Retention and Changes of Soy Isoflavones and Carotenoids in Immature Soybean Seeds (Edamame) during Processing

A. H. Simonne,* M. Smith,† D. B. Weaver,‡ T. Vail,† S. Barnes,† and C. I. Wei§

Department of Nutrition and Food Science, 328 Spidle Hall, Auburn University, Auburn, Alabama 36849, and Department of Pharmacology and Toxicology, UAB Comprehensive Cancer Center Mass Spectrometry Core Facility, University of Alabama at Birmingham, Birmingham, Alabama 35294 and Department of Food Science and Human Nutrition, University of Florida, Gainesville, Florida

Isoflavones and carotenoids in four experimental genotypes and Hutcheson cultivar soybeans were evaluated as a function of processing treatments and maturity. Total isoflavone and carotenoid contents were affected by genotypes and maturity stages ($p < 0.0001$). Total isoflavones ranged from 472 μg/g (in NTCPR93-40) to 2280 μg/g (in Hutcheson). Lutein contents ranged from 895 (in NTCPR93-286) to 2119 (in Honey Brown), and β-carotene ranged from 291 (in Hutcheson) to 491 (in NICPR92-40) μg/100 g. Mean total isoflavone retention percentages in immature Hutcheson soybeans were 46% (boiling), 53% (freezing), and 40% (freeze-drying). Mean retentions of lutein and β-carotene, respectively, were 92 and 73% in frozen, 62 and 62% in boiled, and 34 and 27% in freeze-dried soybeans. Boiling caused a substantial increase in daidzin, genistin, and genistein. The results show that post-harvest changes in total isoflavones and carotenoids in soybeans are influenced by processing methods, but genotype has an effect on isoflavone and carotenoid profiles during seed development.

Keywords: *Isoflavones; edamame; β-carotene; lutein; processing*

INTRODUCTION

Immature soybeans (*Gycine max* (L.) Merrill) refers to soybeans harvested at 80% maturity. This maturity level is equivalent to the R_6 (Fehr and Caviness, 1977) stage of development where the seeds are filled but moisture levels remain at 60−65%. The moisture content of mature soybeans is 10−15% (Rubel et al., 1972). Immature soybeans are often used directly as human food in Asia. Traditionally, the immature soybean pods are picked from the plants, then steamed or boiled in water for up to 20 min, seasoned with salt or spice, and served as an hors d'oeuvre. In addition, shelled immature soybeans can be cooked and served as Qing dou, which is equivalent to green beans, in China, or as edamame in Japan (Liu, 1996). Immature soybeans have characteristics similar to those of immature beans (*Phaseolus lunatus* (L.) *lunatus*) and peas (*Pisum sativum* (L.) *sativum*); thus, they may contain significant amounts of lutein or β-carotene. Monma et al. (1994) studied both the mature and immature seeds of domestic Japanese soybean genotypes and found that green immature soybeans possess carotenoid profiles similar to those of green vegetables. Bates and Matthews (1975) reported levels of β-carotene as high as 0.46 mg/100 g fresh weight in immature soybeans, but the contents in mature beans dropped to 0.12 mg/100 g soaked weight.

Soybeans have been reported to have potential for cancer prevention and suppression (Steele et al., 1995; Kennedy, 1995; Coward et al., 1993; Messina and Barnes, 1991; Adlercreutz et al., 1991) due to a high content of genistein (one of the isoflavones). Genistein, shown in Figure 1, is a naturally occurring inhibitor of tyrosine-specific protein kinases (Akiyama et al., 1987). Therefore, soybeans and soy products currently receive much attention from consumers. In addition to isoflavones, soybeans also contain other components with health benefits and could be regarded as a functional food (Messina and Messina, 1991). As consumption of immature soybeans increases, they could be a significant source of isoflavones and lutein in the U.S. diet.

Because soybeans can be produced in the U.S. only once each year due to their photoperiodic nature, fresh immature soybeans are available only during late summer to early fall. However, processing technologies such as freezing or freeze-drying can be utilized to make immature soybeans available to U.S. consumers year-round. Little is known about the effects of processing techniques on retention and changes in profiles of isoflavones and carotenoids in immature soybeans. Most studies evaluating soy isoflavones have focused mainly on soy products derived from mature soybean seeds such as extruded corn and soy mixtures (Mahungu et al., 1999); raw and cooked soyfoods commonly consumed by Asians and Americans in Singapore and Hawaii (Franke et al., 1999); soy protein isolates (Wang et al., 1998); commercial soybean foods or soy ingredients, traditional soy foods, and second-generation soy foods (Coward et al., 1993; Wang and Murphy, 1994, 1996); defatted soy meal, soy sprouts, soy milk film (Wang et al., 1990);

* Corresponding author. Current address: Department of Food Science and Human Nutrition, University of Florida, P.O. Box 110720, Gainesville, FL 32611-0720. Tel: (352) 392-1978. E-mail: asimonne@gnv.ifas.ufl.edu.

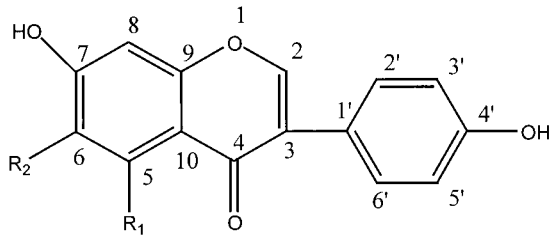
† Department of Pharmacology and Toxicology and UAB Comprehensive Cancer Center Mass Spectrometry Core Facility, University of Alabama at Birmingham.

‡ Department of Agronomy and Soils, Auburn University.

§ Department of Nutrition and Food Science, Auburn University.

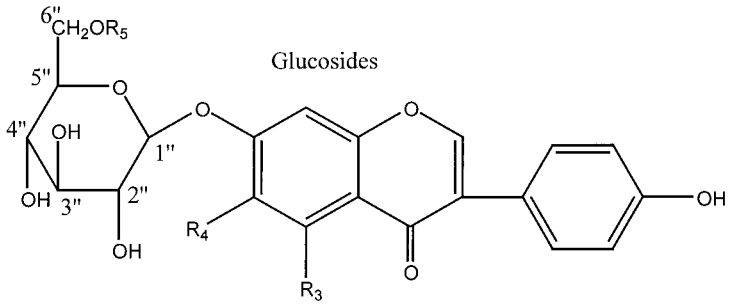
10.1021/jf000247f CCC: $19.00
Published on Web 10/21/2000

Aglycones



R_1	R_2	Compounds
H	H	daidzein
OH	H	genistein
H	OCH_3	glycitein

Glucosides



R_3	R_4	R_5	Compounds
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. Diagram of chemical structures of 12 soy isoflavones.

toasted de-fatted soy flakes (Farmakalidis and Murphy, 1985); and processed soy products (Murphy, 1982).

