A Simplified Method to Quantify Isoflavones in Commercial Soybean Diets and Human Urine after Legume Consumption

Lee-Jane W. Lu,² Lyle D. Broemeling, Milton V. Marshall, and V. M. Sadagopa Ramanujam

Abstract

Reliable and economical quantification of micronutrients in diets and humans is a critical component of successful epidemiological studies to establish relationships between dietary constituents and chronic disease. Legumes are one of the major dietary components consumed by populations worldwide. Consumption of legumes is thought to play a major role in lowering breast and prostate cancer risk. In this study, a simplified method that uses solid-phase extraction and gas chromatography was developed to measure isoflavones at levels down to 10 μg/5 ml. With the use of this method, 12.5 g miso (a soybean paste), 12 ounces Isomil, and 12 ounces soymilk had daidzin/daidzein levels of 2, 5, and 12.4 mg, respectively, and genistin/genistein levels of 3, 6.5, and 13.7 mg, respectively. In these products, most of the isoflavones were present as glucosides. With the same method, urinary levels of isoflavones in six 15-17-year-old females were determined after soymilk ingestion. Each subject was placed on unrestricted nonsoya diets, and three 12-ounce portions of soymilk were given at 12-h intervals. Males excreted 15.02 ± 2.74 (SD) mg of daidzein glucuronides/sulfates [mean recovery, 40.4 ± 7.4% (SD)] by 24 h after the third soymilk ingestion, whereas females excreted 25.56 ± 5.10 mg (68.7 ± 13.7%) of daidzein conjugates, which was more than males (P = 0.02). Males and females excreted 7.73 ± 1.95 mg and 9.11 ± 0.84 mg of genistein glucuronides/sulfates (20% recovery of genistin intake), respectively, in the urine. Most of the isoflavones were excreted within 24 h after ingestion. The relative urinary levels of daidzein to genistin excreted were significantly (P < 0.05) higher in females than males after the third ingestion. The observed sex difference requires more study since two of the females are siblings. Thus, the method described can be used to measure isoflavones in soya products and urinary excretion after soya ingestion.

Introduction

Legumes are consumed widely in the diet, and they have been implicated as protective factors against development of breast and prostate cancer (1–3). With the use of animal models, anticarcinogenic effects have been identified after soybean diet supplementation (reviewed in Refs. 4–7). Isoflavones, particularly daidzein and genistein, are abundant in soya beans and exhibit pleiotropic biological effects. Isoflavones are weak estrogens and also act as estrogen antagonists (8). Furthermore, genistein inhibits cell proliferation (9), induces cell differentiation (10), and inhibits tyrosine kinase (11). These are potential mechanisms by which legumes can reduce cancer incidence.

Procedures for the analyses of plant estrogens have been described (12–22). Typically, HPLC coupled with UV (14–22), fluorescence (15), or amperometric detection (18, 19) has been used. Additionally, GC quantification of isoflavones by FID or mass spectrometry detection have been described (12, 13, 19, 21, 23–29). With these methods, prepurification of samples by ion exchange- or reversed-phase chromatography are required prior to chromatographic analysis and typically involves multistep purification processes.

In epidemiological studies, quantification of dietary consumption has traditionally relied on recall questionnaires and dietary records. Errors from recall studies frequently compromise the validity of collected data and subsequent results. A simple method to measure quantitatively dietary exposure would be an important tool in improving epidemiological data analysis. We report here a simplified solid-phase extraction procedure that can quantify isoflavone levels in food products and in human urine after dietary soya consumption.

Materials and Methods

Chemicals and Reagents. Daidzein, genistein, and DHF were obtained from Indofine Chemical Co., Inc. (Somerville, NJ). α-Glucosidase from almonds and β-glucuronidase/sulfatase from Helix pomatia were from Sigma Chemical Co. (St. Louis, MO). ChemElut columns were purchased from AnalytiChem International (Harbor City, CA). BSTFA with 1% TMCS was from Pierce Chemical Co. (Rockford, IL). All other reagents such as ammonium carbonate, ethyl acetate, methanol, and pyridine were from Fisher Scientific Co. (Fair Lawn, NJ). HP-1 columns (100% dimethylpolysiloxane) were obtained from Hewlett Packard Co. (Palo Alto, CA).

Diet. Soymilk used for this study was obtained from Banyan Foods Co. and Plum Flower Foods Co. (both from Houston, TX), Isomil from Ross Laboratories (Columbus, OH), and miso, a mild soybean paste, from Yamabuki (Los Angeles, CA).

