Mass Balance Study of Isoflavones during Soybean Processing

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The effects of processing techniques on the distribution of isoflavones were investigated by manufacturing tempeh, soymilk, tofu, and protein isolate. The manufacturing steps causing significant losses ($p < 0.05$) of isoflavones were as follows: soaking (12%) and heat processing (49%) in tempeh production; coagulation (44%) in tofu processing; and alkaline extraction (53%) in soy protein isolate production. In the production of tempeh, soymilk, and tofu, malonyldaidzin and malonylgenistin decreased after soaking and cooking. Concomitantly, acetyldaidzin and acetylgenistin were generated during heat processing. After fermentation, daidzein and genistein concentrations increased in tempeh, apparently as a result of fungal enzymatic hydrolysis. In protein isolate processing, alkaline extraction caused the generation of daidzein and genistein, probably through alkaline hydrolysis.

Keywords: *Isoflavones; mass balance; soy protein isolate; tempeh; soymilk; tofu*

INTRODUCTION

Soybeans have been incorporated into a popular human diet throughout Asian countries for centuries. Traditionally, soybeans consumed in Asia are usually divided into two groups: fermented and nonfermented. The nonfermented soy foods include fresh soybeans, soybean sprouts, soymilk, tofu, and toasted soy protein flours. Miso, natto, soy sauce, and tempeh are representatives of the fermented soyfoods group (Wilson *et al*., 1992). In the United States, soybeans are principally used in the forms of soybean protein products, such as flours, grits, isolates, concentrates, and textured soy proteins, and are usually employed as ingredients of a wide variety of foods (Berk, 1992). Recent trends have indicated increased consumption of tofu, soymilk, and tempeh in the United States.

Recently, increasing evidence has indicated that soybeans might have cancer-preventive properties, by epidemiological (Goldin *et al*., 1986; Adlercreutz *et al*., 1986, 1991, 1992; Lee *et al.*, 1991), animal (Axelson *et al*., 1984; Barnes *et al.*, 1990; Baggot *et al*., 1990; Sharma *et al*., 1992; Wei *et al*., 1993; Hendrich *et al*., 1994), and *in vitro* studies (Adlercreutz *et al*., 1992; Wei *et al*., 1993). Results from these studies have suggested that the isoflavones in soybeans might be the contributing factors in prevention of cancer. These compounds possess estrogenic (Miksicek, 1993), antioxidative (Wei *et al*., 1993), antifungal (Weidenbörner *et al*., 1990), and aromatase-inhibiting (Adlercreutz *et al*., 1993) activities. Genistein (4′,5,7-trihydroxyisoflavone) is an potent inhibitor of the activities of tyrosine protein kinases (Akiyama *et al.*, 1987) and DNA topoisomerase (Okura *et al.*, 1988). At concentrations in the microgram per milliliter range, genistein has the ability to affect cells by inducing differentiation (Watanabe *et al*., 1991; Honma *et al*., 1991). Genistein has been reported to have antiproliferative effects on the growth of human breast carcinoma (Peterson and Barnes, 1991) and gastric cancer cell lines (Matsukawa *et al*., 1993). These biological properties of isoflavones have drawn a great deal of attention.

In previous studies, in order to determine the dietary levels of isoflavones in common soy foods, we have investigated a wide array of commercial soy-based products and found considerable differences among samples in terms of content and distribution of isoflavones (Murphy, 1982; Wang and Murphy, 1994a). We have proposed that the variety of soybeans, the method of processing, and the addition of non-soy components were the affecting factors. Additionally, we investigated the composition of isoflavones in 11 varieties of soybeans (Wang and Murphy, 1994b). The results have shown that American and Japanese varieties of soybeans had similar isoflavone isomer distribution; however, the total concentrations appeared to fluctuate due to genetics, crop year, and growth location. In the present study, we have examined the effects of different methods of processing on the retention, distribution, and transformation of isoflavones (Figure 1) by conducting mass balance studies during the preparation of four kinds of soybean products, tempeh, soymilk, tofu, and soy protein isolate, from a selected soybean variety, Vinton 81.

MATERIALS AND METHODS

Soybeans used in this study were food-grade Vinton 81, generously provided by Dr. L. A. Wilson of the Department of Food Science and Human Nutrition, Iowa State University. Soybeans from crop year of 1992 were used for making tempeh and 1993 for making soy protein isolate, soymilk, and tofu.

