JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Stability and Bioaccessibility of Isoflavones from Soy Bread during In Vitro Digestion

KELLY R. WALSH,[†] YU CHU ZHANG,[‡] YAEL VODOVOTZ,[‡] STEVEN J. SCHWARTZ,[†,‡] AND MARK L. FAILLA*,[†,§]

Interdisciplinary Ph.D. Program in Nutrition, Department of Food Science and Technology, and Department of Human Nutrition, The Ohio State University, Columbus, Ohio 43210

The impact of simulated digestion on the stability and bioaccessibility of isoflavonoids from soy bread was examined using simulated oral, gastric, and small intestinal digestion. The aqueous (bioaccessible) fraction was isolated from digesta by centrifugation, and samples were analyzed by high-performance liquid chromatography (HPLC). Isoflavonoids were stable during simulated digestion. Partitioning of aglycones, acetylgenistin, and malonylgenistin into the aqueous fraction was significantly ($P < 0.01$) affected by the concentration of bile present during small intestinal digestion. Omission of bile resulted in nondetectable genistein and <40% of total daidzein, glycitein, and acetylgenistin in the aqueous fraction of digesta. Partitioning of these compounds into the aqueous fraction was increased by physiological concentrations of bile extract. These results suggest that micellarization is required for optimal bioaccessibility of isoflavonoid aglycones. We propose that the bioavailability of isoflavones from foods containing fat and protein may exceed that of supplements due to enhanced bile secretion.

KEYWORDS: Isoflavones; soy; bioavailability; bioaccessibility; digestive stability; in vitro digestion; bile; soy bread; micelle

INTRODUCTION

Numerous studies suggest that estrogen-like isoflavonoids present in soy foods promote cardiovascular, skeletal, and postmenopausal health (*1*−*3*). Some data also suggest that these compounds may hinder the progression of certain forms of cancer, although further studies are needed to address the efficacy of isoflavonoids as chemopreventive agents (*4*). Unfortunately, consumption of soy foods by individuals eating a typical western diet remains low. The development of soy-enriched foods represents one strategy for increasing soy consumption. We have recently developed a soy-enriched bread that contains sufficient soy protein per serving to meet the FDA-approved health claim (*5*). The potential health-promoting activity of the bread is in part dependent upon the absorption of its isoflavonoids and their bioactive metabolites.

Isoflavonoids in soy products exist in either the free form (aglycone) or as the acetyl, malonyl, or β-glucoside conjugates. The content of isoflavonoids varies from approximately 0.1 to 3.0 mg/g dry weight among soybean products (*6*−*8*). Conjugation patterns of the isoflavonoids are significantly affected by style and duration of cooking, as well as interactions with other ingredients (*9*). For example, the predominant isoflavonoids in soy flour are the malonyl glucosides. Baking at 190 °C does not alter the total isoflavone content in soy flour, but slight increases in β-glucoside conjugates with proportional declines in the malonyl glucosides of isoflavonoids have been reported (*10*). Malonyl glucoside isoflavonoids are also degraded more rapidly during cooking in the presence of sugar and fat (*10*).

The bioavailability and bioactivity of soy isoflavonoids depends to some extent on the quantity consumed, chemical speciation, and the physical properties of soy foods (*11*, *12*). Previous studies have demonstrated that the aglycone, but not glycosylated isoflavonoids, are transported across the apical membrane of absorptive epithelial cells in the gastrointestinal tract (*13*, *14*). β-Glucosidase activity along the brush border surface in the small intestine and resident microflora in the large intestine convert isoflavonoid glucosides to their aglycone derivatives (*15*, *16*). Within mucosal epithelial cells, isoflavonoids are extensively modified to glucuronide and sulfate conjugates (*17*, *18*). These conjugated compounds can be transferred to the plasma or exported to the intestinal lumen, which confounds determination of their bioavailability (*19*).

Bioaccessibility characterizes the potential of a dietary compound for apical uptake by absorptive epithelial cells, whereas bioavailability generally describes its utilization following transport across the brush border and basolateral membranes (*20*). Information pertaining to factors affecting both the bioaccessibility and the bioavailability of dietary isoflavonoids is limited. The specific aims of the present study were to assess the digestive stability and bioaccessibility of isoflavonoids from a soy-rich bread. Simulated oral, gastric, and small

* To whom correspondence should be addressed. Tel: (614)292-4485. Fax: (614)292-8880. E-mail: failla.3@osu.edu.
[†] Interdisciplinary Ph.D. Program in Nutrition.
[‡] Department of Food Science and Technology.
[§] Department of Human Nutrition.

10.1021/jf0342627 CCC: $25.00
Published on Web 07/03/2003

intestinal phases of digestion were used to determine digestive stability. Bioaccessibility was determined by examining the partitioning of isoflavonoids into the aqueous fraction of digesta. The potential role of micelles as delivery vehicles for aglycones was examined by varying the concentration of bile during the small intestinal phase of the in vitro digestion procedure.

MATERIALS AND METHODS

Supplies. Porcine pepsin, porcine lipase, porcine pancreatin, porcine bile extract, β-glucosidase (almond), daidzein (4′,7-dihydroxyisoflavone), genistein (4′,5,7-trihydroxyisoflavone), and glycitein (4′,7-dihydroxy-6-methoxyisoflavone) were purchased from Sigma Chemical Co. (St. Louis, MO). Daidzin (daidzein, 7-*O*-β-D-glucopyranoside), genistin (genistein, 7-*O*-β-D-glucopyranoside), acetyldaidzin (6″-*O*-acetyldaidzin), malonyldaidzin (6"-*O*-malonyldaidzin), acetylgenistin (6″-*O*-acetylgenistin), malonylgenistin (6"-*O*-malonylgenistin), acetylglycitin (6"-*O*-acetylglycitin), and malonylglycitin (6"-*O*-malonylglycitin) were purchased from LC Laboratories (Woburn, MA). Other reagents were purchased from Fisher Scientific Co. (Fairlawn, NJ); reagents used for high-performance liquid chromatography (HPLC) analysis were HPLC grade. Soy flour was purchased from Archer Daniel Midland Co. (Decatur,IL), soy milk powder was purchased from DevanSoy Farms (Carroll, Iowa), and wheat flour was purchased from General Mills (Minneapolis, MI).

