Rapid HPLC Analysis of Dietary Phytoestrogens from Legumes and from Human Urine (43826)

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Abstract. **Due to growing evidence suggesting that phytoestrogens might protect against various cancers, particularly against breast and prostate cancer, it is important to measure the exposure of populations to these compounds by determining levels in food and in human tissue or body fluids to assess the possible cancer protective properties of these agents.**

Therefore, we developed a simple and fast procedure to extract and simultaneously hydrolyze phytoestrogens and their conjugates from food items, and present a fast and selective high-performance liquid chromatography (HPLC) method for precise determinations of the most common dietary phytoestrogens genistein, biochanin-A, daidzein, formononetin, and coumestrol using flavone as internal standard. For the first time HPLC was applied to measure these phytoestrogens and their most abundant metabolites equol and O-desmethyl-angolensin from human urine.

The proposed methodology has been evaluated for losses due to thermal degradation during extraction and hydrolysis and due to sample handling during the entire work-up including solid phase extraction, and values are given for inter- and intra-assay variability.

We present isoflavonoid levels of most common peas and beans used in "western" and "eastern" diets and compare isoflavonoid and coumestrol levels of raw, canned, and cooked foods which can be used in future epidemiological studies. We also determined human urinary levels with our methodology comparing values before and after soybean intake. [P.S.E.B.M. 1995, Vol 208]

Isoflavonoids and other phytoestrogenic compounds have been shown in many animal and *in vitro* studies to inhibit various processes during carcinogenesis (1) such as mutation (2), proliferation of cancer cell lines (3, 4), promotion (5), or angiogenesis (6). In particular, the observed decrease of tumor numbers in animals after treatment with soy products (7) or after treatment with soy isoflavonoids (8, 9) and the suggestive role of soy products in reducing cancer risk (10) sparked the general interest in these phenolic agents.

Reports about protective properties against cancers in humans, however, are so far only suggestive. Therefore, it is important to measure the dietary exposure of populations to these compounds and determine exact phytoestrogen levels in human body fluids to assess accurately the potential role of these phenolic compounds as cancer protective agents.

Phytoestrogens occur in foods almost entirely as a complex mixture of glycosides and glycoside esters (11–14) requiring complicated procedures for their analysis. An easier way of analyzing these compounds is to hydrolyze the conjugates and monitor the formed aglycones. Phytoestrogens in human urine, occurring mainly as glucuronide and sulfate conjugates (15–17) have been analyzed after hydrolysis exclusively by gas chromatography coupled mass spectrometry (GC/MS), recently in combination with selected ion monitoring (18–23). This methodology is very sensitive but requires many preparative steps including column chromatography and derivatization, and is also very time consuming and costly, and demands expensive instrumentation and profound analytical experience.

We developed a simple and fast procedure to ex-

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tract and hydrolyze isoflavonoids and their conjugates from foods and present a rapid and selective high-performance liquid chromatography (HPLC) method for the precise determination of daidzein, genistein, formononetin, biochanin-A, and coumestrol (Fig. 1) using flavone as an internal standard. For the first time, we analyzed these dietary phytoestrogens and the metabolites equol and O-desmethyl-angolensin (Fig. 1) from human urine by HPLC in a procedure slightly modified from the protocol developed for foods. The proposed methods were evaluated for inter- and intra-assay variability and for losses due to thermal degradation during extraction and hydrolysis, and during solid phase extraction.

We present isoflavonoid levels of most common legumes used in "western" and "eastern" diets and compare levels of raw and cooked foods of potential use in future epidemiological studies. We also determined human urinary phytoestrogen levels with the developed methodology and compare values of 10 individuals (6 females, 4 males) before and after intake of 40–96 g roasted soybeans.

Material and Methods

Apparatus. HPLC analyses were carried out on system "Gold" chromatograph with an auto sampler 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). Optical density readings were obtained from a DU-62 spectrophotometer (Beckman Fullerton, CA). C-18 reversed-phase solid-phase extraction columns were from PGC Scientific (Gaithersburg, MD).

Chemicals. Methanol, hydrochloric acid, acetic acid, 96% ethanol, dimethylsulfoxide (DMSO), and all solvents used for HPLC and optical density readings were analytical grade or HPLC grade from Fisher (Fair Lawn, NJ). Butylated hydroxytoluene (BHT), glucosidase (from almonds), sodium acetate, biochanin-A, and glucuronidase/sulfatase (from *Helix pomatia* Type HP-2S) were purchased from Sigma Chemicals Co. (St. Louis, MO). Daidzein, formononetin, and genistein were obtained from ICN (Costa Mesa, CA) and flavone from Aldrich (Milwaukee, WI). Equol and O-desmethyl-angolensin were a kind gift from Dr. H. Adlercreutz (University of Helsinki).

