HPLC Analysis of Isoflavonoids and Other Phenolic Agents from Foods and from Human Fluids (44231)

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Abstract. **A fast, precise and selective diode array HPLC method is presented for the extraction and analysis of soy isoflavonoids from foods and from human urine, plasma, and breast milk in support of mechanistic and epidemiologic studies assessing the potential cancer protective role of soya or isoflavones. Solid phase or solvent extraction was chosen for isolation, and enzymatic or acid hydrolysis procedures were used for aglycone production depending on the matrix to be analyzed. C-18 reversed-phase HPLC was applied to selectively separate and quantitate daidzein (1),[2] glycitein (3),[2] and genistein (4),[2] including their malonyl (a)[2] and acetyl (b)[2] esters, and their mammalian metabolites equol (6)[2] and *O*-desmethylangolensin (7),[2] as well as formononetin (2),[2] biochanin-A (5),[2] and coumestrol (8)[2] using a gradient elution system. UV absorbance scans and authentic standards were applied for identification purposes, additional to fluorometric monitoring, electrochemical detection, and GC/MS analysis after trimethyl silylation. Detection limits of 20-μl injections were found to be 1.09, 0.53, 3.28, and 1.00 pmoles for daidzein, genistein, equol, and *O*-desmethylangolensin (DMA), respectively, by monitoring at the individual compound's absorption maximum. The proposed method was applied to monitor isoflavone levels in soy foods and in human plasma, urine and breast milk after challenge with roasted soybeans. Implications of the presented results on the potential activity of isoflavones to prevent cancer by exposing newborn infants to these agents are discussed.**

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Cancer prevention by isoflavonoids (1–3) such as daidzein (1) and genistein (4) has been suggested to be due to multiple effects connected with the inhibition of processes during carcinogenesis such as radical scavenging, antioxidant (4), antiestrogenic (5–8), antimutagenic, antiproliferative (9), differentiation-inducing, (10) and angiogenesis-inhibiting (11) activities. A 100% long-term survival was achieved in leukemic mice by treatment with genistein linked to an antibody against B-cells (12). This added another important item to the growing list of potent anticancer effects of isoflavonoids. The relevance of exposure of isoflavonoids at an early period in life was shown previously by the ability of genistein to reduce breast cancer incidence and tumor numbers when only three doses were given to newborn mice (13). Also, the role of isoflavonoids at reducing cancer risk (1, 14) especially, breast cancer risk (15), was suggested due to lower cancer rates observed in populations with high isoflavone exposure through soy consumption (16, 17).

Dietary exposure of isoflavonoids to humans is generally attributed to soy food consumption (17). Daidzein (1) and genistein (4) were the major isoflavones found in these foods with combined levels averaging 0.2% relative to dry weight (18, 19). However, these concentrations vary depending on a variety of factors such as environmental, genetic, harvesting, and processing conditions (18, 20–23).

Traditionally, GC/MS was applied to determine soy isoflavones and their metabolites in human biological fluids including urine (9, 24, 25), plasma (25), and feces (26, 27). Recently, HPLC was introduced to measure these analytes in human urine (29, 30) allowing the measurement of a variety of phytoestrogens including aglycones and conjugated analytes in one run. Compared to GC/MS this HPLC method required fewer steps for sample preparation and analysis and demanded less technician time and less expen-

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[2] The numbers and letters in parentheses refer to code numbers assigned these compounds and used in Figure 1.

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sive instrumentation. Although HPLC has been applied in previous studies to determine isoflavone levels in plasma (30, 31), glycitein and *O*-desmethylangolensin were not included in these assays and conditions were not evaluated for human specimen. In support of future studies assessing the potential cancer protective role of soya or diets containing isoflavones, we present a fast, simple, and accurate HPLC technique to determine isoflavonoid levels in soy foods and human urine, plasma, and breast milk.

Materials and Methods

Apparatus. HPLC analyses were carried out on a system 'Gold' chromatograph with an auto sampler model 507, a dual channel diode-array detector model 168 (all units from Beckman, Fullerton, CA), a fluorescence detector model FD100 (GTI/SpectroVision, Concord, MA) and a Coulochem II-5200 electrochemical detector (ESA, Bedford, MA) using a 5011 coulometric cell. Absorbance readings were obtained from a DU-62 spectrophotometer (Beckman, Fullerton, CA). Evaporation was performed with a Savant AS 160 speed-vac (Farmingdale, NY) at room temperature. GC analysis was carried out with a Hewlett-Packard model 5890 using a mass selective detector 5971A and electron impact ionization at 70 eV.

Chemicals. Methanol, hydrochloric acid, acetic acid, 96% ethanol, dimethyl sulfoxide (DMSO), ethyl acetate, and all solvents used for HPLC and absorbance readings were analytical grade or HPLC grade from Fisher Scientific (Fair Lawn, NJ). Butylated hydroxytoluene (BHT), sodium acetate, genistin, and glucuronidase/sulfatase (isolated from Helix pomatia type HP-2S) were purchased from Sigma Chemical Co. (St. Louis, MO). Daidzein and genistein were obtained from ICN (Costa Mesa, CA), flavone from Aldrich (Milwaukee, WI), coumestrol from Serva (New York, NY) and β-glucuronidase isolated from *Escherichia coli* (200 U/ml) and arylsulfatase isolated from Helix pomatia (1–5 U/ml) were purchased from Boehringer Mannheim (Indianapolis, IN). Equol and *O*-desmethylangolensin were purchased from Dr. K. Wähälä, University of Helsinki, Finland.

Participants. One Caucasian and one Chinese woman between 32 and 34 years of age, both in Week 15 postpartum, of normal height and weight, nonsmoking, not on any medication including hormones or dietary supplements, without particular dietary patterns (e.g., vegetarians), and in good health donated breast milk, blood, and urine for the present study. One 39-year-old male subject of normal height and weight, nonsmoking, not on any medication or dietary supplements, without particular dietary patterns and in good health donated blood and urine after consumption of 5, 10 and 20 g of roasted soybeans. During the studies, the participants did not consume any alcohol and maintained their usual diet except for the intake of roasted soybeans during intervention. All procedures of the protocol followed were in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 1983.

Collection of Foods. Soy foods from Hawaii and Singapore were obtained from 'open' markets or from local stores, frozen immediately after purchase, and kept frozen until analysis. The same food items from different sources were collected on the same day.

Collection and Handling of Human Milk, Blood and Urine. Milk was collected at the time of breastfeeding from the opposite breast starting 12 hr before the first soybean intake. Milk samples were stored in plastic vials between +6°C and −4°C. Isoflavones in milk were found to be stable for at least 5 days when kept in this temperature range.

Overnight fasting blood was drawn into heparin-containing vacutainers and kept on ice until centrifuged for 30 min at 4°C and 850 g. The supernatant plasma was either worked up immediately or stored at −70°C.

Urine samples were stored in disposable bottles containing 0.2 g boric acid and 0.1 g sodium ascorbate to control for bacterial contamination and for degradation of analytes. After mixing and volume determination, each urine sample was transferred into 50-ml disposable plastic tubes, and stored between −4°C and −20°C. All urine collection times were recorded including times of previous voids for adjustment purposes.

Extraction of Isoflavones from Soy Foods. Soy foods were freeze-dried, homogenized *via* mortar and pestle or blender, and 1.0 g dry powder was extracted by acid hydrolysis (18) yielding total aglycone content or with aqueous methanol at room temperature yielding conjugates and genuinely present aglycones. In the latter case the dry powder was sonicated for 10 min in 80% aq methanol (v/v) containing 20 ppm flavone as internal standard followed by stirring for 2 hr in a sealed container. After centrifugation, a clear aliquot of this solution was diluted 1:1 with 0.2 *M* acetate buffer (pH 4), and 20 μl were injected into the HPLC system.

