JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Variations in Isoflavone Levels in Soy Foods and Soy Protein Isolates and Issues Related to Isoflavone Databases and Food Labeling

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The reliability of databases on the isoflavone composition of foods designed to estimate dietary intakes is contingent on the assumption that soy foods are consistent in their isoflavone content. To validate this, total and individual isoflavone compositions were determined by HPLC for two different soy protein isolates used in the commercial manufacture of soy foods over a 3-year period ($n = 30$/isolate) and 85 samples of 40 different brands of soy milks. Total isoflavone concentrations differed markedly between the soy protein isolates, varying by 200−300% over 3 years, whereas the protein content varied by only 3%. Total isoflavone content varied by up to 5-fold among different commercial soy milks and was not consistent between repeat purchases. Whole soybean milks had significantly higher isoflavone levels than those made from soy protein isolates (mean ± SD, 63.6 ± 21.9 mg/L, $n = 43$, vs 30.2 ± 5.8 mg/L, $n = 38$, respectively, $p < 0.0001$), although some isolated soy protein-based milks were similar in content to "whole bean" varieties. The ratio of genistein to daidzein isoflavone forms was higher in isolated soy protein-based versus "whole bean" soy milks (2.72 ± 0.24 vs 1.62 ± 0.47, respectively, $p < 0.0001$), and the greatest variability in isoflavone content was observed among brands of whole bean soy milks. These studies illustrate large variability in the isoflavone content of isolated soy proteins used in food manufacture and in commercial soy milks and reinforce the need to accurately determine the isoflavone content of foods used in dietary intervention studies while exposing the limitations of food databases for estimating daily isoflavone intakes.

KEYWORDS: Phytoestrogens; isoflavones; soy protein isolates; soy milk; soy foods; HPLC analysis

INTRODUCTION

Consumer interest in the health benefits of soy foods is at an unprecedented high. This is primarily the result of approval by the U.S. Food and Drug Administration (FDA) and, more recently, the Joint Health Claims Initiative (JCHI) in the United Kingdom to allow food manufacturers to make a health claim for soy protein's ability to lower the risk for coronary heart disease (*1*), based on compelling evidence for its hypocholesterolemic effects (*2*) and a wealth of clinical and nutritional studies showing a variety of physiological effects of diets including soy protein (*3*, *4*). Although the FDA failed to recognize the role of isoflavones in the hypocholesterolemic effect of soy protein-based foods, the discovery two decades ago that concentrations of isoflavones in the urine of adults consuming modest amounts of soy foods far exceeded endogenous estrogen levels (*5*, *6*) was a major catalyst to the current renaissance in the clinical and nutritional interest in soy. This observation led to the hypothesis that the low incidence of hormone-dependent diseases in countries where soy foods are a staple is related to high dietary intakes of isoflavones. In addition to showing affinity for estrogen receptors (*7*), isoflavones have the ability to behave as antioxidants, inhibit enzyme systems, and influence transport proteins and cell signaling pathways by their inhibitory effects on tyrosine kinases and growth factors (*8*−*10*).

Isoflavones have been extensively investigated, and as a consequence many commercial functional foods are now incorporating soy isoflavones. There is also a large market in "over the counter" (OTC) isoflavone supplements targeting women's health (*11*). Although a case for soy and its isoflavones can be made in the prevention of many diseases, the manner in which the commercialization of soy isoflavones is proceeding is far outstripping the rate at which evidence-based clinical data are able to support many of the health claims. The level of isoflavone incorporation into foods and supplements has been rather arbitrarily chosen, driven more by an attempt to pack as

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10.1021/jf026199b CCC: $25.00
Published on Web 05/29/2003

much isoflavone into the product as possible rather than by experimentally established physiological needs based on knowledge of their pharmacokinetics (*11*−*15*). Foods and soy beverages are being promoted containing >100 mg of isoflavones per serving, and advertising commercials have been aired for products on the basis that they have the highest levels of isoflavones. There is no consistency, and considerable confusion, over the present way soy food products are being labeled with their isoflavone content, and in our opinion there is a dire need to step back and consider many crucial issues, including the development of food databases. In an attempt to highlight these we have performed analytical studies to determine the extent of variability in the isoflavone levels of a wide range of different commercial soy milks and in industrial preparations of soy protein isolates used by the food industry to formulate soy foods. Discussed are the crucial issues relating to dietary isoflavone needs and the problems facing the consumer, health professional, and industry in dealing with the rapid growth in demand for phytoestrogen-rich foods.

MATERIALS AND METHODS

Sources of isoflavone standards: daidzin ($C_{21}H_{20}O_9$), CAS Registry No. 1431-39-6 (catalog no. 02-1096, Indofine Chemical Co., Belle Mead, NJ, or equivalent); daidzein ($C_{15}H_{10}O_4$), CAS Registry No. 486-66−8 (catalog no. DO101, Indofine Chemical Co., or equivalent); genistin ($C_{21}H_{20}O_{10}$), CAS Registry No. 529-59-9 (catalog no. 02-1050 Indofine Chemical Co., or equivalent); genistein ($C_{15}H_{10}O_5$), CAS Registry No. 446-72-0 (catalog no. G-103, Indofine Chemical Co., or equivalent); glycitin ($C_{22}H_{22}O_{10}$) CAS Registry No. 40246-10-4 (Plantech United Kingdom, Reading, U.K.); glycitein ($C_{16}H_{12}O_5$) CAS Registry No. 40957-83-3 also Indofine (Plantech United Kingdom); equilenin ($C_{18}H_{18}O_2$) (catalog no. E0400, Sigma-Aldrich Fine Chemicals, St. Louis, MO).

