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Review

Bioavailability of isoflavones

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Abstract

Isoflavones are disease protective components of soybeans. Isoflavone metabolism and bioavailability are key to understanding their biological effects. Isoflavone glucuronides, dominant biotransformation products in humans that are more hydrophilic than isoflavone aglycones, activate human natural killer cells in vitro but are less toxic to NK cells than the parent aglycones. Gut microbial isoflavone metabolites have also been identified, but remain to be well characterized. Gut transit time (GTT) seems to be a significant determinant of isoflavone bioavailability because women with more rapid GTT (<40 h) experienced 2–3-fold greater absorption of isoflavones than did women with longer GTT (>65 h). Isoflavone metabolism varies a great deal among individuals, thus limiting the quantitative value of urine or plasma isoflavones as biomarkers of soy ingestion. Defining and lessening interindividual variation in isoflavone bioavailability, and characterizing health-related effects of key isoflavone metabolites are likely to be crucial to further understanding of the health benefits of isoflavones.

Keywords: Reviews; Bioavailability; Isoflavones

Contents

1. Introduction

Intake of isoflavones, especially those found in soybeans and soybean-derived foods, has been associated with lesser risk of some types of cancer [1]. For example, 24-h urinary excretion of daidzein (one of the major isoflavones in soy) from Japanese women was about 10-fold greater than daidzein excretion from recent Asian women immigrants to Hawaii and breast cancer rates are greater in the immigrant women than in Japanese women [1]. Isoflavone content of human plasma or urine serves as a biological marker of soy intake because iso-

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flavones are unique constituents of soybeans. No other commonly consumed human foods contain appreciable amounts of isoflavones. The isoflavones in human urine can be quantified relatively readily, as can plasma isoflavones if the isoflavones are ingested in amounts of 10 mg or more per day. Such amounts are presumably needed for biological effects and found in soybean foods. The ability of isoflavones to prevent cancer and other chronic diseases depends on absorption, metabolism, distribution to target tissues, and excretion of these compounds, i.e., their bioavailability. Although this topic has been reviewed recently [2–6], new information is continually added concerning isoflavone bioavailability. Developing better, easier, cheaper, and more rapid methods of isoflavone analysis in human body fluids is likely to be crucial to progress in assessing the roles of these compounds in human health. Better understanding of the kinetics of these compounds, their mechanisms of absorption, distribution, utilization and excretion is needed as well. Identification of key bioactive isoflavone metabolites and more thorough characterization of the role of gut microflora in the metabolism of isoflavones are also important. The current status of research on these topics will be addressed herein.

2. Recent advances in methods for detection and quantification of isoflavones in human body fluids

Isoflavones were first quantified in human urine by gas chromatography [7,8]. Lundh et al. [9] developed a C_{18} reversed-phase high-performance liquid chromatography (HPLC) method for analysis of isoflavones in bovine plasma and urine, using UV detection of isoflavone aglycones after enzymatic hydrolysis of the presumed major isoflavone metabolites by glucuronidase/sulfatase. The method had 73–91% recovery for equol and daidzein, respectively, in urine, and 92–105% recovery of daidzein and equol from plasma, and could detect 0.4 ng daidzein and 13 ng equol/ml blood and 130 ng daidzein and 4000 ng equol/ml urine. Our laboratory made minor adaptations to this method for detection of human plasma and urine isoflavones, and also developed a method for fecal isoflavone analysis, all using UV detection [10]. Subsequently, we adapted an internal standardization method from Murphy et al. [11], using the isoflavone synthetic precursor, 2,4,4′-trihydroxydeoxybenzoin (THB). Recoveries of spiked isoflavones from urine, plasma and feces ranged from 60 to 85%, with standard deviations for recovery for each isoflavone of no more than 7% of the mean recovery [12]. Isoflavone detection limits were about 120 ng/ml. Franke and Custer [13] also developed a reversed-phase HPLC method for analysis of isoflavones including the daidzein metabolite, *O*-desmethylangolensin (ODMA), after enzymatic hydrolysis of isoflavone metabolites (similar to Ref. [9]). They used flavone as an internal standard. Their reversed-phase solid-phase extraction method showed isoflavone recoveries of about 100%. The UV detection method permitted determination of 1.3–2.4 ng/ml of daidzein and genistein, respectively, and 151–201 ng/ml of equol and ODMA, respectively. Gamache and Acworth [14] reported plasma, urine and tissue isoflavone analysis with reversed-phase HPLC and coulometric detection, giving limits of detection of 1–2 ng/ml, and recoveries of 85–95%, with intra-assay precision of 2–4% relative standard deviations (RSDs). This method also involved a simple extraction method of incubation with glucuronidase/sulfatase followed by mixing with ethanol, centrifugation, drying of supernatant and redissolution in methanol–water. This method provided greater sensitivity for detection of equol than the method of Franke and Custer [13], but the two methods were quite similar in sensitivity for daidzein and genistein. Additional metabolites of isoflavones were recently identified in human urine by gas chromatography–mass spectroscopy (GC–MS) [15]. Dihydrodaidzein, dihydrogenistein, 6-hydroxy-ODMA and *cis*-4-equol were detected. Isotope dilution liquid chromatography electrospray (LC-ES) MS and MS–MS have been used to detect tissue (and serum) genistein in rats fed diets of varying isoflavone contents [16]. In these methods, a deuterated genistein internal standard was added and solid-phase extraction preceded chromatography. Limits of detection of genistein, measured after deconjugation with glucuronidase/sulfatase, were 10–20 pg/mg tissue for LC–ES-MS and 3–8 pg/mg for MS–MS. Recovery of genistein standard from tissues ranged from 40 to 78%, and precision of analysis was 1–9%