In a parallel experiment 20 μl of a 1:1 mixture of 80% aq methanol (v/v) containing 20 ppm flavone and 0.2 *M* acetate buffer (pH 4) were injected into the HPLC system in the same batch for internal standard recovery calculation purposes.

Extraction and Enzymatic Hydrolysis of Isoflavones from Human Urine. Urine was extracted as described previously (29). In brief, 20 ml clear urine 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 preconditioned C-18 SPE column followed by washing with 2 ml acetate buffer and eluting the analytes with 100% methanol.

The eluate was dried by speed-vac at room temperature and incubated for 1 hr at 37°C after redissolving in 1.0 ml 0.2 *M* phosphate buffer (pH 7.0) and mixing thoroughly with 50 μl β-glucuronidase (31) and 50 μl arylsulfatase. Subsequently, the enzymes of the hydrolyzed samples were inactivated by addition of 0.9 ml 100% methanol. Samples were analyzed immediately or stored at −20°C and analyzed by HPLC after equilibration to room temperature, vortex-

mixing, and centrifugation at 850 g for 5 min. Additional concentration was achieved by partitioning the isoflavones from the hydrolyzed sample into ethyl acetate. The combined organic phases were dried under nitrogen and redissolved in 150 μl mobile phase and 50 μl 0.2*M* acetate buffer (pH 4) before injecting 20 μl into the HPLC system.

In a parallel experiment 200 μl flavone (60 ppm in EtOH 96%) were mixed with 0.9 ml buffer and 0.9 ml methanol in the same batch for internal standard recovery calculation purposes.

Enzymatic Hydrolysis and Extraction of Isoflavones from Human Milk. In this procedure, 2–4 ml human milk equilibrated to room temperature were mixed with 25 μl flavone (120 ppm in EtOH 96%), 50 μl β-glucuronidase (from *E. coli;* 200U/ml) and 50 μl arylsulfatase (from Helix pomatia; 5U/ml) followed by stirring for 1hr at 37°C. This sample was extracted three times with 2 ml ethyl acetate (ACS certified), and the organic phases were combined after centrifugation followed by drying under nitrogen. The dry extract was redissolved in 150 μl methanol by vortex mixing followed by the addition of 50 μl 0.2 *M* acetate buffer (pH 4). After centrifugation 20 μl of clear sample were injected into the HPLC system.

In a parallel experiment 25 μl flavone (120 ppm in EtOH 96%) mixed with 130 μl methanol and 45 μl 0.2 *M* acetate buffer (pH 4) were injected into the HPLC system in the same batch for internal standard recovery calculation purposes.

Enzymatic Hydrolysis and Extraction of Isoflavones from Human Plasma. Human plasma (1.0 ml) equilibrated to room temperature was mixed with 0.25 ml 0.5 *M* triethylamine acetate (pH 7.0), 80 μl β-glucuronidase (from *E. coli;* 200U/ml), 80 μl arylsulfatase (from Helix pomatia: 5U/ml) and 20 μl flavone (120 ppm in EtOH 96%) followed by stirring for 17 hr at 37°C in a sealed container. After addition of 0.25 ml of 10% aqueous trichloroacetic acid, this sample was extracted three times with 2 ml ethyl acetate (ACS certified), and the organic phases were combined after centrifugation followed by drying under nitrogen. The dry extract was redissolved in 150 μl methanol by vortexing followed by adding 50 μl 0.2 *M* acetate buffer (pH 4) and sonicating for 30 sec. After centrifugation 20 μl clear sample were injected into the HPLC system.

In a parallel experiment 20 μl flavone (120 ppm in EtOH 96%) mixed with 135 μl methanol and 45 μl 0.2 *M* acetate buffer (pH 4) were injected into the HPLC system in the same batch for internal standard recovery calculation purposes.

Trimethylsilylation. Dry milk extracts or crystalline standards were dissolved in 0.1 ml MSTFA/imidazole (100:2; v/w) and incubated for 15 min at 60°C prior to GC/MS analysis (32).

Chromatographic Conditions. For solid-phase extraction (SPE) C-18 reversed-phase columns were obtained from PGC Scientific (Gaithersburg, MD).

All HPLC analyses were carried out on a NovaPak C18 (150 × 3.9 mm i.d.; 4 μm) reversed-phase column (Waters; Milford, MA) coupled to an Adsorbosphere C18 (10 × 4.6 mm i.d.; 5 μm) direct-connect guard column (Alltech; Deerfield, IL). Elution was performed at a flow rate of 0.8 ml/min with the following linear gradient: A = acetic acid/water (10:90;v/v); B = methanol/acetonitrile/dichloromethane (10:5:1;v/v/v); B in A (v/v): 5% for 5 min, from 5%–45% in 20 min, from 45%–70% in 6 min and from 70%–5% in 3 min with equilibration for 15 min before subsequent injection. Analytes were monitored by diode array detection at 260 nm and 280 nm and coulometrically at +500 mV during the entire HPLC run simultaneously. Observed peaks were scanned between 190 and 400 nm.

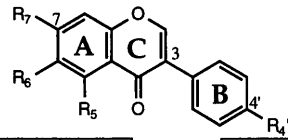
Gas chromatography was performed by injecting 3 μl trimethylsilylated sample onto a HP Ultra-1 capillary column (17 m × 0.2 mm i.d., film thickness 0.11 μm) using helium as the carrier gas at a flow rate of 1.0 ml/min with a 1:10 split. The following temperature program was used: initial temperature = 180°C, rate = 10°C/min, final temperature = 320°C. Signals were registered in the selected ion monitoring (SIM) mode with the following masses determined after analysis of standards: daidzein = m/z 398, 383; genistein = m/z 471, 399, 228; equol = m/z 386, 192; *O*-desmethylangolensin = m/z 459, 281.

Standard Solutions and HPLC Calibration Curves. Standard stock solutions were prepared by dissolving 1–3 mg of the crystalline compound in 20 μl DMSO followed by addition of methanol to give 2–5 molar stock solutions. Compounds with less than 95% purity as determined by HPLC analysis were discarded. The concentration of the stock solutions was determined by absorbance readings as reported previously (29).

Concentrations of analytes from milk and urine were calculated from peak areas obtained after HPLC analyses using the slopes of the calibration curves obtained from serial dilutions of standards. Milk levels were expressed as nanomoles per liter (n*M*) after adjustment for internal standard recovery. Urinary excretion rates were expressed as nanomoles per hour (nmoles/h) after adjustment for time period between urine collection and previous void (hr), urine volume (ml), and internal standard recovery.

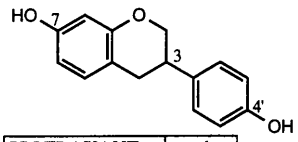
Results and Discussion

Our previously developed HPLC system (33) was improved by applying a gradient elution system consisting of methanol, acetonitrile, dichloromethane and 10% aqueous acetic acid. This led to more efficient separation of analytes, especially the isoflavone conjugates present in soy foods (Fig. 2 and 3). Also, isoflavones and their metabolites including equol (6) and *O*-desmethylangolensin (7) found in humans after exposure to soy foods (25, 29) could be separated more selectively. This method was also applicable to separate and quantitate other flavonoids and phenolic acids implicated in dietary chemoprevention (34) such as quercetin, kämpferol, myricetin, apigenin, luteolin, naringenin,

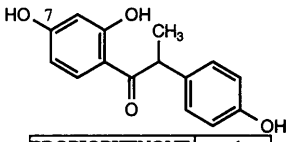


ISOFLAVONES	code°	R_5	R_6	R_7	R_4'
Daidzein	1	H	H	OH	OH
Formononetin	2	H	H	OH	OCH_3
Glycitein	3	H	OCH_3	OH	OH
Genistein	4	OH	H	OH	OH
Biochanin-A	5	OH	H	OH	OCH_3

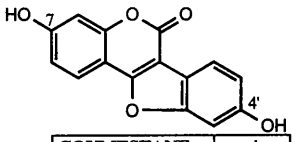
7-O-CONJUGATES	code°	R_7
IN FOODS		
7-O-(6"-O-malonyl)-glucoside	a	
7-O-(6"-O-acetyl)-glucoside	b	
7-O-glucoside	c	
IN HUMAN BODY FLUIDS		R_7 and/or R_4'
7-(and/or4')-O-glucuronide*	d	
7-(and/or4')-sulfate*	e	



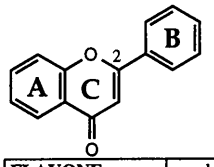
ISOFLAVANE	code°
Equol*	6



PROPIOPHENONE	code°
O-Desmethyl-angolensin*	7



COUMESTANE	code°
Coumestrol	8



FLAVONE	code°
Flavone (internal standard)	1s

Figure 1. Molecular structure of analytes.

hesperetin, catechin and gentisic, anisic, coumaric, ferulic, and caffeic acid (data not shown).