Currently, there has been a strong movement of public breeding programs in the United States and Canada to produce niche market soybean varieties with special traits and high value products for direct human consumption (Carter and Wilson, 1998). These include varieties for production of tofu, organic natto, and special tofu, and others such as immature soybeans as a vegetable. The niche varieties are mostly for small-volume, high-value specialty uses. The prices for the niche varieties can be 2−5 times higher than the price of commodity soybeans (Carter and Wilson, 1998). Thus, this kind of market could be valuable for small soybean producers in the southeastern part of the United States. Most scientific information on soy isoflavone contents were done on soybean varieties grown in the midwestern United States (Eldridge and Kwolek, 1983; Kudou et al., 1991; Wang and Murphy, 1994; Hoeck et al., 2000) or in Japan (Tsukamoto et al., 1995). Those varieties of soybeans were adaptable to the midwestern regions where massive commodity soybeans are produced; however, small soybean producers in the southeastern part of the United States may be better off relying on the specialty soybeans. Simonne et al. (2000) have developed acceptable freeze-dried soybean snacks for American consumers from some specialty soybeans grown in the southeastern United States. Therefore, the objectives of this study were (1) to evaluate the effects of genotypes and development stage (R_6 and R_8 stages) on isoflavone profiles of soybean varieties bred for the southeastern United States, and (2) to determine the effect of processing techniques (boiling, freezing, and freeze-drying) on retention and profile changes of isoflavones and carotenoids in immature soybeans (R_6).

MATERIALS AND METHODS

Soybean Genotypes. One commercial field soybean cultivar (Hutcheson), three advanced soybean experimental genotypes (NTCPR93-286, NTCPR92-40, and NTCPR-5157), and a land race genotype from Nepal (Honey Brown) were grown in central Alabama (in May 1997) following the recommendations of the Alabama Cooperative Extension Service. Colors of the seed coat at R_6 stage were green in Hutcheson, NTCPR93-286, and NTCPR92-40, greenish-yellow in NTCPR-5157, and greenish-brown in Honey Brown. At the R_8 stage, all the soybean seeds were yellowish-cream color, except Honey Brown which was brown.

For immature soybeans, the plants were hand-harvested (in September) from the field at the R_6 stage of development and transported to a cool room (15°C). On the same day, pods were hand-picked from the plants and hand-shelled to minimize damage. The shelled immature soybeans were divided into several 100-g portions for various treatments (untreated control, blanching, boiling, freezing, and freeze-drying), and

each treatment was completed in duplicate. For mature beans, the plants were left in the field and were harvested mechanically (in October) with a small-plot combine.

Processing Treatments. *Blanching.* A 100-g portion of immature soybeans was placed in a cheesecloth bag and dipped for one min in a 1500-mL beaker filled with one liter of boiling water. The bag was then cooled in ice water and blotted dry in preparation for chemical analyses or subsequent treatments such as freezing or freeze-drying.

Boiling. A 100g portion of immature soybeans was placed in a 250-mL beaker, and water was added to make a 1:1 (w/v) bean-to-water ratio. The beaker was then placed on a laboratory hot plate set at medium heat to boil for a total of 35 min including a five-min come-up time. After being drained, the soybeans were blotted dry in preparation for further chemical assessments.

Freezing. A 100-g portion of immature soybeans was blanched in boiling water for one min to inactivate enzymes, then cooled in ice. Once blotted dry, the beans were packed in an airtight commercial Ziploc brand polyethylene bag, and placed in a −80 °C freezer for 1 h to ensure quick freezing, then placed in a −20 °C freezer for two weeks before analytical assessments.

Freeze-Drying. A 100-g potion of immature soybeans was blanched in boiling water for one min, then cooled in ice. Once blotted dry, the beans were placed in a 150-mL beaker and placed in a −80 °C freezer for 1 h to ensure quick freezing. The beaker containing frozen beans was covered with cheesecloth and then placed in a chamber of a lab freeze-drier (Lab Conco Lyphlock 6) to dry at −50 °C and 10 microns Hg pressure to a constant weight. Total drying time was estimated to be 18−26 h for each load, consisting of five to eight 100-g samples. The chamber was covered with aluminum foil to prevent possible photo degradation of chemical components in the immature soybeans.

Analysis of Isoflavones. Isoflavone contents and profiles were determined in all soybean genotypes at both the R_6 and R_8 stages. The effect of processing on isoflavones in immature soybeans was evaluated only in the Hutcheson variety because it contains the highest levels of isoflavones at R_6 when compared to other soybean varieties used in this study.

Extraction. Soybean samples were ground using a coffee grinder. However, occasionally, with wet samples such as the fresh or frozen soybeans, additional grinding with a porcelain mortar and a pestle was employed. The finely ground soy samples (0.5 g) were then mixed with 5 mL of 80% aqueous methanol. Fifty μL of an internal standard solution of the sodium salt of fluorescein (20 mg/mL) in 80% aqueous methanol was added, and the mixtures were tumbled for 2 h at 4 °C. After the extraction, the samples were centrifuged for 5 min at 5000*g*. The supernatant (10 μL) was then injected into a reversed-phase HPLC system (Barnes et al., 1994; Coward et al., 1993). All sample preparation was conducted under yellow light (fluorescent 40W, GE).

HPLC Analysis. The HPLC system consisted of a gradient pump, a 22 cm × 4.6 mm C_8 column (Aquapore C_8, Brownlee [Applied Biosystems], Perkin-Elmer, Foster City, CA), a photodiode array detector, and a HPChem Station data-processor program. A linear reversed-phase HPLC gradient consisted of solvent (A)10% acetonitrile in water with 0.1% trifluoroacetic acid (TFA), and solvent (B)10% water in acetonitrile with 0.1% TFA. Following injection of each sample, solvent B was increased from 0 to 30% over 30 min, followed by a step gradient to 100% B. Prior to the next run the system was equilibrated with solvent A for at least 15 min. The flow rate was 1.5 mL/min. UV spectra (225 to 400 nm) were recorded and area responses (Figure 2) were integrated by HPChem Station software (Hewlett-Packard Co., Wilmington, DE); however, a wavelength of 262 nm was utilized for quantitative analysis of isoflavones. Detection limits for daidzein and genistein were 185 and 100 ng/mL, respectively.

