Isoflavones in Soy-Based Infant Formulas

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Six of the major soy-based infant formulas marketed in the United States were assayed for their isoflavone levels. Samples were taken from the east coast, midwestern, and west coast regions of the United States. Isoflavone levels were variable across brands probably due to different amounts of soy isolate used in product formulation. Total isoflavones ranged from 214 to 285 μg/g of dry formula or $\approx$25−30 μg/mL of reconstituted formula.

Keywords: *Phytoestrogens; genistein; glycitein; daidzein*

INTRODUCTION

Soy-based infant formulas have been marketed in the United States for over 60 years as safe and important feeding options for infants. These formulas have been fed to millions of infants with no evidence of harmful effects. However, there has been no systematic evaluation of isoflavone levels in these soy-based foods. There are only four studies in the literature (Setchell et al., 1997; Setchell and Welsh, 1987; Lu et al., 1995; Nguyele et al., 1995) with any analysis of isoflavone levels in infant formula. We have been involved in the development of a database of isoflavone levels in foods, commercial ingredients, and soybeans. Soy-based infant formulas will be a component of that database.

A number of phytochemicals possess cancer-prevention properties that may inhibit tumor initiation, prevent oxidative damage, and/or affect steroid hormone or prostaglandin metabolism to block tumor promotion (Caragay, 1992). There is a growing literature on the health protective benefits gained by consumption of soy foods. Isoflavones have been implicated in the prevention of breast, prostate, and colon cancers (Messina and Barnes, 1991), in lowering of the risk of cardiovascular disease (Anderson et al., 1995), and in the improvement of bone health (Bahram et al., 1996). The interest in soybean components has been so strong that two international conferences have been convened to report the state of knowledge in the field (Messina, 1995; Setchell, 1997). The beneficial effects of isoflavones may be due, but only in part, to the weak estrogenic activity of the isoflavones. Genistein, daidzein, and their glucosidic forms, daidzin and genistin, are 100 000 to 500 000 times less potent estrogens than estradiol (Farmakalidis and Murphy, 1985). In adults, plasma concentrations of isoflavones do not exceed 13 μM (Barnes, 1995a) even on a high soy isoflavone diet. Japanese adults are estimated to consume up to 12 mg/day (Fukutake et al., 1996). In contrast, Americans consuming a traditional Western diet may consume only 1−3 mg/day (Barnes et al., 1995).

The three isoflavones in soybeans and soy products, genistein, daidzein, and glycitein, occur in four possible forms: the free phenolic form, the glucoside, the malonyl glucoside, and the acetyl glucoside (Wang and Murphy, 1994a,b). The bioavailability of the isoflavones is apparently affected by the gut microflora of the consumer (Xu et al., 1994). There does not appear to be a difference in the bioavailability of the glucosides or free forms (Farmakalidis and Murphy, 1985; Xu, 1995). The effects of processing alter the distribution of the forms and can result in the loss of some isoflavones through leaching and removal of undesirable fractions. The distribution of the isoflavone yields a picture of the processing history of a particular soy product (Wang and Murphy, 1996). We have developed a routine HPLC method to evaluate isoflavones in soy products (Song et al., 1997a) and have employed it to examine the isoflavone levels in commercially available soy-based infant formulas.

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METHODS AND MATERIALS

Samples. Infant formulas were purchased in the Baltimore, MD, metropolitan area, Ames, IA, and San Francisco, CA, in 1995. Soy infant formulas obtained were Mead-Johnson's Prosobee, Gerber's, Ross Laboratories' Isomil, Weyth's Nursoy, Mead Johnson's Enfamil, and Carnation's Alsoy and were different lot numbers per each brand (n = 3). All formulas were obtained as the dry instant formula except Weyth's Nursoy from a west coast location, which was available only in liquid form. The liquid formula was freeze-dried prior to sampling and analysis. Enfamil was not available in the Baltimore area.

Standards. All chemicals were of reagent grade from Fisher Scientific unless noted otherwise. The internal standard, 2,4,4′-trihydroxydeoxybenzoin (THB), genistein, and daidzein were synthesized with resorcinol, 4-hydroxyphenylacetic acid, boron trifluoride etherate, *N*,*N*-dimethylformamide (DMF), and methanesulfonyl chloride from Sigma Chemical Co. (St. Louis, MO). Milli-Q system (Millipore Co., Bedford, MA) HPLC grade water was used.

