

Isoflavonoid and Lignan Phytoestrogens as Dietary Biomarkers^{1,2}

Johanna W. Lampe³

Fred Hutchinson Cancer Research Center, Seattle, WA 98109

ABSTRACT Isoflavones and lignans are biologically active plant-food constituents that have potential chemopreventive properties. Quantitation of isoflavones and lignans in humans is necessary to establish the benefits and risks of exposure to these compounds in populations and to determine which components of a mixed diet contribute to the exposure. Isoflavones and lignans are metabolized by colonic bacteria to more biologically active metabolites; thus both the parent compounds and the metabolites are measured routinely. Isoflavonoids (genistein, daidzein, dihydrodaidzein, *O*-desmethylanangolensin and equol) and lignans (enterolactone, enterodiol, matairesinol and secoisolariciresinol) can be quantified in various body fluids. Typically, high concentrations of isoflavonoids in urine and serum are associated with soy consumption, and high concentrations of lignans are associated primarily with intake of whole grains and other fiber-containing plant foods. Controlled feeding studies and nutritional epidemiologic studies demonstrate a linear dose response between dietary intake and urinary excretion of isoflavones. Lignan excretion is associated positively with dietary fiber intake as well as with diets that are on average higher in fiber and carbohydrate and lower in fat; thus lignans have also been proposed as a marker of healthier dietary patterns. The complex interactions between the colonic environment and the external and internal factors that modulate it contribute to significant variation in serum and urinary phytoestrogen levels among individuals. Understanding these sources of variation is important to be able to use these measures effectively as dietary biomarkers. *J. Nutr.* 133: 956S–964S, 2003.

KEY WORDS: • biomarkers • diet assessment • epidemiology • isoflavonoids • lignans • nutrition • phytoestrogens

Phytoestrogens are plant-derived compounds that have estrogenic properties. The term “phytoestrogens” includes primarily two groups of compounds, isoflavones and lignans, that are present in significant amounts in the human diet. Although identified originally for their weak estrogenic activity, many of these compounds have a variety of other biologic activities that may influence disease risk (1,2). Thus monitoring dietary exposure to these compounds is desirable for reasons beyond just their hormonelike effects. Additional compounds and other classes of phytochemicals (e.g., coumestrol, resveratrol and phytosterols) also have been identified as nonsteroidal estrogens (3–5). However, their use as dietary biomarkers has not been investigated, thus this review focuses on the isoflavones and lignans.

To date much of the emphasis of phytoestrogen research has centered on the potential cancer-preventive effects of iso-

flavones and lignans. However, animal studies and in vitro work support the potential for these phytoestrogens to influence risk factors for the major chronic diseases through a variety of mechanisms (2). These include but are not limited to antiviral and antibacterial, antioxidant, antiproliferative and antiangiogenic activities and inhibition of effects of cytokines and growth factors [reviewed in (2) and (1)]. Intervention studies in humans demonstrate the capacity of isoflavones to modulate risk factors for cardiovascular disease (6–8) and maintain bone mass (7) and of lignans and isoflavones to alter estrogen metabolism (9–11). Despite the great enthusiasm for the beneficial effects of these compounds, some research suggests that potential risks have not been examined sufficiently (12–16). The eagerness with which the public has embraced soyfoods and phytoestrogen supplements is resulting in a major population-based experiment, which could have wide-ranging health consequences, possibly both positive and negative. The ability to monitor objectively exposure to these compounds may be very important in understanding the health effects of these dietary constituents.

Isoflavones are found primarily in members of the Leguminosae family and occur in varying amounts in legumes consumed by humans. Foods such as soy, lentils, beans and chickpeas are sources of isoflavones, however, soybeans are one of the few foods that contain appreciable amounts of isoflavones (17). Lignans are more ubiquitous. They are diphenolic precursors of the polymeric lignins, occur typically in vascular plants and are found in roots and rhizomes and the woody

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³ To whom correspondence should be addressed. E-mail: jlampe@fhcrc.org.

parts, stems, leaves, seeds and fruits. The oilseeds (flax, soy, rapeseed), whole-grain cereals (wheat, oats, rye), legumes and various vegetables and fruit (particularly berries) are rich sources of lignans (18,19). The isoflavones and lignans are associated with the protein and nonstarch polysaccharide fractions, respectively; the oils of phytoestrogen-containing plant foods are not significant sources of these compounds (19).

