PHYTOESTROGENS IN FOODS

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I. INTRODUCTION

Phytoestrogens in foods are a source of intense interest in academic, food and nutraceutical industry research due to their involvement in health protective effects for a variety of chronic human diseases. The term phytoestrogens is used to describe a group of plant chemicals that apparently elicit an estrogen-like biological response. This term is used widely in the literature without the rigor probably needed to define a chemical as an estrogen. Isoflavones, lignan metabolites, the *Fusaria* mycotoxins in the zearalenone family, coumestans, phytosterols, flavonoids (flavonones, flavones, flavonols) and phenolic acids have been termed phytoestrogens. This review will focus on those chemicals from plant foods that fit a multipart definition for an estrogen. Kurzer and Xu (1997) reviewed this area recently. Our review will concentrate on research conducted since their 1997 review.

II. DEFINITION OF PHYTOESTROGENS

Estrogens play an important hormonal role among all vertebrates. Animal estrogens are exclusively steroidal compounds with the principal physiological estrogen in most species being 17-β-estradiol. Many plants produce chemicals that possess estrogenic activity in animals and are defined as phytoestrogens. The rigor with which one uses the term "estrogenic activity" will set the parameters for what one can call a phytoestrogen. Although no one series of assays can be universally applied to all potential estrogens, evaluation of an estrogen needs to consider both estrogen receptor binding as well as biological availability in the test species. Therefore a combination of *in vitro* and *in vivo* assays that yield dose-response relationships are necessary in identifying phytoestrogenic activity (Reel *et al.*, 1996). These assays can be grouped into: (1) reproductive tract response; (2) non-reproductive-tract target tissue response; (3) estrogen receptor binding; and (4) estrogen receptor-dependent transcriptional expression. Reel *et al.* (1996) proposed a decision tree to determine if a compound is an estrogen. An estrogen receptor binding assay is conducted initially with the test estrogen and compared to estradiol or diethylstilbestrol (DES). If a positive response is observed, an *in vitro* estrogen receptor-dependent transcriptional activity is determined. A negative response in the estrogen receptor binding assay suggests the compound is unlikely to be a direct acting estrogen or antiestrogen. The negative result can be confirmed with the estrogen receptor-dependent

transcriptional assay. A positive response in the estrogen receptor-dependent transcriptional assay suggests the compound is a suspected pro-estrogen. A negative response suggests a potential estrogen antagonist. The suspected pro-estrogen compounds should be tested in an *in vivo* assay for estrogenic activity in either an uterotrophic assay or a vaginal cornification assay. A positive response in either of these assays indicates the compound is an estrogen. The dose and route of administration will affect the outcomes of these *in vivo* tests and should mimic the human situation as much as possible. The assay tree proposed by Reel *et al.* (1996) thus includes the processes of absorption, disposition, metabolism and excretion that can be missed in an *in vitro* assay alone.

Thus, the chemicals found in plant foods that have met the criteria described above as phytoestrogens are the isoflavones, the lignans and the coumestans. The estrogenic mycotoxins in the zearalenone family will not be considered in this review since they are of fungi rather than plant origin. All phytoestrogens are diphenolic compounds with structural similarities to 17-β-estradiol or DES (Figure 1). Although other flavonoids and other phytochemicals have been called phytoestrogens, they do not apparently possess the ability to yield positive responses in the estrogen activity decision tree. A number of flavonoids, such as phloretin, naringenin, kaempferol and apigenin, have been called phytoestrogens because of their estrogen receptor binding activity and estrogen receptor-dependent transcriptional activity (Miksicek, 1993). However, some, such as apigenin, are not apparently absorbed from the gut (Hendrich *et al.*, 1999), or have not been evaluated for a positive response in an *in vivo* estrogen assay.

III. CHEMISTRY OF PHYTOESTROGENS

A. ISOFLAVONES

Over 870 isoflavones have been identified in plants (Harborne, 1994). Surprisingly, the number that humans are exposed to in their foods is extremely small. The phytoestrogenic isoflavones in human foods are genistein, daidzein, and glycitein from soybeans, and biochanin A and formononetin from alfalfa and clover sprouts and garbanzo beans (or chick peas) (Figure 2). These isoflavones are found in nominal amounts in other legume foods. These isoflavones typically are present in plants as β-glucosides and predominately as the 6″-O-malonyl-β-glucoside. Aglucons are produced from the seed's β-glucosidases during seed imbibition and germination and by microbial β-glucosidases during soy food fermentation.