Seven isoflavone standards were used in the calibration curves. Genistein (Gein) was synthesized according to the method of Chang et al. (1994). Daidzein was synthesized according to our modification of Chang's method; 4 mL of BF_3-Et_2O was added to a beaker containing 200 mg of THB in 10 mL of DMF, and the mixture was heated in a microwave oven (Kenmore U88) for 30 s at medium energy. Methanesulfonyl chloride (4 mL) was then added to the mixture, and it was heated for an additional 70 s at medium in the microwave oven. Four hundred milliliters of cold H_2O was added to the reaction mixture, giving a light yellow precipitate. The precipitate was washed with water and recrystallized from methanol. Genistin (Gin), daidzin (Din), and acetylgenistin (AGin) were from previous work in our laboratory (Wang and Murphy, 1994a). Glycitin (Glin) was purified using the method of Farmakalidis and Murphy (1985) from soy germ (Schouten USA). Glycitein (Glein) was purified from a 0.1 N HCl

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hydrolysate of soy germ (98 °C for 2 h) by adding acetonitrile (ACN) and filtering. The filtrate was dried by rotary evaporation and redissolved in 100% ethanol and chromatographed on a Sephadex LH-20 (Sigma) column (1 × 50 cm) with 50% ethanol as the eluent. The glycitein peak was collected and freeze-dried. The identity and purity of glycitein was confirmed by HPLC retention times in combination with UV spectral analysis, melting point determination, and mass spectrum analysis. Isoflavone standard solutions were prepared on the basis of UV maximum absorbance and molar extinction coefficients (Ollis, 1962). The purity of the standards was based on the percentage peak area according to the Beckman System Gold software, and final concentrations were adjusted based on these purities. For isoflavones without pure standards, the malonyl forms, and acetyldaidzin and acetylglycitin, the appropriate standard curves were adjusted on the basis of molecular weight differences as confirmed by our previous work with most of these standards (Wang and Murphy, 1994a,b).

Internal Standard. The internal standard, THB, was synthesized according to a modification of the procedure of Chang et al. (1994). Resorcinol (2.9 g) was added to a mixture of 4-hydroxyphenylacetic acid (2 g) and boron trifluoride etherate (4.5 mL). This mixture was refluxed for 10 min, cooled, and treated with 30 mL of saturated sodium acetate and 15 mL of saturated sodium bicarbonate. The yellow precipitate was filtered and washed with water and then chloroform and dried to give yellow crystals. The crystals were redissolved in 100% ethanol and chromatographed on Sephadex LH 20 with 50% ethanol as the eluent. A single peak was collected giving >99% THB.

Quality Control. Quality control measures included the following: analysis of authentic standards each day food samples were analyzed; recoveries of Gin, Gein, Dein, and THB on five different soyfood matrices were conducted monthly; and analysis of four reference materials weekly over the lifetime of this project (24 months). Precision and accuracy estimates were made on the basis of these quality control measures. Any significant deviations (>5%) from baseline of these reference materials would lead to system inspection and correction before any further samples would be analyzed. All data were corrected for recovery based on internal standard.

Isoflavone Analysis. Dry infant formulas were extracted as 2 g samples in 10 mL of acetonitrile, 2 mL of 0.1 N HCl, and 7 mL of water in a 125 screw-top Erlenmeyer flask with stirring for 2 h at room temperature. The extracts were filtered and taken to dryness on a rotary evaporator at <30 °C. The residues were dissolved in 80% HPLC grade methanol. An aliquot was filtered through a 0.45 μm PTFE filter (Alltech Associates, Deerfield, IL) and analyzed by HPLC. A Beckman System Gold chromatograph with a Model 507 autosampler, a Model 126 dual pump, a Model 168 photodiode array detector, and an IBM 486 computer using Beckman System Gold HPLC data processing software (version 8, 1993) was used. A YMC-pack ODS-AM-303 column (5 μm, 25 cm × 4.6 mm) was employed for chromatographic separations and maintained at 34 °C with a column heater. A linear gradient was composed of A (0.1% acetic acid in water) and B (0.1% acetic acid in acetonitrile). The gradient profile was modified from that of our previous paper (Wang and Murphy, 1994a) to reduce analysis time. After injection of a 20 μL sample, the system was maintained at 15% B for 5 min, then increased to 29% in 31 min, and then to 35% in 8 min. The system was recycled to 15% B at the end of 45 min. The flow rate was 1.0 mL/min for the first 5 min, then increased to 1.5 mL/min for the next 40 min, and returned to 1.0 mL/min for recycle. The UV absorbance was monitored from 200 to 350 nm. UV spectra were recorded and peak areas were integrated using Beckman System Gold software.