During the past decade, analysis of foods for lignans and isoflavones (2,19–23) has led to the compilation of several databases (24–26) and the development and modification of food-intake questionnaires to estimate intake of these various compounds (27–29). As is the general case for measurement of nutrient intakes, accurate determination of dietary phytoestrogen intake is limited not only by the constraints of intake-measurement methodologies but also by the problems associated with establishing the phytoestrogen content of foods. The lignan or isoflavone concentration can vary substantially within a food according to variety, crop season, location and processing methods (30,31). Furthermore, given the ever-growing list of preprepared foods, functional foods and dietary supplements that are available to consumers, a food-intake instrument may be hard pressed to capture fully the intake of these classes of compounds. Measuring lignans and isoflavones in biologic samples provides another approach to estimating human exposure to these compounds, however, this presents its own set of issues and challenges.

Measurement in biologic samples

Levels in humans. Isoflavones and lignans have been quantified in many types of biologic specimens including urine (32,33), feces (34,35), serum and plasma (36, 37), nipple aspirate fluid (38) and prostatic fluid (39). They have also been measured in breast milk (40), umbilical cord plasma (41), amniotic fluid (41) and in several tissues in humans (38) and animals (42,43).

Isoflavone concentrations in human breast tissue (obtained at surgery) have been shown in one study to be comparable to those found in serum with supplemental soy increasing both breast tissue and serum daidzein concentrations (38). Given that the collecting of tissue samples from humans can be difficult, requires invasive procedures and often deters individuals from participating in studies, it would be helpful to establish as soon as possible whether measuring concentrations in tissue is necessary or whether serum concentrations are a sufficient surrogate for tissue exposure.

Phytoestrogen concentrations are higher in fluid collected from breast (38) and prostatic (39) ducts compared with serum or plasma, which is suggestive of accumulation of these compounds within the ducts. In a cross-sectional observational study, prostatic duct fluid concentrations of daidzein correlated positively with plasma concentrations among men in Hong Kong, but plasma and duct fluid concentrations of enterolactone were not correlated (39). In women, nipple aspirate fluid concentrations of isoflavones and lignans did not correlate well with circulating levels and did not respond to 2-wk soy supplementation. Therefore, nipple aspirate fluid concentrations are probably not accurate indicators of recent exposure (38). Ductal fluid concentrations may prove useful for monitoring long-term cumulative exposure, however to date, this has not been investigated.

Isoflavones and lignans in urine and serum or plasma are probably most pertinent to the application of their measurement as dietary biomarkers in human population-based studies. Several reviews have summarized the accumulated data on the physiologic ranges of isoflavones and lignans in urine

and plasma that have been observed in various populations worldwide and in relation to disease risk (1,44–46). In the U.S.A. alone, wide ranges in urinary isoflavonoid excretion, which are dependent in part on dietary behaviors, have been reported: daily daidzein excretion [$\mu\text{mol/d}$; geometric mean (range)] values were 0.28 (0 to ~14) in women from a multiethnic population in California (47); 0.56 (0.04–29.48) in a predominantly Caucasian population in Minnesota (48); and 0.37, 3.64 and 1.42 in premenopausal women who consumed omnivorous, macrobiotic or lacto-ovovegetarian diets, respectively (44). By comparison, a daidzein level of 2.58 $\mu\text{mol/d}$ (range, 1.60–5.25) was reported for men and women who consume a traditional Japanese diet (49). Urinary enterodiol and enterolactone excretion values [$\mu\text{mol/d}$; geometric mean (range)] were 0.69 (0 to ~5.5) and 4.31 (0–22.7) among the California women of Caucasian origin (47), 0.45 (0.02–29.31) and 2.12 (0.14–32.75) in the Minnesota group (48) and 0.24 and 1.92, 7.10 and 19.9, and 1.03 and 4.97 in premenopausal women who consumed omnivorous, macrobiotic and lacto-ovovegetarian diets, respectively (44). Lignan excretion among the Japanese appears to be lower: urinary enterodiol and enterolactone levels were 0.27 (0.09–3.39) and 0.50 (0.04–3.25) $\mu\text{mol/d}$, respectively (49).

Populations that consume a Western diet typically have very low circulating levels of isoflavones. Reported plasma concentrations (nmol/L; geometric mean) of daidzein and genistein were 4.2 and 4.9 in Finland and 2.1 and 5.7 in the U.S.A. (50,51), whereas among six men who consume a traditional Japanese diet, mean daidzein and genistein concentrations [geometric mean (range)] were 163 (58–924) and 248 (90–1204) nmol/L, respectively (50). Mean plasma lignan concentrations (enterodiol plus enterolactone) appear to be in the range of 10–270 nmol/L in studies where plasma was collected from individuals that consumed their usual diets (46). However, as with urinary levels, wide ranges in plasma enterolactone concentrations—from below the detectable limit to 1,078 nmol/L—have been reported (36). Generally under habitual dietary conditions, enterolactone is the lignan in highest concentration in plasma (37,50,52), however, this varies among groups studied in that enterodiol concentrations higher than enterolactone have also been reported (38,53).

