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Profiles of Phytoestrogens in Human Urine from Several Asian Countries

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Intake of a diet rich in phytoestrogens has been associated with a decreased risk for hormone-dependent cancers in humans. Biomonitoring of phytoestrogens in human urine has been used to assess the intake of phytoestrogens. Although studies have reported phytoestrogen levels in urine specimens from the United States and Japan, little is known of human intake of phytoestrogens in other Asian countries. In this study we determined the concentrations of seven phytoestrogens, namely, enterolactone, enterodiol, daidzein, equol, *O*-desmethylangolensin (*O*-DMA), genistein, and coumestrol, in 199 human urine samples from three Asian countries, Vietnam (Hanoi and Ho Chi Minh), Cambodia (Phnom Penh), and India (Chennai and Kolkata), using a simple, sensitive, and reliable liquid chromatography (LC)−tandem mass spectrometry (MS/MS) method. The residue levels of phytoestrogens in urine samples from the three Asian countries were compared with the concentrations in 26 urine samples from Japan (Ehime) and 16 urine samples from the United States (Albany), analyzed in this study. Among the phytoestrogens analyzed, isoflavones such as daidzein and genistein were predominant in urine samples from Vietnam; samples from Cambodia and India contained higher concentrations of enterolactone than isoflavones. Urinary concentrations of isoflavones in samples from Hanoi, Vietnam, were notably higher than the concentrations in samples from Cambodia, India, and the United States and similar to the concentrations in samples from Japan. The lowest concentrations of daidzein and the highest concentrations of enterolactone were found in urine samples from India. Concentrations of equol and *O*-DMA, which are microbial transformation products of daidzein (produced by gut microflora), were notably high in urine samples from Hanoi, Vietnam. The ratios of the concentration of equol or *O*-DMA to that of daidzein were significantly higher in samples from Hanoi than from Japan, indicating high biotransformation efficiency of daidzein by the population in Hanoi. High concentrations of equol, in addition to isoflavones, in urine have been linked to reduced breast cancer risk in previous studies, and, thus, the Vietnamese population may have potential protective effect against breast cancer. This study suggests that the dietary intake and profiles of phytoestrogens vary considerably, even among Asian countries.

KEYWORDS: Phytoestrogens; human urine; biomonitoring; isoflavones; cancer; Asia; LC-MS/MS

INTRODUCTION

Phytoestrogens are naturally occurring diphenolic substances that are categorized into three main classes: isoflavone, lignan, and coumestan. Isoflavones, such as genistein and daidzein, are found in legumes, especially soybeans, as glucosides (*1*, *2*). Glucosides are hydrolyzed by intestinal bacteria to form aglycones, which are biologically active compounds, and are absorbed by enterocytes (*3*). The aglycones can be further metabolized; for example, daidzein can be transformed into *O*-desmethylangolensin (*O*-DMA) or into equol. Secoisolariciresinol and matairesinol are lignan dimers and are readily converted into lignans, enterodiol and enterolactone, by gut microflora (*1*, *4*). The phytolignans are present in flaxseed, grain breads, vegetables, tea, and fruits (*1*). The main dietary source of coumestrol is legumes (*2*). The aglycones of phytoestrogens can be further converted, in enterocytes or hepatocytes, to β-glucuronide or sulfate conjugates, which circulate in the blood or are excreted in urine and feces.

Phytoestrogens can affect human health, due to their estrogenic potentials. Phytoestrogens interact with estrogen receptors α (ERα) and β (ERβ), because of their structural similarity to 17β-estradiol, and they function as estrogen agonists or antagonists. It has been

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demonstrated that several phytoestrogens, such as genistein and coumestrol, have higher affinity for ERβ than for ERα (*5*, *6*). Transcriptional activities in a transient gene expression assay (*6*) suggested that phytoestrogens can act as estrogen agonists in animal bodies. In vitro assays and in vivo animal studies, as well as epidemiological studies (mainly on breast cancer), have reported estrogenic or antiestrogenic potentials for phytoestrogens, especially genistein (*1*, *2*, *4*, *7*, *8*). Most of the studies have shown that phytoestrogens have a protective action against breast cancer. Interestingly, studies have also shown that the effects of phytoestrogens differ in the presence of high endogenous estrogen levels (such as during pregnancy), moderate levels (such as during premenopausal life), or low levels (such as during childhood and postmenopause) (*4*, *7*).

Mortality rates due to breast cancer in Asian countries are low compared to rates in Western countries, and this pattern has been related to dietary preferences (*9*). The relatively low breast cancer rates in Asian women were suggested to be due to the intake of large amounts of phytoestrogens in the traditional Asian diet. Many epidemiological studies have examined the relationship between breast cancer rates and consumption of, or residue levels of, phytoestrogens, mainly isoflavones (*1*, *7*, *8*). Nevertheless, the findings from the epidemiological studies are inconclusive with respect to the protective action of phytoestrogens against breast cancer. So far, most of the studies on human intake of phytoestrogens have been conducted in Western countries such as the United States and the United Kingdom. The studies in Asia have mainly focused on Japan. Information on phytoestrogen intake in humans from other Asian countries is scarce, although the general populations in Asian developing countries consume large quantities of legumes, grains, flax, and vegetables, which are the main sources of phytoestrogens.

In this study, we determined, using liquid chromatography (LC)−tandem mass spectrometry (MS/MS), the concentrations of seven phytoestrogens, namely, enterolactone, enterodiol, daidzein, equol, *O*-DMA, genistein, and coumestrol, in human urine collected from Vietnam, Cambodia, and India. Concentrations of phytoestrogens in human urine and blood have been previously measured by gas chromatography (GC)−mass spectrometry (MS) (*10*, *11*), LC-MS (*12*, *13*), and LC-MS/MS (*14*−*17*). LC-MS/MS methods are less cumbersome (no need for derivatization) than GC-MS methods and are fast, sensitive, and selective. Urinary concentrations of phytoestrogens are highly correlated with serum levels and with dietary intakes (*18*−*20*), and hence urine is a reliable biospecimen to assess human exposure to phytoestrogens, without the need for invasive phlebotomy.

