Genistein, Daidzein, and Their β-Glycoside Conjugates: Antitumor Isoflavones in Soybean Foods from American and Asian Diets†

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A method is described for the separation and analysis of isoflavone β-glycoside conjugates and aglucones in various foods derived from soybeans. After initial extraction using 80% aqueous methanol and defatting of the extract with hexane, the isoflavones were analyzed by gradient elution reversed-phase high-pressure liquid chromatography. Their structures were confirmed by fast atom bombardment ionization mass spectrometry and by proton nuclear magnetic resonance spectroscopy. The results reveal that most Asian and American soy products, with the exception of soy sauce, alcohol-extracted soy protein concentrate, and soy protein isolate, have total isoflavone concentrations similar to those in the intact soybean. Asian fermented soy foods contain predominantly isoflavone aglucones, whereas in nonfermented soy foods of both American and Asian origin isoflavones are present mainly as β-glycoside conjugates. Since the much larger estimated daily intake of these isoflavones by Asians compared to Americans is similar on a body weight basis to the isoflavones in soybean-containing diets which inhibit mammary tumorigenesis in animal models of breast cancer, it is possible that dietary isoflavones are an important factor accounting for the lower incidence and mortality from breast cancer in Asian women.

INTRODUCTION

The concept of reducing cancer risk by chemoprevention has become an important aspect of current cancer research (Boone *et al.*, 1990). The anti-estrogen, tamoxifen, introduced to therapeutically prevent the metastatic growth of breast cancer, is being studied as a possible chemopreventive agent for breast cancer (Powles *et al.*, 1989; Love, 1991). Compounds in the diet that have properties similar to, or are antagonists of, the physiologic estrogens may also have a role in reducing cancer risk. Two of these so-called phytoestrogens, lignans and isoflavones, have been suggested to play a role in the prevention of estrogen-dependent breast cancer (Setchell *et al.*, 1984; Barnes *et al.*, 1990; Adlercreutz *et al.*, 1991) and of colon cancer (Setchell *et al.*, 1981). This hypothesis has been supported by our subsequent data showing that soy, specifically containing isoflavones, inhibits tumor numbers in classical animal models of breast cancer (Barnes *et al.*, 1990, 1993). Furthermore, we have recently shown that two isoflavones, genistein (5,7,4′-trihydroxyisoflavone) and daidzein (7,4′-dihydroxyisoflavone), inhibit the growth of human breast cancer (Peterson and Barnes, 1991) and prostate cancer (Peterson and Barnes, 1993) cell lines in culture, but by mechanisms independent of inhibition of the binding of steroids to their receptors.

Although flavonoids are found in plants, vegetables, and flowers in a bewildering display of biosynthetic prowess, isoflavones such as genistein and daidzein are found in just a few botanical families. This is because of the limited distribution of the enzyme chalcone isomerase [which converts 2(*R*)-naringinen, a flavone precursor, into 2-hydroxydaidzein] largely to tropical legumes. As a result, isoflavones are a very minor part of the modern American (M. Messina, unpublished data) or British diets (Jones *et al.*, 1989). Genistein and daidzein (Figure 1) and their β-glucoside conjugates are present in high concentrations (up to 3 mg/g) in soybeans (Walz, 1931; Walter, 1941; Eldridge, 1982; Price and Fenwick, 1985). Recent studies by Kudou *et al.* (1991) have shown that the 6″-*O*-malonylglucosides are the principal conjugated forms of these isoflavones in soybean hypocotyl. In addition, in toasted defatted soy flakes, Farmakalidis and Murphy (1985) have reported the presence of 6″-*O*-acetylglucosides.

In the Asian countries, soy is used in many foods—in Taiwan, the average human consumption is 35 g/day per capita (M. Messina, unpublished data). Lee *et al.* (1991) have recently shown a strong correlation between the intake of soy protein and a reduction in the risk of breast cancer in premenopausal, but not postmenopausal, Singapore Chinese women. Furthermore, soy ingestion has also been shown to significantly alter hormonal characteristics of healthy, premenopausal women in a manner which is beneficial with regard to risk factors for breast cancer (Cassidy *et al.*, 1993). Finally, the amount of isoflavones in urine is correlated to soy intake in both European (Setchell *et al.*, 1984; Axelson *et al.*, 1984) and Japanese people (Adlercreutz *et al.*, 1991). Therefore, soybeans are a potentially important link between diet and cancer risk.

Asians consume soybeans in many forms, including soy milk, tofu, and fermented products, such as miso, soy sauce, and tempeh. There have been only a limited number of reports of the isoflavone concentrations and composition

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† These studies were supported in part by grants from the American Cancer Society (BC-599) and the American Institute for Cancer Research (87B64).

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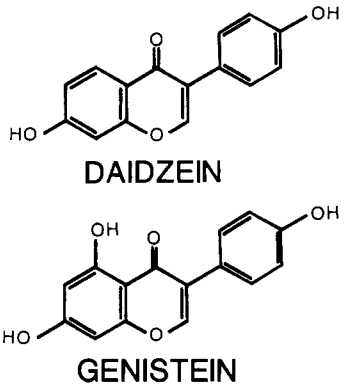

Figure 1. Chemical structures of the isoflavones measured in this study.

in these products. Since the consumption of soy-based foods is increasing in the United States, we describe here an analytical procedure for the determination of the concentrations and composition of isoflavones in a variety of American and Asian soy-containing foods and food products.