Isoflavone Standards. Genistein and genistin were isolated from soy molasses as previously described in Peterson and Barnes (1991). Daidzein and daidzin were purchased from LC Laboratory (Woburn, MA). Calculations of individual isoflavones in samples were based on methodology described in Barnes et al. (1994). All data are expressed as unconjugated aglucones after a conversion by appropriate correction factors, thus allowing comparison of the isoflavone content independently of the chemical form(s) present.

Assessment of Carotenoids. Carotenoids contents and profiles were determined in all immature soybeans at R_6, but not at R_8, because literature indicated that carotenoid content diminished with maturity (Monma et al., 1994). All sample preparation for carotenoid analysis was also conducted in a room equipped with yellow light to prevent any possible photodegradation.

Initial Carotenoids. Initial carotenoid content is defined as the carotenoid content before freezing and freeze-drying. Soybean seeds were blanched in boiling water for one min to inactivate enzymes, then ice cooled. Once blotted dry, the seeds were homogenized in a food processor for only 30 s to avoid carotenoid degradation. Samples were saponified at 70 °C for 30 min under a nitrogen atmosphere (Thompson, 1986). Carotenoids were extracted from the sample using hexane containing 0.01% BHT (antioxidant). The extracts were then evaporated with nitrogen to dryness and redissolved in a mobile phase containing acetonitrile/methanol/tetrahydrofuran (20/28/2, v/v/v) (Bushway, 1986). The prepared extracts were then injected into an HPLC system composed of an injector (Rheodyne, model 7125, Cotai, CA), a pump (Shimadzu, model LC-10AS, Norcross, GA), a column (C_{18} 218TP54, 5 μm, 4.6 × 25 cm, Vydac, Hesperia, CA), a UV−Vis detector (Shimadzu, model SPD-10AV), a photodiode array detector (Shimadzu, model SPD-M10AVP) with SPD-M10AVP software, a column oven (Shimadzu, model CTO-10ASVP), and an integrator (Shimadzu, model Chromatopac CR501), connected in series. The flow rate of the mobile phase was set at 1 mL/min, and the column oven was set at 30 °C. For quantitation, the UV−Vis detector was set at 450 nm, and chromatogram (Figure 3) was recorded on the integrator. Simultaneously, the photodiode array detector was set between 390 and 800 nm for scanning of carotenoids (Figure 3). Electronic chromatograms were recorded on a computer for spectral comparison and identification, in addition to the retention time. The concentrations of individual carotenoids in soybean extracts were determined using peak areas relative to the corresponding standards. Dilutions were prepared when necessary to keep the absorption in the linear range of the standard curve for individual carotenoids. Standard curves ranged from 4 to 250 ng for lutein and β-carotene. Specific carotenoids were identified by matching the retention time and further confirmed by spectral overlay with the respected standard.

Carotenoid standards (lutein and β-carotene) were obtained from Sigma Chemical Company (St. Louis, MO). Concentration and stability of carotenoid standards were monitored spectrophotometrically using a diode array spectrophotometer (Beckman DU 640, Schaumburg, IL); wavelengths were 445 nm for lutein and 450 nm for β-carotene with absorptivities of 2550 for lutein (in ethanol) and 2480 for β-carotene (in methylene chloride) (Davies, 1976).

Proximate Analysis. Moisture of soybean seeds at various stages (raw, blanched, and boiled) were determined using an AOAC method in oil seeds (AOAC, 1995) to allow composition expression on a dry weight basis.

Statistical Analysis. Statistical analysis was conducted using the SAS package (SAS Institute, Cary, NC). Analyses of variance using the general linear model (GLM) were conducted, and differences between the sample means were analyzed by Duncan's multiple range test at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Isoflavone Distribution. Total isoflavones in the soybean varieties range from 472 μg/g in NTCPR93-40 to 2280 μg/g in Hutcheson (Table 1). The experimental soybean genotypes and the land race genotype from Nepal contained lower total isoflavones than the commercial soybean Hutcheson ($p < 0.0001$). Within the experimental genotypes and the land race, NTCPR93-