MATERIALS AND METHODS

Chemicals and Reagents. Enterolactone (purity = 95%), enterodiol (95%), daidzein (98%), equol (99%), genistein (98%), coumestrol (97%), 4-methylumbelliferone, 4-methylumbelliferyl β-glucuronide, and β-glucuronidase/sulfatase (type HP-2, from *Helix pomatia*, ≥100000 units/mL glucuronidase and ≤7500 units/mL sulfatase) were purchased from Sigma-Aldrich (St. Louis, MO), and *O*-DMA (purity = 98%) was obtained from Plantech U.K. (Reading, U.K.). Stable isotope-labeled daidzein ([$^2H_3$]-daidzein) and genistein ([$^2H_4$]-genistein) were purchased from Cambridge Isotope Laboratory (Andover, MA). Analytical grade methanol (MeOH), methyl *tert*-butyl ether (MTBE), and ammonium acetate were obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ), and ethyl acetate was from Honeywell International Inc. (Morristown, NJ). Deionized (DI) water was generated with a NANOpure Diamond ultrapure water system (Barnstead International, Dubuque, IA) and had a resistance of 18.2 MΩ cm.

Standard Solution. Stock solutions of phytoestrogens, 4-methylumbelliferone, and 4-methylumbelliferyl β-glucuronide were prepared at 1 mg/mL in MeOH; the stock for coumestrol was prepared in 5% dimethyl sulfoxide (DMSO) in MeOH. Stock solutions of [$^2H_3$]-daidzein and [$^2H_4$]-genistein were prepared at 10 μg/mL in MeOH. These stock solutions were stored at −20 °C in a dark room. The calibration standards, ranging in concentration from 0.5 to 200 ng/mL, with 50 ng/mL of [$^2H_3$]-daidzein and [$^2H_4$]-genistein, were prepared from each of the stock solutions through dilution with MeOH, whenever a new batch of samples was injected into LC-MS/MS. Standards of [$^2H_3$]-daidzein and [$^2H_4$]-genistein (2.5 μg/mL) were prepared from each of the stock solutions, for use as internal standards, and a standard of 4-methylumbelliferyl β-glucuronide (10 μg/mL) was prepared from the stock solution, as a deconjugation standard.

Sample Collection. In this study, we analyzed 199 urine samples stored at −25 °C in the Environmental Specimen Bank (es-Bank) of Ehime University, Matsuyama, Japan (*21*); these samples had been collected from adults in Hanoi, Vietnam, in September 2002 [male, n = 31 (20−78 years old); female, n = 32 (21−73 years old)], in Ho Chi Minh, Vietnam, in November 2006 [male, n = 14 (21−74 years old); female, n = 14 (33−74 years old)], in Phnom Penh, Cambodia, in December 2000 [male, n = 13 (21−48 years old); female, n = 24 (21−46 years old)], in Kolkata, India, in December 2005 [male, n = 16 (27−62 years old); female, n = 23 (20−70 years old)], and in Chennai, India, in December 2006 [male, n = 18 (26−55 years old); female, n = 14 (20−48 years old)]. Phytoestrogen levels measured in urine samples from the three Asian countries were compared by analyzing 42 urine samples stored at −20 °C at the New York State Department of Health (NYSDOH), which had been collected from Caucasians in Albany, NY [male, n = 10 (24−63 years old); female, n = 6 (23−48 years old)] during February−March 2005 and in September 2009 and at the Center for Marine Environmental Studies (CMES), Ehime University, Japan [male, n = 15 (22−54 years old); female, n = 11 (21−35 years old)] in March 2005. Institutional Review Board approvals were obtained from the NYSDOH for the analysis of urine samples. Although sex, age, and occupation were identified for all of the donors, records for the dietary habits or the time of the last meal at sample collection were not available.

Sample Preparation. After urine samples were thawed at 4 °C, a 500, 100, or 20 μL aliquot of urine was transferred into a 15 mL polypropylene (PP) tube using a tip ejector variable volume micropipetr. To bring all volumes up to 500 μL, we added 400 μL of DI water to the 100 μL urine samples and 480 μL of DI water to the 20 μL urine samples. Then, 50 ng (20 μL of 2.5 ppm solution) of [$^2H_3$]-daidzein or [$^2H_4$]-genistein was spiked as internal standard, and 100 ng (10 μL of 10 ppm solution) of 4-methylumbelliferyl β-glucuronide was spiked as a deconjugation standard. After gentle mixing, 300 μL of β-glucuronidase/sulfatase buffer, containing 2 μL of enzyme in 1 mL of 1 M ammonium acetate, was added. After vortexing, the samples were incubated overnight (12−16 h) at 37 °C. It has been previously demonstrated that deconjugation within 12−16 h ensured completion of hydrolysis while not introducing any changes in analyte levels (*17*). Then, 3 mL of 50% MTBE/ethyl acetate was added, and the mixtures were shaken for 30 min using a reciprocating shaker and centrifuged at 3000*g* for 2 min. The organic phase, containing phytoestrogens, was transferred into a new 15 mL PP tube, and the aqueous phase was extracted one more time with 3 mL of 50% MTBE/ethyl acetate. The organic phases were combined, evaporated to near-dryness using N_2, and redissolved with 1 mL of MeOH. After sonication for 10 s and centrifugation at 3000*g* for 2 min, the solution was transferred into a 1.5 mL amber vial for LC-MS/MS analysis.

LC-MS/MS Conditions. An API 2000 electrospray triple-quadrupole mass spectrometer (ESI-MS/MS; Applied Biosystems, Foster City, CA) equipped with an Agilent 1100 series HPLC system (Agilent Technologies Inc., Santa Clara, CA) was used for the measurement of phytoestrogens. The negative ion multiple reaction monitoring (MRM) mode was used, and the MRM transitions monitored were 297 → 107 (confirmation ion; 119) for enterolactone; 301 → 106 (133) for enterodiol; 253 → 91 (132) for daidzein; 241 → 119 (93) for equol; 257 → 108 (80) for *O*-DMA; 269 → 133 (63) for genistein; and 267 → 266 (91) for coumestrol. Nitrogen was used as both curtain and collision gas. MS/MS parameters were optimized for each phytoestrogen, by infusion of a 1 μg/mL standard solution. The MRM transitions monitored for [$^2H_3$]-daidzein, [$^2H_4$]-genistein, and 4-methylumbelliferone were 256 → 92, 272 → 135 (64), and 175 → 119, respectively.

