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A Simplified HPLC Method for the Determination of Phytoestrogens in Soybean and Its Processed Products

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By using the proposed procedure, phytoestrogens (daidzein, genistein, coumestrol) can be isolated from soybean and its processed products and subsequently quantitated by HPLC without defatting and cleanup of the samples prior to assay. The samples are extracted with acetonitrile–water, and the extract is filtered through a glass fiber filter. The analytes in the filtrate are in turn separated by HPLC on a C_{18} column and quantified by spectrometry. The method is sensitive to 2 ppm of isoflavones with UV detection and 0.5 ppm of coumestrol with fluorescent detection. The recoveries of phytoestrogens in spiked samples ranged between 75 and 110%. The rapidity, simplicity, and low cost of the method make feasible the assay of large numbers of samples in a regulatory laboratory.

Soybean and its processed products have been used as food in the Orient for centuries. They are known to contain high amounts of protein composed of all the essential amino acids. Soybean oil is also known to be rich in ω-3 fatty acids and fat-soluble vitamins. The carbohydrates of soybean are largely polysaccharides and indigestible fiber, which reduces the diseases of the lower gastrointestinal tract. The soybean has been, therefore, acclaimed as a health food. In the United States, people are now eating more health cautiously. Processed soy products such as tofu, soy milk, soy sauce, etc., are commonly sold in the oriental food store, as well as the local supermarket, and the consumption of these products continues to increase. However, soybeans contain estrogenic compounds, isoflavones, and coumestanes, and there is a significant carry-over of the soy phytoestrogens into its processed products (Murphy, 1982). This means more exposure to phytoestrogens by the consumer. These compounds after ingestion can induce estrus in immature animals or interfere with the normal reproductive processes (Thomson, 1975; Morley et al., 1966). Besides, coumestrol has been suspected to be a tumor-promotor (Verdeal et al., 1980). In order to assure that food containing appreciable quantities of phytoestrogens are not used for human consumption, the occurrence of phytoestrogens in soybeans and its products thus merits careful scrutiny. As a result, the search for more effective and simple monitoring methodologies are needed.

Naim et al. (1974) described a gas–liquid chromatographic (GLC) procedure for the quantitation of soybean genistein and daidzein after the preparation of the trimethylsilyl derivatives. West et al. (1978) developed a high-performance liquid chromatographic (HPLC) method for the analysis of soybean genistein and 4′,6,7-trihydroxyisoflavone. Since HPLC methodology can directly analyze these compounds in free and conjugated forms in samples without the need for derivatization, a number of HPLC methods have been developed during the past decade for the determination of soybean isoflavones (West et al., 1978; Murphy, 1981, 1982; Eldridge, 1982b) and coumestrol (Lookhart et al., 1978, 1979). Murphy (1982) investigated various extraction solvents for isoflavones and coumestrol with and without H_2O or hydrochloric acid and found that acetonitrile with H_2O or hydrochloric acid was superior to all other solvent systems. But, the recoveries for daidzein and coumestrol were lower than 63% for the best extraction systems. Recently, Pettersson and Kiessling (1984) described

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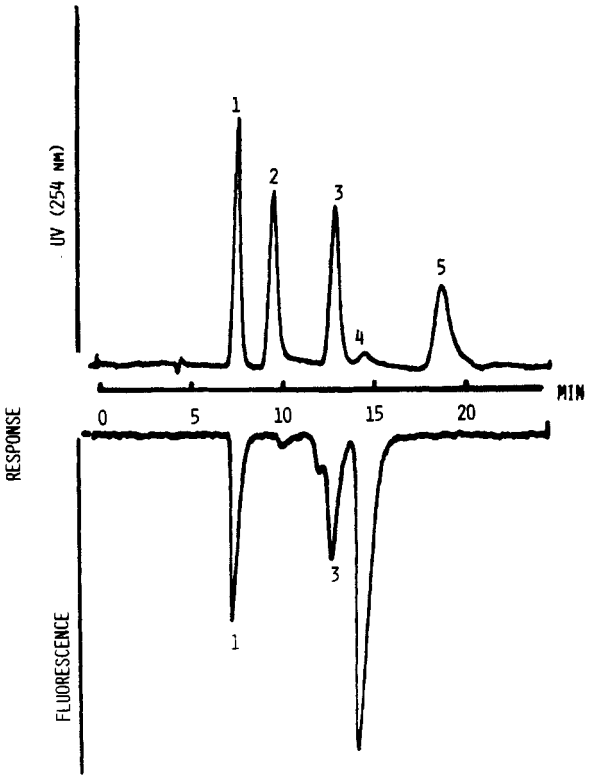

Figure 1. Chromatogram of phytoestrogen standards: 1, daidzein (30 ng); 2, genistein (30 ng); 3, formononetin (30 ng); 4, coumestrol (6 ng); 5, biochamin A (30 ng).