EXPERIMENTAL PROCEDURES

Apparatus. In this study the following were used: a Buchi rotavapor, Model R110; a LKB Ultraspec UV–visible wavelength spectrophotometer; a Perkin-Elmer Series IV high-pressure liquid chromatograph with a ternary solvent delivery system, UV–visible wavelength detector, and data station; a Varian MAT 311A mass spectrometer retrofitted with an Ion Tech saddle-field atom gun providing a beam of fast atoms of xenon (8 keV with a 1-mA current); and a GE 300 wide-bore spectrometer (NT series) with an 1180e processor and a 293c pulse programmer providing a resonance frequency of 300.1 MHz.

Reagents. HPLC grade methanol and acetonitrile and the disodium salt of fluorescein were used without further purification. Double-distilled water used was filtered through a nylon membrane of 0.45 μm.

Soybean chips, various soy flours, soy protein concentrates, soy protein isolates, high-fiber fractions from soybeans, and soy molasses (a concentrated aqueous ethanol extract of defatted soybean flour) were provided by the Archer Daniels Midland Co. (Decatur, IL). Soy molasses contained 45% solids by weight (34.6% carbohydrate, 3.2% protein, 3.1% fat, and 4.2% ash), as analyzed by Hazelton Laboratories, Madison, WI. Tofu prepared by the δ-gluconolactone method (Morinaga Nutritional Foods Inc., Los Angeles, CA) and fermented soy sauce (Kikkoman) were obtained from a local grocery store. Tofu prepared by calcium salt-induced coagulation (Tree of Life, St. Augustine, FL), tempeh, soy milk, chocolate malted soy milk, iced soy milk (Tofutti and Ice Bean), soy cheese, miso, soy flour, soy nuts, and soy powder were all obtained from a national foodstore specializing in vegetable-based food products. Barley miso and rice miso were provided by American Soy Products, Inc., Saline, MI. Miso and several forms of soybean paste (*Doen Jang*) were purchased from a Korean foodstore.

Isoflavone Standards. *Isolation.* Genistin and daidzin, the β-glucoside conjugates of the isoflavones genistein and daidzein, were isolated from soy molasses according to the method of Walter (1941). Genistein was prepared by hydrolysis of genistin under reflux for 6 h in methanolic HCl. Daidzein was prepared by hydrolysis of daidzin with β-glucosidase in a 0.1 M sodium acetate buffer, pH 5.0. The crude daidzein and genistein were recrystallized three or four times from hot 60% aqueous ethanol.

Mass Spectrometry. Molecular weights of the isoflavones were determined by fast atom bombardment ionization mass spectrometry using a glycerol matrix.

^{1}H Nuclear Magnetic Resonance (NMR) Spectra. Isoflavones were dissolved in DMSO-d_6. ^{1}H NMR spectra were obtained with 8K data points, 2800-Hz sweep width, 3.7-s repetition rate, and 75° sweep angle; spectra were internally referenced to tetramethylsilane at 0.00 ppm.

Extraction. Isoflavones in solid foods (analyzed in triplicate), to which 1.25 mg of fluorescein was added as an internal standard, were extracted into 80% aqueous methanol (10 mL/g) by stirring 1 h at 60 °C. The other soy products (miso, soy milk, soy paste, and tofu) were extracted whole and also after freeze-drying. The mixture was centrifuged (10 min at 2500*g*) and the supernatant decanted into a round-bottom flask. The pellet was resuspended in 80% aqueous methanol (2 × 5 mL) and centrifuged, and the supernatants were combined and taken to dryness using a rotary evaporator. The dried extracts were then redissolved in 50% aqueous methanol (5 mL), and lipids were removed and discarded by partitioning into hexane (4 × 20 mL). The aqueous methanol phase was evaporated to dryness on a rotary evaporator and the dried residue dispersed in 10 mL of 80% aqueous methanol. An aliquot of the mixture was centrifuged at 14000*g* for 2 min in an Eppendorf microfuge just prior to analysis by HPLC.

High-Pressure Liquid Chromatography. Separation of isoflavones was achieved by HPLC on a 30 cm × 0.45 cm Brownlee Aquapore C_8 reversed-phase column with a mobile phase consisting of a gradient of 0–46.4% acetonitrile in 0.1% (v/v) aqueous trifluoroacetic acid at a flow rate of 1.5 mL/min. The concentration of acetonitrile increased by 2.25%/min. The eluting components were detected from their absorbance at 262 nm. Concentrations of the isoflavones were calculated from standard curves of the area responses for authentic isoflavone standards normalized to the constant amount of fluorescein added to each sample. The concentrations were expressed either as milligrams per gram of whole food or as milligrams per gram of dry weight.