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2 To whom requests for reprints should be addressed, at Department of Preventive Medicine and Community Health, University of Texas, 700 Strand, 2.102 Ewing Hall, 1110, Galveston, TX 77555-1110.

The abbreviations used are: GC, gas chromatography; DHF, 7,4′-dihydroxyflavone; FID, flame ionization detection; BSTFA, N.O-bis(trimethylsilyl)trifluoroacetamide; TMCS, trimethylchlorosilane.
Study Design and Subjects. Six healthy teenagers, 3 males and 3 females, from the Houston-Galveston area were recruited for this study. Two female subjects (15 and 17 years old) from India were siblings; the third female was a 17-year-old Hispanic. Two Caucasians and one Taiwanese male were also 17 years old. Enrollment of subjects with diverse ethnicity complied with NIH guidelines for inclusion of human subjects. Additional studies will be needed to determine if ethnicity influences isoflavone excretion, which is not the purpose of present study. No efforts were made to restrict dietary intake prior to soymilk consumption or during the study, and all subjects were provided with the same lot of soymilk. Subjects were instructed to take three 12-ounce portions of soymilk at 12-h intervals, i.e., one drink at time 0, 12 h, and 24 h into the study. In all subjects, except one male and one female, initial soymilk ingestions and the first 12-h collections were performed at night. This project was approved by The Institutional Review Board of the University of Texas Medical Branch and by the Human Use Committee of Ball High School (Galveston, TX). Oral and written informed consent were obtained.

Urine Collections. Urine sampling for the 6 teenagers began soon after each soymilk dose and continued until the beginning of the next soymilk dose 12 h later. Urine samples were obtained for 36 h after the third and last soymilk dose. Urine was kept in an ice chest after collection and stored at −20°C until analyzed. The voids for each 12-h period were pooled and provided a total of 5 sequential 12-h samples from each subject. Twelve-h urine samples were also obtained before soymilk consumption began.

Urine Sample Preparation. To determine total levels of isoflavones (free and conjugated forms), 50–200 μg of DHF were added as an internal standard to 20-ml urine. The pH of the urine was adjusted to 5.0 with 2 m sodium acetate, and 0.2 ml (22,800 units) β-glucuronidase/sulfatase from Helix pomatia was added. The urine was incubated for 24 h at 37°C. To quantify unconjugated isoflavones, β-glucuronidase/sulfatase digestion was omitted.

Sample Preparation from Dietary Sources. Isoflavone (100 ml), soymilk (100 ml), or miso (12.5 g suspended in 100 ml water) were mixed with 500 ml of methanol, refluxed for 2 h, and filtered as described (16). The methanol filtrate was dried under vacuum in a rotary evaporator. The residue was redissolved in 100–200 ml of water, and an aliquot (10–20 ml) was mixed with 100–200 μg of DHF (internal standard) and extracted 3 times with 20 ml of hexane. The hexane layers were discarded, and residual hexane in the aqueous layer was removed by evaporation under vacuum. To determine total isoflavone levels (aglycones and glucoside conjugates), the aqueous layer was adjusted to pH 5.0 with 2 m sodium acetate and incubated with 580 units (120 mg) of β-glucosidase at 37°C for 24 h. To determine the levels of isoflavone aglycones, another portion of the aqueous layer (20 ml) after hexane wash was mixed with 50–100 μg DHF without β-glucosidase digestion. Aqueous solutions with or without β-glucosidase digestion were purified further by ChemElut chromatography before quantification by GC-FID.

ChemElut Column Chromatography. Urine (20 ml) with or without β-glucuronidase/sulfatase digestion, or soybbean diet extracts (20 ml) with or without β-glucosidase digestion prepared as described above were adjusted to pH 7 with solid ammonium carbonate and placed onto ChemElut columns. Isoflavones were eluted 3 times with 20 ml of ethyl acetate. Ethyl acetate was removed under vacuum with a centrifuge concentrator equipped with a −105°C refrigeration trap (Jouan, Inc., Winchester, VA). Residues were taken up in methanol, and a portion of the methanol solution was used for silylation. Prior to silylation, methanol was removed under vacuum.

Sample Preparation for GC Analysis. Dry residues from biological samples were mixed with 200 μl of a mixture of 50% BSTFA containing 1% TMCS in pyridine and incubated for 2 h at 90°C. The resulting solution was used directly for GC analysis with the use of an HP-1 column [30 m × 0.53 mm (inside diameter) × 0.88 μm film thickness]. Chromatography was performed with an HP5890 GC equipped with an HP7673A autosampler and HPChem station software. The injector and detector temperatures were 275°C and 350°C, respectively, with a purge time of 1 min. The initial oven temperature was maintained at 85°C for 4 min, increased to 275°C at 30°C/min, and maintained at 275°C for an additional 25 min. The carrier gas was nitrogen with a flow rate of 20 ml/min.