LC-MS/MS Procedure. Ten microliters of urine extract was injected onto a Thermo BETASIL C18 (50 mm length × 3 mm internal diameter,

Table 1. Recoveries of Phytoestrogens from Diethyl Ether, Ethyl Acetate, or 50% MTBE/Ethyl Acetate Extraction, After Spiking of 50 ng/mL into Three Urine Samples

		diethyl ether			ethyl acetate			50% MTBE/ethyl acetate		
	added amount (ng/mL)	mean value	SD	recovery (%)	mean value	SD	recovery (%)	mean value	SD	recovery (%)
enterolactone	50	36.8	0.96	72.2	36.0	0.86	73.0	40.2	0.99	80.4
enterodiol	50	37.5	1.0	75.7	38.5	0.75	78.3	40.3	0.60	80.6
daidzein	50	34.0	0.57	68.7	35.1	1.0	68.1	37.3	0.96	74.6
equol	50	33.0	0.76	64.3	32.5	0.76	63.7	36.2	0.56	72.4
O-DMA	50	35.5	0.50	71.0	35.1	0.69	68.6	38.3	0.37	76.6
genistein	50	35.8	0.90	69.9	33.8	0.96	66.2	37.4	0.78	74.8
coumestrol	50	33.8	0.68	66.5	33.4	0.45	65.8	36.8	0.30	73.6

5 μm particle diameter) chromatographic column serially connected with a guard column (Thermo Electron, Bellefonte, PA), at a flow rate of 300 μL/min. The mobile phase was MeOH (solvent A) and 2 mM ammonium acetate, including 10% MeOH (solvent B); the gradient started at 10% solvent A and ramped to 99% solvent A in 8 min and was held for 4 min before reverting back to 10% solvent A.

Data Analysis. The analytes were quantified by an isotope dilution method, when the retention times of phytoestrogens in urine matched with those of the standards to within ±0.05 min. [$^2H_3$]-Daidzein was used for the quantification of enterolactone, enterodiol, equol, *O*-DMA, and coumestrol, because no isotopically labeled standard for these phytoestrogens was commercially available. An interference peak adjacent to the peak for [$^2H_4$]-genistein was found in some urine samples, when [$^2H_4$]-genistein was monitored at 272 → 135. Therefore, the confirmation MRM transition (272 → 64) of [$^2H_4$]-genistein was also monitored for identification and quantification of genistein. If the peak area ratio of native phytoestrogens to [$^2H_3$]-daidzein or [$^2H_4$]-genistein in urine exceeded that of the calibration standard with the highest native concentration, 200 ng/mL (including 50 ng/mL of [$^2H_3$]-daidzein and [$^2H_4$]-genistein), the sample was reanalyzed using a smaller sample volume, 20 or 100 μL. Data processing was performed with the Analyst 1.4.1 software package. Statistical analyses were conducted with Statistica v. 06J (StatSoft Inc., Tulsa, OK). The Mann−Whitney *U* test was used to evaluate the statistically significant differences in phytoestrogen concentrations existing between males and females and for examination of concentration ratios of equol or *O*-DMA to daidzein, between Hanoi (Vietnam) and Ehime (Japan) samples. Spearman's rank correlation coefficient was calculated to measure the strength of association between age and phytoestrogen concentrations. The statistical significance of differences in concentrations of phytoestrogens, among Hanoi (Vietnam), Ho Chi Minh (Vietnam), Phnom Penh (Cambodia), Kolkata (India), Chennai (India), Ehime (Japan), and Albany (USA), was evaluated by Kruskal−Wallis and median tests. A p value of < 0.05 was considered to be significant. To compare phytoestrogen profiles among the seven sampling locations, principal component analysis (PCA) was performed, after each phytoestrogen concentration in each individual specimen was normalized to the total phytoestrogen concentration in that sample.

RESULTS AND DISCUSSION

Analytical Accuracy and Precision. Linear regression analysis of relative response factors (area of native phytoestrogen/area of [$^2H_3$]-daidzein or [$^2H_4$]-genistein) versus native compound concentrations, ranging from 0.5 to 200 ng/mL, showed correlation coefficients (r) of > 0.99; the linearity of calibration standards was found to be > 0.99, for every batch of 20 samples analyzed. When 10 μL of 0.5 ng/mL standard (i.e., 5.0 pg of enterodiol, daidzein, equol, *O*-DMA, genistein, and coumestrol) or 10 μL of 0.1 ng/mL standard (i.e., 1.0 pg of enterolactone) was injected, the signal-to-noise (S/N) ratio was 3.0 for enterodiol, 4.0 for daidzein, 4.8 for genistein, 5.4 for equol, 6.5 for coumestrol, 9.2 for *O*-DMA, and 3.9 for enterolactone. Thus, the instrumental detection limits were in the range of 1.0−5.0 pg. The limit of detection (LOD) for the analytical method was determined on the basis of the standard deviations from six replicate analyses, using the lowest calibration standard, 0.5 ng/mL. The LODs were calculated as 3S, where S is the standard deviation. The calculated LODs were 0.08 ng/mL for enterolactone, 0.42 ng/mL for enterodiol, 0.35 ng/mL for daidzein, 0.27 ng/mL for equol, 0.17 ng/mL for *O*-DMA, 0.32 ng/mL for genistein, and 0.23 ng/mL for coumestrol. Because of the 2-fold dilution of the samples in the analytical procedure, the actual LODs for samples were 0.15 ng/mL for enterolactone, 0.84 ng/mL for enterodiol, 0.70 ng/mL for daidzein, 0.54 ng/mL for equol, 0.33 ng/mL for *O*-DMA, 0.63 ng/mL for genistein, and 0.46 ng/mL for coumestrol.

In this study, we used a liquid−liquid extraction method to extract phytoestrogens from urine. Diethyl ether has been previously used for the extraction of phytoestrogens (*11*, *12*, *22*). However, when we performed a recovery test using diethyl ether, ethyl acetate, or 50% MTBE/ethyl acetate, by spiking 50 ng/mL levels of phytoestrogens into three urine samples that did not contain any of these free (i.e., without β-glucuronidase/sulfatase) target compounds, the recoveries for diethyl ether (64.3−75.7%) were lower than the recoveries for 50% MTBE/ethyl acetate (72.4−80.6%) and were comparable to those for ethyl acetate (63.7−78.3%) (**Table 1**). Therefore, we selected 50% MTBE/ethyl acetate for the extraction of samples in this study.

A recovery test was conducted, by spiking of two concentrations (10 and 50 ng/mL) of each of the native phytoestrogens and 50 ng/mL of [$^2H_3$]-daidzein and [$^2H_4$]-genistein, into three urine samples that did not contain any of these target compounds, with subsequent passage through the entire analytical procedure without β-glucuronidase/sulfatase. Although the actual recoveries for the native compounds were between 71.5 and 83.4%, the recoveries corrected for the internal standards (isotope dilution) were between 96.7 and 115% (**Table 2**). The coefficients of variation (CVs) of triplicate analyses were between 0.82 and 3.4% (**Table 2**). In addition, variations in phytoestrogen concentrations for 5 days were checked by replicate analyses of a urine sample that contained the seven phytoestrogens. Respective CVs of five replicates were 3.2% for enterolactone, 3.5% for enterodiol, 2.4% for daidzein, 4.4% for equol, 4.1% for *O*-DMA, 1.7% for genistein, and 5.3% for coumestrol (**Table 3**). These results suggest that the method developed for phytoestrogens in urine provides adequate accuracy and precision.

