Quantitation of Phytoestrogens in Legumes by HPLC†,‡

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A fast, sensitive, and precise method is presented for the efficient extraction and quantitation of coumestrol, daidzein, genistein, formononetin, and biochanin A from foods by diode array reversed-phase HPLC analysis using flavone as internal standard. Acid hydrolysis during extraction of foods was chosen to convert the various phytoestrogen conjugates into their respective aglycons, facilitating HPLC analysis and allowing quantitation of total phytoestrogens as aglycons including originally present glycosides, "free" aglycons, and those conjugates which are below the detection limit in food plants. Extraction efficiencies and HPLC conditions were evaluated and optimized, leading to precision and spiking recovery values of 3–8% and 94–104%, respectively, depending on the analyte. Phytoestrogen levels from more than 40 food items, mostly legumes, were determined using this method. High levels of daidzein and genistein were found in soy products and black beans, whereas sprout items were found to be rich in coumestrol and formononetin.

Keywords: *Legumes; soy; isoflavones; phytoestrogens; HPLC*

INTRODUCTION

Phytoestrogens include a wide variety of plant products with weak estrogenic activity (Verdeal and Ryan, 1979; Price and Fenwick, 1985) discovered after isoflavones (Shutt et al., 1967) were found to be responsible for the infertility problems of livestock feeding on forage plants such as subterranean clover (Bennets et al., 1946). Since then, more than 300 plants have been reported to cause estrogenic responses in animals (Bradbury and White, 1954; Farnsworth et al., 1975; Shutt, 1976), and many efforts have been undertaken to screen feeds for these agents (Beck, 1964; Pettersson and Kiessling, 1984) to prevent adverse effects of phytoestrogens on the reproductive system of animals. The growing interest in these compounds, particularly isoflavones, is due to recent findings suggesting that these agents might act as cancer-protective agents (Adlercreutz et al., 1993; Coward et al., 1993) as shown in many cell and animal models and properties often connected with cancer prevention such as antioxidant (György et al., 1964; Ikehata et al., 1968; Murakami et al., 1984; Pratt and Birac, 1979; Jha et al., 1985), radical scavenging (Hatano, 1988), hypolipidemic (Mathur et al., 1964; Sharma, 1979), serum cholesterol lowering (Mathur et al., 1964; Sharma, 1979), antiestrogenic (Barnes et al., 1990; Price and Fenwick, 1985; Martin et al., 1978; Verdeal et al., 1980), and antiproliferative effects (Peterson and Barnes, 1991, 1993; Hirano et al., 1989; Fotsis et al., 1993; Schweigerer et al., 1992). In particular, the observed decrease of tumor numbers in vitro and in vivo after treatment with soy products (Barnes et al., 1990, 1994) or after treatment with daidzein (Jing et al., 1993), one of the major isoflavonoid components in soy items, and the suggestive role of soy products in reducing cancer risk (Messina and Barnes, 1991) sparked the efforts in analyzing phytoestrogens in soy products.

HPLC has emerged as the method of choice for this task due to its speed, precision, and relatively low cost using reversed-phase C_{18} stationary matrices, mostly in mixtures of methanol or acetontrile and aqueous acids or buffers as modifiers. Fluorescence detection (Lundh et al., 1988; Wang et al., 1990; Pettersson and Kiessling, 1984; Kitada et al., 1985) and electrochemical detection (Setchell et al., 1987; Kitada et al., 1985, 1986) were shown to be very useful to increase the sensitivity of commonly used UV detection.

Since glycitin and glycitein occur only in trace amounts in soy foods (Murakami et al., 1984; Kudou et al., 1991), most studies have restricted measurements to the predominant analytes daidzein, genistein, and their 7-*O*-glucosides (Kitada et al., 1985, 1986; Esaki et al., 1990; Matsuura et al., 1989; Murakami et al., 1984). Some studies included coumestrol (Eldridge, 1982; Murphy, 1981, 1982), and very few quantified all of the aforementioned agents (Jones et al., 1989; Setchell et al., 1987). Phytoestrogens occur as glycosides in soy foods (Kudou et al., 1991; Coward et al., 1993), but several authors preferred to measure the total aglycon content including formononetin and biochanin A after hydrolyzing the conjugates to their respective aglycons (Pettersson and Kiessling, 1984; Lundh et al., 1988; Wang et al., 1990; Setchell et al., 1987). Few studies use internal standards to adjust for analyte loss during extraction and separation of phytoestrogens (Eldridge, 1982; Murakami et al., 1984; Jones et al., 1989; Esaki et al., 1990; Wang et al., 1990; Coward et al., 1993). Additionally, the chemical structures of the standards utilized were not related to the analytes and, consequently, the compounds applied in these studies were not well suited as internal standards (Franke et al., 1993).

To our knowledge only one study has analyzed the phytoestrogen content in various foods other than soy items (Jones et al., 1989); however, no detectable levels were reported for the 107 food items analyzed.

We describe a fast, reliable, sensitive, and precise method for the diode array reversed-phase HPLC analysis of the most common isoflavones daidzein, genistein,

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formononetin, and biochanin A (Williams and Harborne, 1989) and of coumestrol, another potent phytoestrogen, after efficient extraction and acid hydrolysis of conjugates. This method was applied to more than 40 widely consumed food items, mostly legumes, since this plant family is known to contain high amounts of these agents (Williams and Harborne, 1989). Flavone, a compound structurally very similar to the analytes, was selected as internal standard among several chemicals tested, and fluorescence detection was used to increase selectivity for the coumestrol analysis. We evaluated the proposed procedure for extraction efficiency, precision, and spiking recovery of phytoestrogens. Additionally we examined the influence of food origin, maturation, and processing, such as boiling and freezing, on phytoestrogen levels and compared the phytoestrogen content of different parts of pods.