Soy Bread (SB) Preparation. Soy-rich bread was developed and produced in our laboratory by incorporating soy milk powder into a standard bread formula and replacing 29% of the wheat flour with soy flour. The bread ingredients were combined, and the dough was kneaded in a 5 quart KitchenAid Mixer (KitchenAid Portable Appliance, St. Joseph, MI), proofed at 50 °C for 1 h in an oven (Blue M Electric Company, Blue Island, IL), and baked at 165 °C for 50 min. The baked SB was allowed to cool at room temperature for 40 min. The macronutrient composition of the SB was 222 mg/g carbohydrate, 22 mg/g fat, 190 mg/g protein, and 447 mg/g moisture. The crust was removed, and crumb samples were taken from the center of each loaf. Samples were stored in sealed polypropylene tubes at −20 °C under nitrogen until analysis.

Preparation of Diluted SB Sample for Reference Starting Material. It was assumed that 1.0 g of SB was equivalent to a 1.0 mL volume for indicated manipulations. Homogenized SB in saline was prepared as a control for the investigation of the effects of in vitro digestion on isoflavonoid stability. SB (10 g) and saline (100 mL) were combined in a 250 mL beaker. The bread was homogenized with a Tissumizer batch dispersing rotor-stator (SDT1800, Tekmar Company, Cincinatti, OH) and dispersing probe (S25KR18G, IKA-Werk, Wilmington, NC) for 30 s at a setting of 85. After the sample was homogenized, the dispersing probe was submerged in clean saline and engaged for 3 s to collect the residual food sample, which was added to the homogenized SB. The beaker used for collection of the residual food sample was rinsed with saline (30 mL), and the contents were pooled with the homogenized SB. The final concentration of the homogenized sample was 50 mg SB/mL. Aliquots (50 mL) were stored at −20 °C under nitrogen until analysis.

In Vitro Digestion. *Oral Phase.* Bread samples were subjected to a modified version of the in vitro digestion method previously described by Garrett et al. (*21*). The primary modification involved the addition of an oral phase to the simulated gastric and small intestinal phases of digestion. The oral phase of digestion was performed in a manner similar to that described by Muir and O'Dea (*22*). Briefly, a single investigator conducted the oral phase for all in vitro digestions to control for potential interindividual variation in saliva composition. After the oral cavity was rinsed with deionized water, 12.5 g of SB (22 °C) combined with 10 mL of saline (37 °C) was chewed 10 times and subsequently expelled into a tared beaker. The oral cavity was rinsed twice with 10 mL of saline (37 °C), and the contents were expelled after each rinse into the beaker containing chewed SB. Saline (45 mL; 37 °C) was added, and the sample was stirred for 5 min at room temperature. The sample was homogenized as described in the reference SB sample preparation and diluted to a final volume of 175 mL with saline. An aliquot (35 mL) was transferred to a centrifuge tube, diluted to 50 mL with saline (50 mg SB/mL), and stored at −20 °C under nitrogen until analysis for determination of the possible impact of the oral phase of digestion on the chemical profile of isoflavonoids in SB.

Gastric and Small Intestinal Phases. The remaining sample from the oral phase of digestion was acidified with 1 mol/L HCl (pH 2.0 ± 0.1). Porcine pepsin (10 mg/mL final concentration) in 100 mmol/L HCl was added, and the sample was diluted to a final volume of 160 mL with saline. Aliquots (40 mL) were transferred to separate 50 mL tubes, blanketed with nitrogen, sealed, and incubated in a shaking (85 rpm) water bath (Versa-Bath S model 224, Allied Fisher Scientific, IN) at 37 °C. After 1 h, the reaction tubes were placed on ice and the pH was immediately increased to 6.0 ± 0.2 with 1 mol/L $NaHCO_3$. Porcine bile extract (2.4 mg/mL final concentration), porcine pancreatin (0.4 mg/mL final concentration), and porcine pancreatic lipase (0.2 mg/mL final concentration) in 100 mmol/L $NaHCO_3$ were added to the reaction tubes. The pH of each tube was increased to 6.9 ± 0.1 with 1 mol/L NaOH, and the final volume was diluted with saline to 50 mL (equivalent to 50 mg SB/mL). All tubes were blanketed with nitrogen, sealed, and incubated in a shaking (85 rpm) water bath for 2 h at 37 °C.

Preparation of the Aqueous Fraction of Digesta. Following the small intestinal phase of digestion, aliquots (10 mL) of digesta were transferred to 15 mL conical tubes and stored at −20 °C under nitrogen until analysis for digestive stability of SB isoflavonoids. Aliquots (12 mL) of digesta were also transferred to Quick-Seal ultracentrifuge tubes (Beckman Instruments, Inc., Palo Alto, CA), heat-sealed (Beckman Instruments, Inc.), and centrifuged at 167 000*g* for 35 min at 4 °C (Beckman L7 ultracentrifuge; 50 Ti rotor). After centrifugation was completed, tubes were placed on ice and the aqueous fraction of the digesta was recovered using a syringe with an 18 gauge needle (Becton Dickinson and Company, Franklin Lakes, NJ). A minimum of 5 mL of each aqueous sample was filtered (0.2 μm Acrodisc syringe filter, Gelman Laboratory) and stored in sealed 15 mL conical tubes at −20 °C under nitrogen until analysis.

Effect of Bile Extract on Isoflavonoid Bioaccessibility. The effect of concentration of bile extract on isoflavonoid bioaccessibility was addressed by varying the amount of bile extract added to initiate the small intestinal phase of digestion. The final concentrations of bile extract examined were 0, 1.2, 2.4 (standard amount (*21*)), and 4.8 mg/mL.

Extraction of Isoflavonoids from Samples. *SB Sample.* SB (0.5 g) was ground to a fine paste and mixed with 100 mmol/L HCl (2 mL), acetonitrile (10 mL), and water (3 mL) in a 50 mL centrifuge tube (*23*, *24*). The mixture was shaken with a multiwrist shaker (Labline Instrument Inc, Melrose Park, IL) on setting 9 for 2 h before centrifuging (IEC HN-SII, Damon/IEC Division, Needhamhts, MA) at 430*g* for 30 min. An aliquot (1 mL) of the supernatant was then transferred to a glass vial, dried under nitrogen at room temperature, and resolubilized in 100% methanol (1 mL) (*25*). The mixture was vortexed (model no. 231, Fisher Scientific, Fair Lawn, NJ) and filtered (0.2 μm syringe filter, Alltech Associates Inc, Deerfield, IL) prior to HPLC injection. The reliability of the extraction method was assessed by extracting after spiking digestion samples with known concentrations of pure isoflavonoids and determining their recovery. Recoveries for all test isoflavonoids exceeded 97%.