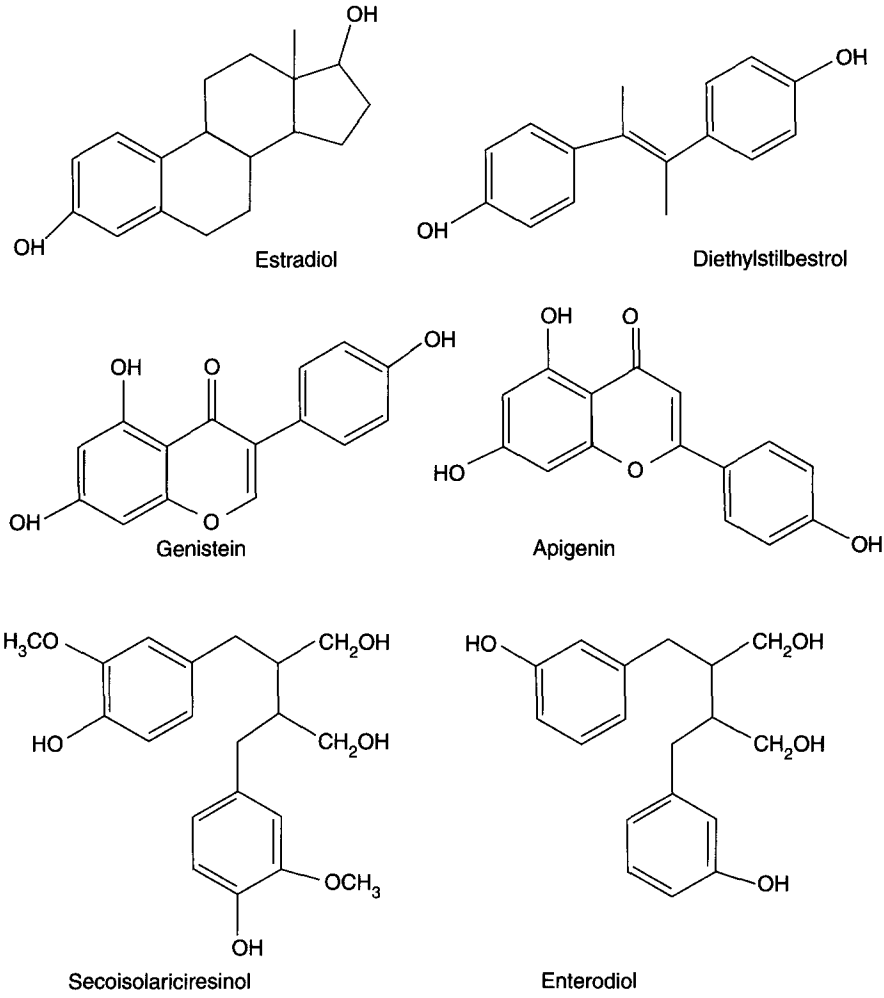

FIG. 1. Estrogens and phytoestrogens.

B. COUMESTANS

Coumestrol is the coumestan found in human foods and it has the highest estrogen activity of the phytoestrogens in foods (Figure 2). In the estrogen receptor binding assay and the mouse uterotrophic assay, coumestrol has approximately 10 times the estrogen activity of the isoflavones (Verdeal and Ryan, 1979). In contrast to its isoflavone relatives, coumestrol is not apparently glycosylated.

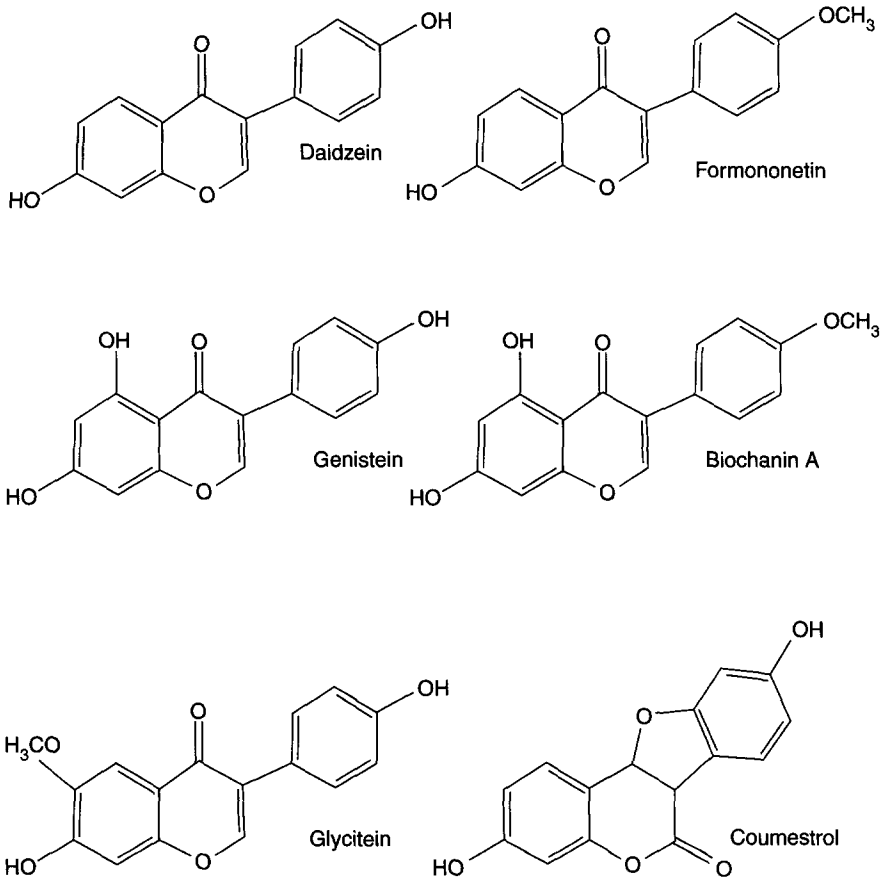

FIG. 2. Phytoestrogens: soybean, chickpea, alfalfa and clover isoflavone aglycons and coumestrol, a coumestan.