EXPERIMENTAL PROCEDURES

Apparatus. HPLC analyses were carried out on a System Gold chromatograph with an autosampler Model 507 and a dual-channel diode array detector Model 168 (all units from Beckman, Fullerton, CA) and a fluorescence detector model FD100 (GTI/SpectroVision; Concord, MA). Absorbance measurements were performed on a DU-62 spectrophotometer (Beckman).

Chemicals. Methanol, hydrochloric acid, acetic acid, 96% ethanol, and dimethyl sulfoxide (DMSO) and all solvents used for HPLC and absorbance readings were of analytical or HPLC grade from Fisher (Fair Lawn, NJ). Butylated hydroxytoluene (BHT), β-glucosidase (from almonds), β-glucuronidase/sulfatase (from *Helix pomatia*), sodium acetate, and biochanin A were purchased from Sigma Chemical Co. (St. Louis, MO). Daidzein, formononetin, and genistein were obtained from ICN (Costa Mesa, CA) and flavone from Aldrich (Milwaukee, WI).

Food Items. Soybean seeds 1 grown in the United States (JFC Co.; San Francisco, CA) were purchased from a local supermarket in May 1993 (batch 1) and in January 1994 (batch 2). Soybean seeds 3 and green peas both grown in Japan were from Savings Co., Japan; the former were roasted according to a traditional Japanese recipe by soaking the seeds for 40 min in water followed by draining for 2 h, roasting for 40 min in an open pan, and toasting again in the oven at 180 °C for 40 min. Frozen soybeans from Taiwan (Shirakiku Co., Honolulu, HI; boiled for 12 min) and raw soybeans were purchased from a local Asian food store. Tofu prepared from U.S.-grown soybean seeds with the $CaSO_4$ coagulation method was obtained from a local manufacturer (Kanai Co., Honolulu, HI). Alfalfa sprouts and radish sprouts were purchased from a local supermarket. Soybean seeds 2 and soy flour were organically grown in the United States (Arrowhead Mill, Hereford, TX) and were obtained from a local health food store together with black bean seeds 1, kidney bean seeds, large lima bean seeds, small lima bean seeds, black-eyed bean seeds, fava bean seeds, small white bean seeds, red bean seeds (boiled for 20 min), pink bean seeds, white navy bean seeds, yellow split peas, broad bean seeds (fried for 7 min), mung bean seeds, green split peas, green peas, round split peas, Chinese peas, lentils, red lentils, urad dahl, masur dahl, kala chana (all from Country Grown Co., CA), organically grown clover sprouts (Aloha Sprout Co., Haleiwa, HI), barley, and sesame. Black bean seeds 2, green split peas, great northern bean seeds, pinto bean seeds, garbanzo bean seeds (all from Golden Grain Co., San Leandro, CA), Chinese peas (boiled for 5 min), and green bean pods (1, raw; 2, boiled for 12 min) were obtained from different local grocery stores.

Standard Solutions, Calibration Curves, and Calculation of Food Levels. Phytoestrogen stock solutions were prepared by dissolving the crystalline standards first in 20 μL of DMSO followed by addition of 96% ethanol to give 2–5 M solutions. The purity of these solutions was checked by HPLC analysis with monitoring at the individual compound's absorption maximum. The purity (percent) of the standard was calculated by dividing the peak area of the compound by all peak areas in the chromatogram and multiplying by 100, assuming that contaminants or byproducts have the same light absorption properties as the standard. Compounds with less than 95% purity were discarded. The concentration of the stock solutions was determined by absorbance readings at the wavelength with maximum absorption (λ_{max}) using molar extinction coefficients (ϵ) (Ollis, 1962) after the stock solutions were diluted to appropriate concentrations with 96% ethanol except for coumestrol, which was diluted with acetonitrile (Wolfbeis and Schaffner, 1980) using the following values: daidzein, λ_{max} = 250 nm, ϵ = 20 893; genistein, λ_{max} = 263 nm, ϵ = 37 154; formononetin, λ_{max} = 256 nm, ϵ = 29 512; biochanin A, λ_{max} = 263 nm, ϵ = 20 893; coumestrol, λ_{max} = 339 nm, ϵ = 22 300. The final stock concentration of each individual standard was calculated using the absorbance reading adjusted for the purity.

Table 1. HPLC and Calibration Parameters of Phytoestrogens Monitored at 260 nm

compound	retention time (min)	k'	slope[a]	intercept[a]	r	concn range (μM)
daidzein	5.4	3.15	1.5326	−0.0088	0.996	0.7–35.0
genistein	8.3	5.38	0.8016	−0.0127	0.998	1.2–52.0
coumestrol[b]	8.8	5.77	2.3653[c]	−0.0229	0.997	0.8–32.0
formononetin	10.5	7.08	0.7354	−0.0181	0.995	1.2–48.0
biochanin A	12.6	8.69	1.0507	−0.0287	0.989	1.0–50.0
flavone[d]	14.4	10.08	*e*	*e*	*e*	*e*

[a] Concentration as a function of peak area units. [b] Responding on fluorometric detection (excitation = 330 nm, emission = 418 nm). [c] Sensitivity higher by a factor of 1.6 when monitored at 342 nm. [d] Internal standard. [e] Not determined.