Chemicals and Reagents. Methanol, acetonitrile (HPLC grade), trifluoroacetic acid (TFA), and ammonium acetate were all of analytical grade and purchased from Sigma-Aldrich Fine Chemicals.

Source of Food Products. *(a) Isolated Soy Proteins.* Thirty separate samples of two different types of soy protein isolates, purchased for the manufacture of commercial soy food products over a 3-year period, were collected. Manufacturer's lot numbers indicated that dates of manufacture were spread fairly evenly over the entire sampling period. The source of these isolated soy proteins has been kept confidential.

(b) Soy Milks. Samples of 40 different brands of soy milk drinks commercially available in Australia and the United States were purchased from supermarkets. The following brands of soy milks were purchased, and the protein source and fat status are indicated in parentheses (fat status is defined as follows: No fat means no added fat; low fat means the fat content is <2.0%; full fat means the fat content is between 3 and 4.0%; high fat refers to a fat content of >4.0%): Bi Lo Lite soy drink (isolate, low fat); Bi Lo soy drink (isolate, full fat); Bowmans soy (isolate, full fat); Edensoy Organic soy milk (whole bean, high fat); Good Life Light (isolate, low fat); Home Brand soy drink (isolate; full fat); Natura soy beverage original (whole bean, full fat); Natures soy (whole bean, full fat); Pacific Organic soy enriched (whole bean, full fat); Pure Harvest Aussie soy (whole bean, full fat); Silk soy milk organic plain (whole bean, full fat); Simply Soy Organics (entire bean, full fat); So Good fat free (isolate, no fat); So Good Lite (isolate, low fat); So Good Now fat free (isolate, no fat); So Good White (isolate, full fat); So Natural (whole bean, full fat); So Natural Lite (whole bean, low fat); So Natural Original (whole bean, full fat); Soy Dream (whole bean, full fat); Soy Life (isolate, full fat); Soy Life Lite (isolate, low fat); Soy Life No Fat (isolate, no fat); Soy Moo fat free (whole bean, no fat); Sungold soy drink (isolate, full fat); Vitalife low fat (whole bean, low fat); Vitalife soy milk (whole bean, full fat); Vitasoy Calci Plus (whole bean, full fat); Vitasoy Calci Plus Lite (whole bean, low fat); Vitasoy Lite (whole bean, light); Vitasoy Regular (whole bean, full fat); Vitasoy Original (creamy) (whole bean, full fat); Vitasoy Creamy Original (whole bean, full fat); Vitasoy Enriched Original (whole bean,full fat); Vitasoy Light Original (whole bean, low fat); Westsoy low-fat soy drink (whole bean, no fat); Westsoy 1% fat Lite (whole bean, low fat); Westsoy Plus soy drink (whole bean, full fat); Westsoy Smart (whole bean, high fat); Zen Don Organic soy milk (whole bean, full fat). Of these brands, a total of 85 individual soy milk samples were analyzed. These products are representative of soy milks made by three distinct processes as follows.

(i) Isolated soy protein milks were made by soaking whole beans, or solvent-extracted soybean flakes, in water to separate soluble materials from the residual okara. Proteins in this extract are concentrated by precipitation at the isoelectric point of the major soy protein fractions and washed with acidified water to decrease the level of unwanted components extracted from the soybean. Thirty-eight individual samples of isolated protein-based soy milks were analyzed.

(ii) Direct extract soy milks were manufactured in a similar way to the soy isolate milks, except that the protein concentration step is omitted. Although the okara (the material from the bean that is insoluble and is separated from the extract) ranges from 30 to 40% of the total soybean used, products made according to this method are usually incorrectly called "whole bean soy milks" by the manufacturers. Forty-three individual samples of soy milks in this category were analyzed.

(iii) "Entire bean" soy milks were manufactured by a process in which no okara is separated from the beans that are used in the manufacture of the milk. Protein and water-soluble materials are present in solution in the product, and the remainder of the bean bulk is treated to reduce particle size to a point where it is organoleptically acceptable. Four ($n = 4$) separate samples of a single brand of "entire bean" soy milk were analyzed.

To assess variability in isoflavone content of the same soy milk brand over time, three brands of soy milk, two isolated soy protein-based (designated brands A and B) and one whole bean variety (designated brand C), were repeat purchased over a 6-month period and analyzed. Finally, a single sample of unprocessed soybeans purchased in Australia was analyzed.

Determination of Isoflavones in Foods and Food Products. All of the isolated soy proteins and soy milks were analyzed in quadruple or greater replicates for isoflavone composition by methodologies described in detail previously (*16*, *17*). Isoflavones were extracted in aqueous methanol, separated by reverse phase HPLC with gradient elution, and measured from their UV absorption.

Extraction of Isoflavones from Food Products. Isoflavones were extracted from ground food samples in hot 80% methanol/water and separated by HPLC chromatography. Ground or freeze-dried sample (3−5 g) was weighed into a round-bottom flask. Methanol/water (80:20 v/v) (~70 mL) was added and swirled to mix. For liquid samples a quantity of liquid that contained from 0.5 to 2 g of solids was weighed. Taking into account the amount of water in the weighed sample, methanol and water were added in quantities so that the final concentrations in the refluxing liquid were in the proportion 80:20 (v/v). The mixture was refluxed on heating mantles for 1 h and allowed to cool. Samples were filtered through Whatman no. 1 filter paper into 100 mL volumetric flasks, washed with 80% methanol/water, and made to volume with 80% methanol. An aliquot (usually 1.0 mL, but greater for samples with low concentrations of isoflavones) of the sample was transferred to a 5 mL disposable glass tube, and 30 μL of equilenin internal standard (60 μg of equilenin) was added. For samples with high fat content the aliquot was transferred into a centrifuge tube and fat was removed by washing twice with hexane. Hexane (4 mL) was added to the extract and vortexed for 30 s. The mixture was centrifuged for 10 min to ensure good separation of the phases. The hexane (upper) layer was removed with suction using a Pasteur pipet. After two washings, the residual lower layer was transferred to a disposable 5 mL tube. The samples were dried under a stream of nitrogen at room temperature without heating. Acetonitrile/10 mM ammonium acetate buffer (1.0 mL) was added to the dried residue and vortexed to mix. The samples were centrifuged for ~15 min, and a portion was pipetted off into an HPLC vial for analysis.