C. LIGNANS

Lignans are a family of polyphenolic constituents of plant cells that must be transformed by gut microorganisms into estrogenic compounds (Figure 3). Secoisolariciresinol, anhydrosecoisolariciresinol, and matairesinol, the predominant lignans, are glycosylated in plants with a variety of carbohydrate moieties and are converted by gut bacteria to enterodiol and enterolactone (Borriello *et al.*, 1985; Liggins *et al.*, 2000c). The plant precursors and the microbial metabolites have estrogenic activity as measured by the estrogen receptor binding (Adlercruetz *et al.*, 1992).

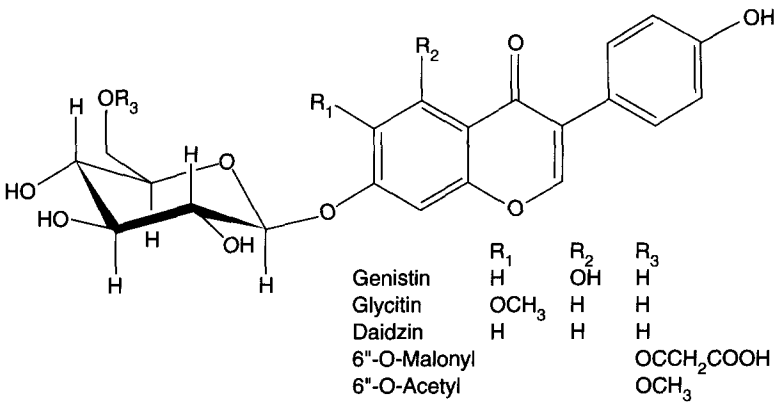

FIG. 3. Soybean isoflavone glucosides.

IV. ROLE IN PLANTS

Before the biology of the phytoestrogens in humans is reviewed, the reason for the presence of these compounds in plants should be briefly discussed. The isoflavones in soybeans appear in all parts of the plant and participate in plant defense, signal transduction and cell-to-cell signaling. In the soybean root, the isoflavone aglucons participate in the induction of the gene in *Bradyrhizobium japonicum*, the nitrogen fixing bacteria of soybeans, to cause soybean root inoculation for the nitrogen fixation steps in these plants (Kosslak *et al.*, 1987). In leaves, the isoflavones apparently are present as a reservoir for rapid phytoalexin synthesis to glyceollin when the plants are attacked by insect and microbial pathogens (Graham and Graham, 1996).

Lignans and lignins are part of the plant cell wall structure and always associated with the cell wall carbohydrates. In food chemistry terms, lignin is considered a three dimensional polymer of phenylpropane units such as syringaldehyde and vanillin. Lignification of the cell walls especially in the xylem contributes to the rigidity and toughness of the cells. Typically, lignins are chemically classified as the noncarbohydrate portion of dietary fiber (Haard and Chism, 1996). Lignans, with structures that appear to be precursors of the polymeric lignins, are actually produced by a separate synthetic route and possess a specific stereochemistry unique to this family (Lewis *et al.*, 1998; Wallis, 1998). Lignans are produced by initial condensation of coniferyl alcohol to (+)-pinoresinol followed by enzymatic reduction to (+)-lariciresinol and (–)-secoisolariciresinol.

V. ANALYTICAL METHODS

The phenolic structure of the isoflavones, lignans and coumestans endows them with significant ultraviolet absorbance characteristics. This has allowed a variety of liquid chromatography techniques to be developed to evaluate the concentration of these components in foods. The Adlercreutz group at the University of Helsinki approached the analysis problem from experience in endocrinology. They have developed an elegant protocol to assay both isoflavones and lignans in plasma and urine and have applied this protocol to foods (Mazur *et al.*, 1998a). Both lignans and isoflavones are eventually measured by gas chromatography-mass spectrometry-selected ion monitoring. This approach is beyond the capabilities of most food analysis laboratories. Mazur *et al.* (1998a) and Liggins *et al.* (1998; 2000a, b) reported an isotope dilution gas chromatography methods for evaluation of isoflavones in foods and nonfood seeds. Liggins *et al.* (1998) reported interassay CVs for genistein and daidzein of 4.7 and 2.7%, respectively. Both methods require derivatization. Johnsson *et al.* (2000) reported a gradient HPLC method for secoisolariciresinol diglucoside analysis for flax with modest pre-HPLC clean-up steps.

The large majority of reported methods for isoflavone and coumestan analysis involve reversed phase HPLC with ultraviolet (uv) absorbance detection. The improvement in availability of photodiode array detectors for HPLC has allowed additional confirmation of suspected HPLC peaks by scanning uv absorbance patterns to compare with library scans of authentic standards. Hendrich and Murphy (2000) recently published a summary of the extinction coefficients reported in the literature for soy isoflavone quantitation. The sensitivity range for these techniques to about 0.1 μM which is adequate for most foods but causes some limitations of this technique's use for plasma but not urine samples. The development of sensitive electrochemical HPLC detectors has lowered the detection limit by one order of magnitude. Given the range of hydrophobicities of the isoflavones, gradient elution rather than isocratic is typically applied using acidified water-methanol or -acetonitrile to resolve the different isoflavone isomers (Murphy, 1981; Farmakalidis and Murphy, 1985a; Wang and Murphy, 1994a, 1994b; Coward *et al.*, 1993; Franke *et al.*, 1994a). Barnes *et al.* (1998) reported analysis of isoflavones and their metabolites utilizing HPLC-mass spectrometry. No derivation is required. Optimization of ion energies in formation and collision of parent ions was essential for detection. Setchell *et al.* (1997) reported isoflavone levels in soy-based infant formula as measured by HPLC and reported total mass of isoflavone without adjustment for molecular weight differences. Song *et al.* (1998) reported quality control measures for soy food isoflavone analysis and