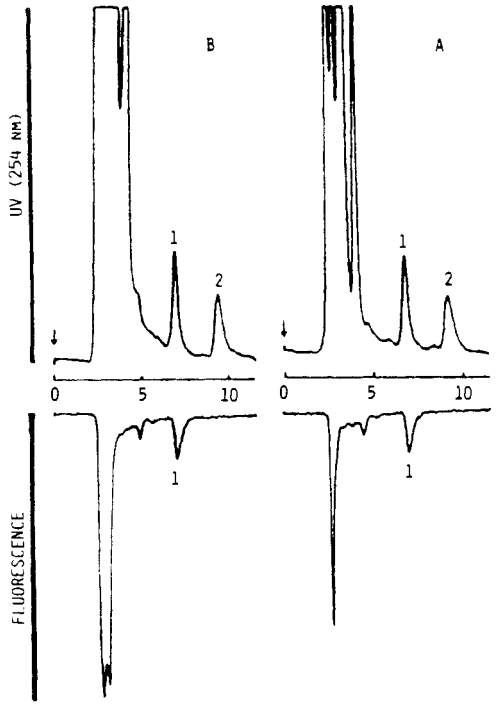

Figure 2. Chromatograms of free phytoestrogens in soybean extracts with (A) and without (B) Sep-pak C_{18} cleanup.

a simplified cleanup procedure for the determination of five main phytoestrogens, genistein, daidzein, biochanin A, formononetin, and coumestrol, in animal feed. In this procedure, a cleanup cartridge was introduced for purifying the crude sample extract. However, the extraction procedure is tedious and time-consuming, and the use of cleanup cartridge makes the method less attractive.

This paper describes a simplified procedure for the extraction, separation, and HPLC quantitation of coumestrol and isoflavones in soybean and its processed products.

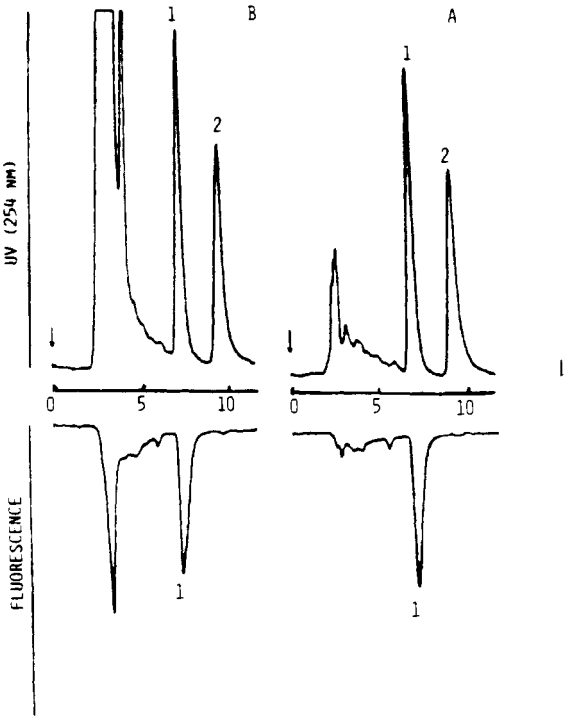

Figure 3. Chromatograms of total phytoestrogens in soybean extracts with (A) and without (B) Sep-pak C_{18} cleanup.

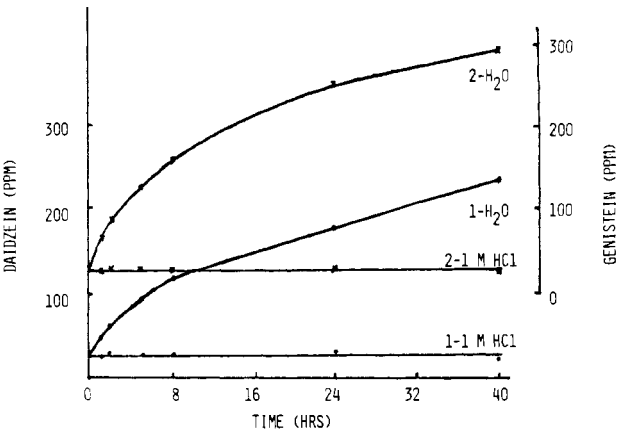

Figure 4. Effect of soaking finely ground soybean with H_2O or 1 M HCl on apparent free phytoestrogen content: 1, daidzein; 2, genistein.