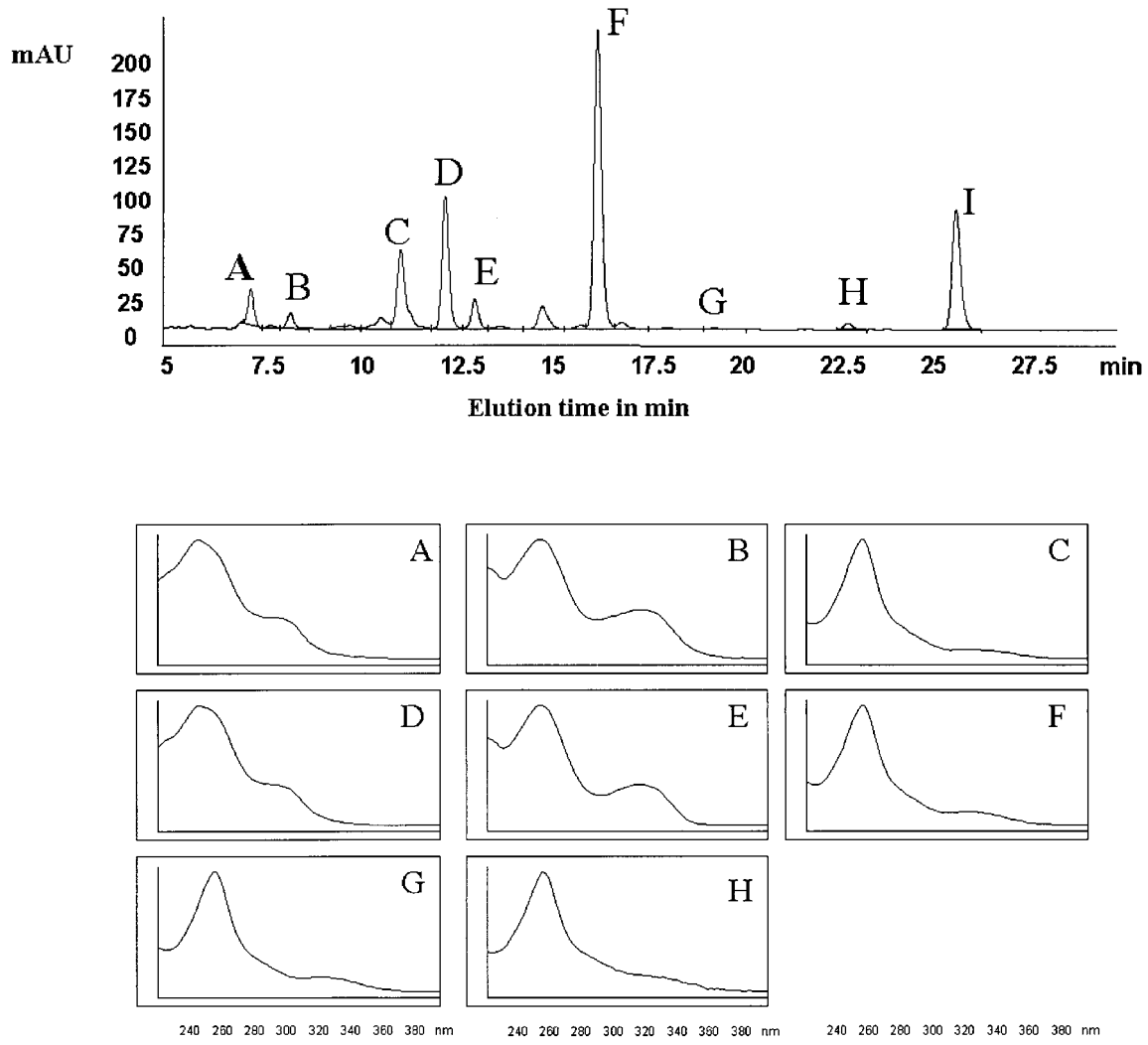


Figure 2. HPLC−DAD chromatogram of *hutcheson* soybean variety and UV spectra to authenticate peak identity of isoflavones. The top panel represents a typical profile of soy isoflavones. Chromatographic conditions were discussed in the Materials and Methods section. The bottom panel represents the UV spectra of each compound. The detector was set to scan in the 220−400 nm range. Each spectrum was compared to that of an authentic standard. The malonyl and acetyl conjugates have spectra that resemble the aglucones and β-glucosides.

286 has the lowest total isoflavones ($p < 0.0001$). These data provide isoflavone information for breeding programs in the southeastern United States.

Because total isoflavones contents in soybeans were affected by genotype and stage of maturity ($p < 0.0001$) and because the interaction between soybean genotypes and stage of maturity was significant ($p < 0.0001$), further statistical analyses were conducted to examine the effects of maturity stages and isoflavone contents (Table 2). It is also noted that malonyldaidzin and glucosides ratios decrease with maturity in all experimental genotypes and a land race (Honey Hrown) soybeans, but remain the same in Hutcheson (Table 2). The experimental soybean genotypes and the land race genotype from Nepal also contained higher total isoflavones at R_6 than at the mature stage (R_8) ($p < 0.0001$) (Table 2); whereas, the result was opposite for Hutcheson variety. Total isoflavones of NTCPR93-40, NTCPR93-286, Honey Brown, and NTCPR93-5157 at the R_8 stage were 31, 46, 50, and 70% of those at the R_6 stage, respectively. In Hutcheson, however, the total isoflavones at R_8 was 126% of that at the R_6 stage. This result in Hutcheson is consistent with the findings by Kudou et al. (1991) in Maple Arrow soybean seeds. Thus, it is possible that isoflavone synthesis pathways in these soybean genotypes are regulated differently. Tsukamoto et al. (1995) reported that growing temperature and growing location affect isoflavone content in soybean seeds.

Wang and Murphy (1994) reported higher total isoflavones (3800−4200 μg/g) in typical mature soybeans grown in the Midwest such as Pioneer and Prize than we found in Hutcheson which is a cultigen commonly grown in the Southeast. Little information exists on isoflavone distribution in soybeans grown in the southeastern United States. According to Wang and Murphy (1994), the Japanese varieties have higher contents of 6"-*O*-malonylglycitin and higher ratios of 6"-*O*-malonyldaidzin than the American soybeans; however, in this