Calibration curves were obtained for each standard with high linearity (r > 0.995) by plotting the standard concentration as a function of the peak area obtained from HPLC analyses with 20 μL injections. For this purpose the stock solutions of the standards were diluted with the mobile phase to nine different concentrations, starting with 25% of the lowest expected concentration and ending with 5 times the highest expected food concentration. Each concentration was analyzed by triplicate injections (Table 1).

Calculation of analytes from food items was performed by using obtained HPLC area units, the slope of the calibration curve, and adjustment for internal standard recovery and thermolability.

Chromatographic Conditions. HPLC analyses were carried out on an Adsorbosphere C_{18} (10 × 4.6 mm i.d.; 5 μm) direct-connect guard column (Alltech, Deerfield, IL) coupled to a Nova-Pak C_{18} (150 × 3.9 mm i.d.; 4 μm) reversed-phase column (Waters, Milford, MA). Elution was caried out at a flow rate of 0.8 mL/min with the following solvent system: A = acetonitrile, B = acetic acid/water (10/90 v/v); 23% A in B (v/v) linearly to 70% A in B in 8 min followed by holding at 23% A in B for 12 min, which equilibrates the system for subsequent injections. Analytes were monitored with the dual-channel diode array detector at 260 and 342 nm simultaneously, and peaks were scanned between 190 and 420 nm for identification purposes. The fluorescence detector was used with a 340 nm excitation filter and a 418 nm emission filter.

Extraction and Acid Hydrolysis of Phytoestrogens from Food Items. One gram of powdered dry or freeze-dried food material was finely dispersed in a mixture of 10 mL of 10 M HCl and 40 mL of 96% EtOH (containing 0.05% BHT as antioxidant and 20 ppm of flavone as internal standard) by stirring and sonicating for 10 min followed by refluxing. After 1, 2, 3, and 4 h refluxing periods, the mixture was cooled to room temperature and ethanol lost during the refluxing was replaced; 1.2 mL of this mixture was centrifuged at 850*g* for at least 10 min, and 20 μL of clear supernatant was injected directly into the HPLC system.

Extraction and Enzymatic Hydrolysis of Phytoestrogens from Food Items. One gram powdered dry food material was finely dispersed in a mixture of 10 mL of water and 40 mL of 96% EtOH (containing 0.05% BHT as antioxidant and 20 ppm of flavone as internal standard) by stirring and

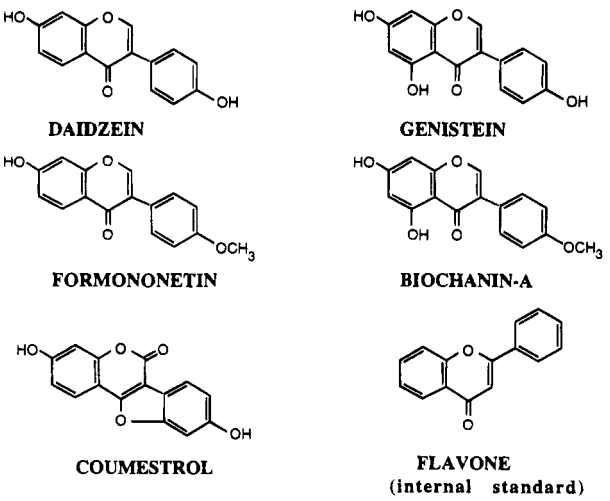


Figure 1. Structures of phytoestrogens analyzed.

sonicating for 10 min followed by refluxing for 3 h. Two milliliters of clear, centrifuged extract was evaporated to dryness under reduced pressure and redissolved in 2.0 mL of 0.1 M acetate buffer (pH 5) containing 2 mg of β-glucosidase and 40 μL of β-glucuronidase/sulfatase (Setchell et al., 1987); 50 μL of this mixture was used for HPLC analysis of "free" aglycons, and the residual 1.95 mL was incubated for 24 h at 37 °C. After centrifugation, 20 μL of clear supernatant was injected directly into the HPLC system for total phytoestrogen analysis.

RESULTS AND DISCUSSION

HPLC Analysis of Phytoestrogens. Among several HPLC columns tested with authentic phytoestrogen standards (Figure 1), a Nova-Pak C_{18} column showed the best selectivity, recovery, and peak shape for daidzein, genistein, coumestrol, formononetin, and biochanin A as shown in Figure 2 (trace A) and Table 1. The use of a Supelcosil LC_{18} column (Supelco, Bellefonte, PA) resulted in poor selectivity and peak shape, and a Spherex 5 C_{18} column (Phenomenex, Torrence, CA) led to extremely low recoveries, probably by binding the analytes to the stationary phase. An acetonitrile mixture with 10% acetic acid was chosen as mobile phase since other modifiers examined (sodium phosphate buffer, pH 5, 0.1 M hydrochloric acid, trifluoroacetic acid) led in combination with acetonitrile and/or methanol and/or tetrahydrofuran to peak tailing, lower selectivity, and/or lower recovery. A fast and steep linear solvent gradient was applied to elute analytes and internal standard, covering a wide polarity range, within 20 min. The analytes were monitored at or very near their absorption maximum (Table 2) with a dual-channel diode array detector at 260 and 342 nm. Coumestrol was selectively detected at 342 nm as well as by fluorescence detection (see Experimental Procedures for details).