MATERIALS AND METHODS

Instrumentation. The HPLC system consisted of Beckman-Altex Model 322 liquid chromatograph pump equipped with a 20-μL injection loop and a Kratos GM 970 fluorescence detector connected in series to a Hitachi 100-40 UV detector. A Waters Associates μ-Bondapak C_{18} (10 μm), 3.9-mm i.d. × 30-cm length, ambient temperature, with guard column packed with C_{18}/Corasil (37–50 μm) was used.

Materials. Soybean and defatted soy flake were purchased from a feed supply company, and processed soybean products were obtained from local groceries. HPLC-grade water and other organic solvents were from J. T. Baker. The isoflavones were purchased from ICN Biochemicals, Inc., and coumestrol was from Eastman Kodak Co. Other reagent-grade chemicals were obtained from Sigma Chemical Co.

Sample Preparation. *A. Procedure for Free Estrogens. (a) Soybean and Soy Milk Film.* Grind soybean, defatted soy flake, or soy milk film in a Microjet 10J mill to a finely divided powder. Weigh 2.00 g of finely ground sample into a 50-mL

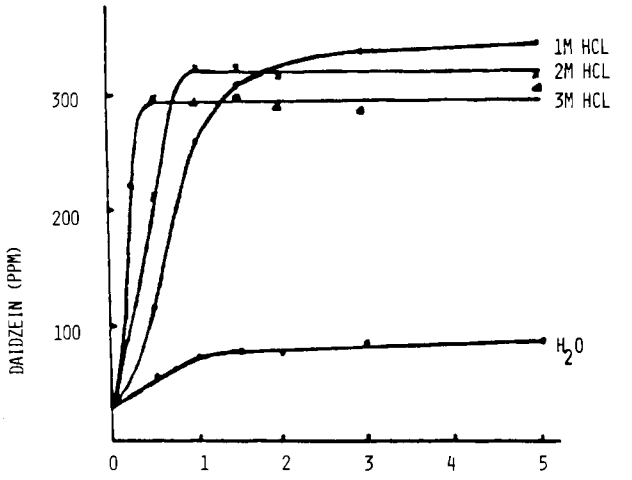

Figure 5. Hydrolysis of conjugated daidzein in soybean with hydrochloric acid at 99–100 °C.

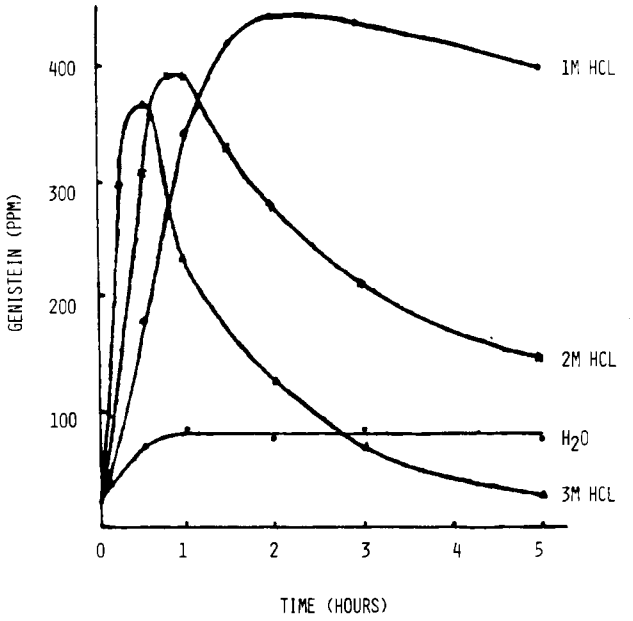

Figure 6. Hydrolysis of conjugated genistein in soybean with hydrochloric acid at 99–100 °C.

glass-stopper Erlenmeyer flask, add 6 mL of 1 M HCl and 24 mL of acetonitrile, and stir for 30 min. Let the mixture settle for a few minutes, dilute 1.0 mL of the supernatant with 1.0 mL of H_2O, and filter with Gelman type A/E size 13 mm glass fiber filter. Use this filtrate for LC analysis or, in the case of high phytoestrogen concentration, properly dilute with the LC mobile phase.