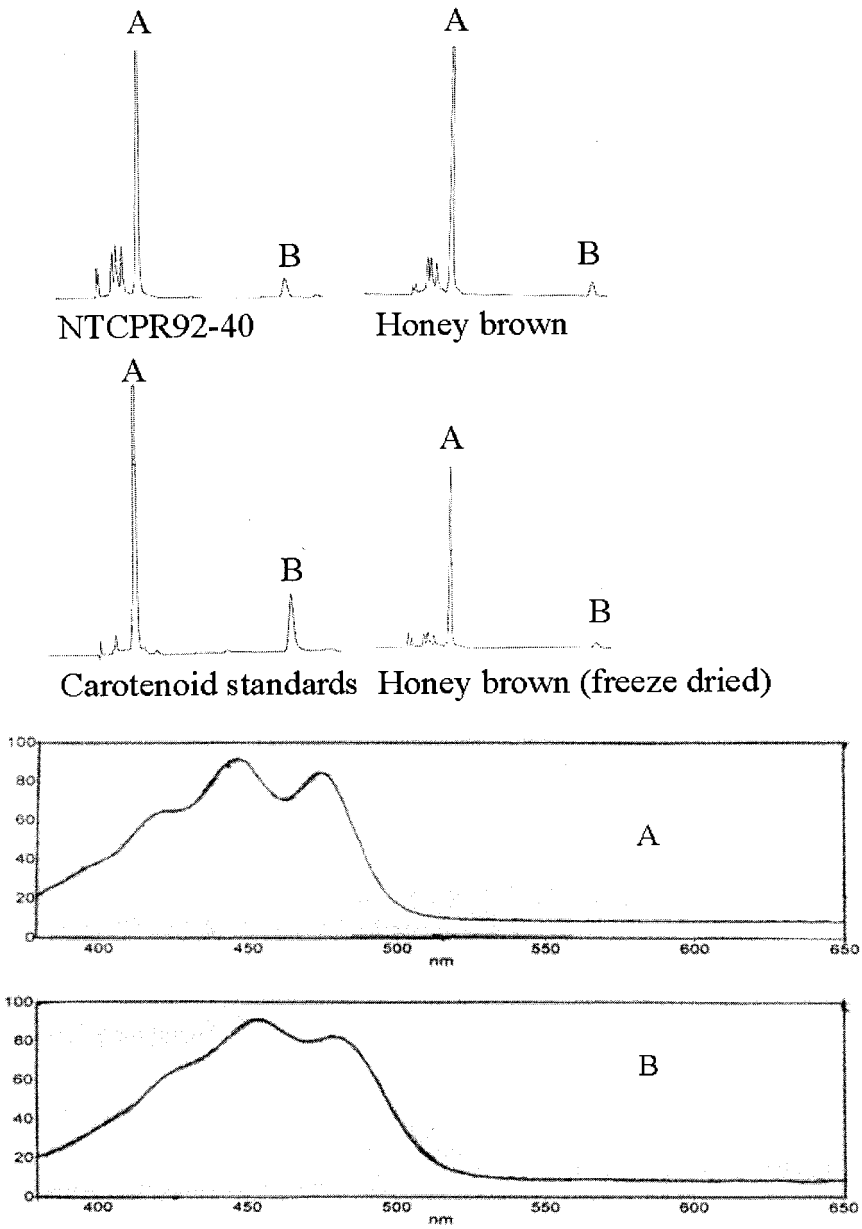


Figure 3. Chromatograms of selected soybean varieties, carotenoid standards, and UV−Vis spectra to authenticate peak identity of caroetnoids in immature soybeans. Chromatographic conditions were discussed in the Materials and Methods section. The detector was set to scan in the 360−650 nm range.

Table 1. Overall Distribution of Isoflavones as a Function of Variety[a]

	isoflavones glucosides			malonyl isoflavones			acetyl isoflavones			isoflavones aglucones			
variety	daid	gly	gen	daid	gly	gen	daid	gly	gen	daid	gly	gen	total[b]
NTCPR93−40	26c	41d	22c	111d	132d	128e	0	0	11a	0	0	0d	472 (292)d
Honey brown	67b	31e	55c	311c	110e	390c	0	0	0d	0	0	0d	964 (391)c
NTCPR93−5157	67b	126a	118b	510b	285a	843b	0	0	7b	0	0	8b	1,965 (408)b
NTCPR93−286	19d	57c	24d	107d	176c	26d	0	0	0d	0	0	5c	414 (261)e
Hutcheson	102a	80b	209a	539a	208b	1113a	0	0	6	0	0	22a	2,280 (307)a
CR	2.2	2	2.6	18.0	5.0	24.0	0	0	0.1	0	0	0.6	52

[a] Values are means of duplicate analysis of each variety at two stages ($n = 4$) and expressed as μg/g dry weight. Concentrations reported in the table are the equivalent of aglucones. Values in column with different letters were significantly different at $\alpha = 0.05$ based on Duncan's multiple range test. CR, critical range; daid, daidzein, gly, glycitein; gen, genistein; [b] Values in parentheses are standard deviations of total isoflavones.

study no particular trend was found in the ratio of malonyl isoflavones and glucosides in immature soybeans. It is possible that these soybeans are from advanced breeding lines which have a mixture of genetic traits from both American and Japanese soybeans. Thus, the distribution pattern may not follow that of

Table 2. Distribution of Isoflavones in Various Soybean Varieties at Immature (R_6) and Mature (R_8) Stages of Development[a]

	isoflavone glucosides			malonyl isoflavones			acetyl isoflavones			isoflavone aglycones			
stages (% moisture)	daid	gly	gen	daid	gly	gen	daid	gly	gen	daid	gly	gen	total[b]
NTCPR93−40													
immature (65.1)	29a	61a	39a	172a	203a	198a	0	0	17a	0	0	0	720 (12.2)a
mature (13.0)	22a	21b	6b	51b	62b	59b	0	0	5b	0	0	0	225 (4.9)b
CR	11.1	11.0	4.7	27	32	5.9	0	0	1.	0	0	0	66
Honey brown													
immature (61.4)	78a	41a	61a	432a	155a	516a	0	0	0	0	0	tr	1283 (25.4)a
mature (13.0)	57a	20b	49a	191b	65b	263b	0	0	0	0	0	tr	644 (2.4)b
CR	31.7	20.6	38.7	56.1	43.6	46.0	0	0	0	0	0	0	251
NTCPR93−5157													
immature (61.1)	66a	171a	143a	596a	410a	913a	0	0	9a	0	0	tr	2308 (4.6)a
mature (13.1)	68a	80b	93b	425b	161b	774b	0	0	6b	0	0	16b	1623 (41.4)b
CR	28.8	20.4	20.9	14.6	15.9	80.1	0	0	1.1	0	0	4.0	331
NTCPR93−286													
immature (60.5)	24a	79a	32a	155a	266a	254a	0	0	0	0	0	0b	810 (3.3)a
mature (13.0)	14b	35b	16b	59b	87b	156b	0	0	0	0	0	10.3a	377 (3.2)b
CR	8.8	8.6	1.5	8.2	6.7	21.0	0	0	0	0	0	1.5	59
Hutcheson													
immature (51.1)	46b	84a	101b	483b	273a	1007b	0	0	9a	0	0	9b	2013 (53)b
mature (13.1)	157a	76a	317a	595a	144b	1219a	0	0	3b	0	0	35a	2545 (76.8)a
CR	11.7	21.6	9.0	46.2	55.4	203.7	0	0	0.8	0	0	9.4	190

[a] Values are expressed as μg/g dry weight. Concentrations reported in the table are the equivalent of aglucones. Values are means of duplicate analysis of each variety at each stage ($n = 2$). Values in column with different letters between mature and immature soybean for each variety were significantly different at $\alpha = 0.05$ based on Duncan's multiple range test. CR, critical range; Tr, trace content; daid, daidzein; gly, glycitein; gen, genistein. [b] Values in parentheses are standard deviation of total isoflavones.

Table 3. Distribution of Isoflavones in Hutcheson Soybean as a Function of Processing Treatments[a]

		isoflavones glucosides			malonyl isoflavones			acetyl isoflavones			isoflavones aglycones			
treatment	% moisture[b]	daid	gly	gen	daid	gly	gen	daid	gly	gen	daid	gly	gen	total[d]
raw	51.1 ± 0.5	46(2)b	84(4)a	101(5)c	484(24)a	273(14)a	1007(50)a	0(0)	0(0)	10(0)a	0(0)	0(0)	9(0)c	2013a
boiling (PG)	60.4 ± 0	101(12)a	66(8)b	207(24)b	123(14)d	74(9)e	281(32)e	0(0)	0(0)	0(0)b	0(0)	0(0)	15(2)b	868cd
boiling (W)	60.4 ± 0	108(11)a	78(8)ab	235(25)a	125(13)d	79(8)e	299(32)e	0(0)	0(0)	0(0)b	0(0)	0(0)	18(2)a	942c
blanching	56.6 ± 0.1	37(4)c	42(5)c	66(7)de	201(23)c	109(12)c	431(49)c	0(0)	0(0)	0(0)b	0(0)	0(0)	0(0)e	886c
freeze-drying	[c]	34(4)c	38(5)c	71(9)d	179(22)c	92(11)d	385(48)d	0(0)	0(0)	0(0)b	0(0)	0(0)	3(0)d	803d
freezing	56.6 ± 0.1	35(3)c	37(3)c	62(6)e	246(23)b	121(11)b	577(54)b	0(0)	0(0)	0(0)b	0(0)	0(0)	0(0)e	1076b
CR		6.3	12.6	6.3	23.7	9.4	35.4	0	0	0.17	0	0	0.25	79.4

