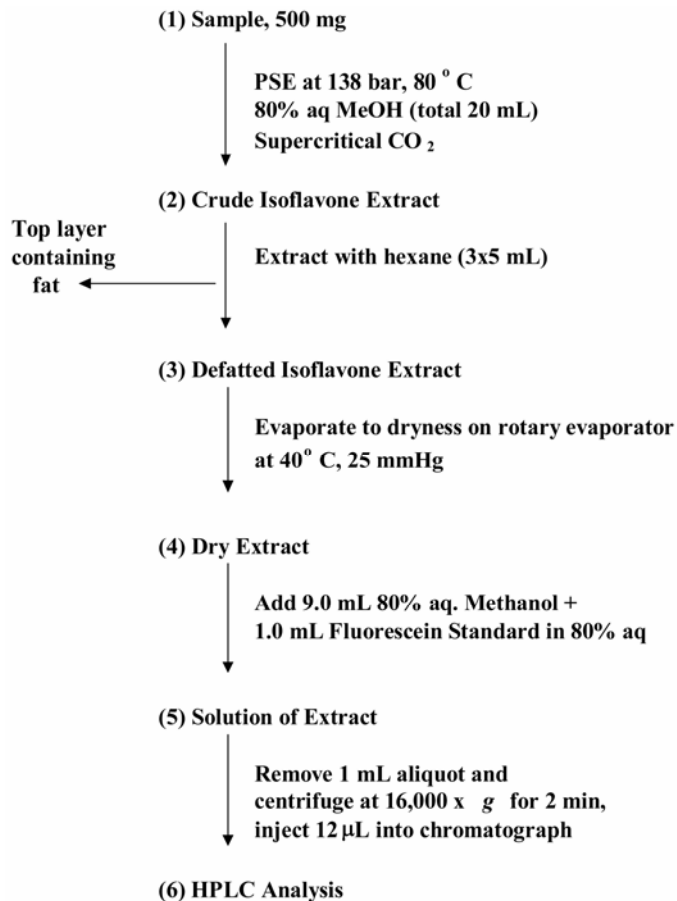


Fig. 2. Flow diagram of pressurized solvent extraction (PSE) genistein extraction workup.

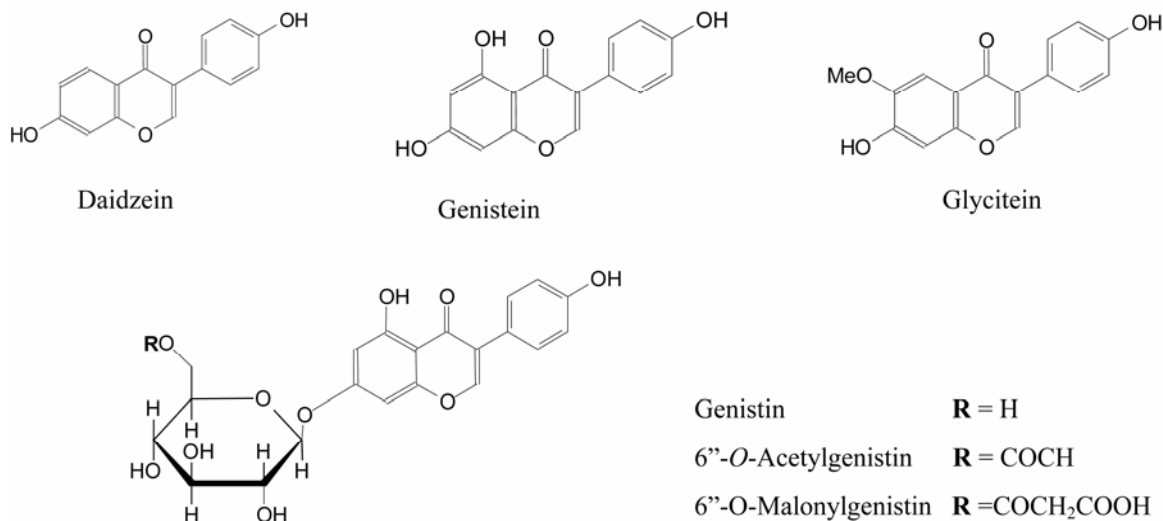


Fig. 1. Structures of daidzein, genistein, glycitein, and genistein glycosidic conjugates.