used of 2,4,4′-trihydroxydeoxybenzoin (THB) as an internal standard for HPLC analysis of foods. Murphy *et al.* (1998, 1999) reported precision and accuracy measurements for isoflavone database development over a 9- and 24-month sampling period, respectively. Recoveries of daidzein and genistein averaged 92% while genistin and THB recoveries were 99%. Coefficients of variation (CV) were evaluated for 12 isoflavone forms in two food matrices stored under two conditions for 9 and 24 months for within-day and between-day precision. Within-day CVs averaged less than 4.8% while between-day CVs were less than 7% except for daidzein (9.6 ± 4.9%) and genistein (11.0 ± 6.0%). Total genistein, total daidzein and total glycitein CVs were ≥ 3.3% and 4.6% for within-day and between-day, respectively.

Franke *et al.* (1999) reported isoflavone levels in soy foods in Singapore and Hawaii for 25 food groups. An external standard (an extract of soy flour) and flavone as internal standard were used. Jeong *et al.* (1998) used isoflavone levels as a quality parameter for fermented soy pastes. King *et al.* (1998) used HPLC to evaluate isoflavone levels in cow's milk with detection levels of 2 ng mL^{-1}. Seasonal variations in levels were observed with peaks of 293 ng mL^{-1} equol occurring in summer months. Equol is one of the mammalian metabolite of daidzein found in plasma and milk. Krishnan (1998) reported genistein in *Apois americana Medikus*, the American groundnut, an indigenous North American tuber, by HPLC analysis with ultraviolet absorbance detection and MS confirmation. Coward *et al.* (1998) monitored effects of baking and frying on soy isoflavone glucoside interconversion by reversed-phase HPLC-mass spectrometry. Mahungu *et al.* (1999) reported effects of extrusion processing on isoflavone distribution using 80% methanol for extractions. They reported lower extraction rates in extruded foods unless rehydrated before extraction. This solvent has previously been reported to incompletely extract the acetylglucosides (Farmakalidis and Murphy, 1985a). Griffith and Collison (2001) have evaluated extraction methods for soy foods and nutritional supplements by HPLC-mass spectrometry and reported similar within-day and between-day CV of analysis to Murphy *et al.* (1999).

Aussenac *et al.* (1998) reported a capillary zone electrophoresis (CZE) method for soybean isoflavones. Mellenthin and Galensa (1998) reported a CZE method to detect soy and lupin protein in meat products by measuring isoflavone content. The same authors (1999) compared HPLC and CZE for isoflavone detection. CZE was recommended for rapid screening but HPLC was less dependent on matrix effects and was more sensitive.

A radioimmunoassay was reported by Lapcik *et al.* (1998) for genistein in serum with intra-assay CV% of 3.5–9.3 and interassay CV% of

6.7–19.7. Cross-reactivity with daidzein (5.8%) and formononetin (2.2%) were reported. An ELISA was reported for daidzein and equol in human plasma with detection limits of 21 pg daidzein per well and 70 pg equol per well by Creeke *et al.* (1998). Bennetau-Pelissero *et al.* (2000) reported development of ELISAs for soy and alfalfa isoflavones with detection limits of 0.3 and 9.0 pmol per well.

There is an AOAC method recently accepted for soy isoflavones involving an alkaline heat extraction procedure in order to convert the malonyl- and acetyl-glucosides into the β-glucosides (Klump *et al.*, 2001). This procedure would require only six standards, the β-glucosides and the aglycons. This method will allow quantitation of "conjugated" and "free" isoflavone forms but would not allow estimation of the individual forms in a food matrix.

VI. SOURCES, FOOD LEVELS AND DATABASES

A. ISOFLAVONES

Isoflavones are found in the highest concentrations in soybeans and soybean foods reflecting the segregation of the enzymes synthesizing these components to one subfamily of the Leguminosae, the Papilionoideae. The isoflavones are synthesized by condensation of a phenylpropane backbone with an additional ring structure from an acetate condensation.

The soybean isoflavones, genistein, daidzein and glycitein, are present in soybeans and in soy foods in four different forms as the β-glucoside, the malonyl-β-glucoside, the acetyl-β-glucoside and as the aglucons (Figure 3). The distribution of these forms depends on the level and type of processing the soy has undergone. The chickpea, alfalfa and clover isoflavones, biochanin A and formononetin, are also present as β-glucosides and malonyl-β-glucosides, aglucons and potentially as the acetyl-β-glucosides. However, fewer reports on the distribution of these forms are found in the literature.