Deconjugation efficiencies, estimated from concentrations of 4-methylumbelliferone formed from 4-methylumbelliferyl β-glucuronide, were nearly 100%. No phytoestrogens were detected in procedural blanks, which consisted of 500 μL of DI water (instead of 500 μL of urine) and were passed through the entire analytical procedure.

Phytoestrogens in Human Urine. Concentrations of phytoestrogens determined in human urine samples are shown in **Table 4**. For samples from each of the seven locations, Hanoi (Vietnam), Ho Chi Minh (Vietnam), Phnom Penh (Cambodia), Kolkata (India), Chennai (India), Ehime (Japan), and Albany (USA), no significant relationship between age and phytoestrogen concentrations was found. Similarly, urinary phytoestrogen concentrations were not significantly different between males and females, although a significant difference ($p = 0.027$) was detected for

Table 2. Recoveries of Phytoestrogens through the Entire Analytical Procedure, After Spiking of either of the Two Concentrations (10 and 50 ng/mL) of Phytoestrogens into Three Urine Samples

	added amount (ng/mL)	mean value	SD	CV (%)	recovery (%)	corrected rec[a] (%)
enterolactone	10	8.34	0.28	3.4	83.4	115
	50	40.2	0.99	2.5	80.4	107
enterodiol	10	8.3	0.15	1.8	83.0	114
	50	40.3	0.60	1.5	80.6	108
daidzein	10	7.15	0.16	2.2	71.5	102
	50	37.3	0.96	2.6	74.6	101
equol	10	7.54	0.11	1.5	75.4	104
	50	36.2	0.56	1.5	72.4	96.7
O-DMA	10	7.56	0.25	3.3	75.6	104
	50	38.3	0.37	0.97	76.6	102
genistein	10	7.31	0.22	3.0	73.1	97.9
	50	37.4	0.78	2.1	74.8	98.3
coumestrol	10	7.18	0.16	2.2	71.8	99.0
	50	36.8	0.30	0.82	73.6	98.3

[a] Recoveries corrected by the isotope dilution method.

enterolactone levels between males and females from Hanoi, Vietnam (**Table 4**). It is likely that urinary phytoestrogen levels are related to an individual's dietary habits and are unaffected by age and sex, as has been shown previously (*20*). The results for the total number of samples from each location, reported in **Table 4**, are discussed below.

Among the seven phytoestrogens, the highest median concentrations of daidzein were found in urine samples from Hanoi (550 ng/mL) and Ho Chi Minh (300 ng/mL), Vietnam, and Ehime, Japan (940 ng/mL), followed by genistein (300 ng/mL for Hanoi, 120 ng/mL for Ho Chi Minh, and 640 ng/mL for Ehime), enterolactone (230 ng/mL for Hanoi, 79 ng/mL for Ho Chi Minh, and 51 ng/mL for Ehime), enterodiol (42 ng/mL for Hanoi, 20 ng/mL for Ho Chi Minh, and 19 ng/mL for Ehime), *O*-DMA (39 ng/mL for Hanoi, 4.6 ng/mL for Ho Chi Minh, and 15 ng/mL for Ehime), equol (26 ng/mL for Hanoi, 1.3 ng/mL for Ho Chi Minh, and 1.5 ng/mL for Ehime), and coumestrol (0.90 ng/mL for Hanoi, 0.81 ng/mL for Ho Chi Minh, and < LOD for Ehime). Urine samples from India contained the highest median concentrations of enterolactone (180 ng/mL for Kolkata and 330 ng/mL for Chennai), followed by enterodiol (30 ng/mL for Kolkata and 37 ng/mL for Chennai), genistein (3.6 ng/mL for Kolkata and 11 ng/mL for Chennai), and daidzein (1.6 ng/mL for Kolkata and 11 ng/mL for Chennai); concentrations of equol, *O*-DMA, and coumestrol were below the LOD (**Table 4**). Median concentrations of enterolactone were also the highest in urine samples from the Unite States (43 ng/mL) and Cambodia (67 ng/mL); next highest for the United States were daidzein (22 ng/mL), enterodiol (7.5 ng/mL), genistein (5.0 ng/mL), equol (2.4 ng/mL), *O*-DMA, and coumestrol (< LOD), whereas the order for Cambodia was daidzein (19 ng/mL), genistein (11 ng/mL), enterodiol (9.4 ng/mL), *O*-DMA (1.1 ng/mL), and equol and coumestrol (< LOD) (**Table 4**). It has previously been observed for urine samples from Western countries such as the United States (*20*, *23*) and the United Kingdom (*10*, *19*) that concentrations of lignans, especially enterolactone, were relatively higher than concentrations of isoflavones. In contrast, studies have found higher levels of isoflavones than

Table 3. Precision of Phytoestrogen Determinations (Nanograms per Milliliter) by Replicate Analyses of a Urine Sample (1 Run per Day for 5 Days)

day	enterolactone	enterodiol	daidzein	equol	*O*-DMA	genistein	coumestrol
1	130	63.2	231	23.4	39.9	282	2.40
2	129	61.9	225	23.1	37.7	277	2.15
3	134	64.8	230	24.0	39.0	285	2.25
4	139	67.9	240	25.8	41.9	290	2.45
5	137	65.5	235	24.5	41.0	287	2.39
av	134	64.7	232	24.2	39.9	284	2.3
SD	4.32	2.29	5.63	1.06	1.65	4.97	0.124
CV (%)	3.2	3.5	2.4	4.4	4.1	1.7	5.3

lignans in Japanese urine and serum samples (*24*, *25*). The patterns of phytoestrogen concentrations in the United States and Japanese urine samples analyzed in the present study were consistent with the patterns seen in previous studies (*20*, *23*−*25*). This correspondence strongly suggests a predominant influence of diet on urinary phytoestrogen levels for each country. For example, dietary isoflavone intakes for U.S. women were estimated as 0.63 mg/day, whereas those for Japanese women were estimated as 46.5 mg/day (*19*). For all countries' samples, in our study, the concentration and detection rate for coumestrol were much lower than for the isoflavones and lignans (**Table 4**). This difference can be attributed to low intake rates of coumestrol via foods, given that coumestrol is present in only a small number of food items (*26*). In a U.S. study of 199 adults, coumestrol was detected in only 9 urine samples (4.5%) (*20*). However, in our urine samples from Vietnam, the detection frequency of coumestrol exceeded 50%, although the median concentration was close to the LOD (**Table 4**). Coumestrol has much stronger binding affinity to ERα and ERβ than do isoflavones; the binding affinity of coumestrol is comparable to that of 17β-estradiol (*5*).