TABLE I
Genistein Equivalents ($\mu\text{g/g}$) Determined by the Pressurized Solvent Extraction (PSE) Method and the Stirring Extraction Method

Soy Sample	PSE			<i>t</i> -grouping ^b	Stir		
	Mean	\pm STD	RSD ^a		Mean	\pm STD	RSD ^a
Flour 1	757	\pm 30	4	C	766	\pm 15	1
Flour 2	1,749	\pm 149	9	A	1,728	\pm 214	12
Flour 3	1,342	\pm 15	1	B	1,266	\pm 160	13
Flour 4	540	\pm 22	4	C	566	\pm 15	3
Flour 5	743	\pm 64	9	C	782	\pm 9	1
Meat substitute	132	\pm 8	6	D	116	\pm 3	3
Nuts	837	\pm 46	6	C	795	\pm 9	1
Protein isolate	732	\pm 34	5	C	674	\pm 28	4

^a Percent relative standard deviation.

^b Samples with different letters are significantly different ($\alpha = 0.05$).

TABLE II
Comparison of Genistein and Glycosidic Conjugates Extracted by the Pressurized Solvent Extraction (PSE) and the Stirring Extraction Methods^a

Sample	Extraction Method	Genistein Conjugates (% total genistein)			
		Genistin	Malonyl Genistin	Acetyl Genistin	Free Genistin
Flour 1	PSE	34.28a	5.74a	59.23a	0.75a
	Stir	23.65b	5.88a	69.77b	0.70a
Flour 2	PSE	43.04a	3.95a	52.23a	0.77a
	Stir	28.13b	5.57a	65.50b	0.80a
Flour 3	PSE	42.91a	4.73a	50.88a	1.49a
	Stir	42.98a	5.08a	50.76a	1.18a
Flour 4	PSE	41.14a	16.1a	41.26a	1.51a
	Stir	32.26b	18.31a	48.16b	1.27b
Flour 5	PSE	51.45a	5.72a	40.79a	2.04a
	Stir	39.82b	5.21a	53.24b	1.73a
Meat substitute	PSE	46.86a	16.27a	32.60a	4.26a
	Stir	39.21b	17.71a	38.39b	4.69b
Nuts	PSE	86.23a	10.52a	0.49a	2.76a
	Stir	40.37b	30.78b	26.28b	2.57b
Protein isolate	PSE	56.43a	2.83a	36.88a	3.87a
	Stir	33.67b	3.95a	58.41b	3.97a

^a Values within samples and a molecular class with different letters are significantly different ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Methods of Extraction and Chromatography

Compared with the method of Barnes requiring stirring with extraction solvent for 60 min, the PSE extraction procedure required 20 min and required no filtration. Total time for the chromatographic separation and column reequilibration was 40 min. Recovery experiments utilized ground soy that was spiked with three levels of THB. The recovery of THB from the ground soy was satisfactory ($\leq 86\%$ recovered).

Comparison of the Extraction Methods

Levels of genistein extracted by the PSE extraction method closely matched those of a 1-hr stir extraction method. The same extracting solvent, 80% aq methanol, was employed for each method. Table I shows the mean normalized levels of genistein (genistein equivalents) found in the various soy samples when extracted by the PSE and the stirring methods. It is important when reporting the amount of genistein extracted that the value is corrected to the aglycone weight (Song et al 1998). It has been shown that the gut bacterial enzymes remove the conjugated sugar from genistein, and it is generally believed that the aglycone form of the molecule passes through the intestinal wall (Setchell et al 1984). There were no significant differences in the amounts of genistein extracted by the PSE high pressure and the atmospheric pressure methods (Table I). However, Table II shows significant differences in the conjugate forms of genistein extracted by the two methods, except for ground soy sample 3. Heat used during PSE extraction caused significantly less acetylgenistin to be present in extracts when compared with the atmospheric extraction method where ambient temperature was used.

The change in the forms of genistein was due to heat-induced deesterification of the acetylgenistin to genistin, except for soy nuts. When soy nuts were subjected to the PSE extraction method, deesterification of both acetyl genistin and malonyl genistin occurred (Table II).

CONCLUSIONS

PSE offers a rapid alternative to the atmospheric extraction method to recover genistein from soy flour and soy foods. Other advantages of the PSE extraction are automated operation and the fact that no filtration step is required. Disadvantages of the method are the initial cost of purchasing the extraction instrument and the need for a greater volume of methanol per sample extraction.

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[Received December 9, 2005. Accepted August 27, 2006.]