There are a number of reports of isoflavones in other legume and non-legume based foods. However, the concentrations are typically 100 to 1000 less than those found in soybeans and chickpea foods. Table I lists foods containing significant amounts of isoflavones (μg/g food) and their concentration ranges from the reports published after 1996. Table II provides a summary of foods containing minor amounts of isoflavones (ng/g food). Besides soy, alfalfa and clover sprouts, and garbanzo beans, a few other food items are reported to contain isoflavones, although at concentrations usually about 1000 times lower than soy, including hops in

TABLE I

FOODS CONTAINING SIGNIFICANT CONCENTRATIONS OF ISOFLAVONES[a] (μg/g)

Food	Concentration range	Reference
Soybeans	720–2370	1
Soy flours, defatted	610–2440	1
Soy sprouts	250–530	1
Soy protein isolate	465–1993	1
Soy protein concentrate		
water washed	20–318	1
ethanol washed	612–1670	1
Texturized soy protein	44–2956	1
Hydrolyzed soy protein (HSP)	127–1621	2
Tuna (packed in water with HSP)	22	2
Doughnuts	85–100	2
Pancake mix	171	2
Soy milk	13–211	1
Low fat soymilk	17–86	2
Tofu	79–635	1
Low fat tofu	194–200	2
Tempeh	69–625	1
Miso	227–892	1
Natto	464–870	1
Soy based infant formula	202–316 (25–30 μg mL^{-1})	1; 4
Edamame (green immature soybeans)	1354–1860	1
Soybean (vegetable) oil	0	1
Bacon, meatless	121	1
Chicken analog	146	1
Harvestburgers®	82.2	1
Soy cheeses	33–593	1
Soy hotdog and breakfast sausage	34–150	1
Soy sauce	12.7–23.0	1
Commodity hamburgers with soy	5.5–29	1
Clover sprouts	0–23	1
Alfalfa sprouts	0–2610	1
American groundnut	3.5–8.4	3
Green beans	1.5	1
Kidney beans	0.1–4.1	1
Lima beans	0–3.7	1
Pinto beans	0.0–11	1
Red beans	3.1	1
White beans	7.4	1
Navy beans	4.3	3
Broadbeans (fava)	0.3	1
Garbanzo beans	0.0–19	1
Cowpeas	0.0–18	1
Kala Chana seeds	6.4–12.6	1

TABLE I *(continued)*
FOODS CONTAINING SIGNIFICANT CONCENTRATIONS OF ISOFLAVONES[a] (μg/g)

Food	Concentration range	Reference
Mung beans	0.0–7.0	1
Peanuts	1.3–2.9	1
Peas	0–80	1
Pigeon peas (red gram)	2–5.6	1
Black gram	6.4–17	3
Sunflower seeds	0.3	1

[a] Total moles of genistein, daidzein, glycitein, biochanin A and formononetin multiplied by the aglucon molecular weight.
References: 1. Beecher *et al.*, 1999; Liggins *et al.*, 2000a; Liggins *et al.*, 2000b. 2. Unpublished data, Murphy. 3. Mazur *et al.*, 1998a, 1998b. 4. Knight *et al.*, 1998.

beer (De Keikeliere *et al.*, 1997) and dry cherries (Wang *et al.*, 1999). Liggins *et al.* (2000b) reported isoflavones in 56 types of vegetables, but none has concentrations greater than 10 $\mu g\ g^{-1}$ except for soy-based foods. Most vegetable foods reported by Liggins *et al.* (2000b) contained less than 1 $\mu g\ g^{-1}$. Other nonfood herbal items containing isoflavones reported in the literature with the largest number containing Kudzu root with its predominate isoflavones, daidzin and puerarin (Rong *et al.*, 1998a; Rong *et al.*, 1998b; Pei *et al.*, 1999; Okamura *et al.*, 1999).

There are a few reports in the literature comparing isoflavone levels in glyphosate-tolerant soybeans and their genetic parents (Taylor *et al.*, 1999; Padgette *et al.*, 1996). These authors reported no differences in isoflavone levels were observed between genetically modified soybeans and their parent lines. Lappe *et al.* (1999) tried to suggest that genetically modified soybeans contained different levels of isoflavones than common varieties. However, they did not evaluate parent lines nor obtain their soybeans from uniform growing environments. Environmental or growing conditions have a large effect on isoflavone levels reported in soybeans (Wang and Murphy, 1994a; 1994b; Tsukamoto *et al.*, 1995; Hoeck *et al.*, 2000). The range of concentrations reported by Lappe *et al.* (1999) for their samples was well within the range reported for traditionally derived soybeans.

A database on isoflavones in foods became available in 1999 and is being routinely updated as literature reports appear (Beecher *et al.*, 1999). This database reports total daidzein, total genistein, total glycitein and total isoflavones on a mg per 100 g food basis, the minimum and maximum value and the standard error of the mean for 140 foods. The totals are the sum of the moles of each isoflavone form multiplied by the molecular weight of the aglucon form. The authors of this database evaluated the

TABLE II

FOOD CONTAINING MINOR AMOUNTS OF ISOFLAVONES[a] (ng g^{-1})

Food	Concentration range	Reference
Apricots	43	1
Black eyed peas	451–860	2
Cherries	32	1
Cranberries	38	1
Currants	2245	1
Figs	19–69	1
Fruit cocktail	3	1
Lentils	250–399	2
Lima beans	380–1172	2
Mango, raw	71	1
Mango, canned in syrup	22	1
Melon, cantaloupe	4	1
Melon, galia	10	1
Melon, yellow honeydew	26	1
Passion fruit	174	1
Peaches, canned in syrup	15	1
Pears, canned in syrup	12	1
Peas	81–410	1
Plums, Victoria	75	1
Prunes	128–164	1
Raisins, California	1836	1
Strawberries	40–51	1
Chestnuts	12–68	1
Coconut, fresh	186	1
Hazelnuts	240	1
Peanut butter	98	1
Peanuts	122–1456	1, 2, 4
Sesame seeds	54	1
Barley	217	2
Teas and coffees	1–2340	3
Beer	340–7830[b]	5

[a] Total moles of genistein, daidzein, glycitein, biochanin A and formononetin multiplied by the aglucon molecular weight.