HPLC Separation of Individual Isoflavones and Their Glycoside Conjugates. HPLC was carried out on a 4.6 mm × 250 mm C18 reverse phase column with 5 μm particle size using an injection volume of 10 μL and a flow rate of 1 mL/min. Mobile phases were prepared as

follows. Solvent A (90% acetonitrile) was prepared by the addition of 100 mL of ultrapure distilled water to 900 mL of acetonitrile, mixing, and filtering through a 0.45 μm membrane. Solvent B (10 mM ammonium acetate/TFA buffer) was prepared with 1.5416 g of ammonium acetate in ~2 L of ultrapure distilled water, adding 2 mL of TFA (0.1%) and bringing the final volume to 2 L with water. The solvents were filtered through a 0.45 μm filter. Gradient elution was used using 90% acetonitrile and ammonium acetate/TFA buffers. Chromatograms were run for 2 min with 100% elution of solvent A (90% acetonitrile) followed by a linear gradient arriving at 50% solvent A and 50% solvent B (ammonium acetate/TFA buffer) 24 min after the start of the chromatogram. This proportion was held for a further 5 min and then returned to 100% solvent A over a 5 min period. The column was then washed for a further 6 min with solvent A before injection of the next sample. Isoflavones were detected from their ultraviolet absorbance at 260 nm and their identity confirmed from the characteristics of their absorption spectrum using a diode array as detector or by electrospray ionization mass spectrometry.

Calculation of Isoflavone Levels. The amount of isoflavone present was calculated on the basis of sample peak area calibrated against peak area of standards. Equilenin was used as an internal standard to correct for losses during the workup of the samples after the extraction step, and the peak area ratio of the isoflavone to the internal standard was interpolated against a calibration plot of known concentrations of isoflavones. Acetyl and malonyl forms are unavailable as pure standards, and therefore calculation of the concentrations of these isoflavones assumed unity responses in molar absorption with the respective β-glycosides. The amount of the *aglycon* form of the isoflavone in each peak was calculated by adjusting for molecular weight differences of the individual conjugates, and the total amount of daidzein, genistein, and glycitein equivalents in the sample was obtained by summing all forms in which these isoflavones were found. The total isoflavone content was calculated as the sum of these three forms. All data throughout are expressed as *aglycon* equivalents. The intra-assay reproducibility of the method for total isoflavone content determined from replicate analyses of the same soy isolate or soy milk was 5.6% expressed as coefficient of variation. The standard error of estimate for the average of duplicate determinations was determined using the one-way analysis of variance function of the Statgraphics version 2.0 statistical analysis package (Manuguistics Inc., Rockville, MD). The standard deviation was converted to a percentage of the mean value, and this percentage was averaged over the whole set of samples analyzed.

RESULTS

Isoflavone Composition of Isolated Soy Proteins. To study variations in the level of retained isoflavones over long periods of time, 30 separate samples of two different soy protein isolates, purchased over a 3-year period for the manufacture of soy food products, were analyzed by HPLC.

The predominant isoflavones, identified from their retention indices and confirmed by photodiode array detection (*18*) and electrospray ionization mass spectrometry (*19*, *20*), were the β-glycosides and their corresponding acetyl esters of daidzein and genistein. Glycitein and its conjugates were also present, accounting for 5−30% of the total isoflavone content. Detectable levels, although extremely low, of malonyl glycosides were present, whereas the aglycons were relatively minor constituents in common with most unfermented soy protein foods (*16*). The total isoflavone concentration, expressed as aglycon equivalents after correction for the mass of the sugar moiety, in the two different types of soy protein isolates was very different, and both samples of isolates varied considerably over the 3-year period (**Figure 1**).

The variation in the proportions of daidzein, genistein, and glycitein, when expressed as aglycons, over the 3-year period for a selection of the soy protein isolates is shown in **Figure 2**. The mean (± SD) ratios of genistein/daidzein (i.e., the sum of all the isoflavone forms) over the 3-year period for all of the isolated soy proteins analyzed were 3.8 ± 0.87 and 3.7 ± 0.63 for type 1 and 2 soy protein isolates, respectively.

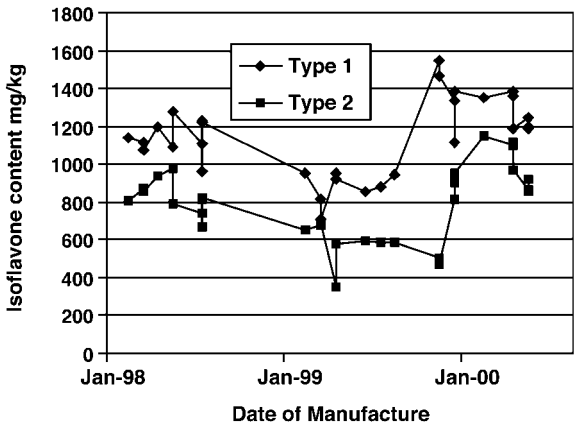

Figure 1. Variation in the total isoflavone content of two different types of isolated soy protein samples used by the food industry over a 3-year period.