(*b*) *Soy Curd, Paste, and Sprout.* Blend 20.0 g of sample with 60 mL of 1 M HCl in a high-speed blender. Weigh out 8.00 g of the blended sample (equal to 2.00 g of original sample) into 50-mL glass-stopper Erlenmeyer flask, add 24 mL of acetonitrile, and shake for 1 min. Let the mixture settle for a few minutes, and proceed as in (a).

(*c*) *Soy Drink and Sauce.* Weigh out 2.00 g of sample into a 50-mL glass-stoppered Erlenmeyer flask, add 6 mL of 1 M HCl and 24 mL of acetonitrile, and shake for 1 min. Let the mixture settle for a few minutes, and proceed as in (a).

B. Procedure for Total Estrogens. Weigh out 2 g of sample into a 150-mL glass-stoppered Erlenmeyer flask, add 24 mL of 1 M HCl, and heat inside a steam bath (98–100 °C) for 2 h. Cool, add 96 mL of acetonitrile, and shake for 1 min. Let the mixture settle for a few minutes and proceed as in (a).

C. Determination of Water Content. Weigh 10.0 g of sample in a sample vial. Prefreeze the sample at −20 °C in a deep freezer. Place the vial into a freeze-drying flask. Connect the flask to the vacuum manifold under a pressure of 1 Torr or lower until dry. Transfer the freeze-dried sample to a 110 °C oven for 1 h. Weigh the sample again, and calculate the water content.

D. Germination of Soy Sprout. Soak soy seeds with water for 5–6 h. Place onto a piece of cheesecloth inside a sieve and cover with a cardboard. Sprout soaked soy seeds at 20 ± 2 °C. Rinse with lukewarm water three to four times daily and spray with 0.033% cetalkonium chloride (benzyldimethylhexadecylammonium chloride) aqueous solution after each rinsing to prevent microorganism growth.

Assay Conditions. Equilibrate the LC column at ambient temperature, and stabilize the detectors with mobile phase (methanol—1 mM ammonium acetate, 6:4) at a flow rate of 1 mL/min for $^1/_2$ h before assay. Set the UV detector at 254 nm and fluorescence detector at 365 nm for excitation, and use a 418-nm emission filter. Inject 20 μL of mixed standard solution, and record chromatograms from both UV and fluorescence detectors.

Inject 20 μL of sample extract, and record chromatograms. Identify peaks by retention time or standard addition, and calculate phytoestrogen content in the sample (correct for dilution factor if needed):

Procedure A

$$\text{free estrogen (mg/kg)} = \frac{\text{peak area (or height)}}{\text{response factor}} \times 30$$

Procedure B

$$\text{total estrogen (mg/kg)} = \frac{\text{peak area (or height)}}{\text{response factor}} \times 120$$

RESULTS AND DISCUSSION

All five phytoestrogens in mixed standard solution eluted within 16 min with base-line resolution (Figure 1). To ensure good resolution, a column having 4500 theoretical plates or more should be used. Sample assay should not be started until good resolution of standards in the mixture is attained. Calibration curves can be constructed by using either peak area or height vs corresponding phytoestrogen concentration and response factors calculated. Linear responses were obtained for five phytoestrogens and ranged from 0.1 to 10 μg/mL (equivalent to 0.1–10 mg of phytoestrogen/kg of sample).

To improve the extraction efficiency of coumestrol, defatting the sample with petroleum ether before extraction has been proposed for soy meal and lipid-rich compound feed (Lookhart et al., 1979; Pettersson and Kiessling, 1984). Five samples of whole soybean meal from different lots were tested to study the effect of petroleum ether on the extraction of soybean phytoestrogens. However, results indicated that of the five phytoestrogens investigated only daidzein and genistein were found in all the sample extracts. The studies also indicated that the use of petroleum ether neither increased the amount of daidzein and genistein in the extract nor enhanced the extraction of trace amounts of coumestrol. Therefore, the use of petroleum ether for defatting is excludible.

Figures 2 and 3 show the chromatograms of samples treated with and without Sep-Pak C_{18} cleanup. This cleanup step removed some of the coextractants appearing in the peaks prior to phytoestrogen peaks. However, these coextractants did not cause any interferences in assaying phytoestrogens. Thus, this cleanup step was eliminated.