Detection limits (Table 3) obtained from authentic standards were found to be extremely low, even lower than those found for carotenoids (Franke et al., 1993), although the latter agents possess higher extinction coefficients. This might be explained by the much lower background noise observed in the proposed system for phytoestrogens when monitoring takes place at 260 nm compared to the conditions used for the carotenoid analyses. Coumestrol showed a 1.6-fold lower detection limit when monitored at its absorption maximum (342 nm); a further decrease of detection limit is possible by using fluorescence detection at higher pH of the mobile phase since the maximum 436 nm emission intensity of the coumestrol monoanion occurs at pH 8 (Wolfbeis and Schaffner, 1980).

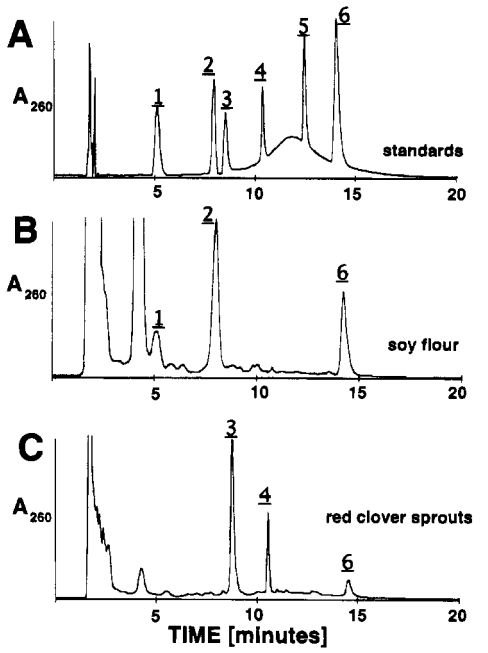


Figure 2. HPLC trace of phytoestrogen standards (A) and extracts from soy flour (B) and red clover sprouts (C) monitored at 260 nm. Peak identification: 1, daidzein; 2, genistein; 3, coumestrol; 4, formononetin; 5, biochanin A; 6, flavone (internal standard). Analyte concentration (mg/L) in trace A: 1, 4.05; 2, 2.38; 3, 3.74; 4, 3.17; 5, 2.42; 6, 9.01. Analyte concentration (mg/L) in trace B: 1, 15.94; 2, 16.80; 6, 16.03. Analyte concentration (mg/L) in trace C: 3, 116.09; 4, 34.45; 6, 17.16.

Table 2. Absorption Maxima of Phytoestrogens Determined with the Proposed Diode Array HPLC Method

compound	absorption maxima (nm)
daidzein	260 sh,[a] 300
genistein	258, 290 sh, 328
coumestrol	260 sh, 301, 342
formononetin	260 sh, 300
biochanin A	266, 332 sh
flavone	252, 293, 310 sh

[a] sh, shoulder.

Table 3. Detection Limits[a] of Phytoestrogens Analyzed with the Proposed HPLC Method

			ng/g	
analyte	nM	ng/mL	*b*	*c*
daidzein	5.15	1.31	65.5	13.1
genistein	8.75	2.37	118.3	23.7
coumestrol	25.70[d]	6.89[d]	344.7[d]	68.9[d]
formononetin	7.25	1.95	97.2	19.5
biochanin A	13.0	3.70	184.8	37.0

[a] Determined with a 20 μL HPLC injection at a signal to noise ratio of 5 and monitoring at 260 nm. [b] Data calculated for 1 g of food material extracted in 50 mL. [c] Data calculated for 5 g of food material extracted in 50 mL. [d] Detection limit lower by a factor of 1.6 when monitored at 342 nm.

Calibration curves with extremely high linearity were obtained from all analytes ($r > 0.995$) in the concentration range expected for food extracts (Table 1).

Extraction Efficiency and Evaluation of Phytoestrogens Using Soy Flour. Aqueous ethanol (77%) was chosen as extraction solvent since phytoestrogens occur in soybeans originally almost entirely (>95%) as glycosides and malonyl esters (Kudou et al., 1991;

Coward et al., 1993), and consequently polar solvents have been recommended for efficient extraction (Pettersson and Kiessling, 1984; Murphy, 1981; Setchell et al., 1987; Coward et al., 1993). Extraction yields of phytoestrogens from soybeans gave 11% higher values when refluxing was used compared to shaking at room temperature (Kudou et al., 1991), and flavonoids were found to give optimal yields when refluxed in an aqueous polar organic solvent (Keinänen, 1993). Additionally, 80% aqueous ethanol was recommended as solvent system for flavonoid extractions because it gave the best efficiency and safety when refluxing was applied as extraction method (Keinänen, 1993).

Acid hydrolysis (Pettersson and Kiessling, 1984; Wang et al., 1990) converting originally occurring phytoestrogen conjugates (Walz, 1931; Walter, 1941; Kudou et al., 1991) into their respective aglycons was chosen to measure all conjugated and "free" analytes in one step. Consequently, various conjugates that may be below the detection limit add to the amounts of free aglycons after hydrolysis, leading to final amounts more likely to be above the detection limit. Additionally, HPLC is facilitated using hydrolyzed samples due to the reduced number of analytes. Acid conditions may also destroy unwanted coextractives interfering with the detection of analytes and increase recoveries by efficiently destroying proteins bound to analytes.