Little work has been done to assess the long-term reliability of phytoestrogen measurements in biologic samples. One study of 60 women (both pre- and postmenopausal) in New York measured phytoestrogens in serum samples collected from women at three different time points over a 3-y time period (51). The median enterodiol concentration was ~1.5 nmol/L, and over one-third of the serum samples had concentrations < 1.00 nmol/L, which was the sensitivity level of the assay. Enterolactone concentrations were higher, with only 2% of the assays < 1.00 nmol/L. On average, 28% of the daidzein and 8% of the genistein concentrations were < 1.00 nmol/L. Concentrations of equol and O-desmethylangolensin (ODMA)⁴ were very low, with approximately two-thirds of the samples being < 1.0 nmol/L. Overall, the reliability coefficients were low (< 0.4) for all compounds except for enterolactone (0.55). The higher reliability coefficient for enterolactone likely reflects regular intake of the appropriate precursors. These data point to potential problems in using plasma phytoestrogen concentrations, particularly isoflavones, as dietary biomarkers in populations that consume Western diets.

⁴ Abbreviations used: FID, flame-ionization detection; GC/MS-SIM, gas chromatography/mass spectrometry in selected-ion monitoring mode; HPLC, high performance liquid chromatography; ODMA, O-desmethylangolensin

Phytoestrogen metabolism. Phytoestrogen metabolism in humans involves the action of intestinal microflora as well as enzymatic modification by the conjugating enzymes, UDP-glucuronosyltransferases (UGT) and sulfotransferases and possibly the cytochromes P450. Lignans and isoflavones are present in plants predominantly as glycosides, although plant foods that include a fermentation step in the processing or preparation have an increased proportion of aglycones (1). In soy, the major isoflavone glycosides are genistin, daidzin and glycitin (20). Some legumes also contain biochanin A and formononetin, which are precursors of genistein and daidzein, respectively (22). The two dietary lignans, secoisolariciresinol and matairesinol, are derived from coniferyl alcohol (54) and are immediate precursors of the mammalian lignans enterodiol and enterolactone, respectively (55). Studies suggest that there are probably additional mammalian lignan precursors (2,56).

Upon ingestion, the sugar moieties of isoflavone and lignan glycosides are hydrolyzed by gastric hydrochloric acid, β -glucosidases in foods and human gut bacterial β -glucosidases, thereby releasing the aglycones (57). These are absorbed or can be metabolized further in the gut to additional metabolites (Table 1). The importance of the microflora in the metabolism of lignans and isoflavones has been well demonstrated by the observations that antibiotic administration prevents production of the metabolites (58), individuals without an intact colon have low plasma and urinary lignan levels (59) and infants fed soy formulas during the first 4 mo of life when gut microflora are underdeveloped do not excrete appreciable amounts of equol, a metabolite of daidzein (60).

TABLE 1

Common dietary isoflavones and lignans and metabolites¹

Parent compound present in plant foods	Intestinal bacterial metabolites	Human metabolites
Isoflavones		
Genistin; genistein	Genistein; dihydro-genistein; 6'-hydroxy-O-desmethylangolensin; <i>p</i> -ethylphenol	Sulfates, glucuronides and sulfoglucuronides of genistein and metabolites
Daidzin; daidzein	Daidzein; dihydrodaidzein; dehydroequol; 2-dehydro-O-desmethylangolensin; tetrahydrodaidzein; O-desmethylangolensin; equol	Sulfates, glucuronides and sulfoglucuronides of daidzein and metabolites
Glycitin; glycitein	Glycitein	Sulfates, glucuronides and sulfoglucuronides of glycitein
Lignans		
Secoisolariciresinol diglycoside	Secoisolariciresinol; enterodiol; enterolactone	Monohydroxylation products of enterolactone; sulfates, glucuronides and sulfoglucuronides of metabolites
Matairesinol	Enterolactone	Monohydroxylation products of enterolactone; sulfates, glucuronides and sulfoglucuronides of metabolites

¹ Modified from existing references (64,113,115).

Absorbed lignans and isoflavones and their metabolites are conjugated by sulfotransferases and UDP-glucuronosyltransferases in intestinal epithelium and liver (61). They are excreted in urine and bile. The conjugated forms that are reexcreted through the bile duct can undergo enterohepatic recycling: deconjugation by bacterial β -glucuronidases and sulfatases may promote reabsorption, further metabolism or degradation in the colon (62). Although intestinal microbes play a primary role in the metabolism of plant lignans, there is evidence that additional metabolism beyond glucuronidation and sulfation occurs in the mammalian host. Hepatic microsomes from humans as well as rats are able to hydroxylate enterolactone and enterodiol at both the aliphatic and aromatic positions (63). To date, products of aromatic monohydroxylation at the *para* and *ortho* positions have been identified in human urine, whereas products of aliphatic hydroxylation have not been detected (64). It remains to be determined whether the lack of these aliphatically hydroxylated compounds is because they are not produced *in vivo*, are metabolized further or are lost during the extraction process. Although these metabolites have not been quantitated accurately, it is estimated that they account for < 5% of the total urinary lignans at least with high intakes of lignan precursors (64). Thus the amount of and the interindividual variation in production of these hydroxylated compounds may not contribute significantly to the wide ranges in urinary and plasma enterodiol and enterolactone levels that are observed in the various flaxseed supplementation studies.