[b] ng L^{-1}.

References: 1. Liggins *et al.*, 1998. 2. Mazur *et al.*, 1998a. 3. Mazur, 1998. 4. Mazur *et al.*, 1998b. 5. Lapcik *et al.*, 1998.

quality of the data in each literature citation and assigned a quality score or confidence code of a, b or c with a = best to the data as suggested by Mangels *et al.* (1993) for the carotenoid database. The methodology criteria for inclusion in this database were relaxed because it is the first of its kind for isoflavones. This database expands upon the database originally

published by Reinli and Block (1996) and includes glycitein levels since this isoflavone also has estrogenic activity (Song *et al.*, 1999).

The USDA-Iowa State University Isoflavone Database (Beecher *et al.*, 1999) includes a summary of the biochanin A and formononetin levels in 41 foods ranging from 0.0 and trace to 13 220 mg per 100 g for red clover seeds. The highest value for a typical food containing these isoflavones is for alfalfa and clover mixed sprouts at 5.8 and 2.2 mg per 100 g wet weight basis for formononetin and biochanin A, respectively.

B. LIGNANS

In contrast to isoflavones, the lignans are quite widely distributed in the plant kingdom in foods that humans consume and are the result of a single condensation of two phenylpropane units (Figure 4). The lignan content of foods has not been well characterized yet. However, there are more data in the literature since the Kurzer and Xu (1997) review. Table III summarizes lignan composition of food. Lignans in foods represent an excellent example of the requirement for the metabolism of phytochemicals prior to absorption in humans. In fact, the metabolites, enterodiol and enterolactone were discovered in urine before the plant precursors, secoisolariciresinol and matairesinol, were identified (Setchell *et al.*, 1980; Borriello *et al.*, 1985). Some researchers use gut bacterial incubation methods to determine lignan concentrations in foods (Thompson *et al.*, 1991). Secoisolariciresinol and matairesinol probably do not account for all the sources of dietary lignans. Recently, Liggins *et al.* (2000c) have reported shonanin, an anhydride of secoisolariciresinol, in a variety of plant foods. However, they did not quantify shonanin separately from secoisolariciresinol. Rickard *et al.* (1996) could only account for 20% of the urinary enterodiol and enterolactone production when comparing rats fed purified secoisolariciresinol diglycoside to rats fed flaxseed with same precursor dose. This difference may account for the differences in reported lignan levels in foods. Flax seed is the plant food with the highest concentrations of lignan precursors at 3.7 mg g^{-1} (Mazur *et al.*, 1998) to 13.3 mg g^{-1} secoisolariciresinol in flax reported by Johnsson *et al.* (2000). Other food seeds contain lignans ranging from 0.1 μg g^{-1} in clover seeds to 19 μg g^{-1} in lentils (Thompson, 1991; Mazur *et al.*, 1998a, 1998b; Liggins *et al.*, 2000c). Many other plant sources contain surprising amounts of lignans as lignan precursors. Coffee and teas, both green and black, are a source of lignans (Mazur *et al.*, 1998b). Black and green teas averaged 16.2 and 2.00 μg g^{-1} (dry weight basis) secoisolariciresinol and matairesinol, respectively, while coffees average 5.6 μg g^{-1} (dry weight basis) secoisolariciresinol. Blackberries (*Rubus fructicosus*), strawberries (*Fragariax ananssa*), lingonberry