Detection limits for daidzein (1), genistein (4), equol, and *O*-desmethylangolensin using a 20 µl injection volume and monitoring at the analytes' absorption maximum were found to be 1.09, 0.53, 3.28 and 1.00 pmoles, respectively (Table I). The present system led to later retention times and thereby, to lower peak heights. Consequently, detection limits calculated using peak heights were found to be slightly greater compared to the HPLC system used in our earlier report (29). Detection limits of analytes isolated from human body fluids were similar to those listed in Table I due to concentration steps during extraction (see Materials and Methods) and due to the application of greater injection volumes. Finally, coulometric detection at +500 mV lowered detection limits for most analytes by a factor of 2 to 6 compared to diode-array monitoring (Fig. 4, Table I). Coulometric response patterns obtained at various voltages can also be used for analyte identification purposes (Paul Gamache; paper enclosed in this issue).

We found similar peak areas using the present system when monitoring at the absorbance maximum of the analytes compared to our previous elution system (33). Therefore, the present HPLC system showed equal sensitivity compared to the previous system, as evidenced by the nearly identical calibration curve slopes (data not shown).

Analytes were identified using internal and external authentic standards in addition to spiking experiments. Most importantly, HPLC peaks of isoflavonoids were identified by comparison of absorbance patterns obtained by diode array detection (33) with those reported recently (20). UV scans of conjugated isoflavones isolated from soy foods were found to have identical absorbance patterns relative to those of their respective aglycones (Fig. 3) which is in excellent agreement with established knowledge about the influence of 7-*O*-substituents of flavonoids not changing flavonoid chromophores (35). Finally, correct peak assignments (Fig. 2) of the glucosyl esters and glucosides of daidzein, glycitein, and genistein (Fig. 1) were reconfirmed based on comparison to HPLC results of an authentic toasted soy sample provided by Dr. S. Barnes (University of Alabama). Assignment by spiking experiments (Fig. 2) and diode array detection was identical with that using mass spectrometry (23).

Isoflavones present in soya as glucosides (23, 36) were extracted from soy foods with simultaneous acid hydrolysis in order to produce aglycones (33). This avoids the need of chromatographic separation of complex mixtures and is recommended for food-screening purposes (18). Due to the relevance of information about conjugate patterns in soy foods regarding pharmacokinetic and anticancer properties (14), we extracted soy foods also at room temperature in order to include conjugated isoflavones in the assay. Compared to ethanol or other solvents such as tetrahydrofuran or acetonitrile, 80% aqueous methanol was found to yield best extraction efficiencies without leading to degradation,

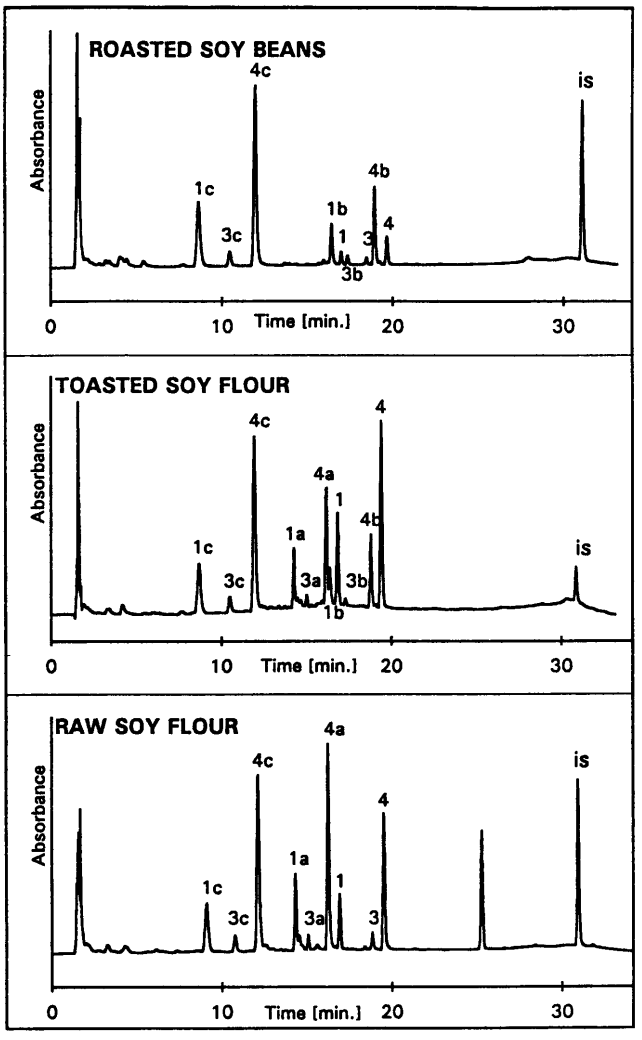


Figure 2. HPLC trace of extracts of roasted soybeans, toasted soy flour (23) after spiking with daidzein (**1**) and genistein (**4**), and raw soy flour. Flavone used as internal standard (**is**) elutes at 31 min.

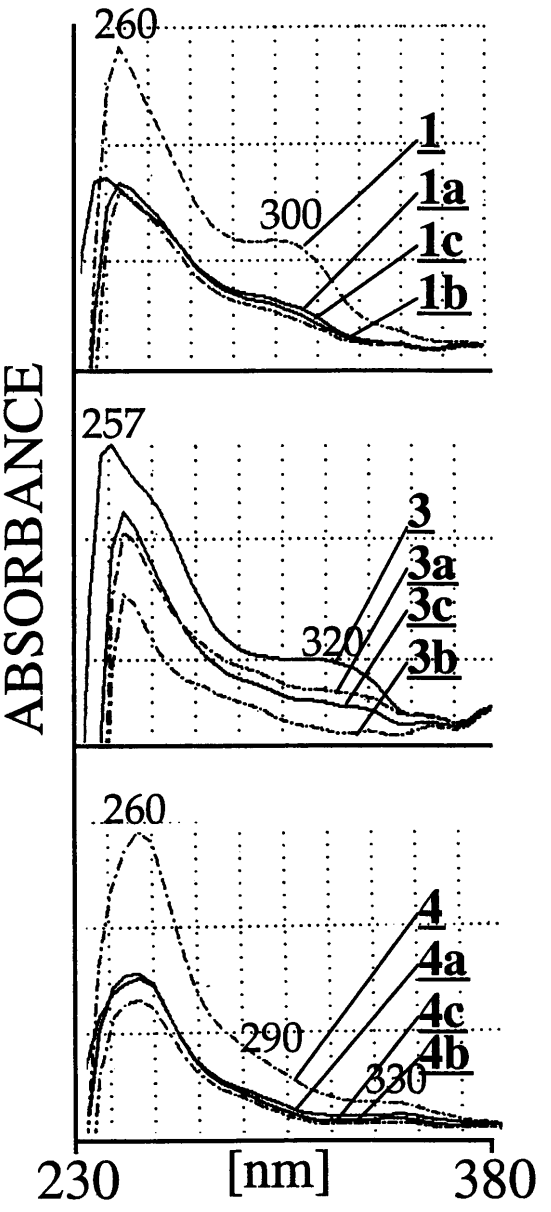


Figure 3. Absorbance scans of unconjugated and conjugated daidzein (**1** and **1a–1c**), glycitein (**3** and **3a–3c**) and genistein (**4** and **4a–4c**) obtained by diode array detection after HPLC separation of toasted soybeans (**a** = malonylglucoside, **b** = acetylglucoside, **c** = glucoside; structures given in Figure 1)

which is in good agreement with earlier reports (19). Extraction using cold 80% aqueous methanol resulted in nearly identical total daidzein (101%), genistein (104%), and glycitein (95%) levels compared to extraction with acid hydrolysis when soy flour was analyzed. The 5%–9% intra- and interassay variability of the major analytes (Tables II and III) confirms the validity of the presented procedure.