Extraction efficiencies were optimized by varying refluxing period and hydrochloric acid concentration (Figure 3). Although 1 h of refluxing with 3.0 M HCl gave excellent yields for daidzein and refluxing for 3 h with 1.0 or 1.5 M HCl gave high yields for genistein, only refluxing for 1 and 3 h in the presence of 2 M HCl gave maximum yields for daidzein *and* genistein, respectively. Refluxing in 77% aqueous ethanol without added acid resulted in 3% yield of free aglycon relative to total aglycon content, in good agreement with earlier reports (Murphy, 1982; Wang et al., 1990; Coward et al., 1993). Enzymatic hydrolysis using the extract obtained with 77% ethanol was found to be slightly less effective than acid hydrolysis (Figure 3) and was abandoned.

These results show that a significantly longer refluxing time in more concentrated acid is required for optimum extraction efficiency of all analytes compared to earlier studies (Wang et al., 1990). The extraction procedure optimized using soy flour was applied for the analysis of all other food items in this study, although it cannot be excluded that items different from the examined soy flour will have different extraction efficiencies. Due to possible variations of extraction efficiencies in different foods, hourly aliquots of all items studied were analyzed during the entire 3 h extraction period and only the highest concentration calculated among the three values is reported in Table 4.

Further purification of extracts by defatting with petroleum (Pettersson and Kiessling, 1984), freezing (Murakami et al., 1984), solid phase extraction (Pettersson and Kiessling, 1984; Setchell et al., 1987), or phase separation (Dziedzic and Dick, 1982; Lane and Newman, 1987) was found to be unnecessary, since interfering compounds were not eliminated by these procedures and the HPLC performance was not negatively influenced, even after injection of approximately 400 crude extracts obtained from food items analyzed in this study. In fact, defatting powdered soy foods with hexane prior to extraction resulted in 30–40% lower yields for daidzein and genistein.

During extraction, the ratio of solvent volume (milliliters) versus food material (grams) was never lower than 10 in the protocol applied, which is the recommended value for exhaustive extractions of isoflavonoids (Coward et al., 1993). Ratios as great as 500 were found to have no influence on extraction efficiencies or reproducibility in this study.

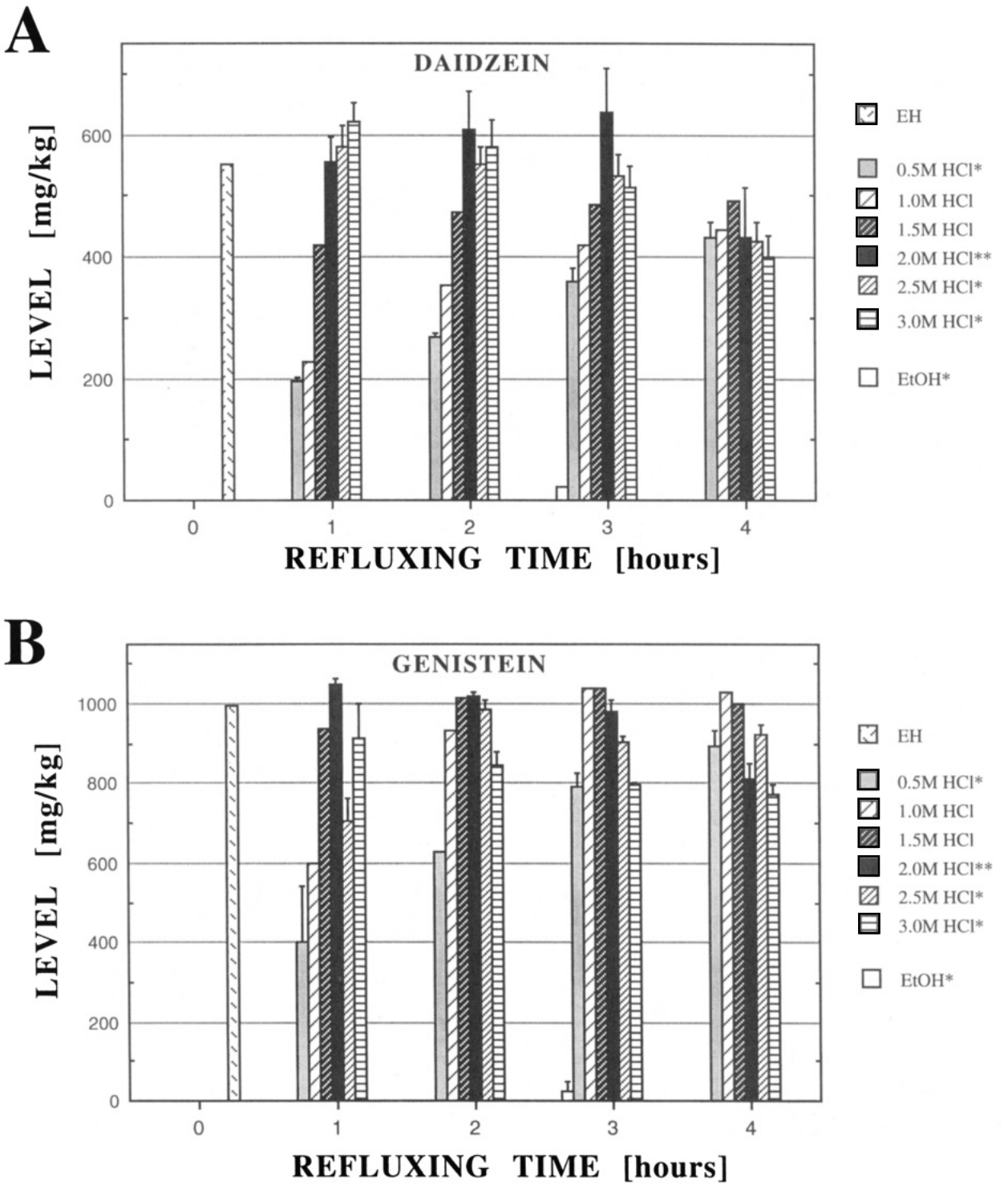
Precision and spiking recoveries listed in Table 5 confirm the validity of the proposed procedure, in particular considering the fact that excellent values for interassay precision were obtained by two different analysts.