Food Items. In the following list, the term *bean* refers to an entire fruit (i.e., pod). *Hull* and *seed* specify the part of the fruit investigated. 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 40 min in water followed by draining for 2 hr, 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 $CaSO_4$ precipitation method was obtained from a local manufacturer (Kanai Co., Honolulu, HI) and alfalfa 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 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 Down to Earth, Honolulu, HI), organically grown clover sprouts (Aloha Sprout Co., Haleiwa, HI), barley, and sesame. Green split peas, great northern bean seeds, black beans, pinto bean seeds, garbanzo bean seeds (all from Golden Grain Co., San Leandro, Ca.), Chinese peas (boiled 5 min), green beans (boiled 12 min), and black soybeans 1 and 2 were obtained from a local grocery store.

Standard Solutions, Calibration Curves, and Calculation of Phytoestrogen Levels. Phytoestrogen stock solutions were prepared by dissolving the crystalline standards first in 20 μl DMSO followed by addition of 96% ethanol to make 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 (%) 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 optical density readings at the wavelength with maximum absorption (λ_{max}) using molar extinction coefficients (ϵ) (24, 25) after diluting the stock solutions to appropriate concentrations with 96% ethanol except for coumestrol which was diluted with acetonitrile (26) using the following values: daidzein (λ_{max} = 250 nm; ϵ = 20893), genistein (λ_{max} = 263 nm; ϵ = 37154), formononetin (λ_{max} = 256 nm; ϵ = 29512), biochanin-A (λ_{max} = 263 nm; ϵ = 27542), coumestrol (λ_{max} = 339 nm; ϵ 22300), equol (λ_{max} = 281 nm; ϵ = 6761), and O-desmethyl-angolensin (λ_{max} = 320 ϵ = 7586). The final stock concentration of each individual standard was calculated using the optical density reading adjusted for the purity.

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 five times the highest expected food concentration. Each concentration was analyzed by triplicate injections.

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 thermal degradation in acid.

Chromatographic Conditions. HPLC analyses were carried out on an Adsorbosphere C18 (10 × 4.6 mm i.d.; 5 μm) direct-connect guard column (Alltech, Deerfield, IL) coupled to a NovaPak C18 (150 × 3.9 mm i.d.; 4 μm) reversed-phase column (Waters, Milford, MA). Elution was carried out at a flow rate of 0.8 ml/min with the following solvent system for food analyses: A = acetonitrile, B = acetic acid/water (10/90; v/v); 23% A in B (v/v) linearly to 70% A in 8 min followed by holding at 23% A for 12 min which equilibrated the system for subsequent injections. For urine analyses the mobile phase gradient was modified to: 20% A in B (v/v) for 16 min, then 70% A for 14 min and again 20% A for 10 min. Analytes were monitored with the dual channel diode array detector at 260 nm continuously, at 280 nm during equol elution and at 342 nm during coumestrol elution. Peaks were scanned between 190 and 600 nm. The fluorescence detector was used with a 340-nm excitation filter and a 418-nm emission filter to selectively monitor coumestrol.

Extraction and Acid Hydrolysis of Phytoestrogens from Food Items. One to five grams powdered dry or freeze dried food material were finely dispersed in a mixture of 10 ml 35% HCl and 40 ml 96% EtOH (containing 0.05% BHT as antioxidant and 20 ppm flavone as internal standard) by stirring and sonicating for 10 min followed by refluxing. After 1 hr, 2 hr, 3 hr, and 4 hr refluxing periods the mixture was cooled to room temperature and ethanol lost during the refluxing was replaced. Of this mixture, 1.2 ml were centrifuged at 850*g* for at least 10 min, and 20 μl clear supernatant were injected directly into the HPLC system.

In a parallel experiment, 100 μl 10 *M* HCl and 400 μl 96% EtOH containing 0.05% BHT as antioxidant and 20 ppm flavone as internal standard were mixed and kept in an amber HPLC vial at −20°C until HPLC analysis. After equilibration to room temperature the standard was injected into the HPLC system to determine the 100% internal standard level.

Extraction and Enzymatic Hydrolysis of Phytoestrogens from Food Items. One to five grams powdered dry food material were finely dispersed in a mixture of 10 ml water and 40 ml 96% EtOH (containing 0.05% BHT as antioxidant and 20 ppm flavone as internal standard) by stirring and sonicating for 10 min followed by refluxing for 3 hr. Two milliliters clear, centrifuged extract were evaporated to dryness under reduced pressure and redissolved in 2.0 ml 0.1 *M* acetate buffer (pH 5) containing 2 mg glucosidase and 40 μl glucuronidase/sulfatase (27). Of this mixture, 50 μl were used for HPLC analysis of "free" aglycones and the residual 1.95 ml were incubated for 24 h at 37°C. After centrifugation, 20 μl clear supernatant were injected directly into the HPLC system for total phytoestrogen analysis.

Collection and Handling of Urine. Over a period of 72 hr, overnight urine samples were collected and times of collection and previous void were recorded for adjustment purposes. Urine samples collected overnight during a period of 6–8 hr over three consecutive days were stored in three separate 2 l disposable urine bottles containing 0.4 g sodium azide and 0.3 g sodium ascorbate to control for bacterial contamination and for degradation of analytes. After mixing and volume determination each urine sample was transferred into five 25 ml disposable plastic tubes and stored at −20°C until analyzed.