Tempeh Production. Tempeh was prepared by the method of Hedger (1982). A procedure for making tempeh is illustrated in Figure 2. Soybeans (100 g) were washed and then soaked in tap water (330 mL) for 10−12 h at room temperature (24 °C). The soaked beans were drained and suspended in fresh water and dehulled by hand. After dehulling, the beans and approximately 1200 mL of water were placed in a glass beaker and heated on a hot plate with frequent stirring, brought to the boil, and boiled for 20 min. Immediately the beans were drained thoroughly through a sieve and spread over a clean tray already surface sterilized with alcohol. The beans was stirred with a spatula to promote evaporation of excess water and allowed to cool to 35−40 °C. A 10 g of inoculum of *Rhizopus oligosporus* (culture 22959, American Type Culture Collection, Rockville, MD) was added to the boiled beans, and the whole mass was mixed thoroughly. The

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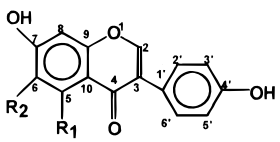
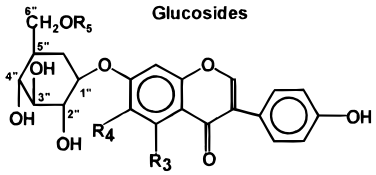
Aglycones



R_1	R_2	Compound
H	H	Daidzein
OH	H	Genistein
H	OCH_3	Glycitein

Glucosides



R_3	R_4	R_5	Compound
H	H	H	Daidzin
OH	H	H	Genistin
H	OCH_3	H	Glycitin
H	H	$COCH_3$	6"-O-Acetyldaidzin
OH	H	$COCH_3$	6"-O-Acetylgenistin
H	OCH_3	$COCH_3$	6"-O-Acetylglycitin
H	H	$COCH_2COOH$	6"-O-Malonyldaidzin
OH	H	$COCH_2COOH$	6"-O-Malonylgenistin
H	OCH_3	$COCH_2COOH$	6"-O-Malonylglycitin

Figure 1. Structures of the isoflavone isomer in soybean foods.

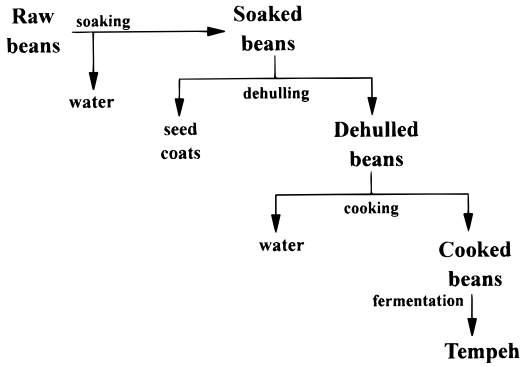


Figure 2. Flow diagram for processing of soybeans to tempeh.

beans mixed with fungi were placed in a new plastic bag previously pierced at 1−2 cm intervals all over its surface. The contents were formed into a cake about 2 cm thick and incubated in the dark at 37 °C for 22 h. After fermentation, tempeh was frozen and stored at −10 °C.

Soymilk and Tofu Production. The pilot-plant method of soymilk and tofu processing was according to Chen (1993). A flow sheet for a soymilk and tofu production is shown in Figure 3. Soybeans (600 g) were soaked in tap water for 13 h at room temperature, washed, and ground twice in a Model MC 15 Microcut (Stephan Machinery Corp., Columbus, OH) with 4 L of tap water. The slurry was cooked at 95 °C for 7 min, with an additional 1 L of tap water in a steam-jacketed kettle. The cooked slurry was filtered through a fine mesh sack and squeezed by hand to separate soymilk from the water-insoluble residue, okara. In order to produce tofu, the volume of the collected soymilk was recorded and the solids content was measured by using refractometer. The addition of tap water was necessary to adjust the solids content of soymilk to 5%. The amount of food-grade coagulant ($CaSO_4 \cdot H_2O$) was determined by the soymilk solids level and the final soymilk volume (Johnson, 1984). The soymilk was then transferred back to the steam-jacketed kettle and heated to 85 °C with frequent stirring. After the coagulant was added, 3 min was

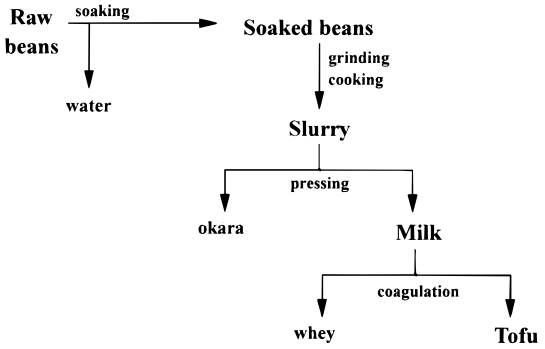


Figure 3. Flow diagram for processing of soybeans to soymilk and tofu.