Murphy (1981) reported that the addition of water and/or hydrochloric acid to methanol, acetone, and acetonitrile greatly improved the extraction efficiency of genistein, daidzein, and coumestrol. Pettersson and Kiessling (1984) found that extraction of silage with acetonitrile and hydrochloric acid gave much smaller quantities of isoflavones than extraction with 75% ethanol. However, the addition of hydrochloric acid enhanced the extraction of isoflavones. In our work with whole soy-

Table I. Recoveries of Phytoestrogens Added to Soybean, Soybean Sprout, and Dry Spiced Tofu

	soybean			soybean sprout			dry spiced tofu		
estrogen	content, μg/g	added, μg/g	rec, %	content, μg/g	added, μg/g	rec, %	content, μ/g	added, μg/g	rec, %
daidzein									
free	23.7	25.0	97.5 ± 7.7		30.0	78.9 ± 3.8	185.3	100.0	94.9 ± 9.1
total	306.5	400.0	95.5 ± 5.3	137.8	100.0	88.8 ± 14.2	253.4	250.0	103.7 ± 7.6
genistein									
free	26.3	25.0	100.8 ± 4.1	2.6	30.0	99.8 ± 1.0	251.2	100.0	97.7 ± 7.2
total	427.9	400.0	89.7 ± 1.1	112.6	100.0	87.2 ± 12.1	421.5	250.0	112.2 ± 8.4
formononetin									
free		25.0	87.4 ± 2.9		30.0	78.7 ± 2.7		100.0	95.1 ± 1.4
total		400.0	90.5 ± 5.4		100.0	92.7 ± 7.9		250.0	102.8 ± 3.2
coumestrol									
free		5.0	81.3 ± 2.6	3.8	6.0	74.2 ± 3.8		20.0	88.9 ± 1.4
total		80.0	79.9 ± 0.9	9.2	20.0	78.7 ± 7.1		50.0	87.3 ± 2.4
biochanin A									
free		25.0	110.6 ± 11.1		30.0	91.2 ± 11.0		100.0	98.1 ± 4.4
total		400.0	103.1 ± 10.7		100.0	86.3 ± 4.8		250.0	100.7 ± 8.9

Table II. Phytoestrogen Content Found with the Proposed Method and the Method of Pettersson and Kiessling (1984) in Soybean and Its Processed Products

	phytoestrogen content, mg/kg, dry basis					
	daidzein		genistein		coumestrol	
sample	free	total	free	total	free	total
soybean (whole)						
proposed	21.2	314.1	27.6	430.2		
Pettersson's	20.8	375.9	21.8	469.6		
defatted soy meal						
proposed	106.2	614.1	56.4	678.4		
Pettersson's	73.1	641.2	58.5	683.7		
soy milk film						
proposed	268.5	488.3	365.5	878.8		
Pettersson's	280.3	538.3	389.2	888.5		
soy sprout						
proposed	24.1	1333.5		1458.2	6.3	28.5
Pettersson's	35.4	270.9		281.0	8.2	11.4

Table III. Precision of the Method on Subsamples of Soybean, Soy Milk Film, and Soy Sprout

	daidzein			genistein			coumestrol		
sample	mean	SD	CV, %	mean	SD	CV, %	mean	SD	CV, %
soybean (whole)									
free	23.8	1.7	7.0	23.8	2.1	8.9			
total	306.0	19.4	6.3	402.8	12.6	3.1			
soy milk film									
free	212.5	4.8	2.3	340.6	17.6	5.2			
total	420.5	25.4	6.1	819.0	40.3	4.9			
soy sprout									
free	3.7	0.3	7.9				1.04	0.10	9.1
total	217.0	9.6	4.6	230.4	7.5	3.3	4.53	0.37	8.0

beans, three solvent systems, CH_3CN–HCl–H_2O (80:0.5:19.5), 80% ethanol–HCl–H_2O, and 80% methanol–HCl–H_2O, were investigated for the extraction of free phytoestrogens. Ground soybean was extracted with these systems by stirring for a certain period of time and were analyzed for free phytoestrogens. Our results indicated that these solvent systems produced similar extraction efficiencies. The extraction with acetonitrile–hydrochloric acid was preferred because this system resulted in fast settling of suspending sample particles and less interfering impurities in the extract. It was therefore used for the extraction of free phytoestrogens in all subsequent studies.