Statistical Analysis. Midwestern sample analyses were replicated in triplicate. Statistical evaluation was conducted using the General Linear Models procedure and Student's *t* test with SAS package (version 6.03, 1995, Cary, NC). Differences were considered significant if $p \leq 0.05$.

RESULTS AND DISCUSSION

Figure 1 presents a typical chromatogram obtained in soy-based infant formula analysis resulting in baseline resolution of all soy isoflavones and the internal standard, THB. All data reported in Tables 1 and 2 were corrected for recovery with internal standard (Song et al., 1997a). To evaluate the analytical precision of isoflavone analysis, triplicate analyses of the midwestern-purchased infant formulas were done. The results of these analyses are presented in Table 1 for total isoflavones. The largest percent coefficient of variation was an average of 5% for total genistein for all formulas. These data supported our decision to analyze only one sample from each lot of infant formula for the national survey and still obtain representative data.

An analysis of variance revealed that there were only minor differences in isoflavone concentrations within a brand among sampling locations. The differences were in total genistein, acetylgenistin, and glycitein. There were no other differences in the other 13 forms. Total isoflavone content (the sum of total genistein, total daidzein, and total glycitein) was not different among sampling locations.

Total isoflavone levels were quite similar across brands: 29% daidzein, 59% genistein, and 12% glycitein. Table 2 presents the results of isoflavone analysis for the individual forms of each isoflavone and the total isoflavones adjusted for their molecular weight differences and expressed as the free isoflavone forms (i.e., totals of genistein, daidzein, or glycitein). Simple addition of isoflavone concentrations without this correction will overestimate true isoflavone aglycon concentrations by almost a factor of 2 (Wang and Murphy, 1994a, 1996). Since only the free forms of the isoflavones are absorbed by the gut and exert their potentially protective effects, the concentrations of isoflavones in food products must be expressed in this manner to avoid erroneous conclusions and labeling.

An analysis of variance showed small but significant differences across brands for individual isoflavone forms. The effects of processing to produce soy isolates from soybeans is evident from the distribution of the different forms. The malonyl glucosides are moderately reduced in these formulas compared to being the highest concentrations of isoflavone forms found in raw soybeans (Wang and Murphy, 1994a,b, 1996). The glucosides, daidzin, genistin, and glycitin, are higher than the malonyl forms, indicating the product, either at soy isolate manufacture and/or infant formula formulation, has undergone heat processing. There are appreciable concentrations of the acetyl glucosides, indicating some intensive heat treatment has occurred during processing such as spray-drying. Little hydrolysis of the glycosides appears to have occurred since the genistein, daidzein, and glycitein concentrations are quite low as expected (Wang and Murphy, 1996; Farmakalidis and Murphy, 1985).

The variation among soy infant formula brands observed here may be due in part to the difference in the percent soy isolate used in each brand's formulation. Within a single infant formula brand, it is probable that the isolate used was produced by a single supplier. Some or all of the soy isolates used in preparing these infant formulas may have come from a common supplier. The percent soy isolate in these infant formulas ranges from 14.6 to 21%. When total isoflavone concentrations were adjusted on a percent soy isolate basis (as reported on product label), they were 1271 (Alsoy),