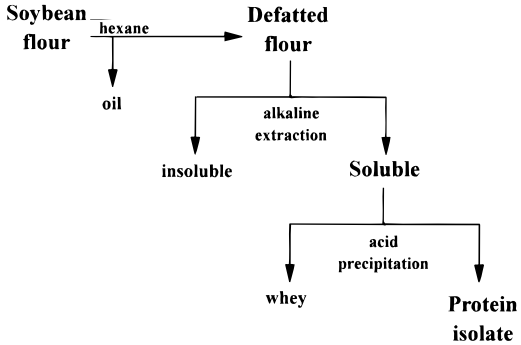


Figure 4. Flow diagram for processing of soybeans to protein isolate.

needed to allow the curds to form. The curds were poured into a cheese cloth-lined, stainless steel box, with perforations on all sides, placed in a larger bucket. The curd was then pressed for a total of 15 min and stored in tap water at 4 °C.

Soy Protein Isolate Production. Soy protein isolates were prepared by following the method described by Wolf (1970). The process for producing soy isolates is outlined in Figure 4. Finely ground soybean flour (50 g) was defatted by using hexane at room temperature. After evaporation of the hexane residue, defatted flours were extracted with dilute sodium hydroxide solution (100 mM) at a pH of 8.5 for 3 h at room temperature. The insoluble residue, containing water-insoluble polysaccharides and protein, was separated by filtrating through Whatman filter paper No. 1 (Micron Separation Inc., Westborough, MA). The clarified extract was adjusted to pH 4.5 with hydrochloric acid to precipitate proteins. The precipitated proteins were removed by centrifugation under 4400*g* for 30 min by using Beckman centrifuge Model J2-21 (Beckman Instruments Inc., Palo Alto, CA). The precipitated proteins were washed and freeze-dried to give the isoelectric protein.

All processing operations for soy foods were carried out in triplicate. The moisture content of all samples was determined by freeze-drying to a constant weight to preserve isoflavone distribution. Higher drying temperatures can change the isomer distribution (Wang and Murphy, 1994a). Data of the moisture content were used for calculation of isoflavone concentration in samples on a dry basis. Samples of the dried residues were used for isoflavone analysis.

Isoflavone Extraction. Freeze-dried samples (2 g) were ground, mixed with 2 mL of 0.1 N HCl and 10 mL of acetonitrile, stirred for 2 h at room temperature, and filtered through Whatman No. 42 filter paper. The filtrate was taken to dryness under vacuum at a temperature below 30 °C. The dried material was redissolved in 10 mL of 80% methanol and then filtered through a 0.45 μm filter unit (Alltech Associates, Deerfield, IL). Filtrate (20 μL) was injected for the HPLC analysis.

HPLC Analysis. The high-performance liquid chromatography (HPLC) system used was described previously (Wang and Murphy, 1994a). A linear HPLC gradient was employed: solvent A was 0.1% glacial acetic acid in H_2O, and solvent B

Table 1. Yield, Moisture, and Isoflavone Amounts[a,b] in Tempeh Processing

step	yield (g)	moisture (%)	total daidzein (mg)	total genistein (mg)	total glycitein (mg)	total (mg)
raw soybeans	100	11.03 ± 0.07	39.2 ± 1.5^{A}	73.9 ± 4.2^{A}	4.8 ± 0.2^{A}	117.9 ± 5.6^{A}
soaked soybeans	229	63.23 ± 0.40	34.4 ± 1.7^{B}	65.2 ± 3.8^{AB}	4.5 ± 0.2^{A}	104.1 ± 5.3^{AB}
soaking water	nc[a]					
dehulled soybeans	208	61.69 ± 0.12	32.0 ± 4.7^{B}	60.7 ± 8.6^{B}	4.3 ± 0.5^{A}	97.0 ± 14.0^{B}
seed coats	21	81.11 ± 1.88	0.9 ± 0.1^{D}	0.5 ± 0.0^{D}	0.2 ± 0.0^{C}	1.6 ± 0.1^{D}
cooked soybeans	176	65.73 ± 0.67	12.1 ± 1.3^{C}	25.1 ± 2.1^{C}	2.1 ± 0.7^{B}	39.3 ± 4.0^{C}
cooking water	nc					
tempeh	167	66.28 ± 0.16	7.8 ± 3.3^{C}	18.6 ± 8.3^{C}	1.5 ± 0.7^{B}	27.9 ± 12.0^{C}

[a] In order to estimate total isoflavone amounts, individual isoflavone glucosides and aglycon forms were normalized for their molecular weight differences and summed. Values represent the mean ± standard deviation; $n = 3$. Values in a column with different superscripts were significantly different ($p < 0.05$). nc, not collected. [b] Calculated on dry basis.