Rehydration of dry sample prior to extraction was helpful in shortening the extraction time (Livingston et al., 1961). Finely ground soybeans were soaked with water (or 1 M HCl) and analyzed at different time intervals for free phytoestrogens. Presoaking with water greatly enhanced daidzein and genistein contents in the extract, and their concentrations increased with soaking time (Figure 4), whereas no increase of daidzein and genistein levels in the extract was observed when ground soybeans were soaked in 1 M HCl. The increase of apparent daidzein and genistein levels in the extract by prehydration with pure water was due probably to the enzymatic hydrolysis of conjugated phytoestrogens in the sample. Therefore, prehydration of soybean (or its processed products) with distilled or tap water before extraction is not recommended for the determination of free phytoestrogens.

Naturally occurring soybean phytoestrogens exist mainly in conjugated forms, e.g. glucosides (Murphy, 1981; Eldridge, 1982b; Pettersson and Kiessling, 1984). The conditions for complete hydrolysis of the bound phytoestrogens were studied. When ground soybean was heated at different temperatures with 1, 2, and 3 M HCl inside a covered steam bath, daidzein concentration in the extract increased with heating time, reached a maximum, and

Table IV. Phytoestrogen Content in Soybean and Its Processed Products

		phytoestrogen content, mg/kg, dry basis							
		daidzein		genistein		formononetin		coumestrol	
sample	water, %	free	total	free	total	free	total	free	total
soybean 1	7.3	25.6	330.6	28.4	461.6	–[a]	–	–	–
soybean 2	6.4	14.1	506.8	16.7	679.8	–	–	–	–
defatted soy mean	8.1	125.0	625.4	59.2	743.7	–	–	–	–
hard tofu	82.8	94.2	168.4	93.1	288.1	–	–	–	–
soft tofu	86.6	84.9	256.5	59.9	392.5	–	–	–	–
dry spiced tofu	68.1	580.9	794.4	787.5	1321.3	–	–	–	–
soy milk skin	6.8	272.7	469.5	384.4	835.9	–	–	–	
soy milk	87.8	22.1	130.3	28.7	91.8	–	–	–	–
soy sauce	66.6	23.5	23.5	17.9	14.5	–	–	–	–
hot soy paste	44.5	104.8	106.9	99.5	40.5	–	–	–	–
sweet soy paste	41.6	36.7	51.3	24.5	5.3				
fermented tofu	69.1	120.8	115.9	130.4	128.1	–	–	–	–
soy sprout[b]	81.0	–	725.3	13.7	592.1	–	–	20.0	48.4
soy sprout[c]	84.2	23.8	1333.5	–	1458.2	–	–	6.6	28.7

[a] None detected. [b] Homemade, 7 days. [c] Bought in food store.

Table V. Phytoestrogen Content of Soy Seed and Sprout Germinated for Various Times

		phytoestrogen content, mg/kg, dry basis					
		daidzein		genistein		coumestrol	
germination time, day	water, %	free	total	free	total	free	total
0	7.5	7.6	723.2	6.2	939.2		2.0
1	60.4	10.7	667.9	10.9	901.0	0.6	1.5
2	62.8	8.7	863.7	4.1	1059.7	1.3	3.2
3	67.6	9.8	834.6		1057.4	6.5	14.8
4	70.0	20.8	857.0	5.4	878.7	10.1	26.4
5	75.7	21.4	932.5	7.6	958.8	15.4	39.7
6	78.6	24.3	1012.1	4.9	890.7	17.5	48.5
7	78.2	19.8	1098.6	7.4	912.4	10.6	36.2
8	85.6	36.1	1754.2	16.0	1132.6	50.1	114.5
9	82.9	42.3	1763.2	22.5	1032.2	49.5	128.1
10	85.4	38.8	1643.2	22.9	1195.2	48.8	119.6

then leveled off (Figure 5), while genistein concentration tended to decrease after reaching the maximum (especially at higher HCl concentration) (Figure 6) due probably to the degradation of the molecule by HCl. This phenomenon was also found in soy curd and sprout (data not shown). As evidenced from Figures 5 and 6, the best conditions for the hydrolysis of phytoestrogen conjugates in soybean and its processed products were found to be in 1 M HCl for 2 h at 98–100 °C.