Total isoflavone levels determined from various soy foods (Table II) were found to be similar in most items analyzed when based on dry weights but lower in fresh and processed items due to the higher water content in the latter items. The ratio of daidzein/genistein/glycitein of approximately 1:1:0.2 was found to be consistent in all items analyzed. However, absolute isoflavone levels varied even within the same food item when collected from different markets (coefficient of variation = 1%–101%, Table II). This high variability is probably due to differences in environmental conditions and genetic dispositions of the food plants (22, 37) suggesting that detailed and specific soy food analyses are required for studies measuring dietary isoflavone exposure. Conjugation patterns of soy isoflavones determined in selected items (Table III) showed that malonate (**a**) and glucoside (**c**) conjugates dominated over acetate (**b**) and aglycones. This agrees with earlier observations (35) and was attributed to the fact that acetates and aglycones are artifacts with the former upon dry heat (23). Fermented soy items suggested to have higher isoflavone bioavailability over unfermented items (38) contained exclusively aglycones in the foods analyzed, which agrees with previous reports (19, 21). Ongoing food analyses in our laboratory hint a 20%–50% reduction of isoflavone levels by boiling of soy foods due to extraction of analytes into the heated water.

Urinary isoflavones were isolated as reported earlier (29) except that glucuronidase and sulfatase preparations

Table I. HPLC Detection Limit and Sensitivity

	UV	ECD		
	Detection limit[a] [n*M*]	Detection limit[a] [n*M*]	Decrease of detection limit[b]	Increase of sensitivity[b]
Daidzein[c]	54.3	15.8	3.43	3.44
Genistein[c]	26.6	13.9	1.91	1.94
Equol[d]	164.2	29.7	5.53	7.90
O-Desmethylangolensin[d]	50.2	85.2	0.59	1.01
Coumestrol[c]	67.4	ND	ND	ND

UV = diode-array detection in the ultraviolet range
ECD = electrochemical detection coulometrically at (+)500 mV
[a] determined by peak height with a 20 µl HPLC injection at a signal to noise ratio of 5
[b] coulometric values relative to UV values
[c] UV values obtained by absorbance at 260 nm
[d] UV values obtained by absorbance at 280 nm
ND = not determined

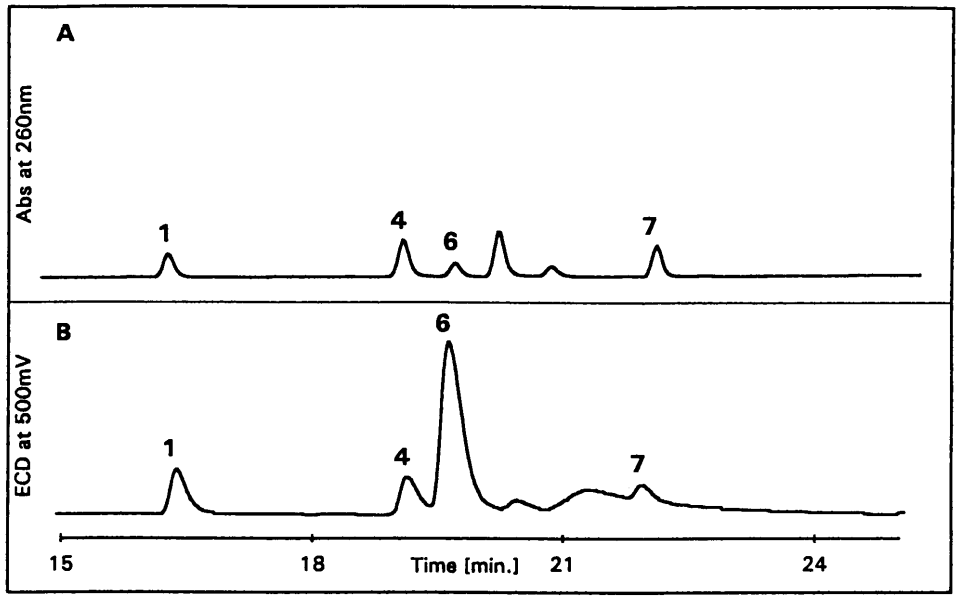


Figure 4. HPLC traces of authentic daidzein (**1**), genistein (**4**), equol (**6**) and O-desmethylangolensin (**7**) monitored at 260 nm (A) and coulometrically at +500 mV (B) shown on the same scale.

with higher purity were used for enzymatic hydrolysis purposes (see Material and Methods). This led to lower enzyme amounts and shorter incubation times required for complete hydrolysis and to less interfering signals in the chromatogram. Reevaluation of extraction procedures revealed that purification of urine by solid-phase extraction is not required for efficient enzymatic hydrolysis and that a variety of organic solvents are suitable for isolation of the analytes. Applying the HPLC system described above to urine extracts resulted in baseline separation of all analytes allowing sensitive identification by diode array detection (Fig. 5). The validity of the technique presented is evidenced by similar results achieved in independent soy intervention studies using HPLC (29) or GC/MS (8, 25) to measure urinary isoflavone levels. According to our experience showing highly variable urinary isoflavone levels intra-individually depending on the amount of soy product consumption (17), epidemiologic studies using urinary isoflavone data should consider collecting urine from the same individual two to three times a week followed by pooling prior to analysis. Although degradation is highly unlikely due to the high stability of isoflavonoids (33), urine samples should be stored in acid pH and at temperatures below −20°C. Preferably, overnight urine should be used since this results in better compliance and facilitates specimen analysis due to higher analyte concentration. This protocol is also recommended since urinary excretion of isoflavonoids is usually completed 48–60 hr after soy intake as observed by us and others (25, 29, 39) and since dietary habits should generally be represented over a period of 1 week.

Isoflavones were analyzed from plasma using the HPLC system described above after enzymatic hydrolysis and solvent extraction (Fig. 6). Thorough evaluation of extraction conditions revealed that enzyme amount, incubation time, and buffer environment were very critical in obtaining good extraction efficiency and precision. Best extraction efficiencies were achieved by addition of triethyl amine (pH 7) to plasma (40) before incubating with not less than 80 µl enzyme/ml plasma over a period of at least 17 hr at 37°C. Daidzein (1.42 nmoles) and genistein (0.74

Table II. Mean Total Isoflavone Levels[a] in Soy Foods [mg/kg]

	n	Daidzein	cv	Glycitein	cv	Genistein	cv
Raw dry soybean[b]	4	913	19%	114	38%	763	12%
Soy flour[b]	2	789	8%	213	9%	1069	1%
Roasted soybean[b]	1	786		168		889	
Toasted soybean[b]	1	860		307		852	
Green soybean pod[c]	2	122	94%			144	62%
Soy protein[c]	2	633	4%			596	79%
Soybean sprout[c]	2	225	20%			305	19%
Raw tofu[c]	3	258	33%			377	21%
Raw tofu[b]	4	139	21%	17	9%	141	13%
Fermented tofu[b]	2	250	11%	50	3%	288	13%
Fermented curd[c]	2	90	3%			192	1%
Soy milk[c]	3	81	34%			78	101%
Soy cheese[c]	2	211	24%			382	1%
Raw foo jook[bd]	4	1160	21%	184	29%	1317	17%
Cooked foo jook[c]	4	182	32%			325	44%
Raw tau kwa[bd]	4	136	13%	20	17%	139	19%
Raw tau pok[bd]	4	163	40%	16	44%	190	41%
Fried bean curd[c]	2	122	21%			307	44%