Digested SB Fractions. Postoral, small intestinal digesta, and aqueous fractions of digesta from simulated digestion of SB were thawed at room temperature and vortexed for 1 min. Aliquots (5 mL) were transferred to 50 mL centrifuge tubes and mixed with 100 mmol/L HCl (2 mL) and acetonitrile (5 mL). Samples were extracted as described above. Extracts were immediately filtered (0.2 μm syringe filter, Alltech Associates Inc.) and analyzed using HPLC.

HPLC Analysis. A Waters 2695 separations module (Milford, MA) and a Waters 2996 photodiode array detector (PDA) were used to quantify isoflavone content. Separation of isoflavonoids was achieved using a Waters Nova-Pak C_{18} reversed-phase column (150 mm × 3.9 mm i.d., 4 μm, 60 Å pore size) with a Nova-Pak C_{18} guard column. The mobile phase consisted of 1.0% acetic acid in water (v/v) (solvent A) and 100% acetonitrile (solvent B) at a flow rate of 0.6 mL/min. The injection volume was 10 μL, and components were eluted using the following solvent gradient: from 0 to 5 min 15% B; from 5 to 36

Table 1. Isoflavone Mass Balance in Soy Ingredients and SB[a]

			ingredients		recoveries[b]	
isoflavonoid	soy flour (nmol/g dry weight)	soy milk powder (nmol/g)	total (nmol/g SB)	SB (nmol/g SB)	individual compounds (%)	family (%)
daidzin	538.5 ± 43.6	874.9 ± 50.9	295.3	298.3 ± 35.5	101	96
malonyldaidzin	815.4 ± 10.6	321.0 ± 38.2	328.1	252.6 ± 0.75	77	
acetyldaidzin	236.0 ± 3.3	85.1 ± 1.2	94.0	42.1 ± 1.2	45	
daidzein	51.5 ± 3.5	534.9 ± 18.5	81.7	175.8 ± 2.3	215	
genistin	1211.0 ± 44.8	1929.6 ± 40.2	659.6	482.2 ± 5.3	73	86
malonylgenistin	1280.0 ± 25.6	589.3 ± 6.0	525.2	403.9 ± 4.0	77	
acetylgenistin	96.3 ± 0.6	64.9 ± 6.4	41.9	39.2 ± 1.4	94	
genistein	92.9 ± 1.9	1140.5 ± 75.8	168.3	271.6 ± 7.9	161	
glycitin	nd[c]	nd	nd	nd	nd	105
malonylglycitin	133.2 ± 6.0	31.7 ± 0.8	51.1	41.1 ± 0.29	80	
acetylglycitin	86.3 ± 3.8	69.8 ± 1.3	38.9	4.09 ± 0.32	11	
glycitein	8.4 ± 0.6	48.2 ± 4.0	8.7	58.4 ± 0.64	671	
total	4549.5	5689.9	2292.8	2069.3	90	

[a] SB was prepared using 249 g of soy flour and 83 g of soy milk powder (dry weight). The final weight (dry) of the loaf was 700 ± 42 g. [b] Recovery refers to the percentage of indicated compounds contributed by the ingredients that are present in the final SB product. [c] nd, not detected.

min 15−29% B; from 36 to 44 min 29−35% B; from 44 to 45 min 35 to 15% B; and reequilibration at 15% B for 5 min prior to the next injection (*26*). The PDA monitored a spectral range of 210−400 nm, and compounds in the eluate were detected from their absorbance at 260 nm.

Isoflavonoid Identification. Retention times and UV absorption patterns of pure isoflavonoid standards were used to identify isoflavonoids in samples following extraction and HPLC analysis (*23, 24*). Identification of aglycones was confirmed by monitoring changes in β-glucoside and aglycone peak areas following enzymatic hydrolysis with almond β-glucosidase (*27*). To do so, prior to drying the SB extract under nitrogen, an aliquot (1 mL) of SB extract was suspended in 1 mL of 100 mmol/L acetate buffer (pH 5.0) containing 1 mg of β-glucosidase. The mixture was incubated at 37 °C in a water bath shaker overnight (*23*). Concentrations of aglycones and β-glucosides were calculated from the standard curves of these compounds. Concentrations of acetyl and malonyl glucosides were calculated from the curves for the corresponding glucoside.

Standard Solutions and Calibration Curves. Stock solutions for the different isoflavonoids were prepared by dissolving 1 mg of the crystalline pure compounds in 80% methanol (100 mL). Each solution was then serial diluted with 80% methanol to prepare a standard curve. The concentration of working solutions was determined using the Beer−Lambert law with UV absorbance readings in the range of 240−360 nm and their molar extinction coefficients in 80% methanol: daidzein $\lambda_{max} = 254$ nm, $\epsilon = 26\,000$; genistein, $\lambda_{max} = 262$ nm, $\epsilon = 37\,300$; glycitein, $\lambda_{max} = 256$ nm, $\epsilon = 22\,387$ (*6, 28*). We applied the λ_{max} and ϵ values of the aglycones to their respective conjugates for mass calculations with the assumption that the absorption spectra of the conjugates are not significantly different from the representative aglycone (*29, 30*). Aliquots of working solutions were then analyzed by HPLC, and the relationship between HPLC peak area and concentration for each standard solution was calculated. These factors were used to calculate the concentrations of isoflavonoids present in the SB, oral phase, digesta, and aqueous fractions of digesta following HPLC analysis.

Statistical Analysis of Data. Four independent in vitro SB digestions were performed, and from each digestion, multiple oral ($N = 3$), digesta ($N = 4$), and aqueous fraction ($N = 4$) samples were analyzed for experiments addressing isoflavonoid stability. Independent digestions with variable concentrations of bile extract were performed, and aqueous fractions ($N \geq 5$) from each digestion were analyzed to characterize the effect on isoflavonoid bioaccessibility. All data are expressed as means ± SEM unless otherwise indicated. Means were compared using one way analysis of variance (ANOVA) followed by Bonferroni posthoc comparison of statistical significance. Linear regression was performed when the bile extract concentration was varied for assessment of bioaccessibility. An α level of 0.01 was established a priori, and results were considered statistically significant when $P < 0.01$. Statistical analyses were performed using SPSS Release 11.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Comparison of Isoflavone Profile of Ingredients and SB. The mass balance study demonstrated that 90% of total isoflavonoids from soy flour and soy milk powder were present in the SB product, indicating a general stability of isoflavonoids during processing (**Table 1**). This result is consistent with previous findings in which normal cooking conditions did not alter the total isoflavone content of soy foods (*31*).