Urinary recovery of daidzein and genistein has been reported in the ranges of 42–62 and 7–18%, respectively (65–70). Conversion of genistein to *p*-ethylphenol, a compound that is a major metabolite of genistein in sheep but is not routinely measured in humans (1,71), may account for the low urinary recovery of genistein in the form of the parent compound and metabolites. Given the relatively low recovery of daidzein and genistein in feces (68,69), it seems likely that there are other isoflavone metabolites that need to be identified and measured to characterize fully the pharmacokinetics of these compounds.

Equol, a bacterial metabolite of daidzein, is only produced by ~30–40% of individuals in response to a soy challenge (72,73). Although the capacity to produce equol appears to be a relatively stable phenotype (57), there is at least one report of individuals who converted from being equol nonexcretors to excretors with chronic soy ingestion (74). This may be the result of a microfloral change or may reflect an inadequate assessment of equol-excretion status at baseline. Several studies have shown that equol does not appear in the urine until ~72 h post-daidzein ingestion, and typically at least 3 d of soy intake are needed to distinguish between excretors and nonexcretors in a population that regularly does not consume soy (72,73).

Phytoestrogen pharmacokinetics. Measured from their plasma appearance and disappearance curves, the plasma half-life for daidzein and genistein has been estimated to be 7.9 h in adults with peak plasma concentrations occurring 6–8 h after ingestion of the pure compounds (1) and soy powder (68). Similarly, excretion half-lives for genistein, daidzein and equol with soy supplementation are ~7, 4 and 9 h, respectively (70,74). Typically all of the urinary recovery of genistein and daidzein from a single time-point intake is complete within 24–36 h, whereas postchallenge urinary recovery of equol and ODMA remains measurable for at least 3 d (75). This has significant implications for monitoring urine or serum to determine isoflavone exposure in populations that may only consume soy once or twice per week.

Isoflavones are present in urine predominantly as conjugates: 75–85% are present as glucuronides, 15–25% as

sulfates and < 1% as free aglycones (76). A more detailed analysis by Adlercreutz et al. (77) suggests that conjugation patterns differ for the various isoflavonoids: ODMA occurred predominantly as a monoglucuronide (97%); equol was found as the monoglucuronide (32–93%), sulfoglucuronide (0–43%), monosulfate (0–15%) and disulfate (0–10%); daidzein was present as the monoglucuronide (79–82%), sulfoglucuronide (6–17%) and free form (1–5%); and genistein was excreted as monoglucuronide (53–76%), diglucuronide (12–26%), sulfoglucuronide (2–15%) and disulfate (1–4%) conjugates.

Few studies have examined lignan pharmacokinetics in humans. These studies are small and of short duration—blood samples are not collected beyond 24 h—and there are no data on lignan disappearance from circulation. Feeding men a baked product that contained 15 g of soybean flour and 15 g of cracked linseed (flaxseed), Morton et al. (78) reported that plasma enterolactone concentrations did not increase measurably until 8.5 h after the men ate the flaxseed. The next blood sample taken was at 24 h. This had the highest enterolactone concentration, but it was also the last blood sample collected. The mean plasma lignan concentration at 24 h was 102 nmol/L (range, 36–146 nmol/L). In a similar study, supplementation of 500 g of strawberries (which contained ~11.7 mg of secoisolariciresinol and 0.6 mg of matairesinol) also resulted in significantly elevated plasma enterolactone concentrations 24 h postingestion (20.6 vs. 10.3 nmol/L); however blood samples were not collected between 8 and 24 h or beyond 24 h (79). In women, significant increases in plasma lignan concentrations (enterolactone plus enterodiol) also were reported by 9 h after consumption of 25 g of flaxseed. These concentrations did not change significantly at 12 or 24 h (53). The mean lignan concentration 24 h postflaxseed dose was 66 nmol/L. In these same women, 7 d of flaxseed supplementation resulted in the total plasma area under the curve being significantly higher on day 8 than on day 1: 1,840 and 1,027 nmol·L⁻¹·h⁻¹, respectively. These studies suggest that because enterodiol and enterolactone are products of microfloral metabolism, appearance of the compounds in plasma is delayed, however, there is substantial variation in this time frame among participants.