When urinary phytoestrogen concentrations for each sampling location were compared, significant regional differences were detected for daidzein, genistein, *O*-DMA, and equol ($p < 0.0001$) and for enterolactone and enterodiol ($p < 0.05$) concentrations. Notably high concentrations of isoflavones were detected in urine samples from Vietnam and Japan (**Figure 1**). Urinary isoflavone concentrations in samples from Cambodia and India were comparable to or lower than the concentrations found for the U.S. samples. Urinary lignan concentrations were relatively higher in samples from India and Hanoi (Vietnam), but the regional differences were smaller than those observed for isoflavones (**Figure 1**). It has been reported that the intakes of isoflavones in Western countries such as the United States (*27*) are considerably lower than intakes in Japan (*28*) and Singapore (*29*) and that these differences are strongly associated with urinary concentrations of isoflavones (*27*−*29*). On the basis of those reports and the urinary phytoestrogen concentrations determined in the present study, it can be said that Vietnamese people consume large amounts of isoflavones and that their intake is comparable to that for Japanese people; in contrast, isoflavone intake in India and Cambodia is similar to the intake in the United States. Thus far, a generalization has been that populations in Asia consume larger amounts of isoflavones than do Western populations. However, our results clearly suggest that certain Asian populations (such as Indians and Cambodians) consume amounts of isoflavones far lower than those perceived levels and, thus, should be differentiated from the high-intake Asian populations.

Patterns and Profiles of Urinary Phytoestrogens. Profiles of phytoestrogen concentrations found in urine samples collected from each of the sampling locations are shown in **Figure 2**.

Table 4. Concentrations (Nanograms per Milliliter) of Phytoestrogens in Human Urine Analyzed in This Study

	daidzein				equol				*O*-DMA				genistein			
	mean	median	range	detection rate (%)	mean	median	range	detection rate (%)	mean	median	range	detection rate (%)	mean	median	range	detection rate (%)
Vietnam (Hanoi)																
male (n = 31)	5700	930	35−93000	100	520	29	<0.54−5700	90	390	39	<0.33−4100	97	2300	450	18−25000	100
female (n = 32)	2800	360	11−27000	100	1300	19	<0.54−21000	78	190	39	<0.33−1400	94	1700	260	8.5−17000	100
total (n = 63)	4200	550	11−93000	100	930	26	<0.54−21000	84	290	39	<0.33−4100	95	2000	300	8.5−25000	100
Vietnam (Ho Chi Minh)																
male (n = 14)	440	370	24−1200	100	68	1.4	<0.54−760	64	20	4.6	<0.33−110	86	180	120	4.4−660	100
female (n = 14)	1300	230	<0.70−8700	93	33	1.0	<0.54−210	57	200	5.5	<0.33-2100	71	590	120	0.88−4900	100
total (n = 28)	860	300	<0.70−8700	96	50	1.3	<0.54−760	61	110	4.6	<0.33-2100	79	380	120	0.88−4900	100
India (Kolkata)																
male (n = 16)	8.9	2.9	<0.70−47	63	<0.54	<0.54	<0.54−0.89	6.3	<0.33	<0.33	<0.33−1.2	19	11	4.4	<0.63−60	94
female (n = 23)	5.2	1.3	<0.70−50	61	<0.54	<0.54	<0.54−1.2	4.3	<0.33	<0.33	<0.33−0.66	8.7	6.0	2.8	<0.63−52	87
total (n = 39)	6.8	1.6	<0.70−50	62	<0.54	<0.54	<0.54−1.2	5.1	<0.33	<0.33	<0.33−1.2	13	8.2	3.6	<0.63−60	90
India (Chennai)																
male (n = 18)	10	7.6	<0.70−43	94	<0.54	<0.54	<0.54−2.5	17	0.55	<0.33	<0.33−3.0	44	16	7.9	3.0−78	100
female (n = 14)	16	12	1.9−42	100	<0.54			0	0.63	<0.33	<0.33−3.5	43	22	12	1.8−100	100
total (n = 32)	13	11	<0.70−43	97	<0.54	<0.54	<0.54−2.5	9.4	0.58	<0.33	<0.33−3.5	44	19	11	1.8−100	100
Cambodia (Phnom Penh)																
male (n = 13)	77	6.4	<0.70−380	85	0.56	<0.54	<0.54−3.5	31	2.3	<0.33	<0.33−12	31	150	4.9	<0.63−920	92
female (n = 24)	85	22	<0.70−570	83	0.67	<0.54	<0.54−7.0	25	5.4	2.1	<0.33−30	63	84	12	<0.63−540	96
total (n = 37)	82	19	<0.70−570	84	0.63	<0.54	<0.54−7.0	27	4.3	1.1	<0.33−30	51	110	11	<0.63−920	95
Japan (Ehime)																
male (n = 15)	2300	1500	27−18000	100	14	1.6	<0.54−160	80	48	17	<0.33−200	93	1300	380	15−12000	100
female (n = 11)	3400	690	14−25000	100	1300	1.4	<0.54−13000	73	200	1.7	<0.33−1600	73	1900	900	16−13000	100
total (n = 26)	2700	940	14−25000	100	580	1.5	<0.54−13000	77	110	15	<0.33−1600	85	1600	640	15−13000	100
USA (Albany)																
male (n = 10)	130	30	<0.70−980	90	2.2	1.8	<0.54−5.2	70	97	<0.33	<0.33−940	40	44	10	<0.63−310	90
female (n = 6)	68	14	2.7−230	100	5.0	2.5	<0.54−21	67	17	<0.33	<0.33−100	17	53	2.4	<0.63−300	83
total (n = 16)	110	22	<0.70−980	94	3.2	2.4	<0.54−21	69	67	<0.33	<0.33−940	31	47	5.0	<0.63−310	88