The primary source of isoflavonoids in the SB is soy flour. Malonyl glucosides (49.0%) and β-glucosides (38.5%) were the prevalent isoflavonoids in soy flour with the aglycones representing less than 4% of the total isoflavone content. The isoflavone composition of soy milk powder differed in that β-glucosides (49.3%) and aglycones (30.2%) were most abundant with the malonyl and acetyl derivatives of daidzin and genistin accounting for only 22 and 17%, respectively, of the total. The differences in the isoflavonoid profile of the soy milk and soy flour likely result from altered processing of the beans (*32*). Soy flour is produced by grinding soybeans into powder, while preparation of soy milk powder involves cooking ground soybeans (95 °C, 7 min) followed by drying liquid soy milk (*33*). The significant water-holding capacity of soy proteins in soy flour may regulate the internal temperature of the dough during baking and protect some of the malonyl glucosides from heat-induced decarboxylation (*31*).

Analysis based on derivative families of isoflavonoids revealed that daidzin was the most stable glucoside with a recovery of 101%. However, the amounts of the three malonyl glucosides in SB were 20−25% lower than those in the starting ingredients. The amounts of acetyldaidzin and acetylglycitin decreased by 55 and 89%, respectively, during preparation of SB, whereas the amount of acetylgenistin was similar in the ingredients and SB. The decreases in malonyl and acetyl glucosides were associated with a nearly 2-fold increase in the amounts of daidzein, genistein, and glycitein in SB as compared to starting ingredients. The high recovery of the daidzein (96%) and glycitein (105%) families in SB from the starting ingredients suggests that the lost malonyl, acetyl, and β-glucosides were converted to their respective aglycone during preparation of SB. However, the 14% decline in total content of compounds in the genistein family suggests that some genistin, malonylgenis-

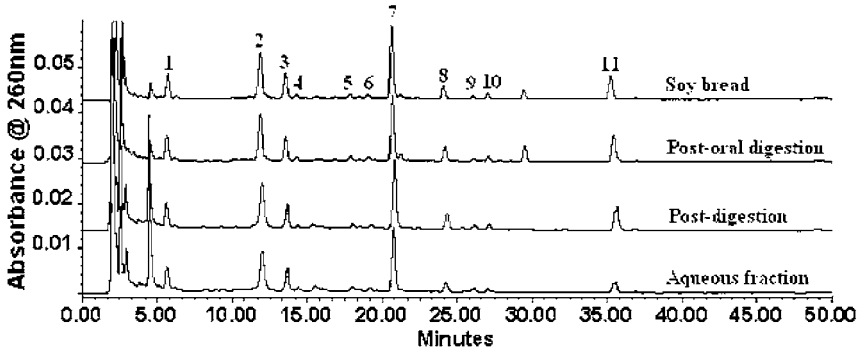

Figure 1. Reversed-phase HPLC analysis of isoflavonoids in starting and digested SB. SB was exposed to simulated oral, gastric, and small intestinal phases of digestion, and the aqueous fraction of digesta was prepared by high-speed centrifugation and filtration as described in the Materials and Methods. Representative chromatograms depict isoflavone profiles of extracts from SB and SB subjected to either oral or complete digestion and the aqueous fraction of the digesta. Peak identification for representative chromatograms are as follows: (1) daidzin, (2) genistin, (3) malonyldaidzin, (4) malonylglycitin, (5) acetyldaidzin, (6) acetylglycitin, (7) malonylgenistin, (8) daidzein, (9) glycitein, (10) acetylgenistin, and (11) genistein.

tin, acetylgenistin, and, perhaps genistein were degraded during SB preparation. Similar results have been observed in the recovery of genistein and its derivatives following heating (*34*, *35*).

Stability and Bioaccessibility of Isoflavonoids from SB During Simulated Digestion. Generally, the bioavailability of a dietary compound is dependent upon its digestive stability, accessibility for uptake across the apical surface of intestinal epithelial cells, and the efficiency of its transepithelial passage for delivery to peripheral tissues. To our knowledge, this is the first study addressing the stability of individual isoflavonoids during simulated digestion. In a recent paper by Andlauer et al. (*36*) on absorption and metabolism of isoflavones in isolated rat small intestine, it was stated that isoflavonoids from tofu were stable during in vitro digestion, although no data were provided. The results reported below address the digestive stability of isoflavonoids from SB, as well as their potential accessibility for cellular uptake.

Daidzein, genistein, their corresponding β-glucosides, and glycitein in SB remained stable during simulated oral, gastric, and small intestinal phases of digestion with greater than 95% recovery of initial amounts (**Figures 1** and **2**). Stabilities of genistein and genistin during simulated gastric digestion are in line with previous findings in which these particular isoflavonoids remained stable following acid incubation for 4 h (*37*). Decreases in malonyldaidzin (**Figure 2a**) and malonylglycitin (data not shown) were associated with slight increases in the amounts of daidzein (**Figure 2a**) and glycitein, respectively, in the digesta. This suggests that some of the glucoside derivatives are converted to their corresponding aglycones during digestion. Neither glycitin nor acetylglycitin were detected after any phase of simulated digestion (data not shown).