Similar to the isoflavones, the major proportion of lignans in urine are conjugated. Enterolactone and enterodiol are excreted primarily as monoglucuronides (95 and 85%, respectively) with small percentages being excreted as monosulfates (2–10%) and free aglycones (0.3–1%) (77).

Measurement of phytoestrogens. Several types of analytic methods have been described in the literature to measure phytoestrogens and their metabolites in body fluids. All have some limitations, including a reduced capacity to measure certain active metabolites, a need for large volumes of urine or plasma, laborious cleanup and derivitization steps, expensive equipment or antibodies and standards that are not commercially available. Choice of method will depend on the type of study, the level of sensitivity required, the amount of biologic sample available, etc. (Table 2).

Chromatographic separation coupled with various detection systems is most commonly used for measuring phytoestrogens in urine and plasma. Methods have been published using gas chromatography/mass spectrometry in selected-ion monitoring mode (GC/MS-SIM) (33,50,80), GC with flame-ionization detection (FID) (81), high performance liquid chromatography (HPLC) with ultraviolet (82), diode array (83) or coulometric electrode array detection (42,84), and LC with MS (76) or electrospray (ES)/MS (85) or MS/MS detection (86). GC/MS-SIM methods, which are adapted from the highly specific and sensitive approaches used for measuring steroid hormones and using deuterated internal standards of the phytoestrogens and their metabolites, have been used effectively for analysis of urine and serum. Rapid and sensitive LC/MS (76) and LC/MS/MS (86) systems allow for isoflavone measurement in small volumes of urine (0.5–1 mL) and plasma (1 mL); however the detection limit for equol using negative single-ion monitoring is ~40 times higher than for the other isoflavonoids (76).

Other methods such as HPLC with diode array detection (83) and GC/FID (81) require a large starting volume of urine (20 mL), which is then concentrated 10–20-fold before analysis. These methods are best suited to studies in which high phytoestrogen concentrations are expected (e.g., interventions using phytoestrogen-containing foods, high-dose pharmacokinetic studies, etc.). Coulometric electrode array

TABLE 2

Detection limits for daidzein and enterolactone using different analytic methods

Reference	Instrumentation	Method detection limit for urine (nmol/L; volume)	Method detection limit for serum or plasma (nmol/L; volume)
Daidzein			
Gamache and Acworth (42)	HPLC-CA ¹	5.19 (0.2 mL)	3.2 (0.2 mL)
Gamache et al. (87)	HPLC-CA	—	3.2 (1.0 mL)
Nurmi and Adlercreutz (84)	HPLC-CA	—	0.90 (0.5 mL)
Franke et al. (116)	HPLC-ECD	—	3.16 (1.0 mL)
Franke et al. (83)	HPLC-UV	0.51 (20 mL)	—
Adlercreutz et al. (50,77)	GC/MS-SIM	7.40 (10 mL)	0.5 (4.0 mL)
Coward et al. (86)	LC/MS/MS-SIM	—	10 (1.0 mL)
Kohen et al. (91)	FIA	2.0 (10 µL)	—
Enterolactone			
Gamache and Acworth (42)	HPLC-CA	5.19 (0.2 mL)	3.2 (0.2 mL)
Gamache et al. (87)	HPLC-CA	—	4.0 (1.0 mL)
Nurmi and Adlercreutz (84)	HPLC-CA	—	0.90 (0.5 mL)
Adlercreutz et al. (50,77)	GC/MS SIM	7.40 (10 mL)	0.5 (4.0 mL)
Adlercreutz et al. (92)	FIA	—	1.5 (200 µL)
Wallace, enterolactone kit insert	FIA	—	0.4 (200 µL)

¹ Abbreviations used: HPLC, high performance liquid chromatography; CA, coulometric array; ECD, electrochemical detection; UV, ultraviolet; GC, gas chromatography; MS, mass spectrometry; SIM, selected-ion monitoring; LC, liquid chromatography; FIA, fluorometric immunoassay.

detection improves sensitivity and can be used to monitor plasma phytoestrogen profiles in samples (0.5–1 mL) from individuals that consume their habitual diets not supplemented with phytoestrogens (84,87).

Immunoassays using radiolabeled, enzyme-linked or fluorescently tagged monoclonal antibodies have been developed to measure genistein (88,89), daidzein (90,91) and enterolactone (92) in serum, plasma or urine. These provide an attractive alternative for the analysis of large numbers of samples, however at present, only the enterolactone fluoroimmunoassay is available commercially.