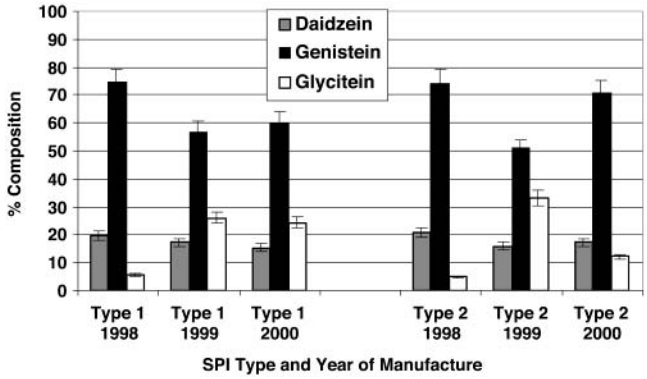

Figure 2. Variation in daidzein, genistein, and glycitein forms of isoflavones in two types of soy protein isolates used in the manufacture of soy foods over a 3-year period.

Soy Milk Beverages. Samples of 40 different brands of commercially available soy milk drinks were analyzed for their isoflavone composition. These included products made from isolated soy proteins, products made directly from whole soybeans using alkaline water extraction and removal of insoluble okara (the so-called "whole bean" milks), and "whole bean" drinks from which no okara is removed (a true "whole-bean" product, which we have called an "entire bean" product to distinguish it from the "whole bean" products that discard close to 40% of the bean as part of the processing method used). The total isoflavone concentration, expressed in aglycon equivalents for each of the products, is shown in **Figure 3**.

There was a large variation in total isoflavone concentration among the different brands of soy milks, but this was especially the case for whole bean soy milks, for which the values varied by up to 5-fold. The mean (± SD) total isoflavone content of soy milks made from isolated soy proteins, 30.2 ± 5.8 mg/L (n = 38), was significantly lower ($p < 0.0001$) than the isoflavone content of whole bean soy milks, which averaged 63.6 ± 21.9 mg/L (n = 43). The mean concentration of the total isoflavones in the four samples of the entire bean variety of soy milk was 36.5 ± 11.5 mg/L (n = 4). The total isoflavone content of whole bean soy milks purchased in the United States was not statistically significantly different from those purchased in Australia (57.7 ± 20.4 mg/L, n = 14, vs 66.4 ± 22.4 mg/L, n = 29, respectively). None of the soy milks purchased from

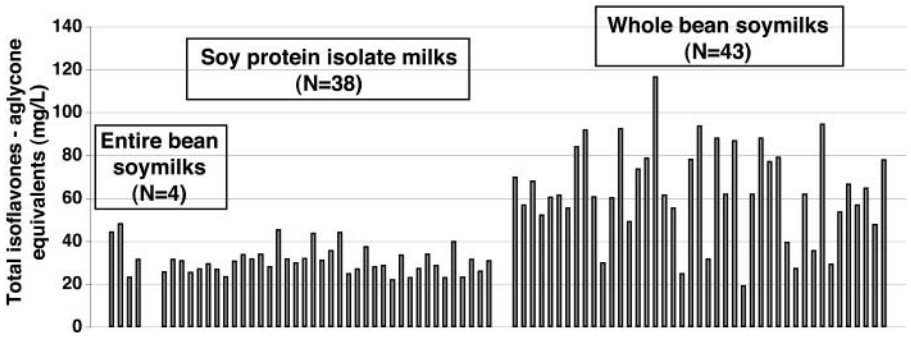

Figure 3. Histogram showing the total isoflavone content of 85 samples from 40 different brands of commercial soy milks grouped according to milk type. Each bar represents the mean values from four or more replicate analyses.

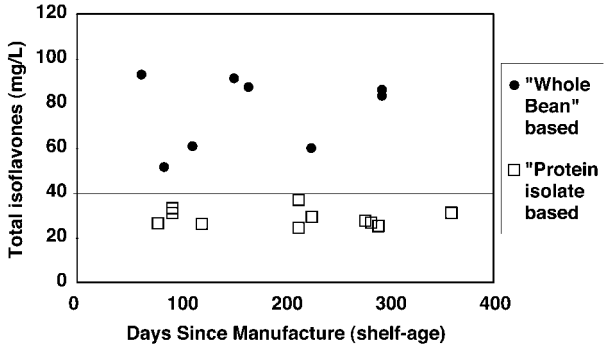

Figure 4. Relationship between total isoflavone concentration and shelf age of a subset of 21 soy milks made from isolated soy protein.

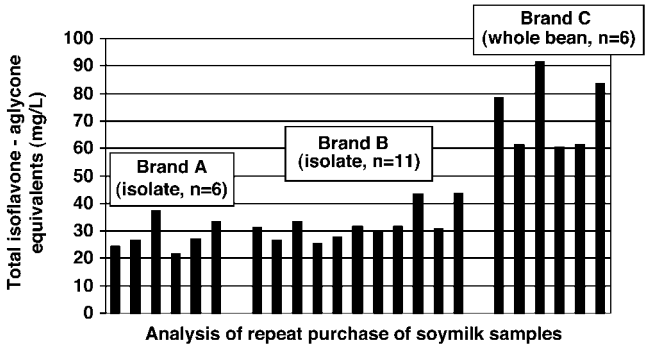

Figure 5. Variation in isoflavone content of three brands of soy milks, two whole bean and one isolated soy protein-based categories purchased over a 6-month period.