RSD. A more rapid method for analysis of isoflavones in human urine was developed, employing time-resolved fluoroimmunoassay [17]. Antibodies were prepared in rabbits to carboxymethyl derivatives of daidzein and genistein coupled to bovine serum albumin. Europium-labeled isoflavones were used to compete with the native isoflavones for binding to the anti-isoflavone antisera. Urine samples were pretreated with glucuronidase/sulfatase, needing only 20–200 μl urine. The urine samples were applied to wells coated with anti-isoflavone antisera, tracer added, and then a fluorescence enhancement solution added before reading the plates with a fluorescence detector. The method had a sensitivity of about 0.1 ng/ml, with intra-assay RSD of about 2–5% and inter-assay RSD of 2–10%. The method showed good correlation with their standard GC–MS method [18] ($r=0.88$ for genistein, $r=0.99$ for daidzein), and will permit more rapid screening of large populations for urinary isoflavone contents. For those interested in developing isoflavone analytical capabilities, a HPLC method with coulometric detection [14] is probably the method of choice (Table 1), being less expensive and easier to institute than the GC–MS [15,18] or immunofluorescence methods [17] and being more sensitive than HPLC–UV methods of similar cost. For very large numbers of samples, and for greatest sensitivity, the immunofluorescence method [17] would be best at this time for the major soybean isoflavones, genistein and daidzein. For sensitivity to equol, GC–MS [18] is somewhat better than HPLC–coulometric detection [14]. What limits of detection are ideal will depend upon further advances in understanding what effects of health significance the isoflavones exert at what concentrations. This remains largely unexplored. It is not yet known if methods that quantify isoflavone metabolites other than the parent aglycones will be important, because the biological effects of such metabolites are almost entirely uncharacterized.

3. Utility of isoflavone contents of human body fluids as biomarkers for chronic disease prevention

The analysis of human body fluids for isoflavones may be helpful in determining the role of isoflavones in preventing various chronic diseases. For example, for soy protein to be effective at lowering blood cholesterol levels in moderately hypercholesterolemic humans, it needed to contain at least ~35 mg total isoflavones/d [19]. No plasma or urine isoflavone contents were determined in that study, and it would be useful to know how well correlated the body fluid isoflavone contents were with cholesterol-lowering effects. Several recent studies have examined relationships among body fluid isoflavones, soy intake, and cancer risk. Dietary questionnaires and interviews assessed soy food intake in a subset of 147 subjects in the Singapore Cohort Study of more than 51 000 individuals. Mean age of