Extraction of Urinary Phytoestrogens. Frozen urine was equilibrated to room temperature, vortex-mixed, and centrifuged at 850*g* for 20 min. Twenty milliliters clear supernatant were mixed with 5.0 ml 0.2 *M* acetate buffer (pH 4) and 200 μl flavone (60 ppm in EtOH 96%) as internal standard and filtered through a C-18 reversed-phase solid phase extraction column preconditioned with 3 ml methanol and 3 ml acetate buffer. After the urine had completely gone through the column and the column had been washed with 2 ml acetate buffer, the phytoestrogens were eluted with 100% MeOH into microcentrifuge tubes to give exactly 2.00 ml. One hundred microliters eluate were kept in an insert of an amber HPLC vial at −20°C until HPLC analysis was carried out (unconjugated phytoestrogens).

Acid Hydrolysis of Urinary Phytoestrogens. The residual 1.9 ml SPE eluate was refluxed for 9 hr with a mixture of 2.1 ml 96% EtOH and 1.0 ml 35% HCl (total of unconjugated and hydrolyzed conjugated phytoestrogens). The hydrolyzed samples were kept at −20°C until HPLC analysis was carried out. The samples were equilibrated to room temperature and were centrifuged at 850*g* for 5 min shortly before HPLC injections.

In a parallel experiment, 200 μl flavone (60 ppm in EtOH 96%) were mixed with 3.8 ml 96% EtOH and 1.0 ml 35% HCl, and were kept in an amber HPLC vial at −20°C until HPLC analysis was performed. After equilibrium to room temperature, the standard was injected into the HPLC system to determine the 100% internal standard level.

Enzymatic Hydrolysis of Urinary Phytoestrogens. The residual 1.9 ml SPE eluate was evaporated by speed vac and incubated for 24 hr at 37°C after mixing thoroughly with 0.9 ml of a freshly prepared mixture of 10 ml 0.2 M acetate buffer (pH 4), 150 mg ascorbic acid, and 500 µl glucuronidase/sulfatase (from *Helix pomatia*) (18). The hydrolyzed samples (total of unconjugated and hydrolyzed conjugated phytoestrogens) were then mixed with 1.0 ml 100% methanol and kept at −20°C until HPLC analysis is carried out. The samples were equilibrated to room temperature and were centrifuged at 850*g* for 5 min shortly before HPLC injections.

In a parallel experiment, 200 µl flavone (60 ppm in EtOH 96%) were mixed with 0.9 ml enzyme containing buffer and 0.9 ml methanol, and were kept in an amber HPLC vial at −20°C until HPLC analysis was performed. After equilibration to room temperature the standard was injected into the HPLC system to determine the 100% internal standard level.

Results and Discussion

A Nova-Pak C18 column in combination with an acetonitrile/acetic acid (10% in water) elution system showed best selectivity, recovery, and peak shape for all analytes of interest among several HPLC columns and solvent system tested using authentic phytoestrogen standards (Fig. 2 and 3). Monitoring was carried out at 260 nm, 280 nm, and 342 nm to achieve sensitive detection at or very near the absorption maximum of the analytes (Fig. 1). Coumestrol was additionally monitored with a fluorescence detector to increase selectivity. Detection limits (Table I) obtained from authentic standards range between 1.3 ng/ml and 4.2 ng/ml and allow sensitive phytoestrogen quantitations. The urinary phytoestrogen metabolites equol and O-desmethylangolensin have significantly higher detection limits but sensitive detection of these analytes was achieved by 10- to 20-fold preconcentration of urine samples through solid phase extraction on reversed-phase columns. The detection limit of coumestrol can be decreased further by using fluorescence detection at higher pH of the mobile phase since the maximum 436 nm emission intensity of the coumestrol mono-anion occurs at pH 8 (26).

Calibration curves with extremely high linearity were obtained from all analytes ($r > 0.995$) in the expected concentration range (data not shown).

Aqueous ethanol (77%) was chosen as extraction medium for foods since phytoestrogens occur in plants mostly as glycosides and their malonyl esters and consequently, polar solvents have been recommended for efficient extractions of these compounds (27, 28). The obtained extracts were hydrolyzed in order to facilitate the chromatographic analysis by reducing the number of analytes. Additionally, conjugates below detection limit will be included with this technique by measuring the sum of the various conjugates as their