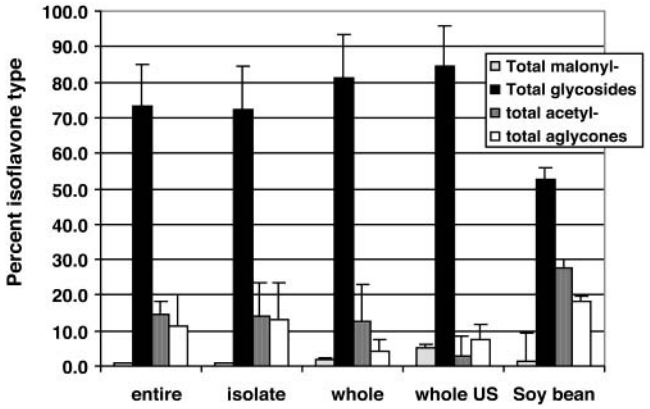

Figure 6. Histogram showing mean (± SD) percentage distribution of glycosidic and aglycon forms of isoflavones in different categories of soy milks and in one variety of soybean.

the United States were of the isolated soy protein variety, so intercountry comparison was not possible for this category of soy milk. No significant difference in total isoflavone content between low-fat and high-fat varieties of soy milk was observed (data not shown). Overall, isolated soy protein-based milks had a much higher proportion of genistein than daidzein compared with whole bean soy milks; the ratio of genistein to daidzein isoflavone forms was 2.72 ± 0.24 versus 1.62 ± 0.47, respectively ($p < 0.0001$).

There was no evidence that the variation in total isoflavone level among the various soy milks was related to the "shelf age" of the milk. **Figure 4** shows a plot of the total isoflavone concentration against the shelf age of the milk, as determined by the interval between its date of manufacture and the date on which the analysis was performed, for a subset ($n = 21$) of whole bean and isolated soy protein-based soy milks for which manufacturing date information was available. There was no trend toward lower total isoflavone concentrations with aging of the soy milk sample within the range of 61−359 days of storage.

The variability in isoflavone content of a typical manufacturer's soy milk was examined by repeat purchase and analysis of multiple samples of the same brand of soy milk over a 6-month period (**Figure 5**). Three brands of soy milks were tested for this purpose. The total isoflavone contents of two different brands of soy milk made from isolated soy protein (brand A, $n = 6$ different samples, and brand B, $n = 11$ different samples) and one brand of whole bean soy milk (brand C, $n = 6$ different samples) varied by as much as 60% over this relatively narrow time span.

Figure 6 shows the mean values for distribution of the isoflavones based on the type of glucose conjugation and according to the category of soy milk. In the case of soy milks, which are subjected in manufacture to an intense heating step, the level of malonyl conjugates was expectedly low. Although the use of heat in the analytical extraction step may lead to degradation of malonyl conjugates, the total isoflavone concentration in soy milks was not significantly different when samples were prepared by either a cold or hot extraction method. For example, when replicate samples ($n = 6$) of three different soy milks were extracted by cold and hot extraction, the final total isoflavone concentrations for each product were 98.8 ± 3.7 and 92.0 ± 3.7 mg/kg ($p = 0.14$), respectively, 210.4 ± 1.3 and 208.1 ± 3.8 mg/kg ($p = 0.84$), respectively, and 40.4 ± 0.7 and 38.6 ± 1.6 mg/kg ($p = 0.35$), respectively. There were marked differences in the distribution of malonyl glucoside, acetyl glucoside, β-glycoside, and aglycon forms of isoflavones (**Figure 6**) among the different categories of soy milks. The proportion of malonyl glucoside conjugates of isoflavones was between 0 and 4.9% of the total in most soy milks, but among the U.S. whole bean milk varieties, 4 of the 14 samples had a

malonyl glucoside content of between 6 and 23% of the total isoflavone content. For interest, data are shown for the relative proportions of isoflavone conjugates in a single sample of unprocessed soybeans. The relatively low proportion of malonyl glucosides in the soybean sample matches the proportions seen in the soy milks and may also be reflective of exposure of the sample to heat during extraction because high proportions of malonyl forms have been reported previously for unprocessed soybeans (*21*−*24*). Acetyl glucoside conjugates were much more prominent and variable, ranging between 0.2 and 40.7% of the total isoflavones present in the soy milks, compared with 27% found in a sample of unprocessed whole soybeans. The free aglycon was found in lower proportions in all of the soy milks than in the unprocessed soybean sample. Proportions range from 0.8 to 17.5% for the soy milks compared to 18% of the total for a sample of unprocessed soybeans. However, it is the proportion of isoflavone present as the β-glycosides that exhibited the greatest differences among the soy milks. The sample of unprocessed soybean had 52% of its isoflavone content in the form of β-glycosides, but this proportion ranged from 53 to 97% among the different types and varieties of soy milks.

DISCUSSION

Soybeans and soy foods contain a large number of components and constituents that are considered to contribute to their health benefits (*25*−*30*), but it is phytoestrogens that has driven much of the resurgence in soy in recent years. This is highlighted by the fact that 85% of the scientific presentations at the recent "4th International Symposium on the Health Benefits of the Role of Soy in Preventing and Treating Chronic Disease" were focused on the role of isoflavones. The food industry has responded to the scientific and clinical data that have emerged on isoflavones by aggressively promoting increased dietary intake of soy protein and isoflavones through the development of classical and functional foods based on soybeans. In some cases, foods are being fortified with isoflavones, and there is a burgeoning industry in phytoestrogen supplements (*11*). A move to "saturate" the food chain with isoflavones raises concerns especially given that there are many unresolved issues relating to what are optimal levels of intake to balance efficacy and long-term safety (*30*−*32*).