Table 2. Effects of Tempeh Processing on the Mass Distribution of Isoflavone Isomers (mg)[a,b]

	glucoside[c]			malonyl glucoside[c]			acetyl glucoside[c]		aglycon[c]	
step	Din	Gin	Glin	Din	Gin	Glin	Din	Gin	Dein	Gein
raw soybeans	28.3^{B}	38.8^{B}	3.7^{A}	40.3^{A}	91.9^{A}	4.6^{A}	1.0^{A}	0.2^{D}	0.9^{B}	1.7^{B}
soaked soybeans	36.8^{A}	54.0^{A}	3.9^{A}	21.6^{B}	56.6^{B}	3.8^{A}	0.1^{C}	0.8^{BCD}	1.0^{B}	2.0^{B}
dehulled soybeans	34.8^{A}	52.8^{A}	3.8^{A}	18.6^{C}	49.6^{B}	3.6^{B}	0.1^{C}	0.9^{BC}	1.3^{B}	1.8^{B}
cooked soybeans	16.8^{C}	32.2^{B}	2.0^{B}	1.8^{D}	7.2^{C}	1.4^{B}	0.5^{B}	2.0^{A}	0.7^{B}	1.2^{B}
tempeh	2.5^{D}	11.6^{C}	1.8^{B}	1.6^{D}	7.5^{C}	0.7^{B}	0.6^{B}	1.5^{AB}	5.1^{A}	7.4^{A}
LSD	4.3	8.5	0.4	2.9	7.4	1.3	0.3	0.7	1.6	1.9

[a] Values represent the mean ± standard deviation; $n = 3$. Values in a column with different superscripts were significantly different ($p < 0.05$). [b] Calculated on dry basis as milligrams of individual isomer. [c] Din = daidzin; Gin = genistin; Glin = glycitin; Dein = daidzein; Gein = genistein.

Table 3. Yield, Moisture and Isoflavone Contents[a,b] in Soymilk and Tofu Processing

step	yield (g)	moisture (%)	total daidzein (mg)	total genistein (mg)	total glycitein (mg)	total
raw soybeans	600	11.03 ± 0.07	59.3 ± 20.0^{A}	124.0 ± 35.0^{A}	33.9 ± 7.7^{AB}	217.2 ± 63.0^{A}
soaked soybeans	1297	60.95 ± 0.48	54.2 ± 31.0^{AB}	114.7 ± 47.0^{A}	27.8 ± 4.1^{B}	196.8 ± 82.0^{A}
soaking water[c]	1063	99.67 ± 0.08	0.5 ± 0.3^{D}	0.3 ± 0.0^{C}	0.2 ± 0.0^{D}	1.0 ± 0.3^{C}
cooked slurry	5976	91.85 ± 0.09	67.9 ± 17.0^{A}	121.6 ± 22.0^{A}	37.7 ± 2.7^{A}	227.2 ± 41.0^{A}
soymilk	5581	93.93 ± 0.09	63.6 ± 12.0^{A}	103.7 ± 16.0^{A}	27.6 ± 1.9^{B}	194.8 ± 30.0^{A}
okara[c]	717	79.08 ± 0.67	4.1 ± 0.8^{D}	14.0 ± 0.6^{BC}	7.8 ± 2.1^{C}	25.9 ± 0.7^{BC}
tofu	1390	82.11 ± 2.21	16.0 ± 1.5^{CD}	40.2 ± 3.4^{B}	14.6 ± 2.9^{C}	70.8 ± 4.7^{B}
whey[c]	5140	98.37 ± 0.13	35.9 ± 1.3^{BC}	50.4 ± 1.8^{B}	9.1 ± 0.1^{C}	95.4 ± 3.2^{B}

[a] In order to estimate total isoflavone amounts, individual isoflavone glucosides and aglycon forms were normalized for their molecular weight differences and summed. Values represent the mean ± standard deviation; $n = 3$. Values in a column with different superscripts were significantly different ($p < 0.05$). [b] Calculated on dry basis. [c] Typical discards from this processing operation.

was 0.1% glacial acetic acid in acetonitrile; following injection of 20 μL of sample, solvent B increased from 15 to 35% over 50 min and then held at 35% for 10 min. The solvent flow rate was 1 mL/min. A Waters 991 series photodiode array detector (Millipore Corp., Marlborough, MA) monitored from 200 to 350 nm. UV spectra were recorded, and area responses were integrated by Waters software. Minimum levels of detections were the same as given in Wang and Murphy (1994a).