Statistics. Statistics were expressed as mean (± SD), and means of continuous variables were compared with the two-sample t test.

Results

Standard Identification. Fig. 1 shows a typical GC-FID profile of trimethylsilyl ethers of daidzein (Rt, 14.4 min), genistein (Rt, 14.9 and 15.1 min), and DHF (Rt, 15.6 min). Genistein produced two peaks: the major peak (>90%) with a Rt of 14.9 min is a tri-trimethylsilyl ether of genistein [M=CH3]3+, m/z = 471, by mass spectral analysis, whereas the small peak eluting at 15.1 min is a bis-trimethylsilyl ether (m/z = 414) of genistein. The areas under both silylated genistein peaks were added for genistein quantification. Among many flavonoids and isoflavones tested for suitability as internal standards, DHF, a structural isomer of daidzein, was chosen because the optimal pH for its recovery with the use of ChemElut solid-phase extraction was similar to that of daidzein and genistein (see below). Additionally, it was well separated from daidzein and genistein. Other flavonoids such as biochanin A, formononetin, and naringenin have retention times closer to those of daidzein and genistein. Apigenin, a structural isomer of genistein, has a longer retention time than genistein and produces two broad GC peaks that renders it unsuitable as an internal standard.

The GC-FID peak areas under the curve per unit compound injected for daidzein and genistein were similar and linear for concentrations ranging from 0.1–2.0 μg/μl (4.5 × 104 peak area unit/μg injected). The peak area for DHF was linear and approximately 80% that of daidzein and genistein. Response factors for genistein and DHF, but not daidzein, tended to decrease if a split injection technique was used. Under our chromatographic conditions, the optimal purge time was determined to be >1 min.

Quality control samples used for analysis were prepared by mixing aliquots of control urine with standards of daidzein, genistein, and DHF. These quality control samples were extracted and chromatographed with each set of samples. The ratios of GC peak areas among known amounts of daidzein, genistein, and DHF standards were used to identify suboptimal GC conditions and to validate the chromatographic results. Fig. 2 illustrates typical calibration curves for known amounts (10–200 μg) of daidzein and genistein added to 20-ml urine aliquots containing 25 μg DHF as internal standard, after extraction, derivatization, and chromatography as described.

Recovery of Daidzein, Genistein and DHF from Urine. Known amounts (10–200 μg) of daidzein, genistein, and DHF were added to control urine (20 ml). The urine pH was adjusted between 5 and 8, and the flavonoids were extracted with ethyl
Daidzein (D) 4',7-Dihydroxyisoflavone
Genistein (G) 4',5,7-Trihydroxyisoflavone

Fig. 1. Ordinate, typical FID gas chromatograms of trimethylsilylated derivatives of daidzein (D), genistein (G), and DHF. Daidzein, genistein, and DHF were derivatized with a mixture BSTFA containing 1% TMCS and pyridine (1:1, v/v) at 90°C for 2 h. The chromatography was performed with an HP 5890 chromatograph and an HP-1 column (30 m × 0.53 mm inside diameter × 0.88-μm film thickness). The injector temperature was 275°C, detector temperature was 350°C, and the purge time was 1 min. The initial oven temperature was kept at 85°C for 4 min, increased at 30°C/min to 275°C, and maintained at 275°C for an additional 25 min. The carrier gas was nitrogen with a flow rate of 20 ml/min.

Fig. 2. Calibration curves of daidzein and genistein determined by GC-FID. Varying amounts (10–200 μg) of daidzein and genistein and 25 μg of DHF as internal standard were added to 20 ml control urine, extracted, derivatized, and analyzed as described in Fig. 1.

Isoflavone Found (μg) vs Isoflavone Added (μg)

Daidzein and Genistein Levels in Miso, Isomil, and Soymilk.
Levels of daidzein and genistein in three different commonly consumed commercial soybean preparations were analyzed as described previously (Fig. 3). According to Adlercreutz et al. (29), the Japanese consumed typically 12.5 g miso/day. The total isoflavone levels in 12.5 g miso were estimated to be 2.06 ± 0.1 mg for both daidzin and daidzein (29.2 ± 5.8% of total) and 2.85 ± 0.03 mg for both genistin and genistein (19.8 ± 9.6% of total). Data (mean ± SD) were obtained from triplicate analyses of the same lot of miso. [Daidzin and genistin are the glucosides (glycones), and daidzein and genistein are the aglycones.]