Accurate knowledge of dietary isoflavone intake in case-control, population, and clinical studies is crucial to better understanding of the role these phytoestrogens play in accounting for any potential beneficial effect of increased incorporation of soy in the Western diet. Efforts have been made to utilize databases on isoflavone composition (*33*−*35*) and soy protein content of foods to estimate daily intakes from dietary recall questionnaires (*36*−*38*). However, the high variability in isoflavone levels in different products, as illustrated here, raises questions about the value of relying on isoflavone composition databases to calculate dietary isoflavone intakes by individuals. At best such databases are useful as a guide to which foods are low, moderate, or rich sources of isoflavones.

Most soy foods consumed in Western countries are based on whole bean products or highly purified proteins extracted from soybeans (*39*, *40*). The results reported here demonstrate a very large variability in total isoflavone concentration of one of the key ingredients used by the food industry to manufacture many soy foods. Isolates prepared by an ethanol wash process generally do not carry significant amounts of isoflavones unless they are fortified with isoflavone extracts. Soy proteins isolated by isoelectric precipitation and the acid wash process do retain a large proportion of the isoflavones in association with the protein. The isoflavone contents of the two different soy protein isolates studied here (**Figure 1**) were found, over a 3-year time period, to be quite different and in both cases highly variable, even though the protein concentration varied by only 3% over this same time period. This indicates that manufacturers of purified soy proteins may be able to control for protein level more easily than for isoflavone content. The two types of isolated soy proteins, which were prepared by different proprietary manufacturing processes, differed in total isoflavone concentration even though the ratio of all forms of daidzein/genistein was similar. For both types of isolated soy protein, maximum isoflavone concentrations, expressed in terms of aglycon equivalents, were 200−300% higher than the minimum values. The relative proportions of the three major forms of isoflavones also varied markedly based on the year of manufacture and mostly with regard to the proportion of glycitein that was present (**Figure 2**). These variations in isoflavone concentration presumably reflect differences in the overall isoflavone content of the soybeans from which the protein is isolated. Previous studies have shown significant variations in soybean isoflavones according to the conditions under which they are grown (*21*, *41*, *42*) or stored (*22*) and the maturity of the soybean (*43*). There are also wide variations among different strains of soybeans, and levels can vary on the basis of geographical locations of harvest (*21*, *23*, *44*−*48*). Such variations make it difficult to produce a whole bean soy milk with consistent isoflavone levels, as is evidenced by our data.

Because the isolated soy proteins analyzed here are used in the commercial manufacturer of soy milk and other soy foods, our findings indicated the likelihood of 2−3-fold variations in the isoflavone content of any single branded product manufactured over a prolonged time span, independent of the total protein content. This was evident from the wide variation in isoflavone content of three different brands of soy milk purchased repeatedly over a 6-month period. Furthermore, the magnitude of the difference in isoflavone content of any particular soy food will also depend on the particular manufacturer. This is evident from the marked differences we observed in the isoflavone content and composition of 85 samples of 40 different brands of soy milk drinks purchased from local supermarkets. These differences were not related to the shelf age (i.e., time interval between date of manufacture and date of analysis) of the sample (**Figure 4**). Isoflavones appear to be relatively stable over time, at least with regard to the total levels. However, it has been reported that interconversion of the individual conjugate forms can occur with storage (*22*). Such interconversion could not be addressed in our study design.

In general, soy milks made from whole soybeans had higher total isoflavone content than those made with soy protein isolates, although the wide variation among brands meant that some brands of soy milks made from isolates deliver as much isoflavone as soy milks defined as whole bean (**Figure 3**). Overall, whole bean soy milks were far more variable in isoflavone content than isolated soy protein-based drinks, varying 5-fold among brands compared with 2-fold for the latter. For clinical studies, what is important is to have available products that are relatively consistent in isoflavone content, and in this regard soy milks made from isolated soy protein do yield more consistent levels. Higher isoflavone levels in whole bean versus isolated soy protein varieties of soy milk have been noted previously, but the differences were not large enough to reach

Table 1. Relationship between Isoflavone (Expressed in Aglycon Equivalents) Levels and Soy Protein Content in a Variety of Soy-Containing Foods

	estimated protein[d] (g/100 g)	total isoflavones (mg/100 g)	isoflavone (mg/g protein)
soy products			
soybean chips[a]	54.2	35	0.6
soy links frozen raw[a]	3.9	15	3.9
natto boiled fermented[a]	46.4	20	0.4
tofu, silken, firm[a]	7.8	27.9	3.6
tempeh[a]	14.9	43.5	2.9
miso[a]	12.5	42.6	3.4
soy cheese, Cheddar[a]	7.2	28	3.9
soy cheese, mozarella[a]	7.7	32	4.2
soy cheese, Parmesan[a]	6.4	36	5.6
textured vegetable protein[b]	50	142	2.8
textured vegetable protein[b]	50	245	4.9
soy milks			
soy milk, isolate based, low isoflavone[c]	3.3	2.5	0.7
soy milk, isolate based, high isoflavone[c]	3.3	4.4	1.3
soy milk, whole bean based, low isoflavone[c]	3.1	2.4	0.8
soy milk, whole bean based, high isoflavone[c]	3.1	11.6	3.7
soy milk, entire bean[c]	3.1	3.6	1.2
soy milk[a]	3.5	9.7	2.8
soy protein materials			
soy protein isolate, aqueous extract, type 1[c]	85	114	1.3
soy protein isolate, aqueous extract, type 2[c]	85	78.7	0.9
soy protein isolate, aqueous extract, type 3[c]	85	103.4	1.2
soy protein isolate, alcohol extract[c]	85	81.9	1.0
soy protein concentrate, alcohol extract[a]	65	12.5	0.2
soy flour, defatted[a]	50	131.2	2.6
soy flour, full fat, raw[a]	35	177.9	5.1
soy flour, full fat, roasted[a]	39	198.9	5.1