RESULTS

Purity of Isolated Isoflavone from Soybeans. *Melting Points.* The uncorrected melting points of the isoflavones were as follows: daidzein, 325 °C (dec) [literature (Yueh and Chu, 1977) 320 °C (dec)]; daidzin, 223 °C (dec); genistein, 297–299 °C [literature (Walz, 1931) 296–298 °C]; genistin, 257–258 °C [literature (Walter, 1941) 254–256 °C].

UV Absorbance. Maximum absorbance occurred at 254 nm for daidzin, genistin, and genistein (molar extinction coefficients of 29.0 × 10^3, 41.7 × 10^3, and 37.3 × 10^3, respectively, in 80% aqueous methanol), and at 250 nm for daidzein (molar extinction coefficient of 26.0 × 10^3 in 80% aqueous methanol).

Mass Spectrometry. Molecular masses of the isolated isoflavones were 270 and 432 daltons (genistein and genistin, respectively) and 254 and 416 daltons (daidzein and daidzin, respectively).

NMR Spectroscopy. Genistein: δ 6.22 (1H, d, J = 2.4 Hz, H_6), 6.38 (1H, d, J = 1.5 Hz, H_8), 6.82 (2H, d, J = 8.4 Hz, $H_{3'}$ and $H_{5'}$), 7.37 (2H, d, J = 8.4 Hz, $H_{2'}$ and $H_{6'}$), 8.30 (1H, s, H_2), 9.57 (1H, s, $C_{4'}$-O*H*), 10.86 (1H, s, C_{7}-O*H*), and 12.93 (1H, s, $C_{5'}$-O*H*). Daidzein: δ 6.81 (2H, d, J = 9.3 Hz, $H_{3'}$ and $H_{5'}$), 6.87 (1H, s, H_8), 6.94 (1H, d, J = 10.5 Hz, H_6), 7.38 (2H, d, J = 8.4 Hz, $H_{2'}$ and $H_{6'}$), 7.97 (1H, d, J = 8.7 Hz, H_5), 8.27 (1H, s, H_2), 9.59 (1H, s, 4′-O*H*), and 10.86 (1H, s, 7-O*H*). These values are consistent with previously published data (Markham and Mabry, 1975). By using DMSO-d_6 as the solvent, the phenolic hydroxyl ^{1}H resonances were observed, whereas addition of D_2O eliminated these resonances. As expected, the ^{1}H NMR spectra of genistin and daidzin lacked the C_7 phenolic hydroxyl proton resonance, the site of attachment of the glucosyl group. Small chemical shift changes (δ 0.1–0.4) caused by the 7-glucosyl group were also observed for protons (H_5, H_6, and H_8) in the A ring.

Optimization of Extraction and Analytical Procedures. *p*-Nitrophenol, estradiol, and fluorescein were evaluated as possible candidates for use as internal standards. Fluorescein was found to be the most suitable

Table I. Isoflavone Concentrations[a] in Asian Primary Soy Materials

		conjugated		aglucones				aglucones, %	
food	basis	genistin	daidzin	genistein	daidzein	total	D/G ratio	genistein	daidzein
soy milk	g	0.130 ± 0.004	0.103 ± 0.006	0.007 ± 0.000	0.011 ± 0.002	0.252 ± 0.012			
	g dry wt	1.680 ± 0.060	1.337 ± 0.087	0.098 ± 0.002	0.141 ± 0.019	3.256 ± 0.168	0.83	5	10
tofu[b]	g	0.249 ± 0.028	0.121 ± 0.010	0.031 ± 0.001	0.016 ± 0.001	0.417 ± 0.036			
	g dry wt	1.215 ± 0.137	0.591 ± 0.046	0.151 ± 0.006	0.0.077 ± 0.005	2.031 ± 0.171	0.49	11	12
tofu[c]	g	0.269 ± 0.004	0.200 ± 0.008	0.015 ± 0.001	0.015 ± 0.000	0.494 ± 0.011			
	g dry wt	2.087 ± 0.030	1.513 ± 0.019	0.116 ± 0.004	0.113 ± 0.000	3.827 ± 0.045	0.74	5	7
soy flour	g	0.741 ± 0.100	0.582 ± 0.077	0.015 ± 0.002	nd	1.338 ± 0.178	0.77	2	0
soy powder	g	1.148 ± 0.103	0.582 ± 0.054	0.014 ± 0.001	nd	1.748 ± 0.156	0.50	1	0
soy nuts	g	1.390 ± 0.039	0.853 ± 0.022	0.066 ± 0.001	0.054 ± 0.001	2.363 ± 0.061	0.62	5	6

[a] mg/g; mean ± 1 SD of triplicate analyses. [b] Tree of Life tofu. [c] Mori-Nu tofu.