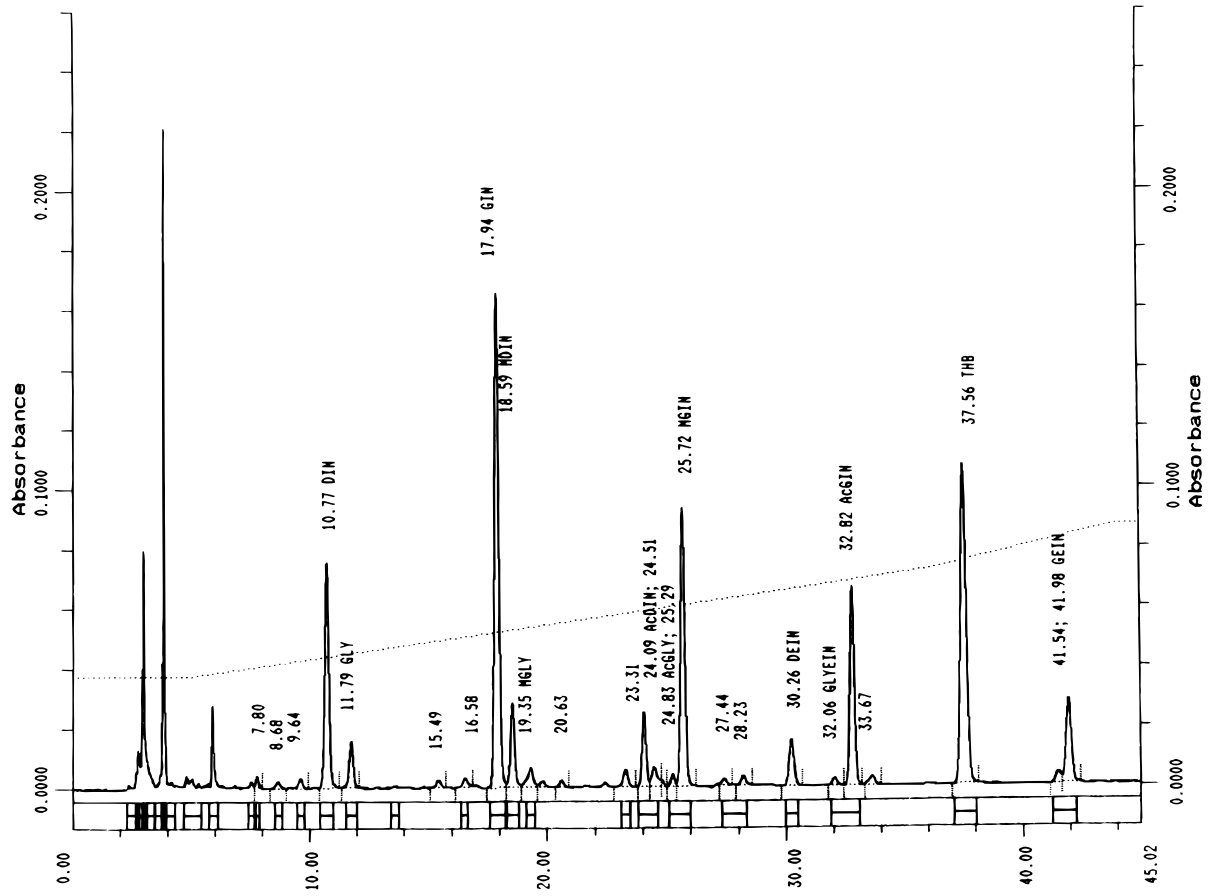

Figure 1. HPLC chromatogram of Enfamil soy-based formula with isoflavone and internal standard peaks resolved. Axis represents UV absorbance at 254 nm. DIN, daidzin; GLY, glycitin; GIN, genistin; MDIN, malonyldaidzin; MGLY, malonylglycitin; AcGin, acetylgenistin; AcDin, acetyldaidzin; AcGLY, acetylglycitin; MGIN, malonylgenistin; DEIN, daidzein; GLYEIN, glycitein; THB, internal standard; GEIN, genistein.

Table 1. Precision of Isoflavone Analysis in Infant Formulas from Midwestern Sample Set[a]

		total daidzein			total genistein			total glycitein		
formula	*n*	*X*	*s*	CV	*X*	*s*	CV	*X*	*s*	CV
Prosobee	3	57	1	2	132	7	5	29	0	0
Gerber	3	62	1	2	140	5	4	29	0	0
Isomil	3	56	3	6	127	7	6	27	1	4
Nursoy	3	63	5	8	139	10	7	28	1	4
Enfamil	3	66	1	2	140	9	6	29	2	7
Alsoy	3	67	2	3	141	6	4	27	0	0

[a] n = number of replicates, X = average, s = standard deviation, CV = coefficient of variation (%).

1329 (Gerber), 1338 (Nursoy), 1356 (Enfamil), 1487 (Isomil), and 1535 μg/g (Prosobee). These isoflavone concentrations were higher than we have reported for commercial soy isolates ranging from 450 to 1100 μg/g (Wang and Murphy, 1994a). Yearly variation in the isoflavone content of soybeans can be different by a factor of 5 (Wang and Murphy, 1994b).

There are only four studies in the literature on isoflavone content of infant formulas (Setchell et al., 1997; Setchell and Welsh, 1987; Lu et al., 1995; Nguyenle et al., 1995) for four of the products we have analyzed: Alsoy, Nursoy, Prosobee, and Isomil. None of these papers report data on glycitein levels except for that of Setchell et al. (1997), who give only an average for five brands. Given the structural similarity of glycitein to the estrogenic isoflavones, genistein and daidzein, we should anticipate this minor isoflavone will contribute to the total phytoestrogen activity of soy foods. We have preliminary evidence that glycitein is as potent an estrogen as daidzein (Song et al., 1997b). Our total genistein compares well with the total reported for the same products in Setchell and Welsh (1987) and Lu et al. (1995). However, our total daidzein concentrations are approximately half of those reported by Setchell and Welsh (1987) and Lu et al. (1995). Nguyenle et al. (1995) used an isocratic HPLC system to evaluate isoflavones in a variety of soy foods but did not resolve the acetyl glucosides from other isoflavones, nor did they consistently resolve the malonyl glucosides from the glucosides. They claim no acetyl forms were present in their products. Others have consistently shown acetyl forms in any heat-processed soy product for which all isoflavone forms are resolved by gradient HPLC (Wang and Murphy, 1994a, 1996; Coward et al., 1993; Song et al., 1997a).