The majority of the methods involve initial enzymatic hydrolysis of the sulfate and glucuronide conjugates in samples thus providing a measure of “total” amounts of the phytoestrogens and their metabolites. A comprehensive analysis of sulfate and glucuronide conjugates of isoflavones and lignans and their metabolites can be obtained using a series of enzymatic hydrolysis steps and subsequent GC/MS or LC/MS to separate and quantitate these compounds in urine (76,77) and plasma (50). These methods are labor intensive and require pure enzyme preparations (e.g., a sulfatase enzyme that is relatively free of glucuronidase activity). Although they may be useful for understanding the pharmacokinetics and mechanisms of action of phytoestrogens, this approach is probably overkill for studies using isoflavones and lignans as biomarkers of dietary exposure. The variation among individuals as a result of conjugation patterns is probably minor compared to the variation due to habitual diet, etc., although this has not been tested under controlled conditions.

Utility of isoflavonoid and lignan phytoestrogens as dietary biomarkers

Although estimated dietary phytoestrogen intakes (5,27) and urinary and circulating concentrations of lignans and isoflavonoids are being used in epidemiologic studies (93,94), few studies have compared concurrently phytoestrogen levels in biologic specimens against dietary phytoestrogen estimates. To date the majority of observational studies that have used isoflavones and lignans as biomarkers have relied on urine collections of varying length and completeness that ranged from spot urines to 3-d collections.

Several studies of populations that typically consume soyfoods have established a strong positive association between soy intake and isoflavone excretion. Soy product intake (estimated from food records) and urinary isoflavones (from a 3-d urine collection) were significantly correlated in a group of 19 men and women that consumed a typical Japanese diet (49). Among Singapore Chinese, urinary daidzein and the sum of urinary daidzein, genistein and glycitein measured in spot urine collections were also associated in a dose-dependent manner with intake of traditional Chinese soyfoods (95). Similarly, among Chinese women in Shanghai, isoflavones in overnight urine collections were also associated with soy intake ($r = 0.5$; $P < 0.001$) (96). Maskarinec et al. (29), studying a multi-ethnic population of women in Hawaii ($n = 102$), showed an association between isoflavones measured in overnight urine samples and soy protein intake, both in the previous 24 h ($r_s = 0.61$; $P < 0.0001$) and in the past year ($r_s = 0.32$; $P < 0.0012$). Franke and Custer (97), also in Hawaii, reported the ability to discriminate, using overnight-urine isoflavone levels, between seven women who consumed tofu more frequently than once a week and 16 women who ate tofu less than once a week. By comparison, even in a predominantly Caucasian population of which $< 30\%$ reported on a food-frequency questionnaire (FFQ) that they consumed soy more than once a month, daily

isoflavone excretion (from a 3-d collection) was significantly associated with immediate soy intake estimated from a 5-d food record ($r = 0.4$) and over the past year estimated by FFQ ($r = 0.25$) (48). On average, urinary isoflavones were also higher in the group of individuals that consumed soy more than once a month compared with those that consumed soy less than once a month or never.

High isoflavone excretion among some individuals with no reported soy intake (48) suggests that on a typical American (U.S.) diet, other food sources contribute substantially to isoflavone intake. The most likely candidates are processed foods that contain soy flour or soy protein but not soybean oil (30); isoflavones from these sources are difficult to detect by the usual self-report methods of monitoring soyfood intake. Other non-soy-based foods are also sources of isoflavones. Daidzein and genistein have been identified in legume sprouts such as clover and alfalfa (22) and in some alcoholic beverages (98). Equol is present in low amounts in cow's milk (99), and milk-product consumption appears to contribute in part to small differences in equol excretion (100). Thus measuring isoflavones in biologic samples may be useful to monitor more completely overall exposure to isoflavones. The efficacy of serum isoflavone concentrations as markers of soy exposure has not yet been established in observational studies.

Two cross-sectional studies (48,49) have reported that equol excretion is associated with meat intake. Because only one-third of individuals have colonic bacteria that are capable of producing equol from daidzein (73), equol would not be expected to be associated with intake of a particular dietary constituent except maybe soy. We cannot determine from these data whether the relationship between urinary equol excretion and meat intake reflects a diet-induced isoflavone-metabolizing profile with improved production of equol from daidzein or instead is due to equol in the meat itself. Adlercreutz et al. (49) hypothesized that consuming more fat and meat creates a colonic environment that is capable of sustaining equol-producing bacteria. However, in a study designed to identify equol excretors using a prescribed soy dose, there was no relationship between fat or animal protein intake and equol-excretor status (73). Another possible explanation for the association between high-fat meat intake and equol excretion is that equol is present in the meat of animals that receive soy-, alfalfa- or clover-supplemented feed (48).