Table 1
Comparison of isoflavone analytical methods

Method	Samples	Detection limits	Special considerations	Refs.
HPLC–UV detection	Urine, plasma, feces	2 ng/ml for daidzein, genistein 200 ng/ml for equol	Equol not readily detectable, standards readily available	[9,10,12,13]
HPLC–coulometric detection	Urine	1 ng/ml for all isoflavones		[14]
GC–MS	Urine, plasma	0.6 ng/ml for all isoflavones, estimated from detection limit of 5 nmol/24 h urine sample	Requires silyl derivatization, ^{14}C-estrone glucuronide and deuterated isoflavone internal standards	[18]
LC–MS	Plasma, tissues	10 ng/g	Requires deuterated internal isoflavone standards	[16]
Time-resolved immunofluorescence	Urine	0.1 ng/ml	Ready screening of large numbers of samples of small sample size (<200 μl), requires antibodies to isoflavones and europium-labeled isoflavones	[17]

subjects (about half men and half women) was 56 years. When frequency of overall soy intake was grouped into those consuming 0–1, 2–3 or 4–5 servings/week, particularly daidzein and total isoflavones excreted in urine showed a significant positive relationship with soy food intake [20]. Soy food intake of 60 women from the control group of the Shanghai Breast Cancer Study was assessed by questionnaire and interview, and isoflavone intake estimated from food composition data previously published. Isoflavone intake was positively associated with daidzein, genistein and total isoflavone excretion in spot urine samples from these subjects [21]. There was a significant positive linear relationship between isoflavone excretion and isoflavone intake, when intake was grouped into three categories. A dietary questionnaire about soy intake was given to 102 women of various ethnic groups in Hawaii and urinary isoflavone samples were analyzed [22]. 24-h soy and isoflavone intake recalls were positively correlated with urinary isoflavone contents ($r=0.61–0.62$). Chinese subjects reported the greatest isoflavone intake during the previous day, but had twofold less isoflavone excretion (nmol/h) than did Japanese subjects, who reported 20% less isoflavone intake than did Chinese in the previous 24 h. Filipino and Caucasian subjects reported similar low isoflavone intakes, compared with Chinese and Japanese, but Caucasians excreted twice as much isoflavone in urine than did Filipinos. Major differences in isoflavone intakes could be discerned by urinary isoflavone excretion, but more subtle differences in isoflavone intakes would be hard to observe by examining urinary excretion. This may be partly due to great interindividual variation in isoflavone bioavailability (perhaps at least tenfold [23]). Five-day diet records and food frequency questionnaires assessed soy food and vegetable and fruit intakes of 98 subjects (half men and half women) [24]. Urinary isoflavones were measured in 72-h samples. Soy food intake was significantly positively associated with urinary isoflavone excretion (r~0.4 for all isoflavones measured, except that no significant association was observed between equol excretion and soy intake). Intakes of fruits and vegetables were not associated with isoflavone excretion. This supports the concept that isoflavone excretion is a signature of soy intake, but the weak correlation coefficients indicate great interindividual variability in isoflavone bioavailability. In a study of 60 case-control pairs of subjects from the Shanghai Breast Cancer Study, urinary excretion of isoflavones from breast cancer patients (urine collected before cancer therapy was initiated) was significantly less than in controls by about one-third [25]. This suggests that a habitual diet including more soy is breast cancer protective. One study attempted to assess whether urinary isoflavone excretion (and hence dietary soy intake) was inversely associated with post-menopausal bone loss [26]. Thirty-two women with bone loss of $<0.5\%$ were compared with 35 women with bone loss of $>2.5\%$ per year over the first 5 years of a 10-year study. Isoflavones were determined in yearly aggregated urine samples, and this did not differ between the groups. Equol excretion was weakly but positively and significantly associated with bone loss during the first 5 years of the study, but not with bone loss over the entire 10 year period. Thus, isoflavones were not protective against bone loss. The ability to produce equol is dependent on adaptation of gut microflora to the presence of daidzein in the diet. This finding deserves further study. Very little is known yet about the effects of isoflavones on bone metabolism. In summary, urinary isoflavones are sufficiently positively associated with soy food intake that measuring these compounds has moderate utility as a biomarker for studying some types of cancer risk reduction. Isoflavones are biomarkers for other types of chronic disease risk remains to be studied sufficiently. Great interindividual variability in apparent isoflavone absorption limits the utility of urinary isoflavone measurements as a valid biomarker of isoflavone or soy food intake to studies of very large numbers of subjects comparing populations with relatively great differences in their soy intake.