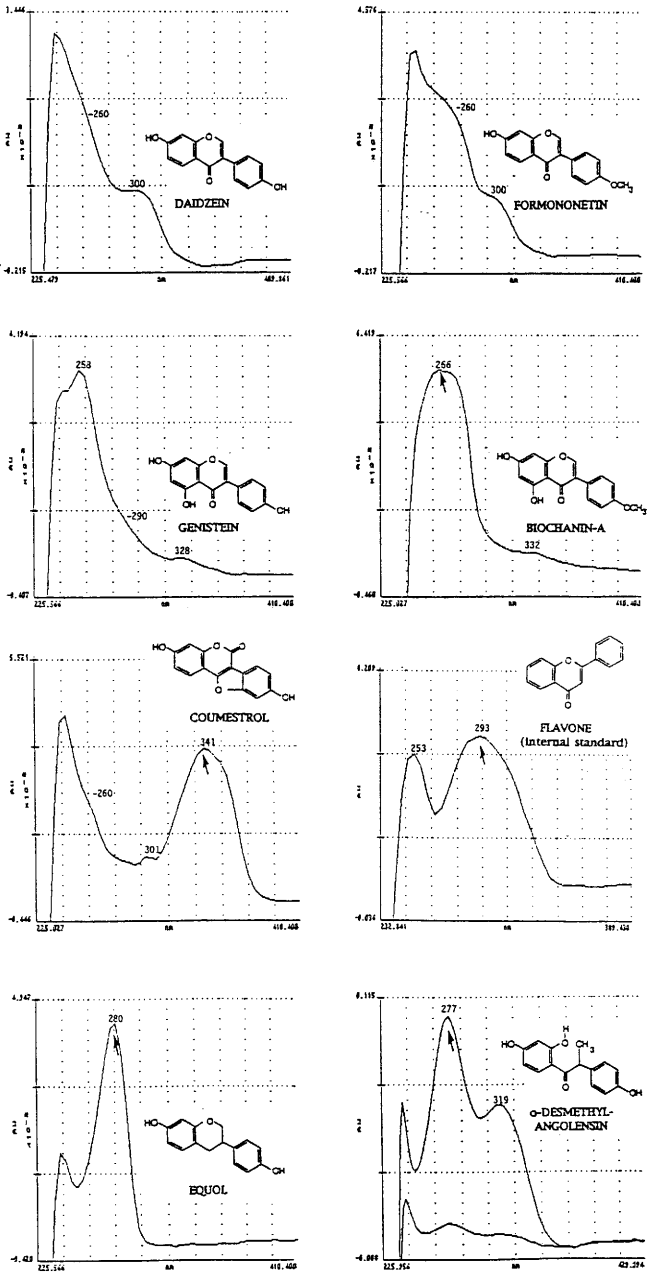

Figure 1. Structure and UV scan of phytoestrogens analyzed.

aglycones leading to amounts of aglycones more likely to be above detection limit. Best extraction efficiencies of soy phytoestrogens were found by 2–3 hr refluxing with 77% ethanol containing 2 *M* HCl (data not shown). Compared to this method, refluxing with 77% aqueous ethanol resulted in 3%–5% yield, which is in good agreement with the "free" aglycone content of soy products (29, 30). Enzymatic hydrolysis with glucosidase/glucuronidase/sulfatase mixtures using the EtOH extracts after removal of the solvent led to 87%–95% yield compared with maximum yields obtained with acid hydrolysis and, therefore, was not utilized. Due to possible variations in extraction efficiencies of other foods than soy items, hourly aliquots of all legumes investigated were analyzed during the 3-hr refluxing