	enterolactone				enterodiol				coumestrol			
	mean	median	range	detection rate (%)	mean	median	range	detection rate (%)	mean	median	range	detection rate (%)
Vietnam (Hanoi)												
male (n = 31)	230	100	0.67−890	100	40	22	<0.84−160	94	1.6	1.2	<0.46−7.4	61
female (n = 32)	500	310	<0.15−1900*[a,b]	97	74	61	<0.84−470	91	1.6	<0.46	<0.46−17	50
total (n = 63)	360	230	<0.15−1900	98	57	42	<0.84−470	92	1.6	0.90	<0.46−17	56
Vietnam (Ho Chi Minh)												
male (n = 14)	150	68	0.56−770	100	24	16	<0.84−66	86	1.8	<0.46	<0.46−16	43
female (n = 14)	210	96	0.81−820	100	55	33	<0.84−330	86	1.4	1.3	<0.46−5.8	64
total (n = 28)	180	79	0.56−820	100	40	20	<0.84−330	86	1.6	0.81	<0.46−16	54
India (Kolkata)												
male (n = 16)	340	230	0.32−1100	100	54	42	3.0−170	100	<0.46			0
female (n = 23)	280	150	0.53−1000	100	36	23	1.2−110	100	<0.46			0
total (n = 39)	310	180	0.32−1100	100	43	30	1.2−170	100	<0.46			0
India (Chennai)												
male (n = 18)	430	360	1.1−1400	100	77	27	<0.84−330	89	<0.46			0
female (n = 14)	400	330	41−1100	100	62	52	2.8−260	100	<0.46			0
total (n = 32)	420	330	1.1−1400	100	70	37	<0.84−330	94	<0.46			0
Cambodia (Phnom Penh)												
male (n = 13)	170	51	<0.15−820	92	18	7.0	<0.84−71	77	<0.46	<0.46	<0.46−4.9	15
female (n = 24)	200	100	1.6−740	100	26	11	<0.84−130	79	0.81	<0.46	<0.46−4.3	42
total (n = 37)	190	67	<0.15−820	97	23	9.4	<0.84−130	78	0.68	<0.46	<0.46−4.9	32
Japan (Ehime)												
male (n = 15)	410	53	<0.15−3500	93	38	30	<0.84−160	93	0.20	<0.46	<0.46−2.9	6.7
female (n = 11)	320	15	0.89−1800	100	24	10	<0.84−99	64	1.6	<0.46	<0.46−9.6	18
total (n = 26)	370	51	<0.15−3500	96	32	19	<0.84−160	81	0.79	<0.46	<0.46−9.6	12
USA (Albany)												
male (n = 10)	220	43	3.3−1100	100	13	3.1	<0.84−98	60	<0.46			0
female (n = 6)	260	140	<0.15−900	83	39	22	<0.84−130	83	<0.46			0
total (n = 16)	240	43	<0.15−1100	94	23	7.5	<0.84−130	69	<0.46			0

[a] Concentrations in females were significantly higher than those in males. [b] *, $p < 0.05$.

Enterolactone in Indian urine samples accounted for 83.6% (Kolkata) and 85.0% (Chennai) of the total phytoestrogen concentrations, whereas daidzein (46.2% for Hanoi, 57.1% for Ho Chi Minh, and 56.5% for Japan) and genistein (25.3% for Hanoi, 22.8% for Ho Chi Minh, and 38.4% for Japan) are the predominant phytoestrogens in Japanese and Vietnamese urine