4. Comparative isoflavone kinetics: influence of isoflavone form and background matrix

The kinetics of isoflavone metabolism may determine what dosing regimens and isoflavone forms are needed to achieve desirable effects while avoiding adverse effects. This remains to be seen, but a few recent studies have examined the kinetics of isoflavones and key metabolites in greater detail than previously reported. Seven healthy Japanese men,

average age 33 years, consumed kinako (baked soybean powder, 60 g, containing 26 mg daidzein and 30 mg genistein) in a single meal after abstaining from isoflavone-containing foods for 6 days [27]. Mean peak plasma daidzein was significantly less than that for genistein, but urinary daidzein excreted was about twice as much as genistein. Two of seven subjects excreted equol in amounts similar to amounts of daidzein excreted in urine. All other subjects excreted only very small amounts of equol. Two of seven subjects, different from the equol excretors, excreted only small amounts of ODMA, but the other five subjects excreted about 1/6th as much ODMA as daidzein. Plasma genistein and daidzein peaked at about 6 h after soy feeding, with a small second peak at about 12 h in some subjects, with isoflavone levels returning to baseline by 48 h, for most subjects. These results confirm previous results in general [10,23], except that Watanabe et al. [27] showed greater plasma genistein than daidzein. This was also noted by Zhang et al. [12], when slightly greater amounts of genistein than daidzein were fed in a single meal, but the dietary proportion of genistein to daidzein of 1.0:0.9 was less than the plasma proportion of genistein to daidzein of 1.0: 0.6. Setchell et al. [28] fed 50 mg purified genistein, genistin, daidzein, or daidzin to groups of 3–6 women. The areas under curve (AUC) for each compound, indicating overall absorption based on plasma concentrations over time, was about 3 μg/(ml h) for daidzein, and about 5 μg/(ml h) for genistein, genistin and daidzin. The time to reach maximal plasma concentration was longer for the isoflavone glucosides than for the aglycones, as deconjugation was needed before absorption of aglycones derived from the glucosides. There has been no observation of absorption of intact isoflavone glucosides. A number of manufacturers are creating fermented isoflavone products, which contain predominantly isoflavone aglycones, whereas most soybean foods contain mostly isoflavone glucosides. The work of Setchell et al. [28] supports the view that genistin is equal to genistein in bioavailability, and daidzin may be more bioavailable than daidzein, in terms of plasma concentrations after a single dose. Piskula et al. [29] showed that daidzein and genistein were absorbed more rapidly than their glucosides, which were observed in blood plasma either a few minutes later than the aglycones or not observed in blood plasma within 30 min after administration, whereas the aglycones were observed in plasma almost immediately after oral dosing. It seems likely that the stomach does not contain enzymes that can hydrolyze isoflavone glucosides, nor are conditions of time and pH sufficient to permit nonenzymatic hydrolysis of isoflavone aglycones. As Setchell et al. [28] indicate, this difference in speed of absorption of isoflavone aglycones vs. glucosides does not disfavor overall absorption of isoflavone glucosides.

The effects of food matrix on isoflavone bioavailability have not been examined in much detail. Three randomized cross-over design studies by our laboratory address this point. Although a diet containing 40 g fiber mostly from wheat significantly decreased overall urinary excretion of genistein and plasma genistein at 24 h after soy feeding (but not at 6 h after soy feeding) compared with a control diet containing 15 g dietary fiber, this decrease was modest (genistein excretion was lesser by about 1/3 after feeding the high fiber diet), and probably of little significance to genistein efficacy [30]. A high fiber diet had no effect on daidzein bioavailability. We also examined relative isoflavone bioavailability when isoflavones were provided from single meals of whole cooked soybeans, tofu, tempeh or tofu, and observed no differences in urinary disposition of isoflavones with type of soy food. We also examined urinary isoflavone disposition when subjects consumed soy milk at each meal, and when meals were provided by us compared with subjects eating ad libitum or self-selecting their meals but eating at regimented times. No differences in isoflavone bioavailability, reflected in urinary excretion of isoflavones, were observed among these different dietary regimens [31]. Based on available data, neither the form of isoflavone ingested nor the background diet or food matrix significantly alter the apparent absorption of these compounds.