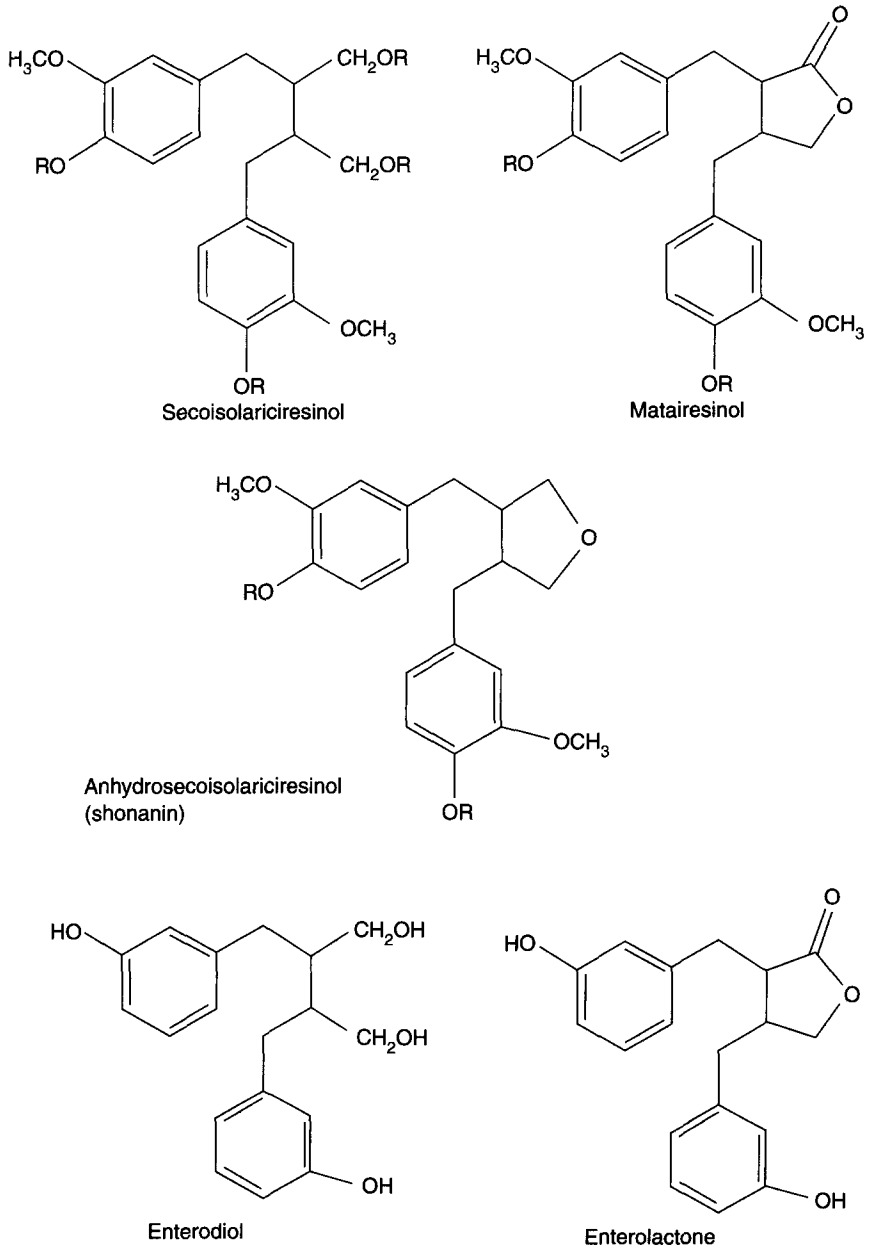

FIG. 4. Lignans in plants and lignan metabolites.

TABLE III

FOODS CONTAINING LIGNAN PRECURSORS, SECOISOLARICIRESINOL, SHONANIN AND MATAIRESINOL[a] AND/OR MAMMALIAN LIGNANS[b] (ng g^{-1} DRY WEIGHT)

Food	Concentration range		
	Seco	Mat	Total bacterial lignan
Oil and legume seeds			
Flaxseed	3,700,000 –13,300,000	10,000	347,520–1,140,300
Soybeans	130–226,000	0	9550
Textured vegetable protein	270,000		
Beans, all varieties	640–30,500	nd–tr	2260–6430
Lima bean	1580–1850	tr	
American groundnut	210–580	20–50	
Garbanzo bean	70–80	0.0	
Peas	30–130	nd–tr	2330
Black gram	450–2400	710–2620	
Black-eyed pea or cowpea	1950	nd	
Mung bean	1720	0	
Peanut	3330	0	1680
Lentils	90–120	nd	19560
Walnut	1630	50	
Hazelnut	1190	40	
Cashew	2570	40	
Caraway seed	2210	57	
Grains			
Rye	470–7200	650	1810
Wheat	81–2800	0	5670
Barley	580	0	1290
Oats	134	tr	3780
Maize	80	0	2630
Rice	160–600	tr	
Triticale			10520
Breads and cereals			
Whole grain breads			3892–117,288
Rye			8326
Wheat			8688
Red River cereal (4% flax)			29322
US Mills Uncle Sam's cereal (20% flax)			174846
Wheat flakes in Uncle Sam's			13394
US Mills Erewhon wheat flakes			13756
Beverages			
Black tea[a]	12,100–26,320	990–4130	
Green tea[c]	7110–27,020	590–2630	
Coffee[c]	5010–7160	nd	
Coffee beans	108,000		

TABLE III *(continued)*

FOODS CONTAINING LIGNAN PRECURSORS, SECOISOLARICIRESINOL, SHONANIN AND MATAIRESINOL[a] AND/OR MAMMALIAN LIGNANS[b] (ng g^{-1} DRY WEIGHT)

Food	Concentration range		
	Seco	Mat	Total bacterial lignan
Fruits			
Blackberry	37,180	225	
Strawberry	15,046	781	7760
Cloudberry	2030	0	
Raspberry	1390	0	
Lignonberry	15,100	0	
Cranberry	10,540	0	
Blueberry	8350	0	
Black currant	3880	0	
Red currant	1653	0	
Bramble berry	37,180	225	
Gooseberry	30,400	58	
Pear			10800
Plum	50	0	7660
Banana	100	0	2840
Orange	768	0	2760
Cantaloupe	1839	0	4260
Apple	tr	0	2290
Avocado	767	160	
Lychee	536	tr	
Papaya	82	0	
Guava	6997	tr	
Lemon	613	0	
Vegetables			
Garlic	3790	36	10500
Squash			63440
Asparagus	65,100		45030
Carrot	1920	30	29320
Sweet potato			10020
Broccoli	4140	230	20720
Leek			13590
Green pepper	1170	70	29570
Turnip			18400
Cauliflower	970	tr	16160
Beet			10600
Snow pea			20790
Iceberg lettuce			26110
Onion	830	80	10270
Chives	12,540	tr	
Green beans			12040
Potato			4090
Brussel sprouts			5440

TABLE III *(continued)*

FOODS CONTAINING LIGNAN PRECURSORS, SECOISOLARICIRESINOL, SHONANIN AND MATAIRESINOL[a] AND/OR MAMMALIAN LIGNANS[b] (ng g^{-1} DRY WEIGHT).