Bioaccessibility refers to the release of a dietary compound from its parent matrix for diffusion through the aqueous milieu bathing the apical surface of absorptive epithelial cells. The aqueous fraction of digesta contains both water soluble components and micellarized lipophiles (*21*). Isoflavonoid β-glucosides are more water soluble than their respective aglycones (*38*). The majority of daidzin (93.7 ± 2.3%), acetyldaidzin (71.4 ± 1.7%), malonyldaidzin (99.0 ± 2.1%), genistin (81.6 ± 1.3%), and malonylgenistin (86.4 ± 2.8%) was present in the aqueous fraction of the digesta following simulated digestion (**Figure 2**). In contrast, only 59.1 ± 2.3 and 33.0 ± 2.3% of daidzein and genistein, respectively, partitioned into the aqueous fraction following simulated digestion. The amount of glycitein (74.5 ± 2.3%) in the aqueous fraction of digesta exceeded that of malonylglycitin (58.8 ± 1.6%). The relatively high recovery

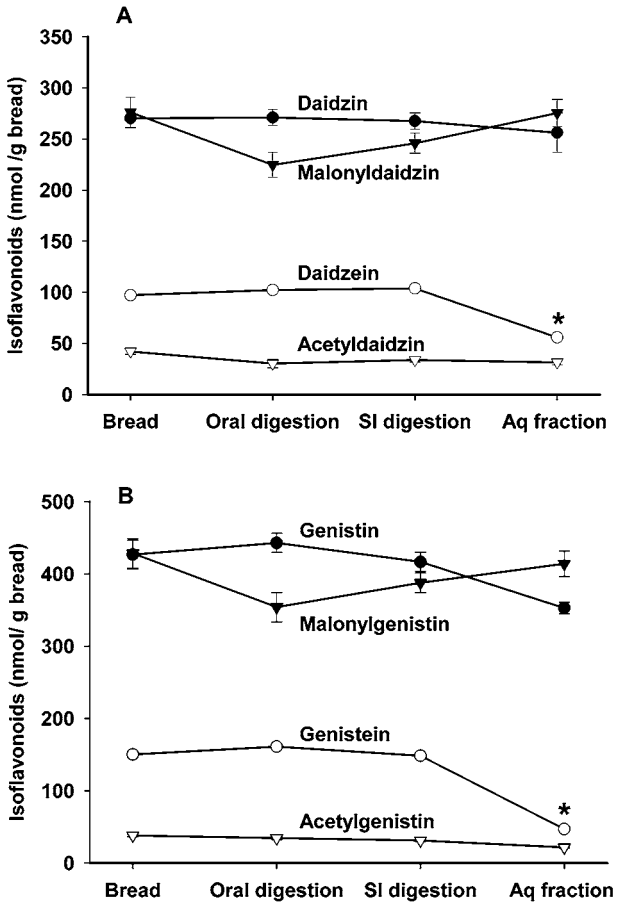

Figure 2. Stability of isoflavonoids in SB during in vitro digestion. Independent digestions of SB were performed, and samples were removed following completion of the oral and small intestinal phases of the procedure. The aqueous fraction of small intestinal digesta was prepared by high-speed centrifugation and filtration as described in the Materials and Methods. Daidzein and its glucoside conjugates (**A**) and genistein and its glucoside conjugates (**B**) in SB and the indicated samples were quantified by HPLC. Daidzein, genistein, and their derivatives were stable during simulated oral, gastric, and small intestinal digestion. Data are means ± SE for starting SB meal ($N = 12$), postoral digestion ($N = 12$), postsmall intestinal digestion ($N = 16$), and aqueous fraction of digesta ($N = 16$). The presence of an asterisk (*) above error bars indicates that means differ significantly ($P < 0.01$) from the starting SB meal.

of glycitein in the aqueous fraction of digesta may be explained by the possibility that more malonylglycitin actually partitioned

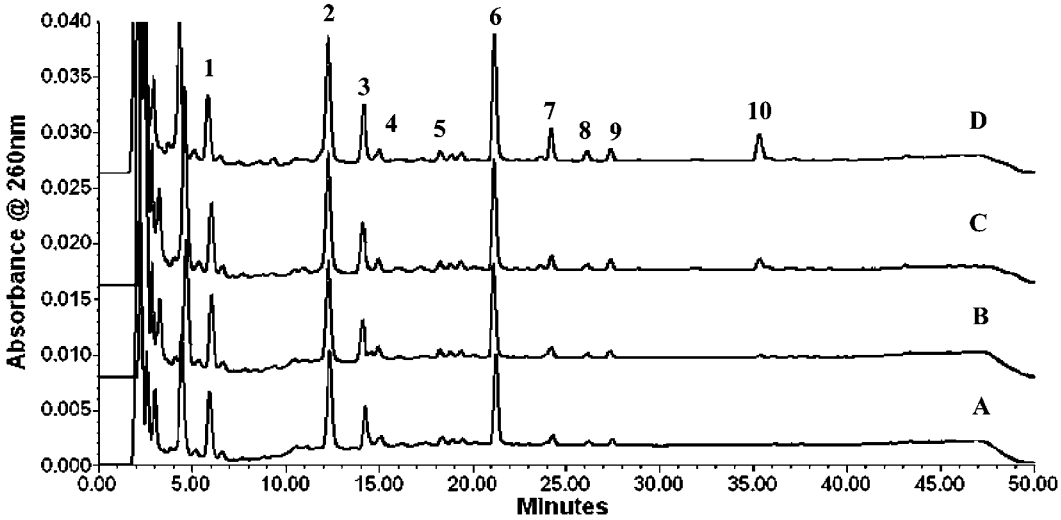

Figure 3. Effect of bile extract content during simulated digestion on isoflavone profile in the aqueous fraction of small intestinal digesta. Independent digestions were performed using 0 (**A**), 1.2 (**B**), 2.4 (**C**), and 4.8 (**D**) mg/mL of bile extract during the simulated small intestinal phase to investigate the effect on isoflavonoid bioaccessibility. Peak identification for representative chromatograms are as follows: (1) daidzin, (2) genistin, (3) malonyldaidzin, (4) malonylglycitin, (5) acetyldaidzin, (6) malonylgenistin, (7) daidzein, (8) glycitein, (9) acetylgenistin, and (10) genistein. Note that the area under the curve for daidzein, glycitein, acetylgenistin, and genistein in the aqueous fraction increases as the concentration of bile extract present during the small intestinal phase of digestion is increased.

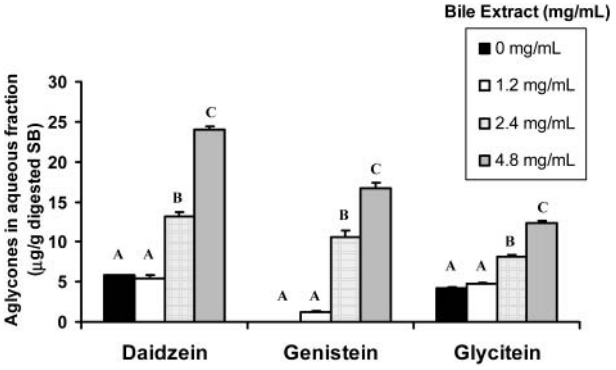

Figure 4. Bile extract increases partitioning of isoflavonoid aglycones from digested SB into the aqueous fraction. The concentration of bile extract regularly used during the small intestinal phase of in vitro digestion was 2.4 mg/mL ($N = 13$). Simulated digestions were also performed with bile extract concentrations at 0.0 ($N = 5$), 1.2 ($N = 5$), and 4.8 ($N = 9$) mg/mL. The aqueous fraction of digesta was prepared by high-speed centrifugation and filtration as described in the Materials and Methods. Means within an aglycone group that do not share a common letter as a superscript differ significantly ($P < 0.01$) from one another as determined by one way ANOVA followed by Bonferroni correction. Data are means ± SEM.

into the aqueous fraction before hydrolysis to its parent aglycone. We next examined the possibility that some of the daidzein, genistein, and glycitein was micellarized in the aqueous fraction of digesta due to the poor solubility of isoflavonoid aglycones in an aqueous matrix.