[a] Levels in foods as consumed and expressed in aglycone units determined by duplicate analysis; within-assay imprecision = 5–9%
[b] Analyzed by extraction at room temperature
[c] Analyzed by acid hydrolysis; no glycitein included in the assay
[d] Tau Kwa = pressed Tofu, Tau Pok = fried Tau Kwa, Foo Jook = skimmed dry supernatant obtained by boiling soybean material
n = number of different food sources
cv = coefficient of variation between different food sources

Table III. Mean Isoflavone Levels[a] of Conjugates and Aglycones in Soy Foods from Singapore [mg/kg]

	n	7-*O*-glucoside of						7-*O*-malonylglucosides of					
		Daidzein	cv	Glycitein	cv	Genistein	cv	Daidzein	cv	Glycitein	cv	Genistein	cv
Soybean	4	256.6	11%	35.2	17%	251.2	9%	604.3	23%	78.5	48%	481.3	16%
Tofu	4	52.3	10%	7.6	23%	60.9	36%	66.2	50%	6.7	36%	63.5	42%
Tau Kwa	4	50.2	24%	7.5	25%	62.4	28%	65.6	14%	10.7	22%	58.8	20%
Tau Pok	4	47.3	42%	5.6	47%	69.9	47%	51.9	48%	3.9	59%	48.0	49%
Foo Jook	4	723.0	27%	135.2	29%	925.9	23%	70.6	37%	2.4	67%	57.0	62%
Fermented Tofu	2	nd	—	nd	—	nd	—	nd	—	0.8	141%	nd	—
INTRA-ASSAY VARIABILITY (*n* = 42)													
cv [%]		6.4%		10.2%		6.1%		7.5%		17.0%		8.5%	

Table III. Continued

	7-*O*-acetylglucosides of						Daidzein	cv	Glycitein	cv	Genistein	cv
	Daidzein	cv	Glycitein	cv	Genistein	cv						
Soybean	nd	—	nd	—	2.6	116%	51.9	22%	nd	—	27.8	30%
Tofu	3.2	29%	0.9	86%	2.4	58%	16.9	66%	2.0	110%	14.3	90%
Tau Kwa	2.9	41%	0.1	200%	2.2	21%	16.9	37%	1.8	71%	15.5	28%
Tau Pok	42.1	46%	3.2	51%	44.7	46%	21.3	34%	3.2	24%	27.3	27%
Foo Jook	35.4	56%	5.5	70%	25.6	31%	331.1	15%	41.0	32%	308.2	10%
Fermented Tofu	nd	—	nd	—	nd	—	250.5	11%	49.1	1%	287.6	13%
INTRA-ASSAY VARIABILITY (*n* = 42)												
cv [%]	9.6%		5.5%		10.5%		7.0%		11.2%		6.3%	

[a] levels expressed in aglycone units determined by duplicate analysis.
n = number of different food sources
cv = coefficient of variation between different food sources
nd = levels below detection limit

nmoles) was spiked into plasma containing 1.33 and 2.27 nmoles, respectively, and led to 85% and 84% recovery rates, respectively. Coefficients of variations for intra-/interassay variability of daidzein and genistein were found to be 8.2%/6.6% and 11.7%/8.4%, respectively. Levels in serum showed no significant difference to those in plasma.

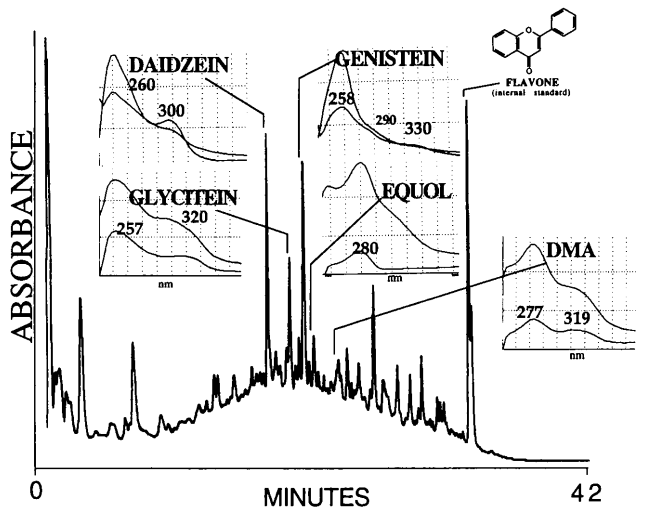


Figure 5. Chromatogram of a hydrolyzed extract obtained from urine of a subject with unknown soy exposure prior to urine collection. The trace monitored at 260 nm shows baseline separations of all analytes. Absorbance scans of peaks depicted in inserts were identical with those from authentic external standards (upper trace in each insert) leading to unambiguous peak assignment. DMA = O-desmethylangolensin.

Analysis of plasma obtained from a male subject 10 hr after intake of 20 g of roasted soybeans shows major HPLC peaks for daidzein and genistein (Fig. 6B). This resulted in unambiguous peak assignment by comparison with authentic standards (Fig. 6C) and by diode array detection. Isoflavone metabolites were not observed in this plasma sample probably due to the presence of *O*-desmethylangolensin below detection limit and due to the absence of equol as assessed by urinary analysis. Similar to previous urinary analyses (29), excellent dose response correlations of plasma isoflavones were observed in a metabolic study exposing an individual to 5, 10 and 20 g of roasted soybeans during 4 days as depicted in Figure 7A. Elimination of isoflavones from plasma was found to be completed approximately 60 hr after intake of soy foods as shown in Figure 7B. Plasma elimination patterns were found to be very similar to those from urine (Fig. 7A, B). Great variation of plasma isoflavone recovery (5%–10%) was observed by analyzing six plasma samples from three individuals. Genistein to daidzein ratios were highly variable (0.5–2.1) and consistent only within an individual similar to earlier findings from urinary patterns (29). These results suggest that future epidemiological and clinical trials assessing the protective role of isoflavonoids or soy intake at inhibiting chronic diseases would not necessarily require plasma but could be designed with a noninvasive protocol using urine facilitating easier subject recruitment, better compliance, and faster approval from study reviews.

Finally, a method for isoflavone determination in human breast milk was developed in order to assess the natural exposure of newborn babies to these agents (41). The high-protein content of milk required thorough evaluation of extraction conditions. Tests with spiked milk samples revealed that solid-phase extraction and many solvents were not applicable for isoflavone extractions from this matrix. However, ethyl acetate frequently used for the isolation of dietary phenolic (42) and flavonoid (43, 44) compounds, resulted in selective concentration of the analytes in the organic phase and led to higher recoveries compared to tertiary butyl methyl ether. Ethyl acetate isolation led to