Table II. Isoflavone Concentrations[a] in Processed or Fermented Asian Soy Products

		conjugated		aglucones				aglucones, %	
soy product	basis	genistin	daidzin	genistein	daidzein	total	D/G ratio	genistein	daidzein
tempeh	g	0.113 ± 0.028	0.040 ± 0.013	0.164 ± 0.004	0.113 ± 0.007	0.430 ± 0.005			
	g dry wt	0.296 ± 0.063	0.103 ± 0.029	0.434 ± 0.005	0.298 ± 0.009	1.130 ± 0.096	0.55	59	74
miso	g	0.043 ± 0.004	0.035 ± 0.025	0.497 ± 0.029	0.345 ± 0.013	0.920 ± 0.070			
	g dry wt	0.064 ±0.007	0.054 ± 0.038	0.745 ± 0.068	0.516 ± 0.036	1.379 ± 0.149	0.70	92	91
rice miso	g	0.198 ± 0.011	0.000 ± 0.000	0.136 ± 0.000	0.071 ± 0.002	0.404 ± 0.009			
	g dry wt	0.353 ± 0.018	0.000 ± 0.000	0.242 ± 0.001	0.127 ± 0.003	0.721 ± 0.014	0.21	41	100
barley miso	g	0.155 ± 0.020	0.142 ± 0.025	0.239 ± 0.008	0.185 ± 0.007	0.721 ± 0.053			
	g dry wt	0.258 ± 0.032	0.235 ± 0.042	0.396 ± 0.012	0.306 ± 0.009	1.195 ± 0.084	0.83	61	57
Shiromiso soup mix	g	0.267 ± 0.020	0.163 ± 0.028	0.170 ± 0.006	0.108 ± 0.008	0.708 ± 0.059	0.62	39	40
Akamiso soup mix	g	0.319 ± 0.025	0.254 ± 0.044	0.173 ± 0.005	0.136 ± 0.008	0.882 ± 0.080	0.79	35	35
soybean paste	g	0.078 ± 0.014	0.044 ± 0.040	0.251 ± 0.008	0.197 ± 0.009	0.570 ± 0.071			
	g dry wt	0.160 ± 0.030	0.090 ± 0.081	0.514 ± 0.016	0.404 ± 0.019	1.168 ± 0.146	0.73	76	82
soybean paste/rice	g	0.066 ± 0.029	0.085 ± 0.016	0.108 ± 0.004	0.103 ± 0.006	0.362 ± 0.041			
	g dry wt	0.106 ± 0.045	0.136 ± 0.026	0.174 ± 0.008	0.166 ± 0.008	0.582 ± 0.061	1.08	62	55
soybean paste/wheat	g	0.110 ± 0.008	0.094 ± 0.026	0.124 ± 0.014	0.105 ± 0.001	0.433 ± 0.032			
	g dry wt	0.220 ± 0.015	0.189 ± 0.052	0.248 ± 0.028	0.210 ± 0.003	0.867 ± 0.063	0.85	53	53

[a] Expressed as mg/g wet weight or mg/g dry weight; mean ± 1 SD of triplicate analyses. nd, not detected.

Table III. Isoflavone Concentrations[a] in Other Soy Foods

		conjugated		aglucones				aglucones, %	
soy food	basis	genistin	daidzin	genistein	daidzein	total	D/G ratio	genistein	daidzein
soy sauce	g	nd	nd	0.009 ± 0.002	0.014 ± 0.001	0.023 ± 0.003			
	g dry wt	nd	nd	0.036 ± 0.014	0.054 ± 0.013	0.090 ± 0.026	1.50	100	100
soy cheese	g	0.028 ± 0.001	0.021 ± 0.001	0.002 ± 0.001	0.001 ± 0.001	0.050 ± 0.003			
	g dry wt	0.057 ± 0.001	0.043 ± 0.001	0.005 ± 0.001	0.001 ± 0.002	0.105 ± 0.003	0.71	8	2
Tofutti	g	0.022 ± 0.001	0.004 ± 0.006	0.004 ± 0.000	0.001 ± 0.002	0.032 ± 0.008			
	g dry wt	0.064 ± 0.001	0.012 ± 0.016	0.014 ± 0.001	0.003 ± 0.004	0.092 ± 0.020	0.19	18	20
Ice Bean	g	0.060 ± 0.006	0.055 ± 0.007	0.001 ± 0.000	0.001 ± 0.002	0.117 ± 0.014			
	g dry wt	0.184 ± 0.016	0.167 ± 0.022	0.004 ± 0.002	0.004 ± 0.006	0.360 ± 0.004	0.91	2	2

[a] Expressed as mg/g wet weight or mg/g dry weight; mean ± 1 SD of triplicate analyses. nd, not detected.

because its HPLC retention index was distinct from that of any other component in the soy food extracts.

When alcohol-extracted soy protein concentrate (1 g) was "spiked" with known amounts of genistein, genistin, daidzein, and daidzin, dried, and then extracted with several different solvent mixtures (10 mL), the maximum isoflavone recoveries were found for 80% aqueous methanol and 60% aqueous acetonitrile. Recoveries ranged from 90 to 93% and from 86 to 90%, respectively, for these two solvent systems.