Internal Standard. Internal standards are recommended for analyses aimed at high precision and accuracy to adjust for potential degradation or loss of analytes during the various processes involved in the measurements (Franke et al., 1993). Therefore, we searched for compounds having structures similar to those of the analytes, capable of mimicking the fate of the analytes during extraction and HPLC analysis. Flavone was selected as internal standard among several candidates, such as *o*-hydroxyacetophenone, *o*-methoxyacetophenone, propiophenone, *n*-butyrophenone, and 4-chromanone, due to the structural similarity of this compound to the analytes, its elution in an "empty" and "late" part of the chromatogram, avoiding interference with the analytes, and its stability against heat and acids (Figure 4) applied in the extraction procedure.

Thermostability of Analyzed Phytoestrogens. The stability of the five analytes under the conditions established for food extractions was examined by refluxing authentic standards in 77% ethanol containing 2.0 M hydrochloric acid (Figure 4). Only flavone was found to be entirely stable during refluxing for up to 4 h, while biochanin A and genistein degraded by 5% and 13%, respectively, and the daidzein and formononetin peaks increased by 11% and 14%, respectively, after 3 h of refluxing. Therefore, food levels determined with the proposed procedure were adjusted for these changes.

Identification of Extracted Analytes from Foods after HPLC Separation. All analytes detected by HPLC in food extracts were identified by comparing retention times and UV absorption patterns with authentic standards analyzed in the same batch as the food extracts (Table 2) and by comparing UV absorption data with the data given in the literature (Dewick, 1982; Markham, 1982; Williams and Harborne, 1989). Coumestrol was detected with the 342 nm trace and the trace obtained by fluorescence detection, in addition to the 260 nm trace (see Experimental Procedures for details).

Food Levels. The measured food levels of total daidzein, genistein, coumestrol, formononetin, and biochanin A are listed in Table 4 as means of two to six separate analyses. Coefficients of variation between measurements were found to be between 3% and 11%. In general, soy foods and black beans were found to have very high levels of total daidzein and genistein, ranging from 0.3% to 1.4% relative to dry weight. Sprout items, especially clover sprouts, showed extremely high concentrations of total coumestrol and formononetin. Most food items showed little or none of the compounds analyzed in this study, confirming earlier results when none of the 107 examined food items showed any detectable phytoestrogen levels (Jones et al., 1989). Boiling foods did not seem to destroy daidzein or genistein significantly as shown by results obtained



* mean and standard deviation of duplicate analysis
** mean and standard deviation of quadruplicate analysis

Figure 3. Extraction efficiency of daidzein (A) and genistein (B) from soy flour depending on hydrochloric acid concentration varying from 0.5 to 3.0 M in 77% aqueous ethanol and refluxing time. Extracting with 2 M HCl gave maximum yields for both daidzein and genistein with refluxing times of 3 h and 1 h, respectively. Refluxing with 77% ethanol (no acid present) ("EtOH") resulted in 3% yield relative to maximum yields for both analytes. Enzymatic hydrolysis ("EH") with β-glucosidase and β-glucuronidase/sulfatase of the ethanol extract resulted in 87% and 95% yield compared to the maximum yield for daidzein and genistein, respectively.

from black beans, but results from soybeans indicated that roasting causes losses of 15% and 21% for daidzein and genistein, respectively. These losses are probably due to the preparative step prior to the roasting process in which the seeds are soaked and drained, thereby being partly extracted by water (Wang et al., 1990) as opposed to the heat exposure (Coward et al., 1993). The method of production of tofu did not affect the isoflavone levels, as noted earlier (Esaki et al., 1990; Coward et al., 1993), since the dry weight level of tofu was found to be very similar to the one from soybean seeds. Soybean seeds grown in Japan versus those grown in