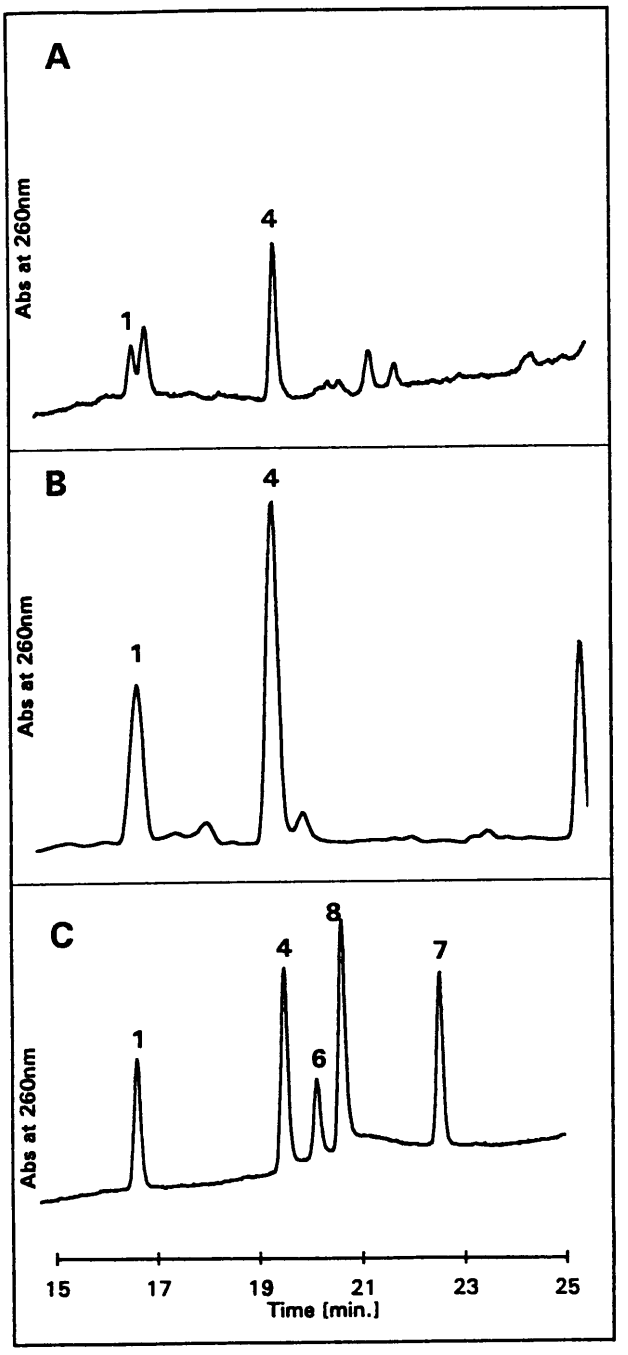


Figure 6. HPLC chromatograms of extracts from (A) human milk and (B) human plasma obtained 18 hr and 10 hr after challenge with 20 g roasted soybeans, respectively. All traces including that from authentic standards (C) were monitored at 260 nm and show a peak for the internal standard flavone at 31 min (not shown). Analyte amount expressed in pmol in trace A, B, and C: daidzein (**1**) = 4, 2739 and 16 pmol; genistein (**4**) = 5, 2334 and 12 pmol; equol (**6**), coumestrol (**8**) and *O*-desmethylangolensin (**7**) were below detection limit in plasma and milk.

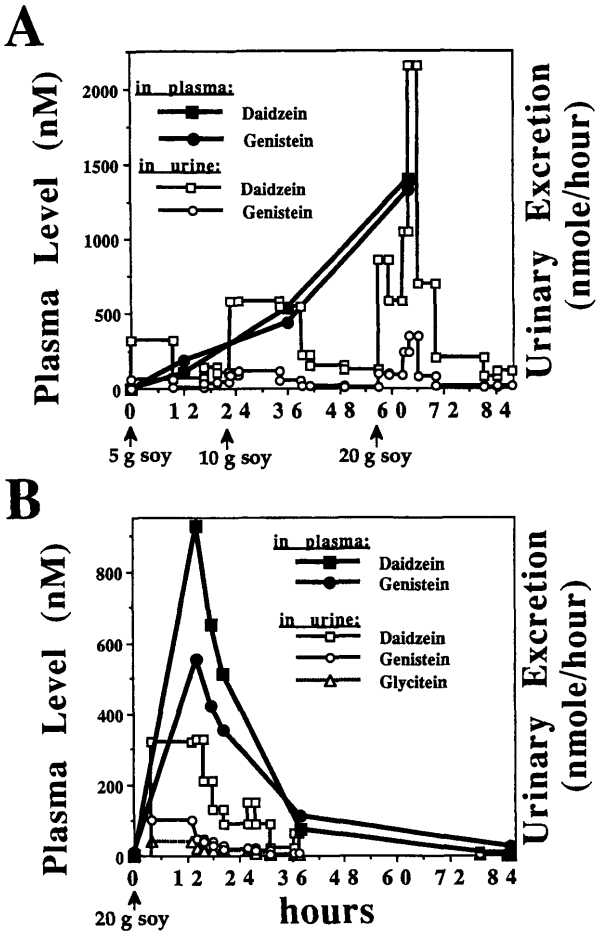


Figure 7. Daidzein and genistein levels in human plasma and urine after challenging a male subject with 5, 10, and 20 g of roasted soybeans (A) and 20 g of roasted soybeans (B), respectively. Blood was drawn 10–12 hr after soybean intake, and urine samples were collected continuously as shown on graphs.

high precision (coefficient of variation = 5%–11%) and spiking recoveries (88%–99%) of isoflavone aglycones from human milk (41) and did not co-extract glucosidic conjugates (data not shown). Therefore, hydrolysis of isoflavone conjugates was required prior to extraction in order to include conjugates in the assay. The lack of detectable isoflavones after extraction without hydrolysis suggested that all isoflavones occur as glucuronide and/or sulfate conjugates in human milk. Analytes were routinely identified by retention times in various HPLC systems (Fig. 6) and by UV absorption patterns obtained by diode-array detection. Additionally, fluorometric (31) and coulometric (45) detection were used to confirm the presence of these agents in human milk. Trimethylsilylation of representative milk extracts followed by GC/MS analysis in the selected ion monitoring (SIM) mode indicated again the presence of daidzein and genistein in human milk due to identical GC retention times and mass fragmentation patterns with those of authentic standards (41). These GC/MS values were also in excellent agreement with those reported previously (28) None of the other soy isoflavones or metabolites shown in Figure 1 were detected in human milk extracts with this GC/MS/SIM method suggesting their absence in human milk after soybean consumption by this particular individual. The exclusive occurrence of the major soy isoflavones in milk is not surprising since glycitein exposure through soy challenge is minor due to its presence at very low levels in soybeans (Table II) (22, 25). A preferential excretion of the metabolites over the parent isoflavones is suggested by the isoflavone to metabolite ratio observed to be much higher in plasma than in urine or feces (26, 28). Since milk is produced by secretory processes from blood (46), the low plasma concentrations of isoflavonoid metabolites might explain their absence in milk.

Soybean challenge led to a fast and dose-dependent response in human milk (Fig. 8) in excellent agreement with results presented recently using GC/MS analysis (47). Maximum milk levels were reached 10–14 hr after soy intake, and baseline levels were reached 2–4 days later depending on the dose. The isoflavone patterns in milk followed those in urine, except with a slight delay (Fig. 8). This is in good agreement with other micronutrients or drugs showing a faster urinary excretion than secretion into milk (46). Most importantly, milk levels of genistein conjugates were found to be higher than those of daidzein conjugates as was observed for plasma levels of this individual (genistein to daidzein ratio = 2:1). In contrast, urinary levels of genistein conjugates were found to be smaller than those of daidzein conjugates in agreement with other studies (24, 25, 29, 30, 38, 48–50). This might be due to the higher polarity of daidzein conjugates favoring its urinary excretion and leading to increasing genistein to daidzein ratios in blood. Secretory processes will consequently result in isoflavone patterns in breast milk to be similar to those in blood.