5. Isoflavone metabolites: influence of host and gut microbial biotransformation

Due to their phenolic nature, isoflavones are candidates for rapid biotransformation at hydroxyl groups by mammalian UDP-glucuronosyltransferases and sulfotransferases in the intestinal mucosa, liver

and other organs. Isoflavone glucuronides and sulfates are rarely measured. Standards of these metabolites are not readily available and toxicological dogma suggests that such metabolites are inactive. But there are examples of glucuronide and sulfate conjugates possessing significant bioactivity. Our laboratory has enzymatically synthesized daidzein and genistein-7-*O*-glucuronides and quantified these metabolites in women after isoflavone feeding, as well as characterizing effects of various isoflavone metabolite mixtures on human natural killer cell activity in vitro [32]. In experiments feeding women single soy meals or soy for 6 days, glucuronide conjugates made up about 70–80% of urinary isoflavones and 50–60% of plasma isoflavones, whereas isoflavone aglycones made up 5% of urinary isoflavones and 20–30% of plasma isoflavones (Fig. 1). The glucuronides were measured directly in comparison with standard glucuronides we prepared, or indirectly after enzymatic cleavage with a pure glucuronidase (not contaminated with sulfatase). The glucuronides predominated to the same extent when single meals were fed or when soy was fed for several consecutive days (data not shown). The glucuronides should be further examined for their biological effects. A mixture of isoflavone aglycones and glucuronides created to simulate plasma isoflavone contents after soy feeding (3:1:0.3:0.1 of daidzein glucuronide–genistein glucuronide–daidzein–genistein) caused enhancement of human natural killer cell activity in vitro similar to each aglycone or glucuronide alone in the same total concentration [33]. Thus, the effects of the isoflavone metabolites were strictly additive. Chang et al. [16]

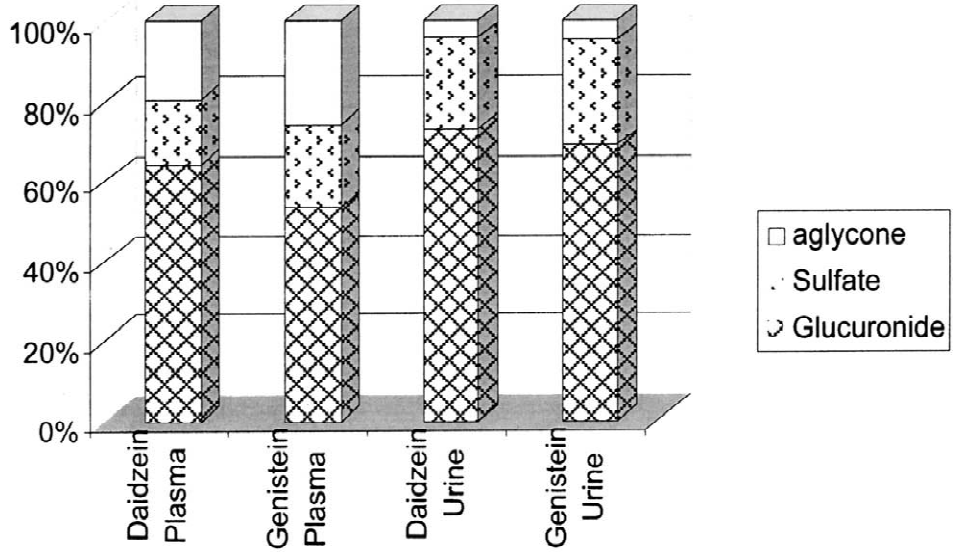

Fig. 1. Percentages of glucuronide and sulfate conjugates and isoflavone aglycones in urine and plasma from women fed soymilk isoflavones. These data are combined from three studies [33] in which women were fed 20 mg daidzein and 25 mg genistein (doses expressed as aglycone equivalents) per day from 25 g soymilk powder given diluted with beverages at breakfast. In study 1, six women were fed soymilk on 2 single days, separated by a 1 week wash-out period. In study 2, five women were fed soymilk on 1 day. In study 3, five women were fed soymilk for 6 consecutive days. Urine was collected for 24 h after breakfast on each day in studies 1 and 2, and on days 5 and 6 of soymilk feeding for 24 h in study 3. Blood was collected at 3 h after breakfast on each of these days for plasma isoflavone analysis. No significant difference was observed in 24 h urinary isoflavone contents across the three studies, nor were 3 h plasma isoflavone contents different across the 3 studies. Samples were analyzed after hydrolysis with β-glucuronidase/sulfatase (H_2 type) or β-glucuronidase (B_3 type). Free aglycone isoflavones were analyzed without hydrolysis. Glucuronide was calculated as (isoflavone aglycone after B_3 hydrolysis−isoflavone aglycone without hydrolysis)×100/[total isoflavone (after H_2 hydrolysis)]. Sulfate was estimated by difference as (total isoflavone after H_2 hydrolysis−isoflavone aglycone after B_3 hydrolysis−isoflavone aglycone without hydrolysis). The percentage of glucuronide form of both daidzein and genistein in urine were significantly higher than in plasma ($P=0.05$ and 0.001, respectively). The percentages of daidzein and genistein aglycones in plasma were significantly higher than in urine ($P=0.001$).