Total isoflavones ranged from 214 to 267 μg/g of dry formula or $\approx$25−30 μg/mL of reconstituted formula. Our values are lower than Setchell's (1997), but it is not clear how he calculated the total isoflavone levels. Arithmetic addition of various glucosidic forms with free forms will overestimate isoflavone concentrations by 50−100% due to the molecular weight difference of the glucose moiety. Since only the aglycon is apparently absorbed, perhaps isoflavone levels in foods should be reported on a mircomolar basis. On a ready-to-drink formula basis, our data represent 8.82, 19.10, and 3.54 μg/mL daidzein, genistein, and glycitein, respectively. Depending on the volume consumed by infants, which will be dependent on age, the child may consume from 5 to 12 mg of isoflavone (kg of body weight)$^{-1}$ day^{-1}. The infant formula isoflavone amounts are greater than typically

Table 2. Isoflavone Distribution in Soy-Based Infant Formulas[a]

	μg of isoflavone/g of dry formula															
													total			
formula	% SPI	Din	Gin	Glin	MDin	MGin	MGlin	ADin	AGin	AGlin	Dein	Gein	Glein	Dein	Gein	Glein	total
Alsoy	21	37^{B}	83^{AB}	17^{AB}	48^{A}	92^{A}	19^{A}	40^{A}	84^{A}	14	10^{BC}	11^{CD}	0	78^{A}	154	27^{A}	267^{A}
Prosobee	17	40^{B}	94^{AB}	18^{AB}	38^{A}	75^{A}	19^{A}	37^{AB}	80^{AB}	13	10^{BC}	12^{BC}	3	73^{AB}	151	30^{A}	261^{AB}
Enfamil	18	76^{A}	85^{AB}	19^{AB}	34^{A}	57^{AB}	17^{A}	37^{AB}	71^{AB}	9	12^{A}	15^{A}	3	70^{AB}	135	28^{A}	244^{ABC}
Gerber	17	38^{B}	65^{B}	18^{AB}	30^{AB}	66^{AB}	18^{A}	32^{AB}	71^{AB}	12	11^{AB}	13^{AB}	3	67^{AB}	126	29^{A}	226^{ABC}
Isomil	15	31^{B}	75^{AB}	17^{B}	32^{AB}	69^{A}	16^{A}	34^{AB}	71^{AB}	11	9^{C}	10^{D}	3	63^{AB}	130	27^{A}	223^{BC}
Nursoy	16	56^{AB}	134^{A}	21^{A}	10^{B}	33^{B}	5^{B}	15^{B}	51^{B}	0	10^{BC}	11^{CD}	3	57^{B}	138	17^{B}	214^{C}
LSD		36	64	3	24	35	8	23	30	18	1	2	8	17	37	9	43

[a] Values in a column with the same superscript are not significantly different at $\alpha = 0.05$. LSD, least significant difference; % SPI, percentage soy isolate reported on label; Din, daidzin; Gin, genistin; glin, glycitin; MDin, malonyldaidzin; MGin, malonylgenistin; MGlin, malonylgenistin; ADin, acetyldaidzin; AGin, acetylgenistin; AGlin, acetylglycitin; Dein, daidzein; Gein, genistein; Glein, glycitein; total Dein, total moles of daidzein × molecular weight of daidzein; total Gein, total moles of genistein forms × molecular weight genistein; total Glein, total moles of glycitein × molecular weight of glycitein; total total sum of total daidzein, total genistein, and total glycitein.

consumed by adult soy foods consumers. However, in the >60 years of use of soy-based infant formula, there is no evidence of any difference in growth and development of children compared to those children consuming cow's milk formula or human milk. Infants are exposed to small amounts of these compounds in the range of 1−2 μM isoflavones in breast milk if the mother is consuming soy foods. It is likely that the potential health protective effects attributed to soy isoflavones may be an advantage to infants consuming soy formulas.

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