Soymilk is an aqueous homogenate of soybeans that is consumed widely by Asians. The lot from Banyan was estimated to contain 12.36 ± 1.23 mg of daidzin (80%) and daidzein (20%) and 13.66 ± 2.83 mg of genistin (80%) and genistein (20%) per 12 ounces (triplicate analyses). We have analyzed in the past 3 years more than 20 lots of soymilk from Banyan and have observed up to a 5-fold difference in isoflavone content (results not shown). The isoflavone content of one other brand (Plum Flower) of soymilk available in the Houston area was 11.03 ± 0.54 mg of daidzin (81%) and daidzein (19%) and 10.95 ± 0.8 mg of genistin (81%) and genistein (20%) per 12 ounce (duplicate analyses of three different lots). Each 12
Ounces of Isomil contained 5.07 ± 0.32 mg of daidzin (65%) and daidzein (35%) and 6.46 ± 0.24 mg of genistin (50%) and genistein (50%).

**Daidzein and Genistein Levels in Urine of Teenagers.** Because soymilk contained more daidzin/daidzein and genistin/genistein than other soybean products surveyed, it was used for studying disposition with teenagers. Daidzin and genistin derived from soymilk were excreted in urine as glucuronide and sulfate conjugates of daidzein and genistein (>90%), respectively. Baseline levels of daidzein and genistein glucuronides/sulfates before soymilk ingestion for this group of subjects ranged from 0 to 200 and from 0 to 350 μg, respectively, per 12-h urine. 

Fig. 4 shows typical GC-FID chromatograms of urine before and after soymilk ingestion. Identities of daidzein, genistein, and DHF peaks in urine after soymilk ingestion were confirmed by GC-mass spectrometry analyses (results not shown).

Fig. 5 shows the levels of daidzein (Fig. 5A) and genistein (Fig. 5B) in each 12-h urine collection. Each subject consumed a single 12-ounce drink at 0, 12, and 24 h into the study. Urine voids within 12 h after each soymilk ingestion were pooled. As shown, excretion of both isoflavones decreased after the fifth collection. There is interindividual variability in urinary levels of daidzein and genistein, with males excreting less daidzein than females at most time points studied. Statistical significance 

\( P = 0.02 \)

was demonstrated between males and females for the fourth collection period (12–24 h after the third drink). For females, daidzein levels also fluctuated with significantly more excretion occurring during the second and fourth collections. Fig. 5B showed that excretion of genistein differed statistically in females and males only during the first 12 h \( (P < 0.01) \).

Fig. 7 shows the total daidzein:genistein excretion ratios as a function of time after soymilk administration. With the exception of the initial 12 h, females consistently had higher urinary daidzein:genistein ratios than did males. Statistical significance can be shown for collections that commenced after the third ingestion of soymilk.

**Discussion**

In this report, we have shown that daidzein and genistein can be recovered easily and conveniently after ChemElut solid-phase extraction in <30 min. ChemElut is composed of diatomaceous material that absorbs and retains aqueous materials, allowing liquid/solid-phase extraction without vortexing or centrifugation. Chromatography with the use of ChemElut columns, unlike ion-exchange or reversed-phase columns, does not require prewashing or conditioning of columns. Such procedures often vary with the type of biological specimen to be analyzed. Furthermore, the capacity of the ChemElut columns (10–500-ml columns available) is determined only by the volume, vis-à-vis, the content of the biological specimen to be
analyzed. Since the extraction is a 1-step process requiring 30 min, it is possible to process at least 30 urine samples/day. The glucuronidase digestion requires typically 24 h, and ChemElut extraction requires 30 min. Drying of ethyl acetate requires ~3 h (most time-consuming step). Derivatization and GC chromatography require 3 h. Thus, to process 1 sample, 2 working days and 3 h of labor are required. We have routinely processed 30 samples within 3 days.

Isoflavones found in several commercial products consist mostly of the glucoside conjugates, daidzin and genistin. Aglycones daidzein and genistein comprised ~20–50% of the recovered isoflavones (Fig. 3), which is consistent with other reports of isoflavone content of soya products (16, 22, 30). Together, these data suggest that humans who consume soy products are primarily exposed to glucoside conjugates of isoflavones.