Representative chromatograms of isoflavonoids present in the aqueous fraction following simulated digestion with 0−4.8 mg/mL bile extract are shown in **Figure 3**. Omission of bile extract during small intestinal digestion resulted in the complete absence of genistein and only 26.1 ± 0.3% of daidzein and 38.9 ± 0.5% of glycitein present in the aqueous fraction of digesta (**Figure 4**). Increasing the concentration of bile extract to 1.2 mg/mL, i.e., 50% of the amount used in the standard digestion procedure, did not significantly ($P > 0.01$) affect the amounts of the aglycones present in the aqueous fraction of

Table 2. Effect of Bile Extract Content on Recovery of Isoflavonoid Glucosides in the Aqueous Fraction of Digested SB[a]

	bile extract concentration (mg/mL) during digestion % total present in the aqueous fraction of digesta			
isoflavonoid	0	1.2	2.4	4.8
daidzin	90.2 ± 2.1[b]	99.2 ± 2.5[b]	93.7 ± 2.3[b]	97.4 ± 1.9[b]
malonyldaidzin	95.0 ± 3.1[b]	90.1 ± 3.6[b]	99.0 ± 2.1[b]	101.6 ± 8.6[b]
acetyldaidzin	67.7 ± 2.3[b]	70.9 ± 2.1[b]	71.4 ± 1.7[b]	75.7 ± 1.7[b]
genistin	66.4 ± 1.7[b]	78.1 ± 2.5[c]	81.6 ± 1.3[c]	81.5 ± 0.7[c]
malonylgenistin	63.9 ± 1.9[b]	72.8 ± 2.2[b]	86.4 ± 2.8[c]	90.1 ± 2.1[c]
acetylgenistin	28.3 ± 0.5[b]	33.8 ± 2.3[b]	52.6 ± 1.3[c]	77.2 ± 1.2[d]
glycitin	nd	nd	nd	nd
malonylglycitin	59.1 ± 0.9[b]	58.6 ± 1.7[b]	58.8 ± 1.6[b]	60.6 ± 7.2[b]
acetylglycitin	nd	nd	nd	nd

[a] The standard concentration of bile extract during the small intestinal phase of in vitro digestion was 2.4 mg/mL ($N = 13$). Simulated digestions were also performed with bile extract concentrations at 0.0 ($N = 5$), 1.2 ($N = 5$), and 4.8 ($N = 9$) mg/mL. Means within a glucoside group that do not share a common letter as a superscript differ significantly ($P < 0.01$) from one another as determined by one way ANOVA followed by Bonferroni correction. Data are mean percentages ± % SEM; nd, not detected.

digesta. However, the quantity of each aglycone in the aqueous fraction increased significantly ($P < 0.01$) when the concentration of bile extract was elevated to 2.4 (concentration used in the standard procedure) and 4.8 mg/mL. These higher concentrations of bile extract are within the range present in the duodenal lumen postprandially (*39*). The amounts of the aglycones in the aqueous fraction of digesta were positively correlated ($R \geq 0.89$) with the concentration of bile extract during small intestinal digestion. The amounts of both acetylgenistin and malonylgenistin in the aqueous fraction of the digesta also were positively correlated ($R \geq 0.85$) with the concentration of bile extract during small intestinal digestion (**Table 2**). These data suggest that at least some portion of isoflavonoid aglycones, as well as the acetyl and malonyl conjugates of genistin, were incorporated into mixed micelles during small intestinal digestion. Murota et al. (*40*) demonstrated that daidzein and genistein had approximately a 2- and 5-fold greater affinity, respectively, for incorporation into liposomal

membranes in aqueous solution than their parent β-glucosides. Furthermore, the affinity of genistein for these large unilamellar vesicles was nearly twice that of daidzein. These findings support our hypothesis that micellarization enhances the bioaccessibility of aglycones during small intestinal transit.

Partitioning of daidzin, its malonyl and acetyl derivatives, and malonylglycitin into the aqueous fraction of digesta was not affected ($P > 0.01$) by changes in the concentration of bile extract during the small intestinal phase of simulated digestion (**Table 2**). Approximately 66% of genistin was present in the aqueous fraction of digesta when bile extract was omitted during small intestinal digestion. Addition of bile extract during the process significantly increased ($P < 0.01$) partitioning of genistin into the aqueous fraction to approximately 80%. The data also suggest that the acetylated derivatives of daidzin and genistin are less bioaccessible than their β-glucoside or malonyl conjugates at all concentrations of bile extract examined (**Table 2**). Recent observations suggest that a portion of isoflavonoid glucosides liberated from the food matrix may diffuse across the unstirred water layer and undergo brush border β-glucosidase activity (*15*, *16*). It is assumed that the generated aglycone then diffuses across the apical surface of the intestinal epithelium (*13*, *14*). Moreover, native flora in the distal small intestine and the large intestine convert isoflavonoid glucosides to aglycones and other bioactive metabolites with the potential for absorption (*8*, *37*, *41*−*43*). Setchell and colleagues (*19*) propose that the glucose moiety of isoflavonoid β-glucosides serves to protect the parent aglycone from microbial degradation during intestinal transit.

Information regarding the bioavailability of isoflavonoids from supplements as compared with that of soy foods is limited (*19*). We found that daidzein was more soluble in the aqueous fraction of digesta and, therefore, more accessible than genistein for potential uptake by absorptive epithelial cells. However, this only represents one of the factors that can affect bioavailability. Previous studies generally have demonstrated greater bioavailability of genistein than daidzein from soy foods or isoflavone supplements administered with a meal (*14*, *19*, *44*, *45*). This may be in part due to the greater lipophilic nature of genistein rendering it more effective in passive diffusion (*46*, *47*). In contrast, Xu et al. (*48*) reported that daidzein was more bioavailable than genistein in women and King (*12*) described similar findings in rats. Daidzein may be more accessible for uptake by enterocytes than genistein when the luminal concentration of bile is low (i.e., in the fasted state) in light of its greater solubility in the aqueous fraction of digesta.