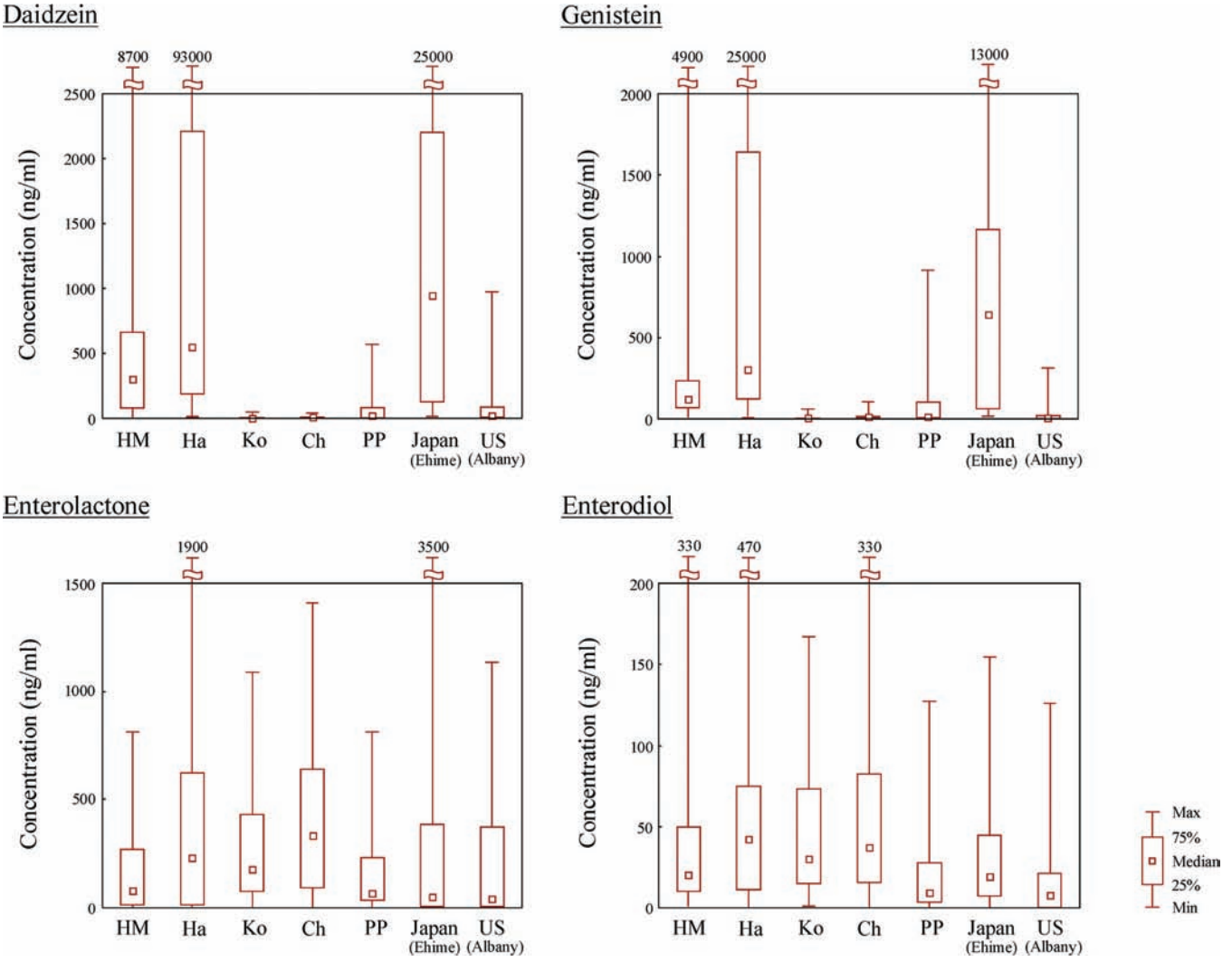

Figure 1. Comparison of phytoestrogen concentrations in human urine among the seven sampling locations. Significant regional differences were detected by Kruskal–Wallis and median tests: $p < 0.0001$ for daidzein and genistein, and $p < 0.05$ for enterolactone and enterodiol. HM, Ho Chi Minh (Vietnam); Ha, Hanoi (Vietnam); Ko, Kolkata (India); Ch, Chennai (India); PP, Phnom Penh (Cambodia).

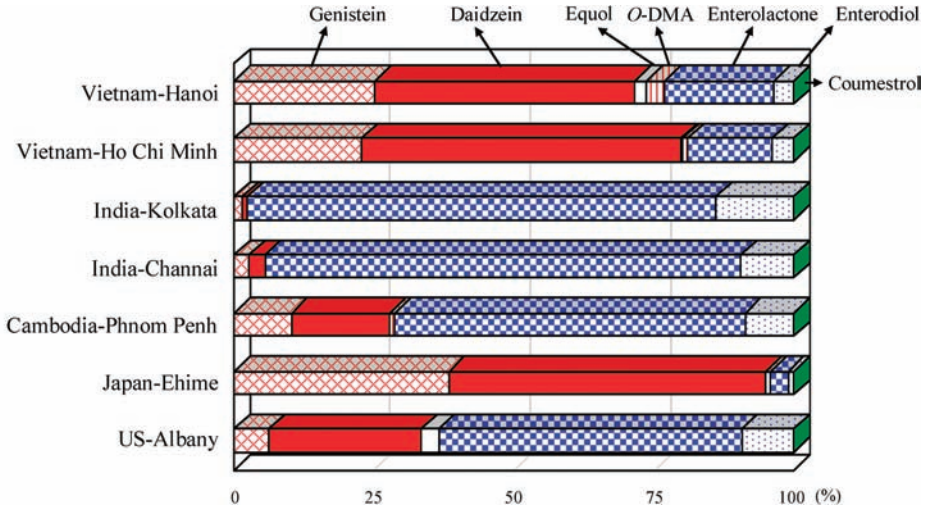

Figure 2. Composition (calculated from median concentrations) of phytoestrogens determined in human urine from Asian countries and the United States.

samples (**Figure 2**). Enterolactone was also dominant in Cambodian (62.5%) and U.S. (54.1%) urine samples, but its proportions were lower than the proportions found for Indian urine samples. The profiles of phytoestrogens in urine samples from the United States were intermediate between those for Japan and Vietnam and those for India (**Figure 2**). These results indicate that the patterns of phytoestrogen intake vary by country, even within Asia; Japanese and Vietnamese people consume very large

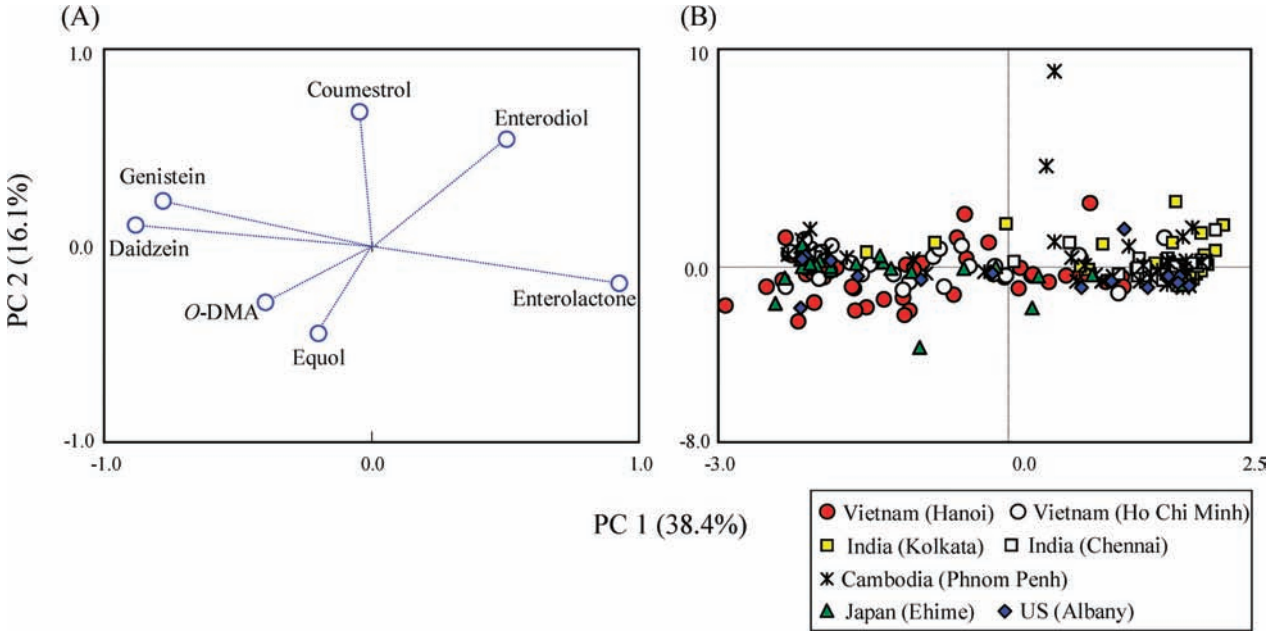

Figure 3. Principal component loading (**A**) and score plots (**B**) analyzed by normalizing each phytoestrogen concentration to the total phytoestrogen concentration in individual specimens from Asian countries and the United States.