[a] Values are means of duplicate analysis of each treatments ($n = 4$) and are expressed as μg/g dry weight. Concentrations reported in the table are the equivalent of aglucones. PG, soybeans ground right after boiling treatment and kept frozen until analysis of isoflavones; W, soybeans kept as whole seed after boiling, then ground right before extraction and analysis of isoflavones. Values in column with different letters were significantly different at $\alpha = 0.05$ based on Duncan's multiple range test. CR, critical range; daid, daidzein; gly, glycitein; gen, genistein. [b] Moisture values were determined in each sample and the mean values were used for conversion of isoflavone into dry weight basis. [c] Moisture values of freeze-dried samples were not determined, but were assumed to be near zero. [d] Values in parentheses are the percent distribution for each isoflavone.

either parental cultivar. It is impossible to compare the values from the two studies because no common variety was used. Furthermore, soybean genotype, crop year, and growth location can influence isoflavone compositions (Wang and Murphy, 1994; Tsukamoto et al., 1995).

Effects of Processing on Soy Isoflavones. Boiling, blanching, freezing, and freeze-drying caused significant reduction in total isoflavones ($p < 0.0001$) (Table 3). Mean retention of total isoflavones was 53% (freezing), 46% (boiling), and 40% (freeze-drying). Freeze-drying appeared to have resulted in the greatest loss (60%) of total isoflavones; however, the initial loss of 56% was due to the blanching, and only 4% was due to the actual freeze-drying process. Because our initial intention was to produce a palatable freeze-dried soybean snack (Simonne et al., 2000), the effect of freeze-drying on raw samples was not examined in this study. Because most processing techniques required a submersion of soybean seeds in boiling water for one min (for blanching) to 35 min (for boiling), it is possible that the loss of isoflavones is due to their leaching into the cooking water. However, we did not analyze isoflavones in the cooking water. Wang and Murphy (1996) reported minimal loss of isoflavones in mature soybean seeds, while the seed coats are intact, during preparation of tempeh, tofu, and soy milk; however, the permeability of the immature seed coat vs that of mature seeds for isoflavones movement is not known.

Wang et al. (1998) reported a 25.8% retention of isoflavones during the production of soy protein isolate; the loss of isoflavones occurred in wash water (21.6%), solid waste (19%), and whey (14.3%), and 19.4% of isoflavones was unrecovered. Although it is impossible to compare the results of this study directly to the results of Wang et al. (1998), the results from these two studies indicate that some leaching of isoflavones occurred during blanching or boiling. However, the extraction of soybean oil did not cause any loss of isoflavones or their glucosides because they are not soluble in hexane (Eldridge and Kwolek, 1983).

Significant ($p < 0.0001$) changes in isoflavones profiles occurred in boiled soybeans. However, the percent distribution of isoflavones in raw, blanched, frozen, or freeze-dried soybean was not different (Table 3). Very little, if any, genistein was found in the immature raw Hutcheson soybeans. Notable increases in content of daidzin (glucoside), genistin (glucoside), and genistein (aglucone) occurred during boiling ($p < 0.0001$). Hy-

Table 4. Carotenoid Contents of Soybean Cultigens[a]

cultigen	moisture (%)[b]	lutein (μg/100 g)	β-carotene (μg/100 g)	β-carotene (RE/100 g)[c]
Hutcheson	56.58 ± 0.12	1092cd	291b	48.5b
NTCPR93−286	62.38 ± 0.33	895d	310b	51.6b
NTCPR92−40	65.85 ± 0.16	1698	491a	81.8a
NTCPR93−5157	63.99 ± 0.12	1428bc	296b	49.3b
Honey brown	63.75 ± 0.45	2119a	418a	69.6a

[a] Values are means of duplicate analysis of each treatment ($n = 4$). Values in column with different letters were significantly different at $\alpha = 0.05$ based on Duncan's multiple range test. [b] Moisture after blanching step. [c] 100% RDA of vitamin A for adult male is 1000 RE, where 1 RE = 1 μg of retinol = 6 μg of β-carotene = 12 μg of other provitamin A carotenoids.

drolysis of the malonyl and acetyl glucosides during boiling probably contributes to the conversion of isoflavone forms, as has been previously noted during soy milk production and cooking of dry soy products (Coward et al., 1998). Mahungu et al. (1999), however, stated that extrusion barrel temperature, followed by moisture content, had the most influence on isoflavone profile. These authors also stated that hydration of a corn and soy mixture without extrusion produced enzymatic glycolysis (by glucosidase action) in the mixture, thus dramatically increasing the aglucone contents. According to some current evidence (Peterson and Barnes, 1991; Messina et al., 1994), genistein appears to be an effective anti-carcinogen; thus, any process that could yield genistein from other forms could be beneficial.

No significant conversion of isoflavones occurred in the blanched soybeans because the heating time (one min) was not long enough to induce such a conversion. Stability of isoflavones during sample handling and preparation was also observed. The boiled soybeans were split into two portions: one portion (PG) was ground immediately after boiling and kept frozen until analysis, whereas the other portion (W) was kept whole during freezing and ground at the time of analysis. A 3% reduction of total isoflavone content was observed in PG soybeans compared to the W soybeans (Table 3). It is possible to prevent this type of loss by grinding samples immediately before extraction, rather than grinding the samples long before analysis.