Table 4. Total Phytoestrogen Levels[a] of Analyzed Food Items

food item[b]	mg/kg of food material				
	daidzein	genistein	coumestrol	formononetin	biochanin A
soybean seeds 1, dry (batch1)	1001.3	1022.7	nd[c]	nd	nd
soybean seeds 1, dry (batch2)	676.4	940.2	nd	nd	nd
soybean seeds 2, dry	700.6	1082.0	nd	nd	nd
soybean seeds 3, dry	1006.5	1382.4	nd	nd	nd
soybean seeds 3, roasted	848.1	1105.5	nd	nd	nd
soybean seeds 4, fresh, raw	90.0	91.7	nd	nd	nd
freeze-dried (64.3% water loss)	252.0	257.0	nd	nd	nd
soybean seeds 5, fresh, boiled	68.5	69.4	nd	nd	nd
freeze-dried (69.5% water loss)	224.7	227.4	nd	nd	nd
soybean seeds 6, fresh, frozen	282.1	315.4	nd	nd	nd
freeze-dried (61.8% water loss)	738.5	825.7	nd	nd	nd
soybean hulls 6, fresh, frozen	nd	18.4	nd	nd	nd
freeze-dried (75.2% water loss)	nd	74.1	nd	nd	nd
soy flour	654.7	1122.6	nd	nd	nd
tofu	113.4	166.4	nd	nd	nd
freeze-dried (86.5% water loss)	840.2	1232.7	nd	nd	nd
black bean seeds 1, dry	698.5	612.2	nd	nd	nd
black bean seeds 2, boiled	269.5	277.1	nd	nd	nd
freeze-dried (65.2% water loss)	774.4	796.4	nd	nd	nd
green beans 1, fresh, raw	nd	nd	nd	1.5	tr[d]
freeze-dried (93.0% water loss)	nd	nd	nd	21.1	tr
green beans 2, fresh, boiled	nd	nd	nd	tr	tr
freeze-dried (93.7% water loss)	nd	nd	nd	tr	tr
large lima bean seeds, dry, raw	nd	nd	14.8	tr	nd
large lima beans seeds, boiled	nd	nd	nd	0.1	nd
freeze-dried (93.7% water loss)	nd	nd	nd	0.2	nd
red bean seeds, dry	nd	3.1	tr	nd	nd
garbanzo bean seeds, dry	nd	nd	nd	nd	15.2
kidney bean seeds, cooked	nd	nd	nd	nd	4.1
freeze-dried (68.6% water loss)	nd	nd	nd	nd	13.2
pinto bean seeds, dry	nd	nd	36.1	tr	5.6
white navy bean seeds, dry	nd	nd	nd	nd	tr
small lima bean seeds, dry	nd	nd	nd	5.5	3.7
great northern bean seeds, dry	nd	nd	nd	nd	6.0
broad bean seeds, fried	nd	12.9	nd	2.1	nd
pink bean seeds, dry	nd	nd	nd	10.5	nd
black-eyed bean seeds, dry	nd	nd	nd	nd	17.3
small white bean seeds, dry	nd	7.4	nd	8.2	nd
yellow split peas, dry	nd	nd	nd	nd	8.6
green split peas, dry	72.6	nd	nd	tr	nd
round split peas, dry	nd	nd	81.1	nd	nd
chinese peas, boiled	nd	nd	nd	nd	93.1
freeze-dried (90.2% water loss)	nd	nd	nd	nd	10.1
kala chana seeds, dry	nd	6.4	61.3	nd	12.6
mung bean seeds, dry	nd	nd	nd	6.1	nd
mung bean sprouts	nd	nd	nd	tr	nd
freeze-dried (92.9% water loss)	nd	nd	nd	tr	nd
clover sprouts	nd	3.5	280.6	22.8	4.4
freeze-dried (95.0% water loss)	nd	69.4	5611.4	456.5	88.1
alfalfa sprouts	nd	nd	46.8	3.4	nd
freeze-dried (93.5% water loss)	nd	nd	720.1	51.7	nd
none of these phytoestrogens were found in the following: green peas, fava beans, Japanese; green peas, red beans boiled; lentils; red lentils; urad dahl; masur dahl; radish sprouts; barley; and sesame					

[a] Means of repeated analyses (two to six times) from dry or freeze-dried item with relative standard deviations between 3% and 11%. [b] Food items with different numbers derived from different sources; beans refer to entire fruit (pod) including hulls and seeds: soybean seeds 1, grown in U.S. from JFC Co.; soybean seeds 2, organically grown in U.S. from Arrowhead Mills Co.; soybean seeds 3, grown in Japan from Savings Co.; soybeans 4, fresh from local market; soybeans 5, fresh from local market; soybeans 6, frozen from Taiwan; tofu from U.S.-grown soybean seeds; soy flour, from organically grown seeds in U.S. (Arrowhead Mills); black beans 1, from Country Grown Co.; black beans 2, from Golden Grain Co.; green beans 1 and 2, from various local stores. [c] nd, not detected. [d] tr, trace (between 60% and 100% of detection limit given in Table 3).

the United States showed roughly the same daidzein levels but were 27% higher in genistein levels. Compared to dry raw soybean seeds, frozen soybean seeds obtained from fresh pods were 20–30% lower in daidzein and genistein; raw soybean seeds from pods stored at room temperature were found to be 75% lower in these analytes. These differences are probably due to the maturation stage since phytoestrogen levels increase with germination (Wong et al., 1965) or maturation of seeds (Kudou et al., 1991) and are most likely not due to the storage temperature since the analytes were shown to be relatively stable against heat (Figure 4). In soybean hulls only 20% of the seed's usual genistein level was found and daidzein was not detected at all. Soybeans grown organically in the United States showed no significant difference in levels for genistein compared

Table 5. Precision and Spiking Recoveries Obtained with the Proposed Method for Phytoestrogen Analysis from Soy Flour

	precision (n = 6)			spiking recovery (n = 4)			
		coefficient of variation (%)					
compound	mean (mg/kg)	within assay	between assay	μg present	μg spiked	recovery (mean)	RSD[a] (%)
daidzein	654.7	2.7	8.2	35.3	44.8	104.7	5.1
genistein	1122.6	2.4	3.8	58.8	40.5	93.7	4.6
coumestrol	*b*	*b*	*b*	*b*	47.5	94.0	4.8
formononetin	*b*	*b*	*b*	*b*	51.3	98.0	1.1
biochanin A	*b*	*b*	*b*	*b*	30.7	101.1	2.5

[a] Relative standard deviation. [b] Not present.