Experiments were carried out to determine the optimum ratio of the volume of the extraction solvent (80% aqueous methanol) to the quantity of food. A range from 0.2 to 1 mg of each isoflavone was present in the soy flour used in this experiment. Consistently higher recoveries were obtained with ratios of solvent (milliliters) to food (grams) equal to or greater than 10:1. Recoveries were lower at ratios below 10 mL of solvent/g of food; however, it was possible to correct for losses using the internal standard, fluorescein. The coefficient of variation for triplicate samples varied from 3.0 to 8.6%, being lower when larger sample sizes (>0.5 g) were analyzed. Similar variation was observed for the values for foods reported in Tables I–IV, being highest (12.0%) in nonhomogeneous foods containing low isoflavone concentrations (>0.4 mg/g) and lowest (5.6%) in powdered materials with isoflavone concentrations greater than 1 mg/g.

Asian Soy Foods. Each of the Asian-style soybean products (soy milk, tofu, soy flour, soy powder, and soy nuts), which were not diluted by the addition of nonsoybean components, had total concentrations of isoflavone (expressed as milligrams per gram of dry weight) in the range 1.3–3.8 mg/g (Table I). When other food components were added to the soybean product, the overall isoflavone concentrations were lower. Fermented soy foods, which are usually prepared by mixing soy with other components such as barley, rice, or wheat, contained isoflavone concentrations that were lower and in the range 0.6–1.4 mg/g of dry weight (Table II). Other soy-based products, such as frozen flavored soy milk (Ice Bean and Tofutti), soy sauce, and soy cheese, had much lower isoflavone concentrations (Table III).

For soy milk (Figure 2A), soy flour, soy nuts, soy powder, and tofu (Figure 2B), the β-glucosidic conjugates were the

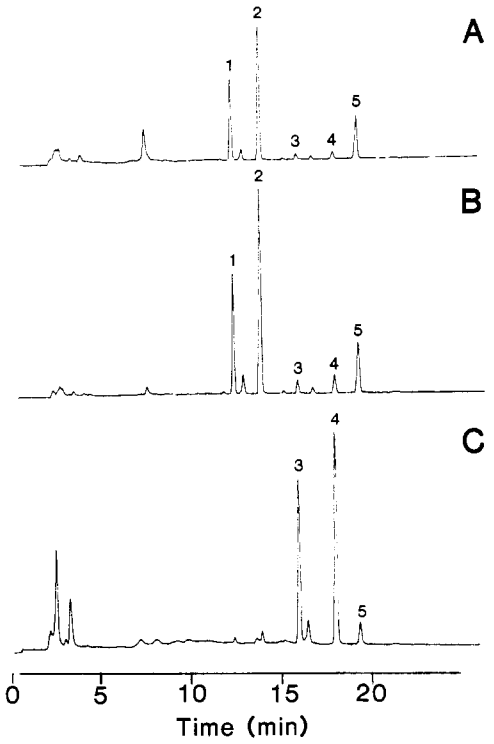

Figure 2. Reversed-phase HPLC chromatograms of extracts of soy milk (A), tofu (B), and miso (C). Note that miso contains only unconjugated isoflavones. Peak identification: 1, daidzin; 2, genistin; 3, daidzein; 4, genistein; 5, internal standard (fluorescein; constant amount added to each sample). Each chromatogram was obtained at the same sensitivity setting; the volumes injected of each extract were adjusted to give similar maximum peak heights.

major forms of isoflavones present. However, in fermented soy foods such as miso (Figure 2C), soybean paste, and tempeh, the unconjugated isoflavone aglucones were the predominant chemical forms.

American Soy Foods. The commercial soy products used in American foods all contained isoflavones (Table IV). Soy flours (Figure 3A), independent of the degree of heating used in their preparation, had consistently high isoflavone concentrations. Soy protein concentrate prepared by extraction with water had an isoflavone concentration (2.7 mg/g) comparable to that of soy flour and many Asian soy products. On the other hand, aqueous alcohol-extracted soy protein concentrate (Figure 3B) contained lower concentrations of isoflavones (Table IV). American soy sauce had the lowest concentrations of isoflavones (Figure 3C). In soy protein isolates, isoflavone concentrations were reduced 3-fold compared with soy flour when the concentration was expressed as milligrams per gram of dry weight and 5-fold when expressed as milligrams per gram of protein (Table IV).

DISCUSSION

By application of the method described and evaluated here, we have comprehensively examined the isoflavone composition of a variety of soybean products typically consumed in American and Asian diets. The data extend results reported by other investigators (Murphy, 1982; Eldridge, 1982; Eldridge and Kwolek, 1983; Farmakalidis and Murphy, 1985; Price and Fenwick, 1985; Setchell *et al.*, 1987; Jones *et al.*, 1989; Wang *et al.*, 1990).

The procedure developed in this study for the analysis of isoflavones in food products is reproducible and reliable and is suited to the measurement of isoflavones in as little as 1 g or 1 mL of the foodstuffs studied. The optimum conditions for the extraction and isolation of the isoflavones from food products were thoroughly investigated using a soy protein concentrate prepared by extraction with hot 65% aqueous alcohol. However, we cannot exclude the possibility that alternative solvent mixtures may be optimal for other soy food matrices. The optimum ratio of the aqueous organic solvent to the food being analyzed was 10:1 (vol/g) or greater. As previously noted (Wang *et al.*, 1990), the use of Sep-Pak C_{18} columns to clean up the extracts was unnecessary (data not shown). Although defatting of the initial extract with hexane did not alter the qualitative or quantitative aspects of the HPLC analysis, it is recommended to prolong the life of the HPLC column.