[a] Data taken from Iowa State University Database on the Isoflavone Content of Foods, relay 1.3 2002, www.nal.usdagov/fnic/foodcomp/isoflav.html. [b] Data taken from ref *23*. [c] Data from Sanitarium Health Food Co. laboratories. [d] Protein contents for items identified as *a* and *b* were estimated from food composition tables as the published data did not include protein content. For items identified as *c*, protein was estimated by the Kjeldahl method using the conversion factor of 6.25 g of protein/g of N.

statistical significance (*47*, *49*). Extraction balance studies show that although some loss of isoflavone occurs during the separation of isolated protein (*40*), natural variations in isoflavone levels due to differences in cultivars, season, growth conditions, and a number of undefined factors are sufficiently large to mask differences due to manufacturing losses. This is apparent from our finding that 5 of the 85 samples of soy milks analyzed had a total isoflavone content of >90 mg/L, and these were presumably either made from isoflavone-rich cultivars of soybeans or were whole-bean varieties that could have been fortified by the addition of isoflavone concentrates to attain such high levels. Identifying specific soy milks that deliver higher levels of isoflavones would be advantageous for clinical studies because it permits smaller volumes of soy milk to be consumed, thereby facilitating compliance in dietary intervention studies. There is, however, no evidence that whole bean soy milks have greater clinical or health effects than isolated soy protein-based varieties, even though lower concentrations of isoflavones are generally found in the latter. Clearly, it is important to measure the isoflavone content of all soy foods used in clinical studies aimed at delivering specific levels of isoflavones. To negate the high variability observed in isoflavone content among and within commercial brands, products used in dietary studies should ideally be derived from a single production lot. In this regard, although labeling of soy foods for isoflavone content is not mandated in the United States and other countries, from December 2002 the Food Standards Australian New Zealand (FSANZ), formerly the Australian New Zealand Food Regulations (ANZFA) have required a statement of the isoflavone content of all foods for which any isoflavone or phytoestrogen composition claim is made. This will be of considerable help to investigators performing dietary intervention studies provided standardized methods for certification of isoflavone levels are implemented.

Standardization and Labeling of Isoflavone Content of Foods. Although there is a general consensus that soy protein and its constituent isoflavones confer health benefits, health professionals are left in a quandary as to what type of soy foods and how much to recommend to consumers because, from the isoflavone and protein perspective, not all soy foods are comparable. Our data show that while the measured protein contents of soy isolates and soy milks may be similar, the isoflavone content is extremely variable (**Table 1**). As a rule of thumb it has generally been recommended that the isoflavone content of any soy food be deduced by assuming there is 1−2 mg of isoflavones/g of protein. This does not always apply, especially if the soy protein has been alcohol washed, and frequently it is not clear from the package labels of soy foods if this is indeed the case. **Table 1** shows the variability in the relationship between isoflavone and protein contents for a selection of the soy milks analyzed in this study as compared with published data for some other soy products and soy ingredients. For much of the published isoflavone data no measurement of the protein content is given. When measured protein levels were not available, we have estimated the protein content from published food composition tables and used this estimate in the calculation of the isoflavone/protein ratio. Differences between the estimated and actual protein contents of the sample analyzed for isoflavones will obviously affect the accuracy of the calculated ratios given in **Table 1**. However, we would not expect the error associated with this calculation to be >10% of its value. The spread of values for the isoflavone/protein ratio, from 0.2 to 5.6, is very much greater than the uncertainty associated with estimating the protein content.

Given the escalation in the commercialization of soy foods there is a critical need for establishing universal guidelines and an agreed industry standard for labeling of isoflavone-rich foods

and isoflavone supplements containing these bioactive components, in a consistent and meaningful way. How can this best be done, given that there is presently no gold-standard reference method for isoflavone determination in foods, and how should data on isoflavone levels be depicted in a way that will be the least confusing to the consumer and health professional? Numerous methods exist for the measurement of isoflavones in food samples (*16*−*18*, *24*, *50*−*52*). Most are based on reverse phase HPLC techniques first developed two decades ago (*53*−*55*) with minor modifications to the extraction and detection techniques. The relative simplicity of these methods means that they should yield similar results, but this is not the case as has been shown in interlaboratory "round-robin" tests of standard soy materials (M. A. Verbruggen et al., "Isoflavone Analysis in Soy and Soy Products—Results of Ring Test", presented at the 4th International Symposium on the Role of Soy in Preventing and Treating Chronic Disease, Abstract B14). More uniform results were obtained in the round-robin study when an identical, defined analytical protocol was used by all participating laboratories (*56*). Before a gold standard can be adopted for measurement, there is a need for certified reference materials and highly purified isoflavone standards.

Although there has been discussion regarding the need for standardizing the analytical method, the biological variability in isoflavone composition of soybeans remains far more significant than the analytical variation in accounting for major differences in reported levels of isoflavones among the same food types. Large variations in both isoflavone content and the type of isoflavone present are observed in near-identical foods, even when processing conditions are quite similar, as is evident from the 85 samples of commercial soy milks analyzed.