At present, regular intake of discrete soyfoods is still relatively low and uncommon in the continental U.S.A. At the same time, soy protein and other soybean derivatives are added frequently to foods. Thus although monitoring exposure to isoflavones using biologic measures offers certain advantages, it also poses a number of challenges. First, unless a person is a regular soy consumer, within-person variability in intake may exceed between-person variability. Second, the half-lives of the isoflavones genistein and daidzein are short (68), therefore, if isoflavone exposure is monitored using urinary or plasma markers, intermittent soy consumption may be severely under- or overestimated.

Intake of total dietary fiber and fiber from grains (estimated by food records) has been most strongly associated with lignan excretion (48,101,102). Adlercreutz et al. (49) also reported that in Japanese men and women, lignan excretion was correlated significantly with intakes of green and yellow vegetables, pulses and beans and boiled soybeans. Lampe et al. (48) reported a significant association between intake of vegetables and fruit and urinary lignan excretion, but this relationship was due exclusively to an association between fruit intake and lignan excretion; there was no association with vegetable intake. This was surprising, given that Kirkman et al. (103) demonstrated previously that vegetable supplementation,

as part of a low-fiber low-phytochemical diet, significantly increased lignan excretion. These data suggest that additional factors need to be considered when examining the relationships between intake of particular nutrients or food groups and lignan excretion. Apart from these few relatively small studies (48,49,101,104), the association between diet and levels of lignans in biologic samples in observational studies has not been investigated. Significantly more work is needed in this area. There were no significant relationships between lignan excretion and intake of dietary fiber or vegetables and fruit when intake over the past year was measured using an FFQ (48). This reflects in part 1) the measurement error inherent in assessing diet by FFQ (105); 2) the variation in lignan content of high-fiber foods; 3) the seasonal and varietal variation in lignan content within a food (31); and 4) the effects of food processing on lignan precursor availability. Given the ubiquitous nature and more regular intake of lignans, one might expect a stronger relationship between dietary constituents and lignans than between soy intake and isoflavones.

Results of several studies also suggest that among individuals consuming a Western diet, plasma and urinary phytoestrogen levels may reflect differences in dietary patterns and possibly more healthy dietary patterns. Adlercreutz et al. (102) showed that there are differences in lignan and isoflavone excretion among groups that consume very different diets (e.g., macrobiotic, vegetarian and omnivorous diets). Compared with omnivores, vegetarians excreted significantly higher amounts of lignans in urine and feces (34,104). Similarly plasma lignan concentrations tend to be higher in vegetarians compared with omnivores (50). Furthermore urinary lignan and phytoestrogen excretion values (lignans and isoflavonoids combined) were greater in individuals who self-reported higher versus lower intakes of vegetables and fruit and on average had higher intakes of dietary fiber and soy foods and lower percent of total energy from fat (48).

Urinary and plasma or serum isoflavone and lignan measures have been used as markers of compliance for dietary intervention trials in which participants consumed their normal diets supplemented with a phytoestrogen source (9,38,66,106) or controlled diets provided by the investigators (11). Urine collections assayed ranged from 72-h (9) to 24-h (66,106) collections. On average, phytoestrogen concentrations in serum and levels in urine reflect intake of the precursor compounds in a dose-dependent manner (9,82,107,108). For example, daily daidzein excretion values were 0.44, 3.65, 5.29 and 9.86 $\mu\text{mol/d}$ with daily soy protein powder intakes of 0, 5, 10 and 20 g (0, 3.25, 6.5 and 13 mg of daidzein), respectively (108). However in these studies, even under controlled dietary conditions, investigators have reported substantial interindividual variation in urinary excretion of lignans and isoflavones. This poses potential problems if these measures are going to be used to gauge dietary compliance at the level of the individual.

Factors affecting measurement of isoflavones and lignans

In theory several factors can affect the measurement of isoflavones and lignans and more specifically can influence the interpretation of phytoestrogen measures as dietary biomarkers. One factor is the effect of dietary constituents and the form of phytoestrogen-containing foods on isoflavone and lignan availability, which has received attention in a few small studies. In short-term trials (e.g., 1-d and single feedings), background diet and the form of the soyfood (69) as well as the addition of wheat fiber (109) appear to have little effect on isoflavone bioavailability. Similarly, a 9-d feeding study that compared

isoflavone administration as tempeh and soy pieces showed modest differences in urinary recovery of daidzein and genistein (110). At the same time, no studies have addressed the influence of chronic dietary patterns on phytoestrogen availability. For lignans, ingestion of ground flaxseed as cooked versus raw did not affect total urinary lignan excretion (53). Because the lignans are closely associated with the fiber matrix of grains and seeds, it is possible that particle size (e.g., coarseness of grind) might influence lignan availability; however there are no data available in this area. Furthermore the bioavailability of lignans from various food sources has not been explored. If the substantial variation in bioavailability of other phytonutrients in relation to vegetable matrices is any indication (111), this also warrants investigation in the case of lignans.