Statistical Analysis. All sample analyses were run in triplicate. Statistical analysis was done by using the SAS package (version 6.03, 1995) developed by the SAS Institute, Inc. (Box 8000, Cary, NC). Analyses of variance using the general linear models (GLM) were conducted. Differences between the sample means were analyzed by Fisher's least significant difference (LSD) test at $\alpha = 0.05$.

RESULTS

Redistribution of Isoflavones during Tempeh Production. Each processing step in making tempeh led to losses in isoflavones. The results show that 100 g of whole soybeans yielded 167 g of tempeh with isoflavone losses of 76% (Table 1). During this process, 12 and 49% of the isoflavones were leached from materials during soaking/dehulling and cooking steps, respectively ($p < 0.05$). Fermentation did not generate major differences from cooked soybeans in terms of the total content of isoflavones. Unfortunately, soaking water and cooking water were not analyzed for isoflavones. Therefore, the isoflavone concentrations for these fractions were estimated by difference.

Table 2 presents the individual isoflavone isomer amounts retained in the materials at different stages during tempeh preparation. Compared to the isoflavone distribution pattern of raw soybeans, soaked soybeans had higher concentrations of the glucosides daidzin and genistin and lower 6″-*O*-malonyldaidzin and 6″-*O*-malonylgenistin. There were insignificant changes in the isoflavone distribution after removal of seed coats, which did not include hypocotyls. A major decrease in the glucosides and the malonylglucosides was found in the cooked soybeans. After fermentation, the aglycons, daidzein and genistein increased in concentration in tempeh with a further decrease of the corresponding glycons daidzin and genistin.

Isoflavone Distribution in Pilot-Plant Soymilk and Tofu Preparation. There was no significant loss of isoflavones in soymilk production. The yield (grams of fresh tofu per gram of dry soybeans) of tofu in this study was 2.32, which is appropriate for this style of traditional tofu, called momem or cotten tofu (Wilson *et al.*, 1992) (Table 3). Tofu contained 33% (based on dry matter) of the isoflavones in the starting material, the raw soybeans. The total mass of isoflavones lost in the soaking water, okara, and whey were 1.0, 25.9, and

Table 4. Effects of Soymilk and Tofu Processing on the Mass Distribution of Isoflavone Isomers (mg)[a,b]

	glucoside[c]			malonyl glucoside[c]			acetyl glucoside[c]		aglycon[c]		
step	Din	Gin	Glin	Din	Gin	Glin	Din	Gin	Dein	Gein	Glein
soybeans	51.7^{AB}	77.2^{BC}	23.8^{A}	52.4^{A}	133.7^{A}	25.8^{A}	0.5^{B}	1.0^{C}	1.0^{E}	5.5^{D}	5.0^{CD}
soaked soybeans	45.1^{B}	63.4^{CD}	23.3^{A}	49.6^{A}	134.0^{A}	24.3^{AB}	1.0^{B}	1.1^{C}	1.0^{E}	4.6^{D}	0.0^{D}
cooked slurry	65.2^{A}	97.9^{A}	24.3^{A}	19.6^{B}	63.8^{B}	20.3^{B}	1.1^{AB}	4.9^{A}	17.6^{A}	24.4^{A}	11.4^{A}
soymilk	65.6^{A}	90.2^{AB}	18.9^{B}	15.3^{B}	48.5^{BC}	14.3^{C}	1.3^{A}	4.2^{A}	15.1^{B}	19.7^{B}	7.9^{AB}
tofu	9.4^{C}	29.5^{D}	9.3^{C}	4.9^{B}	14.7^{C}	6.1^{D}	1.0^{B}	1.7^{B}	7.2^{C}	13.2^{C}	5.5^{BC}
LSD	23.5	24.5	2.8	21.3	45.2	4.7	0.7	0.9	1.9	1.5	4.5

[a] Values represent the mean ± standard deviation; $n = 3$. Values in a column with different superscripts were significantly different ($p < 0.05$). [b] Calculated on dry basis as milligrams of individual isomer. [c] Din = daidzin; Gin = genistin; Glin = glycitin; Dein = daidzein; Gein = genistein; Glein = glycitein.