Milk levels of daidzein and genistein conjugates increased rapidly after soy consumption followed by a rapid decrease and most interestingly, by a subsequent increase

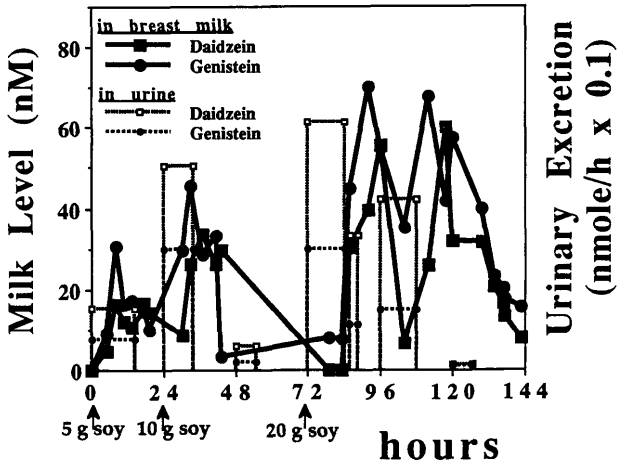


Figure 8. Daidzein and genistein levels in human milk and urine after challenge with 5, 10, and 20 g of roasted soybeans. Milk samples were collected at time of feeding starting at 12 hr before first soybean intake. Six overnight urine samples and two additional samples, 12 hr before and 87 hr after first soybean intake, were obtained to monitor urinary isoflavone excretion.

before reaching baseline levels (Fig. 8). This biphasic elimination pattern has already been observed in animal plasma after treatment with the flavane hydroxyfarrerol (51) or the flavone baicalin (43) and most importantly, in human plasma (52) and urine (39) after soy intervention. This biphasic phenomenon was suggested to be due to enterohepatic circulation (30, 53) a process known to occur with flavonoids (54). Therefore, enterohepatic circulation might also explain the biphasic pattern observed in human milk.

Isoflavone levels of three milk and urine samples of a Chinese woman eating her usual diet including tofu soup once a day for dinner were analyzed with the proposed procedure. Milk levels of daidzein and genistein (80–110 n*M* and 30–50 n*M*) were found to be similar to those observed after challenge with roasted soybeans. This is in good agreement considering the similar total isoflavone exposure from these two food items (18). However, urinary excretion was found to be smaller in the Chinese woman (80–150 nmoles/hr daidzein and 8–33 nmoles/hr genistein) compared to the Caucasian woman during soybean intervention. This may be due to interindividual variation in excretion (25, 39) or to the different food items consumed (38).

In summary, we showed that the diode-array HPLC method presented is applicable to isoflavonoid analysis from foods and from human body fluids helpful for future epidemiologic and clinical trials assessing the role of soy or isoflavonoids at preventing chronic diseases. After moderate soy challenge we found mean total isoflavone levels of 60 μ*M* in urine, 2 μ*M* in plasma and 0.2 μ*M* in breast milk. Although demonstrated exclusively in carcinogen-or cytokine-induced cell and animal models, the anticancer properties of genistein and daidzein are particularly intriguing when considered in combination with the results presented in human milk. Breast feeding is known to be beneficial, not only to the mother by protecting against ovarian and breast cancer, but in particular, to the infant protecting against various diseases including infections (55–57). Cancer incidence and severity is significantly reduced when newborn animals are treated with only three single doses of genistein (13). These findings in combination with the data presented suggest a cancer preventive effect of breast feeding to the offspring when mothers consume soy foods due to exposure of the known anticancer agent genistein (and also daidzein) to the infant. This effect may take place at a very early and most critical developmental period through mother's milk containing these agents in a highly bioavailable form. It is conceivable that the isoflavone conjugates obtained from mother's milk are more bioavailable to the newborn child compared to those from soy foods due to the incompletely developed gut flora (49, 58) preventing hydrolysis of acylated and nonacylated isoflavone glucosides present in soy foods. Analysis of biotransformation products and bioavailable levels of soy isoflavones in the baby are required to explore the risks and benefits further of these compounds in humans.

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1. Messina M, Persky V, Setchell KDR, Barnes S. Soy intake and cancer risk: A review of *in vitro* and *in vivo* data. Nutrition and Cancer **21:**113–131, 1994.
2. Messina M, Barnes S. The role of soy products in reducing risk of cancer. J Natl Cancer Inst **83:**541–546, 1991.
3. Barnes S, Peterson TG. Biochemical targets of the isoflavone genistein in tumor cell lines. Proc Soc Exp Biol Med **208:**103–108, 1995.
4. Wei H, Bowen R, Cai Q, Barnes S, Wang Y. Antioxidant and antipromotional effects of the soybean isoflavone genistein. Proc Soc Exp Biol Med **208:**124–130, 1995.
5. Mousavi Y, Adlercreutz H. Genistein is an effective stimulator of sex hormone-binding globulin production in hepatocarcinoma human liver cancer cells and suppresses proliferation of these cells in culture. Steroids **58:**301–304, 1993.
6. Adlercreutz H, Bannwart C, Wahala K, Makela T, Brunow G, Hase T, Arosemena PJ, Kellis J, Vickery LE. Inhibition of human aromatase by mammalian lignans and isoflavonoid phytoestrogens. J Steroid Biochem Mol Biol **44:**147–153, 1993.
7. Makela SI, Pylkkanen LH, Santti RSS, Adlercreutz H. Dietary soybean may be antiestrogenic in male mice. J Nutr **125:**437–445, 1995.
8. Cassidy A, Bingham S, Setchell KDR. Biological effect of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. Am J Clin Nutr **60:**333–340, 1994.
9. Hirano T, Gotoh M, Oka K. Natural flavonoids and lignans are potent cytostatic agents against human leukemic HL-60 cells. Life Sci **55:**1061–1069, 1994.
10. Constantinou A, Huberman E. Genistein as an inducer of tumor cell differentiation: Possible mechanisms of action. Proc Soc Exp Biol Med **208:**109–115, 1995.
11. Fotsis T, Pepper M, Adlercreutz H, Hase T, Montesano R, Schweigerer L. Genistein, a dietary ingested isoflavonoid, inhibits cell proliferation and *in vitro* angiogenesis. J Nutr **125:**(Suppl):790S–797S, 1995.
12. Uckun FM, Evans E, Forsyth CJ, Waddick KG, Ahlgren LT, Chelstrom LM, Burkhardt A, Bolen J, Myers DE. Biotherapy of B-cell precursor leukemia by targeting genistein to CD19-associated tyrosine kinases. Science **267:**886–891, 1995.
13. Lamartiniere CA, Moore J, Holland M, Barnes S. Neonatal genistein chemoprevents mammary cancer. Proc Soc Exp Biol Med **208:**120–123, 1995.
14. Barnes S, Peterson TG, Grubbs C, Setchell KDR. Diet and Cancer: Markers, Prevention, and Treatment. New York: Plenum Press, p135, 1994.
15. Lee HP, Gourley L, Duffey SW, Esteve J, Lee J, Day NE. Dietary effects on breast-cancer risk in Singapore. Lancet **337:**1197–1200, 1991.
16. Adlercreutz H, Honjo H, Higashi A, Fotsis T, Hamalainen E, Hasegawa T, Okada H. Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet. Am J Clin Nutr **54:**1093–1100, 1991.
17. Adlercreutz H, Markkanen H, Watanabe S. Plasma concentrations of phytooestrogens in Japanese men. Lancet **342:**1209–1210, 1993.
18. Franke AA, Custer LJ, Cerna CM, Narala KK. Quantitation of phytoestrogens in legumes by HPLC. J Agric Food Chem **42:**1905–1913, 1994.
19. Coward L, Barnes NC, Setchell KDR, Barnes S. The antitumor isoflavones, genistein and daidzein, in soybean foods of American and Asian diets. J Agric Food Chem **41:**1961–1967, 1993.