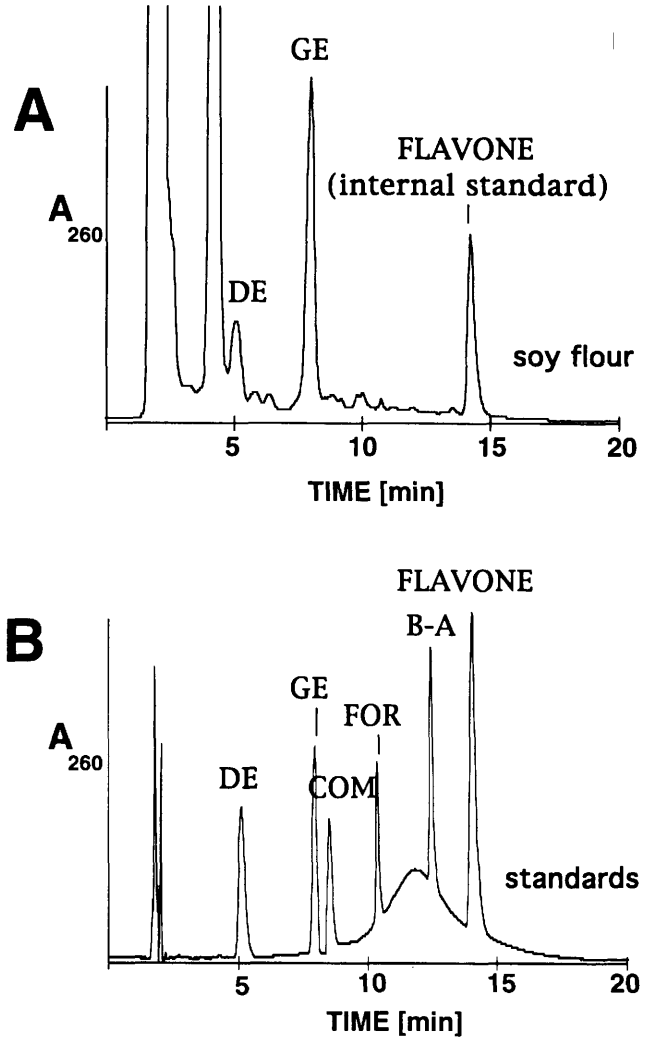

Figure 2. HPLC trace of extracts from soy flour (A) and phytoestrogen standards (B) monitored at 260 nm. A Novapak C18 column (150 × 3.9 mm i.d.; 4 μm) was used as main column with an Adsorboshere C18 guard column (10 × 4.6 mm i.d., 5 μm), and elution was carried out at a flow rate of 0.8 ml/min with the following linear gradient: A = acetonitrile, B = acetic acid/water (10/90; v/v); 23% A in B (v/v) to 70% A in 8 min followed by equilibration at 23% A for 12 min. Peak identification: DE = daidzein, GE = genistein, COM = coumestrol, FOR = formononetin, B-A = biochanin-A, flavone = internal standard. Analyte concentration (μg/ml) in Trace A: DE = 15.94, GE = 16.80, flavone = 16.03; in Trace B: DE = 4.05, GE = 2.38, COM = 3.74, FOR = 3.17, B-A = 2.42, flavone = 9.01.

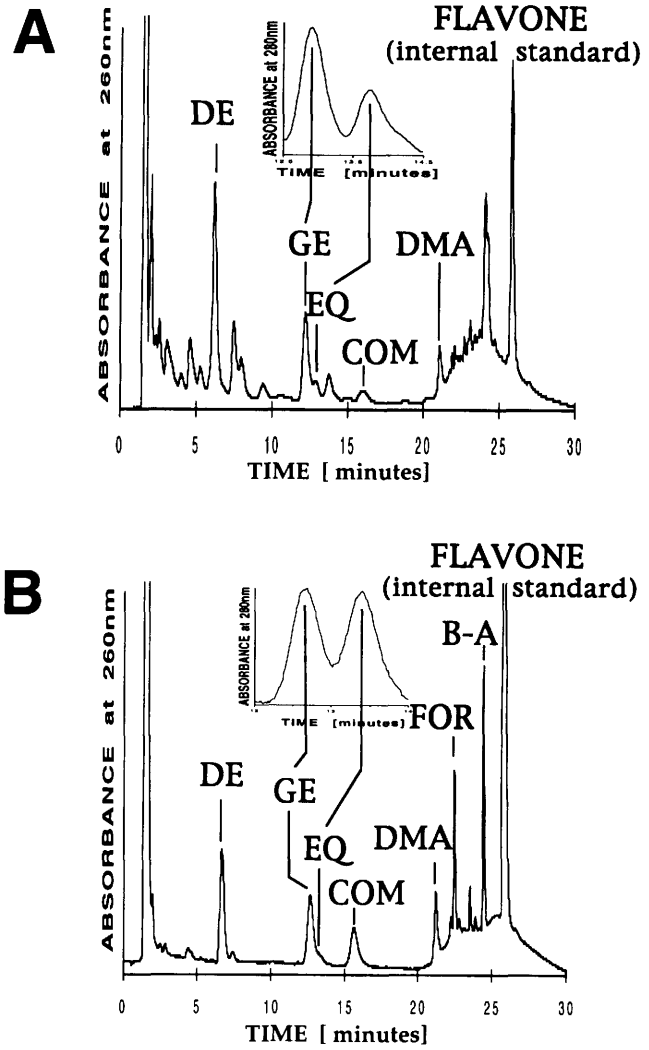

Figure 3. HPLC trace of phytoestrogen extracts from urine (A) and standards (B) monitored at 260 nm and at 280 nm (insert). Column conditions as described in Figure 2. Elution was carried out at a flow rate of 0.8 ml/min with the following step gradient: A = acetonitrile, B = acetic acid/water (10/90; v/v); 20% A in B (v/v) for 16 min and 70% A in B for 14 min followed by equilibration at 20% A for 10 min.

period and only the highest level determined was considered. Further purification of centrifuged extracts prior to HPLC analysis was found to be unnecessary since interfering compounds were separated during the HPLC run and the HPLC system produced highly reproducible results even after more than 400 injections.

Urinary phytoestrogens occurring as glucuronate and sulfate conjugates and mixtures thereof were purified and concentrated by solid phase extraction (SPE) on C18 material without leading to losses of analytes (Table II). Most efficient hydrolysis of the SPE eluate was found by refluxing for 4–9 hr with 77% ethanol containing 2 *M* HCl. The longer times required for complete hydrolysis of urine extracts compared with food extracts was probably due to the known fact of glucuronides being more stable than glucosides at acid treatment. Although enzymatic hydrolysis with glucuronidase/sulfatase mixtures resulted in higher recoveries of all analytes, particularly equol, acid hydrolysis was chosen in this study due to shorter analysis times. Obtained hydrolysates could be injected directly into the HPLC system after centrifugation since, again, interfering compounds were separated during the HPLC run and the chromatographic performance was not negatively affected.