Efficient extraction of these relatively polar isoflavones from foodstuffs requires the use of a polar solvent. In accord with previous studies (Murphy, 1981; Eldridge, 1982; Eldridge and Kwolek, 1983; Setchell *et al.*, 1987; Jones *et al.*, 1989; Barbuch *et al.*, 1989), aqueous methanol (80%) was shown to be the optimum solvent for the extraction of conjugated and unconjugated isoflavones, whereas pure acetonitrile, ethanol, or methanol was a poor organic solvent. Although it has been previously claimed that 80% aqueous acetonitrile was more efficient than 80% aqueous methanol, absolute recoveries were not reported (Murphy, 1981; Farmakalidis and Murphy, 1985). In addition, previously published methods utilized an acidified extraction medium (Murphy, 1981; Farmakalidis and Murphy, 1985; Wang *et al.*, 1990). In whole soybeans (and presumably some, but not necessarily all, soy foods), large amounts of isoflavone 6″-*O*-malonylglucoside conjugates have been reported (Kudou *et al.*, 1991). These malonate ester derivatives, by analogy to malonate esters which are intermediates in many organic syntheses, are prone to decarboxylation to form the corresponding 6″-*O*-acetylglucosides, a reaction stimulated by heat and an acid medium. This may account for 6″-*O*-acetyldaidzin and 6″-*O*-acetylgenistin in toasted soy flakes (Farmakalidis and Murphy, 1985), as was suggested by Kudou *et al.* (1991). Therefore, the apparent pattern of isoflavone glycosidic conjugates measured by HPLC analysis will be a function of the extraction procedure, as well as the processing of individual soy foods. Recent experiments carried out in this laboratory (S. Barnes, unpublished data) suggest that hot extraction procedures also cause deesterification of 6″-*O*-malonylglucosides and 6″-*O*-acetylglucosides, thereby leading to the underivatized β-glucosides (daidzin and genistin) as the predominant forms detected by HPLC analysis, as observed in this and previous studies (Walz, 1931; Walter, 1941; Eldridge, 1982; Price and Fenwick, 1985; Setchell *et al.*, 1987; Jones *et al.*, 1989). Despite these effects, the total isoflavone glycoside concentration was unchanged. Accordingly, the concentrations of daidzin and genistin as determined by HPLC in this study represent the sum of the individual isoflavone glycoside concentrations. New extraction and analytical methods are required that will enable accurate determination of the composition and concentration of isoflavone glycosidic conjugates in soy food materials.

Solvent loss by retention in the insoluble food matrix, or by evaporation, was corrected for by the inclusion of fluorescein as the internal standard. Under the chromtographic conditions employed, fluorescein eluted separately from other UV-absorbing compounds extracted from the foods tested and did not partition into hexane, and its bright yellow-green color served to limit potential problems of losses during the workup procedure. With the exception of Eldridge (1982), who used butyrophenone as an internal

Table IV. Isoflavone Concentrations[a] in Various American Soy Products

	conjugated		aglucones		total			aglucones, %	
soy product	genistin	daidzin	genistein	daidzein	dry wt	protein	D/G ratio	genistein	daidzein
soybean chips	0.356 ± 0.074	0.331 ± 0.058	0.052 ± 0.019	0.065 ± 0.023	0.802 ± 0.172	2.111 ± 0.455	0.97	13	16
soy flours									
Nutrisoy	1.448 ± 0.026	1.161 ± 0.003	0.034 ± 0.000	0.033 ± 0.002	2.678 ± 0.027	5.356 ± 0.054	0.58	2	3
Nutrisoy 7B	1.318 ± 0.009	1.112 ± 0.005	0.053 ± 0.002	0.044 ± 0.001	2.527 ± 0.004	5.054 ± 0.008	0.60	4	4
baker's Nutrisoy	1.300 ± 0.176	1.046 ± 0.117	0.024 ± 0.020	0.019 ± 0.019	2.389 ± 0.332	4.778 ± 0.664	0.56	2	2
toasted Nutrisoy	1.385 ± 0.066	1.093 ± 0.025	0.044 ± 0.011	0.040 ± 0.005	2.561 ± 0.076	5.122 ± 0.152	0.57	3	3
soy concentrates									
water extracted	1.404 ± 0.103	1.180 ± 0.082	0.033 ± 0.002	0.039 ± 0.002	2.656 ± 0.182	3.794 ± 0.256	0.61	2	3
alcohol extracted									
Arcon F	0.087 ± 0.014	0.064 ± 0.007	0.004 ± 0.001	0.004 ± 0.000	0.159 ± 0.022	0.244 ± 0.034	0.75	4	6
Arcon S	0.227 ± 0.059	0.102 ± 0.019	0.069 ± 0.001	0.045 ± 0.002	0.443 ± 0.075	0.682 ± 0.115	0.50	23	31
soy isolate	0.430 ± 0.138	0.232 ± 0.105	0.105 ± 0.011	0.073 ± 0.004	0.848 ± 0.228	0.931 ± 0.250	0.41	20	24
soy isolate	0.589 ± 0.004	0.278 ± 0.001	0.189 ± 0.012	0.102 ± 0.005	1.158 ± 0.012	1.273 ± 0.013	0.35	24	27
soy fiber	0.154 ± 0.004	0.141 ± 0.001	0.114 ± 0.006	0.085 ± 0.002	0.494 ± 0.012		0.84	43	38

[a] Per g dry weight or per g of protein; mean ± 1 SD of triplicate analyses.