Table 5. Yield and Isoflavone Contents[a,b] in Soy Protein Isolate Processing

step	yield (g)	total daidzein (mg)	total genistein (mg)	total glycitein (mg)	total (mg)
soybean flour	50	9.1 ± 0.3^{A}	18.4 ± 1.2^{A}	2.4 ± 0.5^{A}	30.0 ± 1.7^{A}
defatted flour	43	10.0 ± 0.6^{A}	20.0 ± 2.4^{A}	1.7 ± 0.4^{B}	31.7 ± 3.2^{A}
oil[c]	8	0.7 ± 0.6^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{D}	0.7 ± 0.6^{C}
alkaline soluble	949	7.8 ± 2.6^{A}	8.4 ± 2.6^{B}	1.7 ± 0.1^{B}	17.8 ± 5.3^{B}
alkaline insoluble[c]	76	6.0 ± 1.8^{B}	9.0 ± 2.9^{B}	0.8 ± 0.2^{C}	15.8 ± 4.9^{B}
protein isolate	9	6.2 ± 0.6^{B}	7.8 ± 1.0^{B}	0.5 ± 0.1^{C}	14.5 ± 1.5^{B}
whey[c]	865	1.5 ± 0.1^{C}	0.9 ± 0.1^{C}	0.9 ± 0.1^{C}	3.3 ± 0.1^{C}

[a] In order to estimate total isoflavone amounts, individual isoflavone glucosides and aglycon forms were normalized for their molecular weight differences and summed. Values represent the mean ± standard deviation; $n = 3$. Values in a column with different superscripts were significantly different ($p < 0.05$). [b] Calculated on dry basis by freeze-drying to a constant weight. [c] Typical discards from this processing operation.

Table 6. Effects of Processing of Soy Protein Isolate on the Mass Distribution Isoflavone Isomers (mg)[a,b]

	glucoside[c]			malonyl glucoside[c]			acetyl glucoside[c]		aglycon[c]	
step	Din	Gin	Glin	Din	Gin	Glin	Din	Gin	Dein	Gein
soybean flour	4.8^{B}	7.7^{B}	1.5^{A}	10.6^{A}	25.2^{A}	1.6^{A}	0.3^{B}	0.7^{B}	0.5^{B}	0.6
defatted flour	7.7^{A}	11.0^{A}	1.2^{A}	8.6^{B}	24.5^{A}	0.4^{BC}	1.0^{A}	0.4^{B}	0.4^{B}	0.7
alkaline soluble	0.4^{C}	1.2^{B}	0.7^{B}	1.8^{C}	4.1^{B}	0.8^{B}	0.0^{B}	6.6^{A}	5.5^{A}	0.8
protein isolate	1.0^{C}	0.5^{C}	0.0^{C}	0.7^{D}	2.5^{B}	0.1^{C}	0.0^{B}	5.2^{A}	6.2^{A}	0.5
LSD	0.9	1.1	0.3	0.7	2.4	0.5	0.0	2.0	2.5	0.4

[a] Values represent the mean ± standard deviation; $n = 3$. Values in a column with different superscripts were significantly different ($p < 0.05$). [b] Calculated on dry basis as milligrams of individual isomer. [c] Din = daidzin; Gin = genistin; Glin = glycitin; Dein = daidzein; Gein = genistein; Glein = glycitein.

95.4 mg, respectively. The coagulation step contributed to the primary source of loss of isoflavones ($p < 0.05$) in producing this traditional syle of tofu. When the mass of isoflavones in all fractions were summed, a total isoflavone recovery of 196.8 mg was obtained, which was not different from the amount of isoflavones, 217.2 mg, in the starting raw soybeans ($p < 0.05$). This indicates that the isoflavones were not destroyed by the heating step but were fractionated into the okara and whey. Losses in the soaking water were quite small.

The changes in the distribution of 11 isoflavone isomers at different stages during soymilk and tofu production are presented in Table 4. Raw soybeans and soaked soybeans possessed comparable profiles of isoflavones. Cooking of the soy slurry decreased 6″-*O*-malonyldaidzin and 6″-*O*-malonylgenistin and increased the aglycons daidzein, genistein, and glycitein and the glucosides daidzin and genistin. Filtration of the slurry to generate soymilk did not show considerable alteration of isoflavone distribution but caused slight reductions in concentrations. The coagulation of soymilk to produce tofu caused the largest losses of isoflavones in tofu processing but resulted in little change in the distribution of the forms.

Isoflavone Distribution during Soy Protein Isolate Preparation. A 17.8% yield of soy protein isolate was produced from raw soybean flour (Table 5). The procedure for producing soy protein isolate in this study resulted in a significant loss of 53% of total isoflavone contents in the processing steps between the raw material and the protein isolate (Table 5). The alkaline extraction step caused the major losses of isoflavones into the alkaline-insoluble fraction, which is typically discarded. The recovery of isoflavone in all fractions was not significantly different ($p > 0.05$) from the raw soy flour.