Degradation or loss during analyte work-up and measurement are constantly a potential source for errors. Therefore, internal standards are recommended to ensure precise and accurate analyses (31). We searched for compounds with similar structure to the analytes, capable of mimicking the fate of the analytes

Table I. Detection Limits[a] of Phytoestrogens Analyzed with the Proposed HPLC Methods

Analyte	Detection limit				
	(n*M*)	(ng/ml)	(ng/g)[b]	(ng/g)[c]	(ng/ml)[d]
Daidzein	5.15	1.31	65.5	13.1	0.13
Genistein	8.75	2.37	118.3	23.7	0.24
Coumestrol[e]	25.70	6.89	344.7	68.9	0.69
Formononetin	7.25	1.95	97.2	97.2	0.20
Biochanin-A	13.0	3.70	184.8	36.9	0.37
Equol[f]	623.00	150.90			15.09
O-Desmethyl-angolensin	780.10	201.47			20.15

[a] Determined with a 20-µl HPLC injection at a signal to noise ratio of 5 and monitoring at 260 nm.
[b] Calculated for 1 g food material extracted in 50 ml.
[c] Calculated for 5 g food material extracted in 50 ml.
[d] Calculated for 20 ml urine extracted into 2 ml.
[e] Detection limit lower by a factor of 1.6 when monitored at 342 nm.
[f] Detection limit lower by a factor of 3.95 when monitored at 280 nm.

Table II. Mean Recovery of Phytoestrogens After Reversed Phase Solid Phase Extraction

Analyte (0.4–40 µ*M*)	% Recovery *n* = 5	CV[a]
Daidzein	98.3	2.1
Genistein	97.5	3.4
Coumestrol	99.6	4.1
Formononetin	99.4	1.8
Biochanin-A	96.1	1.9
Equol	97.2	5.2
O-Desmethyl-angolensin	101.5	3.1
Flavone	99.5	1.1

[a] CV = coefficient of variation.

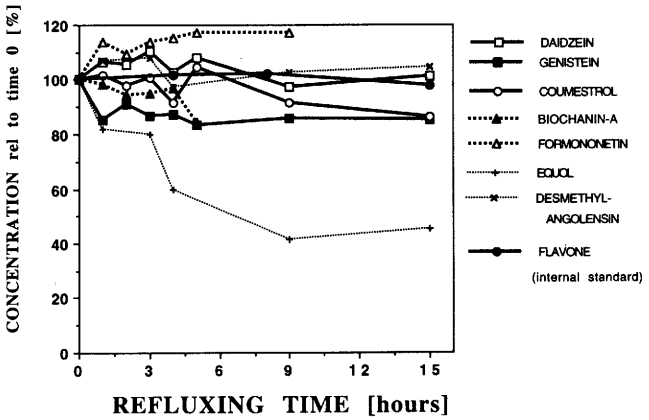

Figure 4. Stability of phytoestrogens after refluxing in 77% EtOH/2 *M* HCl. Only flavone, daidzein, and O-desmethyl-angolensin were stable after heating in 2 *M* HCl. Biochanin-A, genistein, and particularly equol degraded to some extent, and the formononetin peak increased by refluxing in 2 *M* HCl requiring adjustment of final results when acid hydrolysis conditions were used to analyze these compounds in foods or in urine.

during extraction and HPLC analysis, to be used as internal standard. Flavone was selected for this purpose from several candidates because of its excellent structural similarity with phytoestrogens, its elution in an "empty" and "late" part of the chromatogram (Fig. 3) avoiding interference with the analytes, and its stability against heat and acids (Fig. 4) applied during extraction.

The analyte stability during the applied extraction conditions was examined by refluxing authentic standards in 77% ethanol containing 2.0 *M* hydrochloric acid (Fig. 4). Only flavone was found to be entirely stable during this process, while biochanin-A, genistein, and particularly equol degraded and the daidzein and formononetin peak increased. Therefore, levels determined with the proposed procedure were adjusted if

Table III. Precision and Spiking Recovery of Proposed Phytoestrogen Analysis from Foods and from Human Urine

	DE	GE	EQ	COM	DMA	FOR	B-A
Precision							
Soyflour (µg/g)							
Mean {*n* = 5}	636	1037					
CV (%) within assay	2.7	2.4					
CV (%) between assays	9.9	3.8					
Urine (nmoles/hr)							
Range (*n* = 5)	27–701	34–283	52–1787	31–115	56–818		
CV (%) within assay	4.6	2.4	7.7	9.9	4.5		
CV (%) between assays	9.9	11.8	8.0	14.6	11.8		
Spiking recovery							
Soyflour (*n* = 2)							
µg present	35.3	58.8		0.0		0.0	0.0
µg spiked	44.8	40.5		47.5		51.3	30.7
Recovery (%)	104.7	93.7		94.0		98.0	101.1
Urine (*n* = 2)							
nmoles present	9.8	11.2	50.8	41.7	59.1		
nmoles spiked	92.1	74.2	151.1	63.1	90.5		
Recovery (%)	111.4	115.5	115.2	111.2	113.2		

Note. DE = daidzein, GE = genistein, EQ = equol, COM = coumestrol, DMA = O-desmethyl-angolensin, FOR = formononetin, B-A = biochanin-A; CV = coefficient of variation.

the changes were greater than 10% and equol was not considered due to its lability.