Carotenoid Contents and Effects of Processing. Carotenoid contents in immature soybean were affected by genotype ($p < 0.0001$), with mean lutein contents ranging from 895 (in NTCPR93-286) to 2119 (in Honey Brown) and β-carotene from 291 (in Hutcheson) to 491 (in NTCPR92-40) μg/100 g dried weight (Table 4). Among the stable genotypes tested in this study, Hutcheson and Honey Brown contain the lowest and highest amounts of β-carotene, respectively. Hutcheson is a commercial line of soybeans, whereas Honey Brown is a land race line imported from Nepal. The Honey Brown may share some common traits with the Japanese domestic soybeans *Enrei* because they both were developed in the Far East. Monma et al. (1994) also reported higher contents of lutein and β-carotene in *Enrei* at 25 days after flowering (DAF) than at 50 DAF, which is equivalent to the R_6 stage used in our study. Although lutein content in Honey Brown was the highest, from a Western consumer standpoint it is less attractive because the beans become brown upon maturation, despite their good flavor (Simonne et al., 2000). Thus, it is more desirable to use this variety at a less mature stage (30−40 DAF) when the contents of lutein and β-carotene are maximal.

β-Carotene contents found in this study were higher than the values listed in the USDA Handbook 8 (NDB reference no. 11450 and 11451), however, these values are comparable to the reported values in immature seed of *Enrei* (Monma et al., 1994). It is impossible to compare our values to the USDA Handbook 8 values because information on soybean variety for values in the Handbook was not provided. In addition, the Handbook values were based on one or two observations. Literature sources reveal that genotypes play an important role in content of carotenoids in most crops, including soybean.

Table 5. Overall Carotenoid Retention Across Cultigens after Various Processing Treatments[a]

treatment	lutein (μg/100 g)	β-carotene (μg/100 g)	β-carotene RE/100 g)[b]
initial	2003a	549a	91.5a
boiling	1259b	341b	56.8b
freezing	1845a	405b	67.5b
freeze-drying	678c	150c	25c

[a] Values are over means across all genotypes with duplicate analyses ($n = 10$). Values in column with different letters were significantly different at $\alpha = 0.05$ based on Duncan's multiple range test. [b] 100% RDA of vitamin A for adult male is 1000 RE, where 1 RE = 1 μg of retinol = 6μg of β-carotene = 12μg of other provitamin A carotenoids.

Monma et al. (1994) found that lutein was the major carotenoid in common soybeans with a yellow seed coat and a variety having a black seed coat, whereas soybeans with a green seed coat contained several xanthophylls in addition to lutein. Most of the soybean varieties used in our study contain two major carotenoids, lutein and β-carotene (Figure 3), with trace amounts of other xanthophylls. Manoma et al. (1994) also detected trace amounts of β-carotene in a domestic Japanese variety of green soybeans. They reported total carotenoid contents to be higher in green soybeans than in the yellow types; they also correlated carotenoids with chlorophyll contents. Monma et al. (1994) stated that immature green soybeans possess similar carotenoid profiles as the green vegetables, and the amount of β-carotene decreased more rapidly than lutein and chlorophylls during seed maturation. Therefore, the authors suggested that carotenoids in immature soybean seeds may serve as a photo-protective agent in developing seeds, thus making them more susceptible to degradation in the course of seed maturation. Such findings were also reported by Bates and Matthews (1975) with β-carotene in soybeans.

Processing affected carotenoid retention in immature soybeans ($p < 0.0001$); mean retention of lutein and β-carotene, respectively, were (92%, 73%) in frozen, (62%, 62%) in boiled, and (34%, 27%) in freeze-dried soybean seeds (Table 5). Freeze-drying significantly reduced lutein and β-carotene in immature soybeans, but freezing did not. Boiling treatment only reduced carotenoid content by 38%. As carotenoids are not water

soluble, leaching of carotenoid did not occur readily. Immature soybeans are a good source of lutein and a fair source of β-carotene.

The freeze-drying process yields an excellent soybean product in terms of appearance and taste, although the process significantly reduced the amounts of lutein and β-carotene (Simonne et al., 2000). This study expressed the contents of carotenoids on a dry matter basis, thus allowing a comparison with the contents of unprocessed counterpart. Leme et al. (1973) and Fonseca et al. (1972) examined the effects of freeze-drying on the stability of ascorbic acid and β-carotene in fresh fruit products, but they did not take into account the initial water content. Therefore the results led to a conclusion that freeze-drying did not destroy ascorbic acid or β-carotene in the samples.

Our data also showed that the use of blanching is critical for determination of β-carotene and lutein in immature soybeans. β-Carotene was readily degraded to trace amounts, especially in Hutcheson, when the beans were not blanched.

In summary, total isoflavones and carotenoids in soybean were affected by genotype and stage of maturity. Processing such as blanching, boiling, freezing, and freeze-drying affects isoflavones and carotenoids in different manners because of their differences in chemical properties. Blanching and boiling cause major loss in isoflavones due in part to leaching. Boiling (a moist heat) treatment of immature soybean caused substantial increase in daidzin (glucoside), genistin (glucoside), and genistein (aglucone). Freezing did not cause a substantial loss in lutein and β-carotene, but freeze-drying did. Boiling of immature soybeans caused up to 38% loss of lutein and β-carotene. Because sufficient amounts of isoflavones and carotenoids are retained after processing, immature soybeans can provide a significant nutrient contribution in the U.S. diet.

ACKNOWLEDGMENT

The authors thank Dr. Thomas Carter of USDA−ARS at North Carolina State University for providing advanced food-type soybean lines used in this study. We also thank the efforts of Dr. Patricia Murphy, University of Iowa, who served as a reviewer of the manuscript.

ACKNOWLEDGMENT

Parts of this paper were presented at the Institute of Food Technologists annual meetings in 1998 (Simonne and Weaver) and 1999 (Simonne, Barnes, Weaver, and Wei).

LITERATURE CITED

Adlercreutz, H.; Honjo H.; Higashi, A.; Gotsis, T.; Hamalainen, E.; Hasegawa, T.; Okada, H. Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet. *Am. J. Clin. Nutr.* **1991**, *54*, 1093−1100.

AOAC International. *The Official Methods of Analysis*, 15th ed. The Association of Official Analytical Chemists International: Arlington, VA, 1995.

Akiyama, T.; Ishida, J.; Nakagawa, S.; Ogawara, H.; Watanave, S.; Itoh, N. M.; Shibuya, M.; Fukami, Y. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* **1987**, *262*, 5592−5595.

Barnes, S.; Kirk, M.; Coward, L. Isoflavones and their conjugates in soy foods: Extraction conditions and analysis by HPLC−mass spectrometry. *J. Agric. Food Chem.* **1994**, *42*, 2466−2474.

Bates, R. P.; Matthews, R. F. Ascorbic acid and β-carotene in soybeans as influenced by maturity, sprouting, processing and storage. *Proc. Fl. State Hortic. Soc.* **1975**, *88*, 266−271.