indirectly characterized the extent of genistein glucuronide and sulfate conjugates in rat tissues after feeding varying genistein doses, by comparing the amounts of genistein in tissues before and after glucuronidase/sulfatase treatment. Very small amounts of free genistein (aglycone) could be measured with their LC–ES-MS method. Although tissue concentrations of genistein were very small, the percentage of genistein aglycone (of total genistein) ranged from 10 to 100% in males (10% in testes, 100% in brain) and 20–100% in females (20% in thyroid, 100% in uterus). It is possible that very small amounts of this compound, especially its aglycone form, might exert significant effects. The apparent tissue differences in isoflavone metabolism could be important in explaining tissue specificity of isoflavone actions. This remains to be established in vivo. But, for example, genistein and daidzein glucuronides and aglycones have modest natural killer cell activating activity in concentrations of 0.1 μM in vitro [32]. Isoflavone glucuronides and sulfates deserve further study with respect to their bioactivities. But from our set of studies with a few subjects (Fig. 1), glucuronide and apparent sulfate conjugate formation seems to be less variable among subjects than is overall isoflavone absorption.

Not only mammalian but gut microbial metabolism of isoflavones may affect ultimate bioactivities of isoflavones. Equol and ODMA are gut microbial metabolites of daidzein. Only about 1/3 of subjects produce equol, and ODMA production also seems to be variable [27]. Gut microbial metabolites of genistein and glycitein are not well-characterized. Our laboratory showed that overall bioavailability of genistein and daidzein seemed to vary significantly among human subpopulations, characterized as low and high genistein excretors, apparently determined by the extent of gut microbial degradation of isoflavones [3,23,34]. Recently Zheng [35] showed that among 35 Chinese (recent immigrants to the US) vs. 33 Caucasian women, the Chinese subjects had a greater proportion of high isoflavone degraders (25 high degraders vs. 10 low degraders) than did the Caucasians (18 high degraders vs. 15 low degraders). Degradation phenotype was measured by in vitro incubation of fresh human fecal samples with isoflavones in anaerobic Brain–Heart Infusion media, followed by HPLC analysis. Daidzein degradation in high degraders had a half-life of about 3 vs. >12 h in low degraders. Dietary and physical activity questionnaires were administered to these subjects, and gut transit time was measured, as well as plasma, urine and fecal isoflavones after a single dose of soy milk. Although the Chinese subjects ate more red meat and less dairy and had more rapid gut transit time than did Caucasians, the only difference that distinguished high and low isoflavone degraders was gut transit time (GTT), and then only among Chinese subjects. Chinese subjects who were low degraders of genistein also had threefold greater bioavailability of genistein than did Chinese high degraders (6 vs. 2% of ingested dose excreted in urine over 24 h after isoflavone dosing) and the Chinese who were low isoflavone degraders had GTT averaging 40 vs. 65 h for Chinese high degraders. All of the Caucasian subjects, regardless of isoflavone degradation phenotype, had GTT$>$80 h, and all showed genistein bioavailability of about 2% of ingested dose excreted in urine. Thus, gut transit time may alter isoflavone bioavailability in interaction with isoflavone degradation phenotype. The longer the isoflavones are in the gut, the more opportunity for isoflavone degrading organisms to act, and the less isoflavones available for absorption. Isoflavone degradation products and degrading microorganisms remain to be characterized. Gut microbial ecology and gut transit time might exert important influences on bioavailability of other compounds related to isoflavones, such as phenolics in general. This may be an important topic for further investigation, in order to fully characterize biological effects of dietary phenolics and interindividual variability in response to such compounds.

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