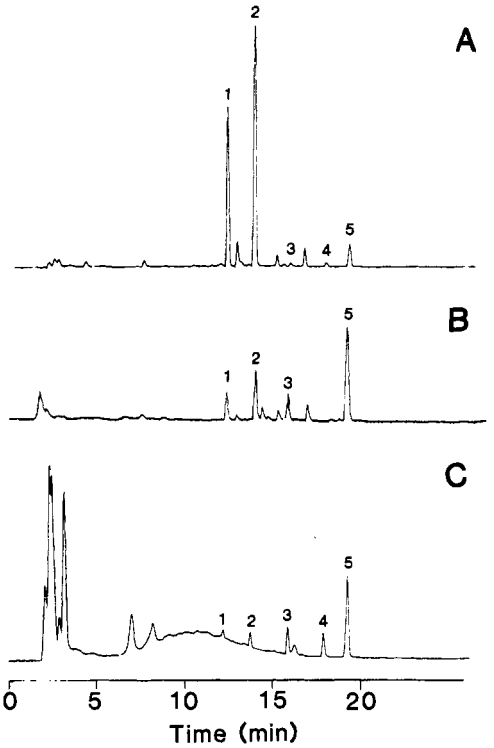

Figure 3. Reversed-phase HPLC chromatograms of extracts of soy flour (A), aqueous alcohol-extracted soy protein concentrate (B), and soy sauce (C). The same sensitivity setting was used for each chromatogram. The amount of fluorescein added to each sample was constant; i.e., the isoflavone content of soy flour vastly exceeds that of the protein concentrate and soy sauce. Peak identification is as in Figure 2.

standard, previously published methods for isoflavones have taken no account of procedural losses.

The isoflavones were separated by reversed-phase HPLC using a gradient of acetonitrile and water, thus permitting analysis of both the β-glucosides and the aglucones in a single chromatographic run. This has particular advantage over methods in which the isoflavone glucosides were calculated by the difference in the concentration of isoflavone aglucones, determined using isocratic HPLC methods, between acid-hydrolyzed and unhydrolyzed samples (Wang *et al.*, 1990). It is an important feature of our analytical method because of the marked differences in isoflavone composition in soy foods. Other investigators have previously used gradient elution reversed-phase HPLC. Murphy (1981) and Eldridge and Kwolek (1983) employed gradients of methanol in water, whereas Koster *et al.* (1983), Jones *et al.* (1989), Matsuura *et al.* (1989), and Kudou *et al.* (1991) all utilized acetonitrile gradients in water. The analyses were carried out at different pHs. Jones *et al.* (1989) used a borate–potassium phosphate buffer, pH 7.5, whereas the other investigators used either trifluoroacetic acid [the present study and Matsuura *et al.* (1989)] or acetic acid (Koster *et al.*, 1983; Kudou *et al.*, 1991).

Application of our analytical method has indicated that manufacture of most American and Asian soy food materials does not result in substantial lowering of isoflavone concentrations. The exceptions were soy sauce, alcohol-extracted soy protein concentrate, soy protein isolates, soy fiber, and foods in which soy was found to be only a minor component.

Soy flour is obtained by grinding dehulled soybeans following the removal of oil by solvent extraction or by extrusion. Isoflavones were not found in soybean oil. During the manufacturing process, soy flour is heated to varying degrees, including toasting, to produce different grades of soy flours. Toasting of soy flour may well have led to formation of more isoflavone 6″-*O*-acetylglucosides by decarboxylation of the 6″-*O*-malonylglucosides than in less heat-treated forms of soy flour. Nonetheless, these glycosidic conjugates were de-esterified according to the extraction procedure employed in the present study; accordingly, we found no evidence that heating had an effect on the isoflavone composition (glucosidic conjugates vs aglucones) or total concentrations determined by our analytical method.

Soy protein concentrate, prepared by extraction of soy flour with hot 65% aqueous ethanol (an excellent solvent for isoflavones), had 10–20-fold lower isoflavone concentrations than most other soy foods. By contrast, soy protein concentrate prepared by hot water extraction (to remove the soluble carbohydrates) at neutral pH retained most of the isoflavones present in soy flour, probably reflecting their strong protein binding and low aqueous solubility.