The lot of Isomil analyzed contained significant amounts of daidzein/daidzin and genistein/genistin consistent with those reported by Setchell et al. (30). However, variable isoflavone content among different lots from the same manufacturer have also been observed for Isomil in our laboratory (results not shown). Because isoflavones are estrogenic, the health impact of isoflavone exposure on infants at these levels deserves further study. Neonatal exposure to sex hormones could alter the development of sexually differentiated behavior, anatomy, and reproductive physiology (31). In newborn rats exposed to genistein, altered postpubertal pituitary response to gonadotropin-releasing hormone was seen (32).

Of the commercial products surveyed, soymilk contained the highest levels of isoflavones, and it was chosen for dietary administration to teenage volunteers. Two of the male subjects participated in similar studies on two separate occasions, and

\[
\text{Cumulative Excretion, mg}\]

\[
\begin{array}{|c|c|c|}
\hline
\text{Time, Hrs} & \text{females} & \text{males} \\
\hline
0 & & \\
10 & & \\
20 & & \\
30 & & \\
40 & & \\
50 & & \\
60 & & \\
70 & & \\
80 & & \\
\hline
\end{array}
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Fig. 5. Daidzein (A) and genistein (B) levels in each 12-h urine collection of 3 males (17 years old) and 3 females (15–17 years old). Each subject ingested 12 ounces of soymilk at 0, 12, and 24 h and collected urine continuously at 12-h intervals. The results shown are total isoflavones in which >99% are conjugated metabolites. P values, comparison between males and females by unpaired t test. Columns, mean; bars, SD.

Fig. 6. Cumulative urinary excretions of daidzein (A) and genistein (B) in 3 males and 3 females after ingestion of soymilk. Consult Fig. 5 for treatments and analyses. Urinary levels from each 12 h sample were used to estimate cumulative levels of excretion. Points, mean; bars, SD.

Fig. 7. Relative levels of daidzein to genistein excretion in urine as a function of time after soymilk consumption. Data from Fig. 6 were used to derive the data shown. Points, mean; bars, SD.
disposition results from the two studies were comparable. Because the subjects were on unrestricted diets, urinary levels of isoflavones may have contained isoflavones from other dietary sources. The females in this study excreted more daidzein glucuronides/sulfates as males. Thus, females had higher daidzein:genistein excretion ratios in urine than did males. One point worth noting is that the two female siblings had strikingly similar kinetics for excretion. Because two of the three female subjects were siblings, the impact of genetic, environmental, or sex-related factors should be determined.

Isoflavone glucosides (daidzin and genistin) are hydrolyzed by β-glucosidase in intestinal bacteria to aglycones (daidzein and genistein; Ref. 33). The observed differences in males and females may also relate to differences in intestinal flora.

Since subjects consumed soymilk prior to each 12 h-collection, isoflavone levels from the first 3 urine collections reflect the balance between the rate of absorption and excretion. The last 2 urine collections (fourth and fifth collections), which occurred 24–36 h after the last dose, presumably reflect only excretion. Isoflavone excretions decreased after the fifth collection, suggesting that isoflavones are excreted rapidly, e.g., 24 h after last ingestion. This result is consistent with that from a study conducted on a metabolic unit. Thus, to monitor isoflavone exposure, a 12–24-h urine collection would identify only soya exposures from the prior day. To validate dietary exposure, multiple samples should be obtained from each subject.

The molar ratios of daidzein/daidzin (12 mg) to genistein/genestin (13 mg) in soymilk are approximately the same. However, we showed that subjects in this study excreted more daidzein than genistein, i.e., daidzein:genistein ratios were consistently >1. The reason for this differential excretion is not apparent and is under investigation.

Urinary isoflavone excretion has been studied by others (27, 29). In these studies, dietary intakes were either not measured or quantified from dietary records. Daidzein and genistein excretion in urine have been reported to be 1 and 3 mg/day, respectively, in Japanese consuming the traditional Japanese diet (29). After consuming a quantity of soya drink commonly consumed by Orientals, our study subjects excreted higher urinary levels of isoflavonoids. Variation in sampling time may account for differences in results obtained. It is also important to know that isoflavone content in soy products can vary among varieties of soybeans, season of soybean harvesting, length of soybean storage, and food processing procedures used (8 and this report). Quantification of soya intake from food records or food frequency alone will not reflect true isoflavone exposure. In this instance, multiple sampling of overnight urine at the time when subjects are known to consume soya may help to further validate isoflavone exposure.

In summary, isoflavones derived from soybeans can be quantified easily in urine or food products with a simplified and economical procedure with the use of solid-phase extractions. The procedure allows quantification of isoflavones from soya intake occurring within 24 h if 12-h urine samples are collected. Differences observed between the male and female subjects in isoflavone excretion patterns require confirmation.

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