Soybean, soy sprout, and dry spiced tofu were spiked, extracted, and analyzed with use of the proposed procedure. The recoveries for daidzein, genistein, formononetin, coumestrol, and biochanin A were 78.9–103.7%, 87.2–112.2%, 78.7–102.8%, 74.2–88.9%, and 86.3–110.6%, respectively (Table I). A comparison study on the extraction efficiency between the proposed procedure and the method of Pettersson and Kiessling (1984) was conducted using soybean, defatted soy meal, soy milk film, and soy sprout as test samples. Since hydration of ground soybean gave higher than actual free estrogen content (Figure 4), the 1-h hydration step for soy meal in the method of Pettersson and Kiessling (1984) was omitted in this comparison study. The results (Table II) indicate that both methods give comparable extraction efficiency for soybean, defatted soy meal, and soy milk film. For soy sprout, the proposed method gave much higher total phytoestrogen content. The reason was not investigated.

Seven portions of samples were picked from the same lot of soybean, soy milk film, and soy sprout, extracted separately, and analyzed for phytoestrogens with the procedure proposed. The coefficient of variations of these tested samples were within 9% (Table III). Phytoestrogens in soybean and its processed products were determined, and the results are presented in Table IV. Significant amounts of daidzein and genistein were found to be carried over into all processed products, indicating that they were neither removed after processing nor modified or degraded by microorganisms during fermentation.

The reason why the total genistein found in soy sauce and soy paste was lower than free genistein was investigated. To make soy sauce or soy paste, flour is always used as raw material and added to soybean before fermentation. The fermentation intermediates of flour (mono-, di-, and tetrasaccharides) were suspected to be responsible for this lowering effect. Genistein standard was mixed with dextrose, fructose, maltose, sucrose, and starchyose. The mixture was treated as described in the assay procedure. It was found that genistein and saccharides formed conjugates showing very high UV absorption with retention times of between 3 and 4 min. The amount of conjugate formed was proportional to the amount of sugar added. These conjugates were eluted off the column together with the trash in the front peak and were not detected.

The phytoestrogen levels in soy sprout obtained commercially varied from batch to batch. Variability in phytoestrogen levels appeared to be affected by stage of growth, length of storage, and storage conditions, such as light and temperature. To investigate the effect of germination on soybean phytoestrogen concentration, soybean seeds were germinated for various times and analyzed at intervals indicated. Results indicated that daidzein and coumestrol concentrations in soy seeds rapidly increased during germination and increased with the increase of

germination time. Only slight changes in genistein concentration were noted (Table V).

The experimental results reported in this paper have demonstrated that defatting with petroleum ether and tedious cleanup steps as described by Pettersson and Kiessling (1984) can be eliminated for the determination of phytoestrogens in soybean and its processed products, thus reducing the analysis time and lowering the analysis cost. The simplified procedure reported here is suitable for use in laboratories where there is a need to assay numerous samples within short time frames.

ACKNOWLEDGMENT

We thank Humberto Guerrero for his suggestion and the supply of cetalkonium chloride used in this study.

Registry No. Daidzein, 486-66-8; genistein, 446-72-0; coumestrol, 479-13-0; formononetin, 485-72-3; biochanin A, 491-80-5.

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Received for review February 7, 1989. Accepted July 12, 1989.

Heavy-Metal Absorption by Perennial Ryegrass and Swiss Chard Grown in Potted Soils Amended with Ashes from 18 Municipal Refuse Incinerators

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Perennial ryegrass (*Lolium perenne*) and swiss chard (*Beta vulgaris* L.) were grown in pots of mardin silt loam soil amended with 5 or 10% by weight of fly ash, bottom ash, or mixtures of both from 18 municipal refuse incinerators representing about one-fourth of all those operating in the United States. The ash and plant material were analyzed for total cadmium, lead, and zinc. The correlation coefficients (r) for the concentration of cadmium, lead, and zinc in the ashes and that in the following crops were, respectively, as follows: ryegrass (first cutting), 0.9964, 0.7600, 0.9699; ryegrass (second cutting), 0.9946, 0.6895, 0.9474; swiss chard, 0.9153, 0.7609, 0.9580. Poor plant growth occurred in a few of the treatments containing ash notably higher in dissolved solids, cadmium, and zinc. The origin and association of heavy metals in refuse ash and their reactions in soils are reviewed.

Currently, there is widespread public opposition to establishing new landfill sites for disposal of municipal solid refuse owing to their unsightliness, cost of maintenance, and potential sources of groundwater pollution. Many U.S. communities are therefore considering construction of incinerators to reduce the mass of solid waste while

0021-8561/90/1438-0190$02.50/0