The effect of processing on the changes in isoflavone isomer distribution is shown in Table 6. The samples of soybean flour before and after defatting showed in no changes in the pattern with the major isomers being daidzin, genistin, 6″-*O*-malonyldaidzin, and 6″-*O*-malonylgenistin. The defatting step did not influence the distribution of these constituents. However, in the alkaline extraction step, the aglycons daidzein, genistein, and glycitein increased in the soluble portion compared to the dry starting materials. Concomitantly, the principal glycosides in the initial soy flour decreased in concentration. As a consequence, the final product, soy protein isolate, contained higher percentages of isoflavone aglycons compared to soy flour.

In contrast to the other three products produced, our soy isolate production utilized no heat processing, and consequently, the distribution of the glucosides and malonlyglucosides did not change appreciably from the raw soy flour. Very little acetyl glucosides were produced. In commercial production of soy isolates, spray-drying or drum-drying may be employed to dry the isolate and would be expected to redistribute isoflavone

isomer composition as a result of heat. In contrast with our freeze-dried isolate, the commercial isolates we have evaluated do have less malonyl glucosides and more acetyl glucosides (Wang and Murphy, 1994a).

DISCUSSION

There was a significant impact on the retention and distribution of isoflavones as a result of different techniques used to produce different types of soy foods. The manufacture of tempeh, soymilk, tofu, and soy protein isolate were based on the different processing techniques, principally, fermentation, chemical coagulation, and pH precipitation. Several common steps were shared for both tempeh and tofu manufacturing, soaking, heat treatment of the cotyledons, and removal of insoluble residues (Figures 2 and 3).

Soaking the soybeans rehydrated their cellular structure and made the beans easier to dehull for tempeh making and reduced the amount of energy required to grind beans for soymilk and tofu production. The beans lost about 10% of isoflavones during soaking due to leaching into the soaking water (Tables 1 and 3). Soaking water contained 34% of the daidzein and 18% of the genistein (data not shown). This result was comparable to Matsuura *et al.* (1989), where daidzein and genistein concentrations increased during soaking of soybeans and were produced maximally at 50 °C and at pH 6.0 for the soaking water. β-Glucosidases were confirmed to be responsible for the production of daidzein and genistein during soaking by addition of a competitive inhibitor, glucono-δ-lactone, to the soaking water (Matsuura *et al.*, 1993). Thus, enzymatic hydrolysis decreased the glucosides in the soybean matrix (Tables 2 and 4).

Heat treatment steps were employed for both tempeh and tofu processing. This heat treatment should inactivate soybean trypsin inhibitors, increase nutritional value, reduce raw or beany flavors, and kill bacteria to prevent undesirable fermentation (Shurtleff and Aoyagi, 1979a). In the tempeh making, cooking could increase the speed of leaching of isoflavones, especially without the barrier of seed coats, and did result in a loss of total isoflavones into the cooking water (Table 1). In contrast, cooking did not influence the isoflavone retention during the making of tofu (Table 3), because cooking water was incorporated in the product. However, cooking did alter the distribution of isoflavones. Malonyl glucosides decreased dramatically (Tables 2 and 4) with an increase in acetyl isoflavone glucosides, 6″-*O*-acetyldaidzin and 6″-*O*-acetylgenistin. The acetyl derivatives might have arisen from corresponding malonyl derivatives during heat treatment, as suggested by Kudou *et al.* (1991). Malonylated isoflavone glucosides appeared to be heat-labile (Park *et al.* 1992; Kudou *et al.* 1991). These observations support the finding that very low levels of malonyl glucosides were present in most commercially processed soy products (Wang and Murphy, 1994a).

Removal of seed coats of soybean in tempeh production is a requirement for enzyme penetration and for proper mycelial growth (Shurtleff and Aoyagi, 1979a). This process caused only a 1.4% isoflavone loss (Table 1). It has been reported that soybean hulls contain no (Kudou *et al.* 1991) or small amounts of isoflavones (<10%), depending on soybean variety (Eldridge and Kwolek, 1983).