In conclusion, daidzein, genistein, their corresponding β-glucosides, and glycitein in SB were stable during simulated oral, gastric, and small intestinal phases of digestion. The present study also suggests that micellarization during the small intestinal phase of digestion enhances the bioaccessibility of isoflavonoid aglycones and especially genistein. Ingestion of foods containing protein and lipids induces release of bile into the duodenum (*49*). Thus, it is possible that the bioavailability of isoflavonoids from foods containing fat and protein exceeds that of isoflavonoid supplements consumed without food. The findings and implications resulting from the described in vitro experiments must now be validated in human studies.

LITERATURE CITED

(1) Setchell, K. D. Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. *Am. J. Clin. Nutr.* **1998**, *68* (Suppl.), 1333S−1346S.

(2) Setchell, K. D. Soy isoflavones-benefits and risks from nature's selective estrogen receptor modulators (SERMs). *J. Am. Coll. Nutr.* **2001**, *20* (5), 354S−362S.

(3) Birt, D. F.; Hendrich, S.; Wang, W. Dietary agents in cancer prevention: flavonoids and isoflavonoids. *Pharmacol. Ther.* **2001**, *90*, 157−177.

(4) Fournier, D. B.; Erdman, J. W.; Gordon, G. B. Soy, its components, and cancer prevention: a review of the in vitro, animal and human data. *Cancer Epidemiol. Biomarkers Prev.* **1998**, *7* (11), 1055−1065.

(5) Food and Drug Administration, Department of Health and Human Services. Food Labeling: Health Claims; Soy Protein and Coronary Heart Disease. *Fed. Regist.* **1999**, *57*, 699−733.

(6) Coward, L.; Barnes, N. C.; Setchell, K. D. R.; Barnes, S. Genistein, daidzein, and their β-glucoside conjugates: antitumor isoflavones in soybean foods from American and Asian diets. *J. Agric. Food Chem.* **1993**, *41*, 1961−1967.

(7) Murphy, P. A. Phytoestrogen content of processed soybean product. *Food Technol.* **1982**, *36* (1), 60−64.

(8) Setchell, K. D.; Cassidy, A. Dietary isoflavones: biological effects and relevance to human health. *J. Nutr.* **1999**, *129* (3), 758S−767S.

(9) Xu, Z.; Wu, Q.; Godber, S. Stabilities of daidzin, glycitin, genistin, and generation of derivatives during heating. *J. Agric. Food Chem.* **2002**, *50*, 7402−7406.

(10) Coward, L.; Smith, M.; Kirk, M.; Barnes, S. Chemical modification of isoflavones in soyfoods during cooking and processing. *Am. J. Clin. Nutr.* **1998**, *68*, 1486S−1491S.

(11) Hendrich, S.; Fisher, K. What do we need to know about active ingredients in dietary supplements? Summary of workshop discussion. *J. Nutr.* **2001**, *131*, 1378S−1388S.

(12) King, R. A. Daidzein conjugates are more bioavailable than genistein conjugates in rats. *Am. J. Clin. Nutr.* **1998**, *68* (Suppl.), 1496S−1499S.

(13) Setchell, K. D.; Brown, N. M.; Zimmer-Nechemias, L.; Brashear, W. T.; Wolfe, B. E.; Kirschner, A. S.; Heubi, J. E. Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. *Am. J. Clin. Nutr.* **2002**, *76*, 447−453.

(14) Izumi, T.; Piskula, M. K.; Osawa, S.; Obata, A.; Tobe, K.; Saito, M.; Kataoka, S.; Kubota, Y.; Kikuchi, M. Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. *J. Nutr.* **2000**, *130* (7), 1695−1699.

(15) Day, A. J.; DuPont, S.; Ridley, S.; Rhodes, M.; Rhodes, M. J. C.; Morgan, M. R. A.; Williamson, G. Deglycosylation of flavonoid and isoflavonoids glycosides by human small intestine and liver β-glucosidase activity. *FEBS Lett.* **1998**, *436*, 71−75.

(16) Day, A. J.; Canada, F. J.; Diaz, J. C.; Kroon, P. A.; Mclauchlan, R.; Faulds, C. B.; Plumb, G. W.; Morgan, M. R.; Williamson, G. Dietary flavonoid and isoflavone glucosides are hydrolyzed by the lactase site of lactase phlorizin hydrolase. *FEBS Lett.* **2000**, *468* (2−3), 168−170.

(17) Liu, Y.; Ming, H. Absorption and metabolism of flavonoids in the Caco-2 cell culture model and a perfused rat intestinal model. *Drug Metab. Dispos.* **2002**, *30*, 370−377.

(18) Hendrich, S. Bioavailability of isoflavones. *J. Chromatogr. B* **2002**, *777*, 203−210.

(19) Setchell, K. D.; Brown, N. M.; Desai, P.; Zimmer-Nechemias, L.; Wolfe, B. E.; Brashear, W. T.; Kirshner, A. S.; Cassidy, A.; Heubi, J. E. Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *J. Nutr.* **2001**, *131*, 1362S−1375S.

(20) Tanumihardjo, S. A. Factors influencing the conversion of carotenoids to retinol: Bioavailability to bioconversion to bioefficacy. *Int. J. Vitam. Res.* **2002**, *72* (1), 40−45.

(21) Garrett, D. A.; Failla, M. L.; Sarama, R. J. Development of an in vitro digestion method to assess carotenoid bioavailability from meals. *J. Agric. Food Chem.* **1999**, *47*, 4301−4309.

(22) Muir, J. G.; O'Dea, K. Measurement of resistant starch: factors affecting the amount of starch escaping digestion in vitro. *Am. J. Clin. Nutr.* **1992**, *56*, 123−127.