Soy protein isolate is prepared by solubilization of proteins (and soluble carbohydrates) from soy flour by an alkaline (pH 9.5) extraction step and the subsequent acid (pH 4.5) precipitation of the extracted proteins. The isoflavone concentrations in soy protein isolate were 4–6-fold lower than in soy flour or soy protein concentrate when expressed as milligrams per gram of protein. Incomplete recovery of the isoflavones from the soy flour during the alkaline extraction step or selective precipitation of the isoflavone aglucones at pH 4.5 may account for the composition and concentrations of isoflavones in the soy protein isolate. The lower daidzein/genistein ratio

(0.4) in the soy protein isolate compared to that in the unprocessed soy flour (0.6) may also be a consequence of the more hydrophilic nature (and hence aqueous solubility) of daidzein compared to genistein.

Some soy foods (soy milk, miso) containing high concentrations of isoflavones are often diluted with other food materials that do not contain isoflavones. The addition of chocolate flavoring to soy milk lowers the isoflavone concentration (milligrams per milliliter) by 33%; however, since the total solids were increased, there was a 3-fold decrease in isoflavone concentration when expressed as milligrams per gram of freeze-dried weight. Tofutti and Ice Bean are both iced soy milks. Tofutti is prepared using soy protein isolate rather than the whole soybean (as is the case for Ice Bean) and consequently has a 4-fold lower isoflavone concentration than Ice Bean. The lower isoflavone concentrations reported in infant formula soy milk (Setchell *et al.*, 1987) also reflect the use of soy protein isolate rather than whole soybeans, which were the source for the soy milks analyzed in the present study.

When miso was diluted with barley or rice, the isoflavone concentrations (1.2 and 0.7 mg/g of dry weight, respectively) were also lower than in undiluted miso (1.4 mg/g of dry weight). Similar effects were also found for soybean paste (diluted with rice or wheat), which had isoflavone concentrations ranging from 0.58 to 1.17 mg/g of dry weight.

Soy sauce, a popular product in Asia and in the United States, is prepared by first fermenting soybean paste with rice and then squeezing the resulting cake to produce a brown liquor. It contains only trace amounts of isoflavones, as has been noted previously (Wang *et al.*, 1990). Murphy (1982) failed to detect isoflavones in soy sauce.

In nonfermented soy foods (soy milk, tofu, soy nuts, soy powder, soy protein concentrate, and soy flour), the isoflavones were present almost exclusively as their glycosidic conjugates, whereas in fermented soy products, such as miso, soybean paste, and tempeh, a large proportion of the isoflavones were in the unconjugated form. Interestingly, when soy milk or tofu becomes contaminated by microorganisms after the container is opened, the proportion of unconjugated isoflavones increases with time (L. Coward and S. Barnes, unpublished data). This may have been the case for the tofu analyzed by Murphy (1982) that contained a high percentage of isoflavone aglucones.

Asians consume the equivalent of 10–35 g of soybeans/day per capita (M. Messina, unpublished data), indicating a daily isoflavone intake of 25–100 mg. This is comparable (on a body weight basis) to the amounts of isoflavones in powdered soybean chip-containing diets that we have recently shown to inhibit the appearance of mammary tumors in a *N*-methylnitrosourea-induced animal model of breast cancer in rats (Barnes *et al.*, 1990).

In contrast to most Asians, the average American or Western European consumer ingests at most only a few milligrams per day of isoflavones. This has been confirmed by several investigators. Setchell *et al.* (1984) showed that the urinary excretion of the isoflavone metabolite equol increased >500-fold when human volunteers included 40 g of soy flour in their diet on a daily basis. In addition, urinary levels of equol are generally very low in most subjects consuming a Western-style diet but are much higher in vegetarians who include soy in their diet (Adlercreutz *et al.*, 1987). Finally, Jones *et al.* (1989) have shown that the isoflavone intake from the British diet is below that believed to be necessary to have a physiological effect.

Since we have shown that isoflavone-containing soybean products prevent the appearance of mammary tumors in rat models of breast cancer (Barnes *et al.*, 1990) and isoflavones inhibit the growth of human breast cancer (Peterson and Barnes, 1991) and prostate cancer (Peterson and Barnes, 1993) cells in culture, these data are consistent with the hypothesis that the low incidence of breast cancer in Asian women is due to their much higher consumption of soybeans containing isoflavones (Setchell *et al.*, 1984; Barnes *et al.*, 1990; Adlercreutz *et al.*, 1991; Lee *et al.*, 1991).

ACKNOWLEDGMENT

We thank the Archer Daniels Midland Co. and particularly Dr. Rasik Daftery and Mr. Ray Parkin for their provision of various soybean products for our analyses and also Mr. Tim Redmond of American Soy Products, Inc. We acknowledge the help of Dr. Vince Waterhous, Department of Chemistry, UAB, who performed the NMR analyses. Dr. Mark Messina, formerly at the Diet and Cancer Branch, Division of Cancer Etiology, National Cancer Institute, Bethesda, MD, kindly provided a summary of an unpublished international survey of soybean consumption. We also acknowledge the dedicated efforts of Dr. Patricia Murphy, University of Iowa, who served as a reviewer of the manuscript.

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Received for review January 27, 1993. Revised manuscript received June 4, 1993. Accepted September 13, 1993.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts*, October 15, 1993.