Bushway, R. J. Determination of α- and β-carotene in some raw fruits and vegetables by high-performance chromatography. *J. Agric. Food Chem.* **1986**, *34*, 409−412.

Carter, T. E.; Wilson, R. F. Soybean quality for human consumption. *Proceedings, 10th Australian Soybean conference.* James, A., Ed.; CSIRO: Brisbane, Australia, 1998.

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

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

Davies, B. H. 1976. Analysis of carotenoid pigments. In *Chemistry and Biochemistry of Plant Pigments;* Goodwin, T. W., Ed.; Academic Press: New York, NY, 1976.

Eldridge, A. C.; Kwolek, W. F. Soybean isoflavones: Effect of environment and variety on composition. *J. Agric. Food Chem.* **1983**, *31*, 394−396.

Farmakalidis, E.; Murphy P. A. Isolation of 6"-*O*-acytylgenistin and 6"-*O*-acetyldaidzin from toasted defatted soyflakes. *J. Agric. Food Chem.* **1985**, *33*, 385−389.

Fehr, W. R.; Caviness, C. E. *Stage of soybean development*. Special Report 80; Iowa Cooperative Extension Service: Des Moines, IA, 1977

Fonseca, H.; Nogueira, N. J.; Lame, J., Jr. Variations in ascorbic acid and β-carotene in freeze-dried fruits. *Solo* **1972**, *64*, 53−59.

Franke, A.; Hankin, J. H.; Yu, M. C.; Maskarinec, G.; Low, S.-H.; Custer, L. J. Isoflavone levels in soyfoods consumed by multiethnic populations in Singapore and Hawaii. *J. Agric. Food Chem.* **1999**, *47*, 977−986.

Hoeck, J. A.; Fehr, W. R.; Murphy, P. A.; Welke, G. A. Influence of genotype and environment on isoflavone contents of soybean. *Crop. Sci.* **2000**, *40*, 48−51.

Kennedy, A. The evidence for soybean products as cancer preventive agents. *J. Nutr.* **1995**, *125*, 733S-734S.

Kudou, S.; Fleury, Y.; Welti, D.; Magnolato, D.; Uchida, T.; Kitamura, K.; Okubo, K. Malonyl isoflavones glycosides in soybean seeds (*Glycine mx Merrill*). *Agric. Biol. Chem.* **1991**, *55*, 2227−2233.

Leme, J., Jr.; Fonseca, H.; Nogueira, J. N. Stability of ascorbic acid and β-carotene in freeze-dried West Indian cherry (*Malpighia punicifola* L.). *Arch. Latinoam. Nutr.* **1973**, *23*, 207−215.

Liu, K. Immature soybean: Direct use for food. *INFORM (Champaign, IL)* **1996**, *7*, 1217−1223.

Mahungu, S. M.; Diaz-Mercado, S.; Li, J.; Schwenk, M.; Singletary, K.; Faller, J. Stability of isoflavones during extrusion processing of corn/soy mixture. *J. Agric. Food Chem.* **1999**, *47*, 279−284.

Messina, M.; Barnes, S. The role of soy products in reducing risk of cancer. *J. Natl. Cancer Inst.* **1991**, 83, 541−546.

Messina, J.; Messina, V. Increasing use of soyfoods and their potential role in cancer prevention. *J. Am. Diet. Assoc.* **1991**, *91*, 836−840.

Messina, M.; Persky, V.; Setchell, K.; Barnes, S. Soy intake and cancer risk: A review of the In Vitro and In Vivo data. *Nutr. Cancer* **1994**, *21*, 113−131.

Monma, M.; Terao, J.; Ito, M.; Saito, M..; Chikuni, K. Carotenoid components in soybean seeds varying with seed color and maturation stage. *Biosci., Biotechnol. Biochem.* **1994**, *58*, 926−930.

Murphy, P. A. Content of processed soybean products. *Food Technol.* **1982**, *36*, 60−64.

Peterson, G.; Barnes, S. Genistein inhibition of the growth of human breast cancer cells, independence from estrogen receptors and the multi-drug resistance gene. *Biochem. Biophys. Res. Commun.* **1991**, *179*, 661−667.

Rubel, A.; Rinne, R. W.; Canvin, D. T. Protein, oil, and fatty acid in developing soybean seeds. *Crop Sci*. **1972**, *12*, 739−741.

SAS Institute. SAS/STAT Guide for Personal Computer. Statistical Analysis System Institute: Carry, NC, 1987.

Simonne, A. H.; Weaver, D. B.; Wei, C. I. Immature soybean seeds as vegetable or snack food: Acceptability by American consumers. *Innovative Food Science & Emerging Technologies*. **2000**, in press.

Steele, V. E.; Pereira, M. A.; Sigman, C. C.; Kelloff, G. J. Cancer chemoprevention agent development strategies for genistein. *J. Nutr*. **1995**, *125*, 713S−716S.

Thompson, J. N. Problems of official methods and new techniques for analysis of foods and feeds for vitamin A. *J. AOAC Int*. **1986**, *69*, 727−738.

Tsukamoto, C.; Shimada, S.; Igita, K.; Kudo, S.; Kokubun, M.; Okubo, K.; Kitamura, K. Factors affecting isoflavone content in soybean seeds: Changes in isoflavones, saponins, and composition of fatty acids at different temperatures during seed development. *J. Agric. Food. Chem*. **1995**, *43*, 1184−1192.

Wang, C.; Ma, Q.; Pagadala, S.; Sherrard, M. S.; Krishnan, P. G. Changes of isoflavones during processing of soy protein isolates. *J. Am. Oil Chem. S*oc. **1998**, *75*, 337−341.

Wang, H.-J; Murphy P. A. Isoflavones composition of American and Japanese soybeans in Iowa: Effects of variety, crop year, and location. *J. Agric. Food Chem*. **1994**, *42*, 1674−1677.

Wang, H.-J.; Murphy, P. A. Mass balance study of isoflavones during soybean processing. *J. Agric. Food Chem*. **1996**, *44*, 2377−2383.

Wang, G.; Kuan, S. S.; Francis, O. J.; Ware, G. M.; Carman, A. S. A simplified HPLC method for the determination of phytoestrogens in soybean and its processed products. *J. Agric. Food Chem*. **1990**, *38*, 185−190.

Received for review February 25, 2000. Revised manuscript received September 1, 2000. Accepted September 7, 2000. This research was partially supported by the Alabama Soybean Producers Association.

JF000247F