Table 6. Comparison of Total Daidzein and Genistein Levels[a] Obtained by the Proposed Method and Previous Studies

food item	food source	daidzein (mg/kg)	genistein (mg/kg)	study
soy flour	USA	655	1123	present study
	USA	658–742	837–939	Coward et al. (1993)[b]
soybean	USA	676–1001	940–1082	present study[d]
	Japan	1007	1382	present study
	Asia	574	935	Coward et al. (1993)
	USA	2060	2040	Kudou et al. (1991)
	USA	341	430	Wang et al. (1990)
	USA	754	1181	Matsuura et al. (1989)
	Europe	706	1000	Pettersson and Kiessling (1984)
	USA	206–548	457–1402	Eldridge and Kwolek (1983)[d]
	USA	22–72	507–664	Murphy (1982)[e]
	USA	256	956	Pratt and Birac (1979)
textured soy	USA	568	568	Setchell et al. (1987)
soy flake	USA	221	280	Setchell et al. (1987)
defatted flakes	USA (Maple Arrow)	1165	1951	Kitada et al. (1986)
defatted flakes	USA	419	1411	Seo and Morr (1984)[f]
defatted flakes	USA	721	1222	Eldridge and Kwolek (1983)
tofu[g]				
(87% water loss)	USA	840	1233	present study
(80–87% water loss)	Asia	438–1036	910–1420	Coward et al. (1993)[h]

[a] Daidzin and genistin levels were converted to daidzein and genistein levels and added to reported aglycon concentrations to give total daidzein and genistein levels. [b] Four brands analyzed. Mean levels (rel standard deviation): DE = 707 mg/kg (5.1%), GE = 891 mg/kg (4.9%). [c] Three different sources analyzed. Mean levels (rel standard deviation): DE = 793 mg/kg (22.8%), GE = 1015 mg/kg (7.0%). [d] Eight varieties analyzed. Mean levels (rel standard deviation): DE = 447 mg/kg (42.1%), GE = 878 mg/kg (39.2%). [e] Two varieties analyzed. Mean levels (rel standard deviation): DE = 47 mg/kg (75.2%), GE = 586 mg/kg (19.0%). [f] Two or three replicates. Mean levels given in table; rel standard deviation: DE = 13.6%, GE = 4.4%. [g] Levels of freeze-dried material. [h] Two brands analyzed. Mean levels (rel standard deviation): DE = 737 mg/kg (57.4%), GE = 1165 mg/kg (31.0%).

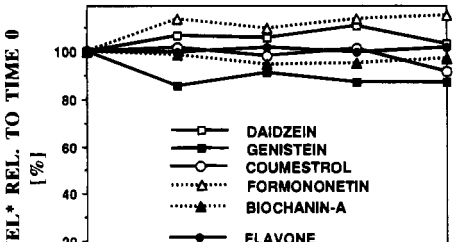


Figure 4. Stability of standards after refluxing with 2 M HCl in 77% aqueous ethanol. Note that only flavone is stable after heating in 77% ethanol/2 M HCl. Biochanin A and genistein degrade by 5% and 13%, respectively, and the daidzein and formononetin peaks increased by 11% and 14%, respectively, requiring adjustment for these changes in food level determinations with this extraction procedure.

to "normally" grown U.S. soybeans but showed 36% lower levels for daidzein. Milling did not affect daidzein or genistein levels since the results of this study showed similar levels for soy flour and organically grown soybeans which both originated from the same source according to the supplier (Arrowhead Mills). A decrease of phytoestrogens during the milling process through the loss of cotyledones or hypocotyls, both shown to differ greatly in isoflavone accumulation (Kudou et al., 1991; Eldridge and Kwolek, 1983), obviously did not occur in this case.

In conclusion, our proposed procedure represents a fast, easy, reliable, reproducible, and sensitive method requiring little technician time to quantitate total daidzein, genistein, coumestrol, formononetin, and biochanin A levels in food items. Up to 10 food items can be analyzed in duplicate per day from one analyst including extraction, HPLC analysis, and data calculation. The presented values for soy flour and soybean seeds compare very favorably with those published recently (Matsuura et al., 1989; Coward et al., 1993; Dwyer et al., 1994) when based on total daidzein and genistein level (Table 6). Differences in total daidzein and genistein levels of soy items comparing our results with other studies (Murphy et al., 1982; Kudou et al., 1991; Table 6) might be due to differences in the analytical procedure. More likely, however, these differences are due to the different origin of the analyzed foods since plant variety, location, harvesting year, and maturity are known to affect isoflavone levels in soybeans (Eldridge and Kwolek, 1983). This is also indicated by variations in levels found in the same laboratory as a function of food origin and food batch (Table 6).

The results presented in this study covering the most likely phytoestrogenic food sources show a wide range of phytoestrogen types and levels, depending on plant species, plant part, maturation, growing conditions, and processing. These data will be very useful in planning epidemiologic trials aimed at evaluating the potential cancer-protective properties of these agents, since exposure data will be available for foods consumed by the study population. However, the effects of origin, i.e., location, growing conditions, and age of food plants, and the known phytoalexin properties of isoflavonoids (Smith and Banks, 1986; Dorr and Guest, 1987) influencing phytoestrogen accumulation must be considered to obtain correct exposure data using published food levels of these agents.

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