In soybeans, or products made from purified soy proteins, there are 12 common isoflavone types. Daidzein, glycitein, and genistein form the "core" isoflavones, and these each form a series of glycosidic conjugates by a condensation between the 4′-hydroxyl group of the aglycon and the C-1 hydroxyl of the glucose. The glucosides so formed are daidzin, glycitin, and genistin, each of which can also exist esterified through carbon C-6 of the glucose with acetic and malonic acids. There is presently no consistency in the manner in which isoflavone levels in foods are being labeled. Some food products have very complex and thorough descriptions of the entire isoflavone composition including all of the individual conjugate forms. This attention to analytical detail is commendable, but overkill, as the public and most health professionals no doubt find it impossible to comprehend or interpret. There is a need to simplify the labeling and, in our opinion, it would be preferable to express the isoflavone content as simply total isoflavones, in *aglycon* equivalents. At the moment many manufacturers are quoting levels in soy foods based on "total isoflavone" including the glucoside portion, because this helps to "boost" the number, giving the appearance of more isoflavone than is actually present, but it is often unclear which format has been used. Ideally, for comparative purposes, units should be expressed in S.I. units, as molar amounts per liter or kilogram. However, because most food ingredients are reported as percentage-based units, for which the public has a better grasp of understanding, it is logical to label isoflavone levels in food as milligrams per 100 g and milligrams per serving. Although the molecular weights of genistein, daidzein, and glycitein differ, the overall difference in not correcting for molar equivalents leads to errors in calculation of only 5−6%, which from a nutritional perspective is negligible.

From an analytical certification perspective, a universal approach to measuring isoflavone levels in foods might be advantaged by subjecting the samples to complete, mild enzymic hydrolysis to convert all of the isoflavone conjugates to their respective aglycon forms, thereby simplifying both the analysis and the interpretation. This approach was used in our early methodology (*17*) and is what happens after they are ingested. Hydrolysis is relatively simple and can be quantitatively achieved by enzymes (*17*, *20*) or, more aggressively, chemically (*52*, *57*). This approach would also circumvent the need for a full range of highly purified isoflavone conjugates as calibration standards, a problem that is difficult to solve, especially with highly labile malonyl and acetyl glucoside conjugates. More recently, a method involving saponification of these labile conjugates to their respective β-glycosides was described (*56*), partly satisfying our contention of the advantages of simplifying the analysis. However, why complete hydrolysis was not advocated is unclear, because this approach would reduce all forms of conjugates to their respective aglycons, and these are considerably easier to measure with accuracy and precision. Levels expressed as total aglycon equivalents would also provide an indication of the potentially maximum bioavailable level of isoflavones in the food, because it is only the aglycons that are absorbed from the intestine. Recent studies show that the glycosides do not cross the enterocyte and that hydrolysis is a crucial first step in their bioavailability in humans (*20*). Therefore, what is important in soy food labeling, apart from the protein content, is knowledge of the potentially maximum bioavailable level of isoflavone being ingested, and the various conjugate forms in which isoflavones exist is largely of academic interest and of little physiological or nutritional relevance given the tremendous capacity of the gastrointestinal tract to efficiently hydrolyze glycosides in adults and infants.

There are conflicting studies about the role of soy and isoflavones in lipid-lowering, as reviewed by Clarkson (*58*). Although isoflavones alone have failed to show significant cholesterol-lowering effects (*59*, *60*) it has been hypothesized from several lines of evidence that the hypocholesterolemic actions of soy protein appear to require the presence of isoflavones. This contention has been based on experiments with alcohol-extracted soy protein with varying amounts of "added-back" isoflavones (*61*−*65*). Other studies are contradictory (*66*, *67*). However, limitations of this approach to "teasing-out" the components responsible have been pointed out (*58*, *68*), most notably the significant alteration to the protein matrix that occurs with alcohol extraction and the coextraction of other components in soy that may act either alone or in concert with isoflavones to influence cholesterol homeostasis. To further complicate the matter, intestinal metabolism, and specifically the propensity toward equol production by some individuals, seems now to be a determinant of the clinical responsiveness to soy isoflavones (*69*). Recent studies indicate that isoflavones probably play a role in reducing lipid peroxidation (*70*−*73*), improving arterial reactivity (*60*, *74*,), reducing blood pressure (*75*, *76*), and influencing proinflammatory cytokines (*77*), factors independent of cholesterol that are important in reducing the risk for cardiovascular disease. Indeed, recent analysis of data from "The Framingham Study" concluded that a high intake of phytoestrogens was associated with a favorable metabolic cardiovascular risk profile (*78*). The consensus of studies is that high intakes of isoflavones are more robust at lipid-lowering. However, we contend that as little as 6−8 g of soy protein (which would translate to 6−16 mg of isoflavone expressed as aglycons) may be sufficient for long-term prevention of cardiovascular disease

as this is the typical range of intake of Japanese adults that have on average much lower serum cholesterol levels than Westerners (*79*). As is evident from our data, the consumption of 25 g of soy protein, the FDA and JHCI's recommended level for heart health, could deliver a range of isoflavone intakes varying from 15 to 142 mg/day depending on the type of soy food consumed or the soy protein source (**Table 1**). This large variation in such a bioactive class of compounds could readily account for some of the inconsistencies in reported outcomes in clinical studies of soy foods. Accurate labeling of food products, using standardized reference methods, would be a positive step forward in helping consumers, health professionals, and those researchers investigating the health attributes of soy in dietary intervention studies.

ACKNOWLEDGMENT

We greatly appreciate the excellent technical assistance of Linda Zimmer-Nechmias, Wayne Brashear, Ph.D., and Susan McCarthy in the analysis of the soy milks and soy protein isolates.

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Received for review December 6, 2002. Revised manuscript received February 27, 2003. Accepted March 3, 2003. This work was partly funded through a grant from the National Institutes of Health (R01CA73328) for studies of the pharmacokinetics of isoflavones in different soy foods.

JF026199B