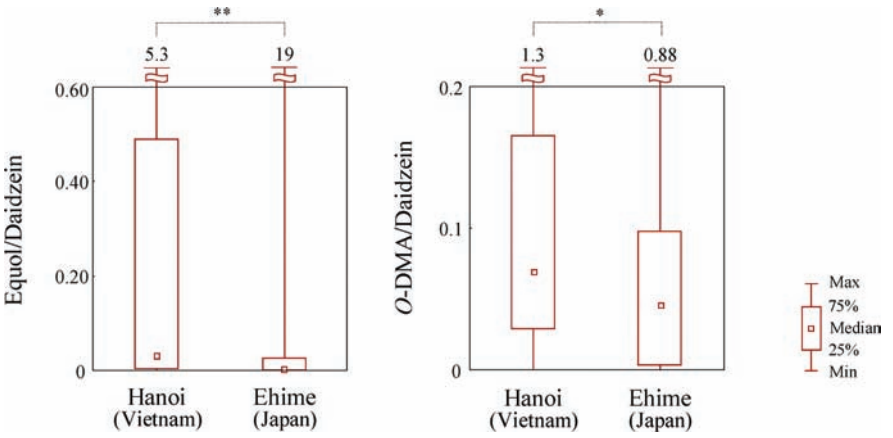

Figure 4. Concentration ratios of equol (equol/daidzein) or *O*-DMA (*O*-DMA/daidzein) to daidzein in human urine from Hanoi, Vietnam, and Ehime, Japan. *, $p < 0.05$; **, $p < 0.01$.

proportions of isoflavones, probably via soy products, whereas Indian and Cambodian people consume large proportions of lignans, via food products such as flaxseed, grain breads, vegetables, teas, and fruits. For a further characterization of urinary phytoestrogen profiles found for each country, we performed principal component analysis (PCA), after normalizing each phytoestrogen concentration in individual sample to the total concentration of phytoestrogens in the sample. The result showed that the first and second principal components (PC 1 and PC 2) accounted for 38.4 and 16.1% of the total variance, respectively (**Figure 3**). Most samples from Japan and Vietnam plotted negatively against PC 1, whereas most specimens from India plotted positively against PC 1 (**Figure 3B**). Considering the high loading factor value (0.929) of enterolactone and negative association of daidzein and genistein with PC 1 (**Figure 3A**), the PCA further supports the dietary differences among the populations studied. Samples from the United States and Cambodia plotted both positively and negatively against PC 1 (**Figure 3B**), implying that the consumption rate of isoflavones and lignans via diet varies among individuals, compared with Japan, Vietnam, and India.

A factor other than dietary intake, metabolism by intestinal bacteria, can also influence an individual's urinary levels of phytoestrogens, especially equol and *O*-DMA, which are transformed from daidzein by gut microflora (*30*). The concentrations and detection rates that we found for equol and *O*-DMA in urine samples from Hanoi were higher than for the Japanese samples, although the median daidzein concentration was low (**Table 1**). When concentration ratios of equol or *O*-DMA to daidzein detected in urine samples from Japan and Hanoi were estimated, as an indicator of the conversion efficiency, significantly higher ratios, especially for equol/daidzein, were found for the samples from Hanoi (**Figure 4**). This result indicates a more efficient biotransformation of daidzein into its metabolites, equol and *O*-DMA, by the population sampled in Hanoi. An earlier study of 24 healthy adults found that the "good" equol producers consumed less fat ($26 \pm 2.3\%$, percentage of total energy) and more carbohydrates ($55 \pm 2.9\%$) than did the "poor" equol producers ($35 \pm 1.6\%$ for fat and $47 \pm 1.7\%$ for carbohydrates) (*31*). Thus, the high equol/daidzein ratios observed for urine samples from Hanoi indicate low-fat and high-carbohydrate intake by this

Vietnamese population. The binding affinity of equol to human ERα and ERβ was found to be similar to that of genistein, but equol induced transcription more strongly than did any other isoflavones, especially by binding to ERα (*32*). Equol inhibited the expression of the estrogen-responsive *pS2* gene in the MCF7 estrogen-dependent breast tumor cell line, suggestive of anti-estrogenic potency (*33*). Biotransformation of daidzein to equol has been proposed as an important factor in the protective effects of phytoestrogens on cardiovascular, bone, and menopausal health and against breast cancer (*34*). A case-control study showed that high urinary levels of equol were associated with a substantial reduction in breast cancer risk (*35*). In a previous study, median urinary excretion of equol was reported to be 97.2 nmol/24 h for case patients (of breast cancer) and 108.6 nmol/24 h for control groups (*35*). Assuming that the 24 h urine excretion volume for women is 1.4 L (*10*), the 24 h average concentration of equol could be estimated as 16.8 ng/mL (69.4 nmol/L) for breast cancer case patients and 18.8 ng/mL (77.6 nmol/L) for control groups. The median concentrations of equol in urine samples from Hanoi (26 ng/mL in **Table 4**) exceeded the control value (18.8 ng/mL), estimated above. Thus, our results indicate that the sampled population in Hanoi comprised "good" equol producers, and this phenotype could have implications for the reduction of breast cancer risk in the population. It has been reported that the rates of hormone-related cancers, such as breast and ovary, for Hanoi's residents were notably lower than the rates for Caucasians in the United States and also lower than the rates for U.S.-resident Vietnamese who had immigrated from Vietnam (*36*). Furthermore, it is noteworthy that the age-adjusted rate (per 100000) of breast cancer for Hanoi's residents (17.5) (*36*) is lower than that for Chennai (26.6) and Kolkata (25.1), India (*37*); we detected much lower concentrations of isoflavones in urine samples from these two Indian cities than from the Hanoi's samples, as described earlier (**Table 4**; **Figure 1**).

Conclusions. In summary, concentrations of phytoestrogens were determined in 199 human urine samples from Vietnam, Cambodia, and India, in addition to 42 samples from Japan and the United States, using a simple, sensitive, and reliable LC-MS/MS method. Our results are useful in assessing any potential protective role for phytoestrogens in hormone-related cancers in populations in these Asian countries. Urinary concentrations of isoflavones in Vietnam are comparable to the concentrations found for samples from Japan, whereas isoflavone concentrations in urine samples from Cambodia and India were notably lower and were comparable to the concentrations found for the U.S. samples. Relatively high concentrations of lignans were found in urine samples from India and Vietnam, but the differences in lignan concentrations among the five countries were significantly smaller than for isoflavone concentrations. Our results on phytoestrogen levels and profiles in urine indicate that the Vietnamese population, especially those from Hanoi, are deriving a potential protective effect against hormone-related cancers, such as breast and ovary, through their high consumption of phytoestrogens. Epidemiological studies on hormone-dependent diseases, in addition to comprehensive monitoring surveys of phytoestrogens in humans, will shed more light on the role of phytoestrogens in the prevention of breast cancer and other related diseases in Asian countries.

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