20. Wang H, Murphy PA. Isoflavone content in commercial soybean foods. J Agric Food Chem **42:**1666–1673, 1994.
21. Wang H, Murphy PA. Isoflavone composition of American and Japanese soybeans in Iowa: Effects of variety, crop year, and location. J Agric Food Chem **42:**1674–1677, 1994.
22. Tsukamoto C, Shimada S, Igita K, Kudou S, Kokubun M, Okubo K, Kitamura K. Factors affecting isoflavone content in soybean seeds: Changes in isoflavones, saponins, and composition of fatty acids at different temperatures during seed development. J Agric Food Chem **43:**1184–1192, 1995.
23. Barnes S, Kirk M, Coward L. Isoflavones and their conjugates in soy foods: Extraction conditions and analysis by hplc-mass spectrometry. J Agr Food Chem **42:**2466–2474, 1994.
24. Adlercreutz H, Fotsis T, Bannwart C, Wähälä K, Brunow G, Hase T. Isotope dilution gas chromatographic-mass spectrometric method for the determination of lignans and isoflavonoids in human urine, including identification of genistein. Clin Chim Acta **199:**263–278, 1991.
25. Kelly GE, Nelson C, Waring MA, Joannou GE, Reeder AY. Metabolites of dietary (soya) isoflavones in human urine. Clin Chim Acta **223:**9–22, 1993.
26. Adlercreutz H, Fotsis T, Lampe J, Wähälä K, Mäkelä T, Brunow G, Hase T. Quantitative determination of lignans and isoflavonoids in plasma of omnivorous and vegetarian women by isotope dilution gas chromatography-mass spectrometry. Scand J Clin Lab Invest **53:**5–18, 1993.
27. Kurzer MS, Lampe JW, Martini MC, Adlercreutz H. Fecal lignan and isoflavonoid excretion in premenopausal women consuming flaxseed power. Cancer Epidemiology, Biomarkers & Prevention **4:**353–358, 1995.
28. Adlercreutz H, Fotsis T, Kurzer MS, Wähälä K, Mäkelä T, Hase T. Isotope dilution gas chromatographic-mass spectrometric method for the determination of unconjugated lignans and isoflavonoids in human feces, with preliminary results in omnivorous and vegetarian women. Anal Biochem **225:**101–108, 1995.
29. Franke AA, Custer LJ. High-performance liquid chromatography assay of isoflavonoids and coumestrol from human urine. J Chromatogr B **662:**47–60, 1994.
30. Xu X, Wang H-J, Murphy PA, Cook L, Hendrich S. Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. J Nutr **124:**825–832, 1994.
31. Lundh TJ-O, Pettersson H, Kiessling KH. Liquid chromatographic determination of the estrogens daidzein, formononetin, coumestrol, and equol in bovine blood plasma and urine. J Assoc Off Anal Chem **71:**938–941, 1988.
32. Donike M. Steroid profiling in Cologne. In: Donike M, Geyer H, Gotzmann A, Mareck-Engelke U, Rauth S, Eds. 10th Cologne Workshop on Dope Analysis. Köln: Sport und Buch Strauβ, pp 73–79, 1993.
33. Franke AA, Custer LJ, Cerna CM, Narala K. Rapid HPLC analysis of dietary phytoestrogens from legumes and from human urine. Proc Soc Exp Biol Med **208:**18–26, 1995.
34. Kelloff GJ, Boone CW, Crowell JA, Steele VE, Lubet R, Sigman CC. Chemopreventive drug development: Perspectives and progress. Cancer Epid, Biomarkers, & Prevention **3:**85–98, 1994.
35. Markham KR, Mabry TJ. The Flavonoids. New York: Academic Press, p46, 1972.
36. Kudou S, Fluery Y, Welti D, Magnolato D, Uchida T, Kitamura K, Okubo M. Malonyl isoflavone glycosides in soybean seeds (Glycine max MERRILL). Agric Biol Chem **55:**2227–2233, 1991.
37. Eldridge AC, Kwolek WF. Soybean isoflavones: Effect of environment and variety on composition. J Agric Food Chem **31:**394–396, 1983.
38. Hutchins AM, Slavin JL, Lampe JW. Urinary isoflavonoid phytoestrogen and lignan excretion after consumption of fermented and unfermented soy products. J Am Diet Assoc **95:**545–551, 1995.
39. Setchell KDR. Estrogens in the Environment. New York: Elsevier, p69, 1985.
40. Coward L, Kirk M, Albin N, Barnes S. Analysis of plasma isoflavones by reversed-phase HPLC-multiple reaction ion monitoring-mass spectrometry. Clin Chim Acta **247:**121–142, 1996.
41. Franke AA, Custer LJ. Daidzein and genistein levels in human milk after soy consumption. Clin Chem **42:**955–964, 1996.
42. Kermasha S, Goetghebeur M, Dumont J. Determination of phenolic compound profiles in maple products by high-performance liquid chromatography. J Agric Food Chem **43:**708–716, 1995.
43. Wakui Y, Yanagisawa E, Ishibashi E, Matsuzaki Y, Takeda S, Sasaki H, Aburada M, Oyama T. Determination of baicalin and baicalein in rat plasma by high-performance liquid chromatography with electrochemical detection. J Chromatogr **575:**131–136, 1992.
44. Lee M-J, Wang Z-Y, Li H, Chen L, Sun Y, Gobbo S, Balentine DA, Yang CS. Analysis of plasma and urinary tea polyphenols in human subjects. Cancer Epidemiology, Biomarkers & Prevention **4:**393–399, 1995.
45. Gamache P. Analysis of phenolics and flavonoids having potential biological activity using HPLC with coulometric array detection. (abstract), National Cancer Institute, Dietary Phytoestrogens: Cancer Cause or Prevention? Herndon, VA, 1994.
46. Lawrence RA. Breastfeeding. St. Louis, MO: Mosby, 1994.
47. Setchell KDR. Proc Soc Exp Biol Med 1997.
48. Hutchins AM, Lampe JW, Martini MC, Campbell DR, Slavin JL. Vegetables, fruits, and legumes: Effect on urinary isoflavonoid phytoestrogen and lignan excretion. J Am Diet Assoc **95:**769–774, 1995.
49. Cruz MLA, Wong WW, Mimouni F, Hachey DL, Setchell KDR, Klein PD, Tsang RC. Effects of infant nutrition on cholesterol synthesis rates. Pediatr Res **35:**135–140, 1994.
50. Baird DD, Umbach DM, Lansdell L, Hughes CL, Setchell KDR, Weinberg CR, Haney AF, Wilcox AJ, McLachlan JA. Dietary intervention study to assess estrogenicity of dietary soy among postmenopausal women. J Clin Endocrinol Metab **80:**1685–1690, 1995.
51. Marzo A, Arrigoni Martelli E, Bruno G. Assay of hydroxyfarrerol in biological fluids. J Chromatogr **535:**255–261, 1990.
52. Barnes S. Serum conjugated and unconjugated isoflavones by capillary reversed-phase HPLC-electrospray ionization-mass spectrometry. NCI Meeting on ''Dietary Phytoestrogens: Cancer Cause or Prevention?''. (abstract) Herndon, VA, 1994.
53. Sfakianos J, Coward L, Kirk M, Barnes S. Intestinal uptake and biliary excretion of the isoflavone genistein in the rat: Evidence for its enterohepatic circulation. J Nutr **127:**1260–1268, 1997.
54. Griffith LA. Mammalian metabolism of flavonoids. In: Harborne JB, Mabry TJ, Eds. The Flavonoids: Advances in Research. London/New York: Chapman and Hall, pp681–718, 1982.
55. The Lancet (editorial). A warm chain for breastfeeding. Lancet **344:**1239–1241, 1994.
56. Savage F. Breastfeeding and SIDS. MIDIRS Midwifery Digest **4:**227–260, 1992.
57. Rogan WJ, Gladen BC. Breastfeeding and cognitive development. Early Hum Dev **31:**181–193, 1993.
58. Setchell KDR, Welch MB, Lim CK. HPLC analysis of phytoestrogens in soy protein preparations with ultraviolet, electrochemical, and thermospray mass spectrometric detection. J Chromatogr **386:**315–323, 1987.