All phytoestrogens detected by the proposed procedure in food and urine extracts were identified by comparing retention times and UV absorption patterns with authentic standards analyzed in the same batch (Fig. 1) and with UV data previously reported (24, 32). Coumestrol was detected at 342 nm additionally to the 260 nm trace and with the trace obtained by fluorescence detection.

Precision and spiking recoveries (Table III) confirm the validity of the proposed procedure, in particular considering the fact that excellent values for inter-assay precision were obtained by two different analysts.

The levels of daidzein, genistein, coumestrol, formononetin, and biochanin-A determined from the most commonly consumed legumes are listed in Table IV as means of two to six separate analyses. In general, soy foods and black soybeans were found to have high levels of total daidzein and total genistein

Table IV. Phytoestrogen Levels[a] of Analyzed Food Items

Food Item[b]	Daidzein	Genistein	Coumestrol	Formononetin	Biochanin-A
			[mg/kg of food material]		
Soy bean seeds 1—dry (batch 1)	1001.3	1022.7	nd	nd	nd
Soy bean seeds 1—dry (batch 2)	676.4	940.2	nd	nd	nd
Soy bean seeds 2—dry	700.6	1082.0	nd	nd	nd
Soy bean seeds 3—dry	1006.5	1382.4	nd	nd	nd
Soy bean seeds 3—roasted	848.1	1105.5	nd	nd	nd
Soy bean seeds 4—fresh, raw	90.0	91.7	nd	nd	nd
freezedried (64.3% water loss)	252.0	257.0	nd	nd	nd
Soy bean seeds 5—fresh, boiled	68.5	69.4	nd	nd	nd
freezedried (69.5% water loss)	224.7	227.4	nd	nd	nd
Soy bean seeds 6—fresh, frozen	282.1	315.4	nd	nd	nd
freezedried (61.8% water loss)	738.5	825.7	nd	nd	nd
Soy bean hulls 6—fresh, frozen	nd	18.4	nd	nd	nd
freezedried (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
freezedried (86.5% water loss)	840.2	1232.7	nd	nd	nd
Black soybean seeds 1—dry	698.5	612.2	nd	nd	nd
Black soybean seeds 2—boiled	269.5	277.1	nd	nd	nd
freezedried (65.2% water loss)	774.4	796.4	nd	nd	nd
Black bean seeds	nd	nd	nd	nd	nd
Green beans 1—fresh, raw	nd	nd	nd	1.5	trace
freezedried (93.0% water loss)	nd	nd	nd	21.1	trace
Green beans 2—fresh, boiled	nd	nd	nd	trace	trace
freezedried (93.7% water loss)	nd	nd	nd	trace	trace
Large lima bean seeds—dry, raw	nd	nd	14.8	trace	nd
Large lima beans seeds—boiled	nd	nd	nd	0.1	nd
freezedried (93.7% water loss)	nd	nd	nd	0.2	nd
Red bean seeds—dry	nd	3.1	trace	nd	nd
Garbonzo bean seeds—dry	nd	nd	nd	nd	15.2
Kidney bean seeds—cooked	nd	nd	nd	nd	4.1
freezedried (68.6% water loss)	nd	nd	nd	nd	13.2
Pinto bean seeds—dry	nd	nd	36.1	trace	5.6
White navy bean seeds—dry	nd	nd	nd	nd	trace
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	trace	nd
Round split peas—dry	nd	nd	81.1	nd	nd
Chinese peas—boiled	nd	nd	nd	nd	93.1
freezedried (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	trace	nd
freezedried (92.9% water loss)	nd	nd	nd	trace	nd
Clover sprouts	nd	3.5	280.6	22.8	4.4
freezedried (95.0% water loss)	nd	69.4	5611.4	456.5	88.1
Alfalfa sprouts	nd	nd	46.8	trace	nd
freezedried (93.5% water loss)	nd	nd	720.1	51.7	nd
None of these phytoestrogens found in:					
Green peas, fava beans, Japan. Green peas, red beans boiled, lentils, red lentils, urad dahl, masur dahl, raddish sprouts, barley and sesame.					

Note. nd = not detected; trace = between 60% and 100% of detection limit given in Table III.

[a] Means of repeated analyses (two to six times) from dry or freezedried 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 US from JFC Co.; soybean seeds 2 = organically grown in US 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 US grown soy bean seeds; soy flour = from organically grown seeds in US (Arrowhead Mills Co.) black beans 1 and 2 = from local grocery store.