During the process of soymilk and tofu production, okara is removed from the slurry, after the slurry has been cooked, by filtering and pressing. Most of the water-soluble nutrients and solids are left in the filtrate (soymilk), and the insoluble residue, the okara, contains most of the fiber. In this study, isoflavone loss was not significantly related to okara (Table 3). It appeared that isoflavones were associated with the soluble components, probably soluble proteins. From the results of recent studies of soyfood isoflavone analysis, it appears that isoflavones were found at higher concentrations in high-protein-containing soyfoods (Murphy, 1982; Coward *et al.*, 1993; Wang and Murphy, 1994a). Coagulation of the soymilk is the most important step in the tofu-making process in order to obtain a product of good quality. In this step, major isoflavone losses in tofu were attributed to the whey (Table 3). During the coagulation, the metallic cation from the coagulant, Ca^{2+}, reacts with the various proteins in the soymilk and precipitate to form curds (Shurtleff and Aoyagi, 1979b). Some protein-associated isoflavones might be released into the whey. The soaking and cooking processing steps in soymilk and tofu production yielded higher concentrations of glucoside and aglycon isoflavones and the production of acetyl glucosides of isoflavones (Table 4).

Dwyer *et al.* (1994) reported similar concentrations of isoflavones in both commercial soymilk and tofu. The type of tofus analyzed in their study was different from the product produced in our study. The Dwyer *et al.* tofus were the aseptic style, where commercial soymilk is coagulated in the package with δ-gluconolactone and no whey is removed. Our tofu was the momen or traditional style, where the whey is discarded after curd production to produce a preferred product (Wilson *et al.*, 1992). In establishing a data base on isoflavone concentrations in foods, it will be critical to understand the types of processing similarly named products have undergone to properly interpret the results of processing on isoflavone concentration and distribution.

Fermentation in tempeh making did not cause a significant loss of isoflavones (Table 1) but generated a different isoflavone distribution (Table 2). Compared to the cooked soybeans in the tempeh production scheme, the final product contained 6.5 times higher aglycons and 57% lower glucosides (Table 2). This was in agreement with the report of György *et al.* (1964) and of Lampe *et al.* (1994). The liberation of aglycons from glucosides occurring during fermentation was the hydrolytic action of β-glucosidase from the fungi (Murakami *et al.*, 1984). However, we did not detect the compound of 4′,6,7-trihydroxyisoflavone (factor 2 in György's paper). Klus *et al.* (1993) found that two tempeh-producing microorganisms, *Brevibacterium epidermidis* and *Micrococcus luteus*, could transform glycitein to 4′,6,7-trihydroxyisoflavone by an *O*-demethylation reaction and that a third tempeh-producing microorganism, *Microbacterium aborescens*, could convert daidzein and glycitein to 4′,6,7-trihydroxyisoflavone by a hydroxylation and by a 6-*O*-methylation reaction, respectively. The genus *Rhizopus*, which was used in the present study, is not capable of performing those reactions (Klus *et al.*, 1993). These aglycons possess antioxidative properties which make tempeh more stable (György *et al.*, 1964).

The preparation of soy protein isolates was different from the other products in this study. Defatting ground soybeans with hexane did not extract isoflavones, in agreement with Eldridge and Kwolek (1983) (Table 5). When defatted flour was extracted by dilute alkaline solution, almost 50% of the isoflavones was lost in the

insoluble portion. The alkaline pH might modify the charges of isoflavone and protein, and then disrupt the association between isoflavone and protein. In the alkaline-soluble fraction, significant concentrations of the aglycons daidzein, genistein, and glycitein were found (Table 6), probably due to hydrolysis by soy glucosidases. The product of soy protein isolate in this study contained no acetylated isoflavone glucosides, which was dissimilar to the several commercial samples analyzed previously (Wang and Murphy, 1994a), probably because freeze-drying, rather than spray-drying, was employed.

The human bioavailability of isoflavones of soy products has been evaluated. According to Xu *et al.* (1994), daidzein was more bioavailable than genistein and approximately 85% of the isoflavones was degraded in the intestine when 12 adult women received single doses of isoflavones in soymilk as part of a liquid diet. There was no difference in the bioavailability of isoflavones from texturized vegetable protein (TVP) or tofu (Xu, 1995), although TVP has a different profile of isoflavone distribution than tofu (Wang and Murphy, 1994a). Fermented soy had a higher urinary isoflavonoid recovery in healthy men than unfermented soy (Lampe *et al.*, 1994). These studies all mentioned that absorption, excretion, and plasma concentration of isoflavones in humans were related to the amount consumed. Our study suggested that processing can affect the retention and distribution of isoflavones in food. Therefore, in order to reduce the loss of isoflavones from soy products, optimizing processing condition would be necessary. Other technologies have been used in soybean processing, including extrusion for TVP, selective extraction to make soybean protein concentrates, and strong pH changes to make spun fibers of soybean protein. Evaluation of these processing methods on isoflavone distribution would be interesting.

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