(23) Murphy, P. A.; Song, T.; Buseman, G.; Barua, K.; Beecher, G. R.; Trainer, D.; Holden, J. Isoflavones in retail and institutional soy foods. *J. Agric. Food Chem.* **1999**, *47*, 2697−2740.
(24) Franke, A. A.; Hankin, J. H.; Yu, M. C.; Masskarinec, G.; Low, S. H.; Custer, L. J. Isoflavone levels in soy foods consumed by multiethnic populations in Sigapore and Hawaii. *J. Agric. Food Chem*. **1999**, *47*, 977−986.
(25) Nguyenle, T.; Wang, E.; Cheung, A. P. An investigation on the extraction and concentration of isoflavones in soy-based products. *J. Pharm. Biomed. Anal.* **1995**, *14*, 221−232.
(26) Griffith, A. P.; Collison, M. W. Improved methods for the extraction and analysis of isoflavones from soy-containing foods and nutritional supplements by reversed-phase high-performance liquid chromatography and liquid chromatography−mass spectrometry. *J. Chromatogr. A* **2001**, *913*, 397−413.
(27) Liggin, J.; Bluck, L. J. C.; Coward, W. A.; Bingham, S. A. Extraction and quantification of daidzein and genistein in food. *Anal. Biochem.* **1998**, *264*, 1−7.
(28) Franke, A. A.; Custer, L. J.; Cerna, C. M.; Narala, K. K. Quantification of phytoestrogens in legumes by HPLC. *J. Agric. Food Chem.* **1994**, *42*, 1905−1913.
(29) Markham, K. R.; Mabry, T. J. Ultraviolet−visible and proton magnetic resonance spectroscopy of flavonoids. In *The Flavonoids, Part I*; Harborne, J. B., Mabry T. J., Mabry, H., Eds.; Academic Press: New York, 1975; pp 45−77.
(30) Markham, K. R. Flavones, flavonols and their glycosides. In *Methods in Plant Biochemistry*; Harbone, J. B., Ed.; Academic Press: London, 1989; Vol. 1, Plant Phenolics, pp 197−235.
(31) Coward, L. S.; Kirk, M.; Barnes, S. Chemical modification of isoflavones in soyfoods during cooking and processing. *Am. J. Clin. Nutr.* **1998**, *68* (Suppl.), 1486S−1491S.
(32) Wang, H. J.; Murphy, P. A. Isoflavone content in commercial soybean foods. *J. Agric. Food Chem*. **1994a**, *42*, 1666−1673.
(33) Wang, H. J.; Murphy, P. A. Mass balance study of isoflavones during soybean processing. *J. Agric. Food Chem*. **1996**, *44*, 2377−2383.
(34) Mahungu, S. M.; Diaz-Mercado, S.; Li, J.; Schwenk, M.; Singletary, K.; Faller, J. Stability of isoflavones during extrusion processing of corn/soy mixture. *J. Agric. Food Chem*. **1999**, *47*, 279−284.
(35) Grun, I. U.; Adhikari, K.; Li, C.; Li, Y.; Lin, B.; Zhang, J.; Fernando, L. N. Changes in the profile of genistein, daidzein, and their conjugates during thermal processing of tofu. *J. Agric. Food Chem*. **2001**, *49*, 2839−2843.
(36) Andlauer, W.; Kolb, J.; Furst, P. Isoflavones from tofu are absorbed and metabolized in the isolated rat small intestine. *J. Nutr.* **2000**, *130*, 3021−3027.
(37) Coldham, N. G.; Darby, C.; Hows, M.; King, L. J.; Zhang, A. Q.; Sauer, M. J. Comparative metabolism of genistin by human and rat gut microflora: detection and identification of the end-products of metabolism. *Xenobiotica* **2002**, *32*, 45−62.
(38) The Merck Index, 12th ed.; Budavari, S., O'Neil, M. J., Smith, A., Heckelman, P. E., Kinneary, J. F., Eds.; Merck Research Laboratories: Whitehouse Station, NJ, 1996; pp 475, 744.
(39) Porter, C. J. H.; Charman, W. N. In vitro assessment of oral lipid based formulations. *Adv. Drug Delivery Rev.* **2001**, *50*, S127−S147.
(40) Murota, K.; Shimizu, S.; Miyamoto, S.; Izumi, T.; Obata, A.; Kikuchi, M.; Terao, J. Unique uptake and transport of isoflavone aglycones by human intestinal Caco-2 cells: comparison of isoflavonoids and flavonoids. *J. Nutr.* **2002**, *132*, 1956−1961.
(41) Xu, X.; Harris, K. S.; Wang, H. J.; Murphy, P. A.; Hendrich, S. Bioavailability of soybean isoflavones depends on gut microflora in women. *J. Nutr.* **1995**, *125*, 2307−2315.
(42) Hur, H. G.; Lay, J. O.; Beger, R. D.; Freeman, J. P.; Rafii, F. Isolation of human intestinal bacteria metabolizing the natural isoflavone glycosides daidzin and genistin. *Arch. Microbiol*. **2000**, *174*, 422−428.
(43) Wiseman, H. The bioavailability of nonnutrient plant factors: dietary flavonoids and phyto-oestrogens. *Proc. Nutr. Soc*. **1999**, *58*, 139−146.
(44) King, R. A.; Bursill, D. B. Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single soy meal in humans. *Am. J. Clin. Nutr*. **1998**, *67*, 867−872.
(45) Watanabe, S.; Yamaguchi, M.; Sobue, T.; Takahashi, T.; Miura, T.; Arai, Y.; Mazur, W.; Wahala, K.; Adlercreutz, H. Pharmacokinetics of soybean isoflavones in plasma, urine, and feces of men after ingestion of 60 g baked soybean powder (Kinako). *J. Nutr*. **1998**, *128*, 1710−1715.
(46) Yu, J.; Cheng, Y.; Xie, L.; Zhang, R. Effects of genistein and daidzein on membrane characteristics of HCT cells. *Nutr. Cancer*. **1999**, *33* (1), 100−104.
(47) Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr*. **2000**, *130*, 2073S−2085S.
(48) Xu, X.; Wang, H. J.; Murphy, P. A.; Cook, L.; Hendrich, S. Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. *J. Nutr*. **1994**, *124*, 825−832.
(49) Otsuki, M. Pathophysiological role of cholecystokinin in humans. *J. Gastroenterol. Hepatol*. **2000**, *15* (Suppl.), 71−83.

Received for review March 18, 2003. Revised manuscript received June 1, 2003. Accepted June 4, 2003. Funds for this research were provided in part by the USDA IFAFS, the Ohio Agriculture Research and Development Center, and the Comprehensive Cancer Center.

JF0342627