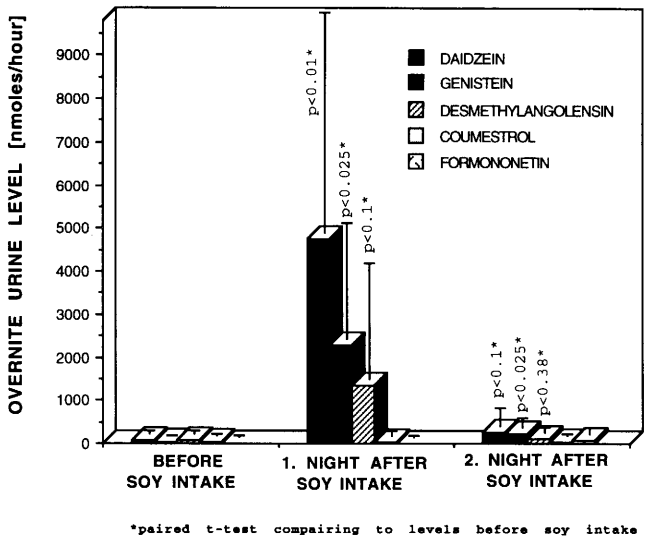

Figure 5. Phytoestrogen levels in human urine determined by HPLC after acid hydrolysis with 2 *M* HCl. The values represent mean phytoestrogen levels (± standard deviation) of 10 healthy individuals (6 females, 4 males) in urine samples collected during the night before intake of 40–96 g soybeans (approx. 42–100 mg genistein and 32–77 mg daidzein) and during the first and second night after soybean consumption. The listed value is the maximum amount obtained after refluxing 4 or 9 hr.

ranging from 0.3% to 1.4% relative to dry weight. Sprout items, especially clover sprouts, showed extremely high concentrations of coumestrol and formononetin. Most food items showed little or none of the compounds analyzed in this study, confirming earlier results when none out of the 107 examined food items showed any detectable phytoestrogen levels (33). Boiling, milling, or processing such as tofu production did not seem to destroy daidzein or genistein significantly, but results from soybeans indicated that roasting caused 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 partly extracting these compounds by water. Compared with dry soybean seeds, frozen soybean seeds obtained from fresh pods were 20%–30% lower in daidzein and genistein, and 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 (34) or maturation of seeds (14) and are most likely not due to the storage temperature, since the analytes were shown to be relatively stable against heat (Fig. 4).

Overnight urine samples of 10 healthy subjects (6 females and 4 males) were collected during the night before intake of 40–96 g roasted soybeans (approx. 42–100 mg genistein and 32–77 mg daidzein) and during the first and second night after soybean consumption. Urinary levels of daidzein, genistein, coumestrol, formononetin, and the metabolite O-desmethyl-angolensin were determined with the proposed method. Concentrations were found to be dramatically increased in the urine samples collected during the first night after consumption and decreased again in the second night urine samples to levels insignificantly higher than baseline concentrations. This indicated a very fast absorption and elimination of these agents. Due to the lack of coumestrol and formononetin in soybeans (Table IV), urinary levels of these analytes did not change in this intervention study. A great variation was found for excreted amounts of the examined soy isoflavones and its metabolite between individuals (Fig. 5) suggesting significant inter-individual differences in absorption rates. Also, the phytoestrogen pattern differed greatly between individuals and changed within individuals from the first to the second overnight urine sample after challenge indicating a highly variable metabolism and excretion rate inter-individually and intra-individually over time. Interestingly, some individuals did not excrete any metabolites while others eliminated greater or lesser amounts without any obvious correlation. These differences might be due to the gut flora known to be responsible for the isoflavone metabolism (35) and, consequently, due to dietary habits. Although not significant because of the small population investigated, no dose response or sex-related differences regarding urinary levels of analyzed phytoestrogens were observed in this intervention study.

Our proposed procedure represents a fast, easy, reliable, reproducible, and sensitive method requiring little "active" laboratory time to quantitate the most common phytoestrogens and their metabolites from food items and from urine. Differences in isoflavone levels of soy items comparing our results with other reports (14, 29) might be due to differences in the analytical procedure or, more likely, are due to the different origin of the foods analyzed, especially in light of the known phytoalexin properties of these compounds (36, 37).

Although most of the investigated analytes were found to be very stable against heat and acid, we recommend enzymatic rather than acidic hydrolysis for future analyses from urine in order to be able to include the metabolite equol in the assay which degrades under acidic conditions.

The results obtained from urine analyses after challenge with soybeans indicate that isoflavones and their metabolites are absorbed extremely fast with great inter-individual qualitative and quantitative variabilities. Since urinary elimination is completed after 2–3 days, multiple sampling of urine over longer period of times is necessary in order to examine exposure of populations to phytoestrogens in future epidemiologic trials assessing the cancer protective role of these agents.

The high activity of daidzein at inducing differentiation, especially in combination with other agents (38) indicates that isoflavones are very promising candidates to act as dietary chemopreventive agents (39,

40) but more work is necessary to study in detail the structural elements and the mechanisms responsible for the observed anticancer effects.

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