Another factor that needs to be considered is colonic environment. Production of the lignans enterolactone and enterodiol and isoflavone metabolites such as equol and ODMA is dependent on metabolism of parent compounds by gastrointestinal bacteria (62). Several of the flaxseed-feeding trials have noted significant interindividual variation in enterodiol-to-enterolactone ratios (53,112): some individuals convert most of the enterodiol to enterolactone, whereas others produce little enterolactone. The specific microbes responsible for the conversion of enterodiol to enterolactone have not been identified. Based on early work in this area, they appear to be facultative anaerobic bacteria present in concentrations of between 10^3 and 10^4 bacteria/g of stool (113). In the case of metabolism of daidzein to equol, the species and/or strains that are involved in this metabolic pathway have not been elucidated either, and to date little work has been done to establish what factors contribute to the capacity to produce equol. Among equol producers, urinary equol can account for 25–30% of the daidzein excreted (108). Thus measurement of the related metabolites (e.g., enterodiol and enterolactone or daidzein plus its metabolites) rather than reliance on a single metabolite presumably would help to minimize the measurement error.

Antibiotic use would be expected to contribute to variation in urinary and plasma phytoestrogen concentrations among individuals. Horn-Ross et al. (47) found no difference in urinary phytoestrogen levels between women who had used (43%) or not used (57%) antibiotics within the year before interview and sample collection. However, antibiotic treatment has been shown clearly to reduce isoflavone and lignan excretion (72,104). To date, the large range and significant variation in phytoestrogen levels in observational studies with relatively small sample sizes probably precludes detecting the effects of antibiotic use.

There are some data to suggest that chronic exposure to isoflavones may change usual plasma concentrations or urinary excretion. In a sample of four individuals, Barnes et al. (61) observed a decrease in plasma concentrations of daidzein and genistein over a 2-wk period. Similarly, Lu et al. (74) reported a decrease in urinary isoflavone excretion in women that consumed soy milk for 30 d, and Xu et al. (9) reported greater excretion of ODMA than daidzein in women that consumed soy protein for 100 d. In an observational study, Chinese and Native Hawaiian compared to Japanese women also excreted lower amounts of isoflavones in an overnight urine collection despite soy protein intakes being similar in the three ethnic groups (29). Hypothetically, these effects could be the result of altered biotransformation enzyme (e.g., UDP-glucuronosyl-transferase and sulfotransferase) expression and/or activity or colonic microfloral metabolism, however, to date this has not been investigated in humans.

The effect of endogenous and exogenous hormones on isoflavone and lignan metabolism and excretion has not been

fully established. Enterolactone was originally identified in the urine of premenopausal women as a compound that was found to fluctuate during the menstrual cycle and to increase significantly during the midluteal phase (32). However, lignan differences between follicular and luteal phases were not detectable in 3-d pooled urine collections (112). Oral contraceptive use was also not shown to influence urinary phytoestrogen excretion in observational studies (47,48); however the number of women in these studies was very small and probably inadequate to provide conclusive data. Additional studies that are in progress should be able to provide useful data in this area.

Sex differences in isoflavone and lignan metabolism and excretion were also reported. Under controlled dietary conditions, isoflavonoid excretion half-lives were longer in women than in men with an initial exposure to soy milk but were shortened in women and lengthened in men with chronic ingestion (114). In this group, women also had higher urinary isoflavonoid recoveries initially and had lower recoveries by the end of the study period. Less controlled conditions and shorter interventions showed no differences in 24-h isoflavonoid excretion (73,103). Higher enterodiol-to-enterolactone ratios in men compared with women were observed in a randomized crossover trial of vegetable feeding (103). Interpretation of these sex-difference results is limited: all the studies were small and provided phytoestrogens as fixed doses and not according to body weight. Because body weight and sex are frequently confounded, it is not clear whether observed excretion differences between men and women are due to physiologic differences in intestinal function or are merely dose effects.

The last decade saw the development of several new methodologies for measuring lignans and isoflavones. Improved levels of assay detection coupled with minimal sample extraction and the capacity to use small amounts of urine, serum or tissue will further the use of lignans and isoflavones as biomarkers in large population-based studies. Additional research efforts are needed to establish more clearly the relationship between phytoestrogen levels and habitual diet in larger population-based studies and to determine in greater detail what contributes to the significant interindividual variation in plasma and urinary levels of lignans and isoflavones. Much of the variation in these measures is attributed primarily to differences in colonic microfloral populations; however the factors that potentially influence these populations and their metabolism have not been studied and seldom are monitored in population-based studies. Factors that may influence endogenous metabolism of phytoestrogens (e.g., conjugating enzymes) also warrant characterization.

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