Food	Concentration range		
	Seco	Mat	Total bacterial lignan
Vegetables			
Boston lettuce			16500
Cabbage	330	tr	8770
Bok choy			12510
Mushroom			5830
Radish	333	30	7210
Celery	1114	35	6380
Cucumber	251	30	5850
Tomato	516	65	3310
Fiddle head fern			2000

[a] Taken from Mazur, 1998; Mazur *et al.*, 1998a; Mazur *et al.*, 1998b; Johnsson *et al.*, 2000; Liggins *et al.* 2000c.

[b] Taken from Thompson *et al.*, 1991; Nilsson *et al.*, 1997; Nesbitt and Thompson, 1997.

[c] Liquid teas and coffees would be 98% water.

(*Vaccinium vitusidaea*), cranberries (*Vaccinium oxycoccos*) and blueberries (*Vaccinium corymbosum*) contain significant concentrations of the lignan precursors ranging from 37 μg g^{-1} for blackberries to 8.4 μg g^{-1} in blueberries (dry weight basis) (Mazur *et al.*, 2000). The content of lignan precursors vary among flax cultivars, growing location and crop year (Thompson *et al.*, 1997) similar to that observed for soybean isoflavones (Wang and Murphy, 1994b). Thompson *et al.* (1997) reported three fold differences in flaxseeds among varieties and crop years with smaller differences observed in growing location effects. Fruits, vegetables and legume seeds contained 50 to 12 540 ng g^{-1} of lignan precursors (Mazur, 1998). Flax seed breakfast cereals and breads appear to be excellent sources of lignans depending on the rate of addition (Nesbitt and Thompson, 1997).

C. COUMESTANS

Coumestrol in human foods is found only in alfalfa, clover and soybean seed sprouts. Coumestrol concentrations have been reported from 0.2 to 184 μg g^{-1} in alfalfa and clover seeds but these seeds are not usually consumed by humans (Lookhart, 1980). Germination apparently causes the synthesis of this phytoestrogen in these seeds as the concentration

increases up to 200-fold with germination time (Buseman, 1996; Lookhart *et al.*, 1979). Knuckles *et al.* (1976) reported coumestrol content of 71 μg/g in soybean sprouts. The USDA-Iowa State University Isoflavone database lists 41 reports of coumestrol in plant foods ranging from not detected to 4660 μg g^{-1} with a median of 0.0 μg g^{-1} (Beecher *et al.*, 1999).

VII. EFFECTS OF PROCESSING

A. SOY ISOFLAVONES

Although there is now a good database on isoflavone content of foods, there are far fewer reports on the effects of processing on isoflavone content and isomer distribution. The first systematic study was conducted by Wang and Murphy (1996) evaluating the mass balance for isoflavones in preparing soymilk and tofu on a pilot plant scale and tempeh and soy protein isolate on a laboratory scale. Production of soymilk involves soaking of soybean seeds from 8–18 h. The soaked beans are homogenized and filtered to remove the fibrous portion, the okara, to produce soymilk. Modest heating to 95°C for 7 min to denature trypsin inhibitors resulted in no loss in total isoflavone content (Figure 5). There were no differences in the concentrations of the β-glucoside forms, but there were decreases in malonylglucosides and small increases in aglycons. The increase in

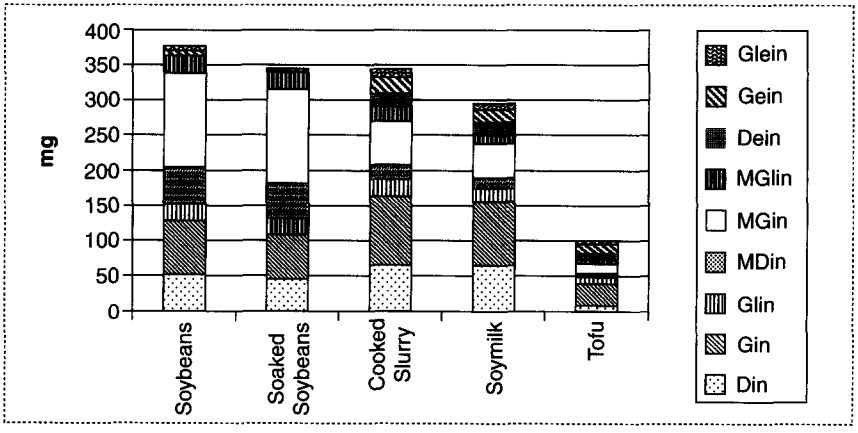

FIG. 5. Mass balance distribution of isoflavones in soy milk and tofu processing for 600 g of soybeans. (Din = daidzin; Gin = genistin; Glin = glycitin; MDin = malonyldaidzin; MGin = malonylgenistin; MGlin = malonylglycitin; Dein = daidzein; Gein = genistein; Glein = glycitein).

aglycons was attributed to the activity of the native β-glucosidases in soybeans prior to cooking of soymilk (Matsuura and Obata, 1993). Coagulation of the soymilk with calcium sulfate at 85°C to produce tofu resulted in 44% loss of total isoflavones to the tofu whey. The resulting distribution of isoflavone forms in tofu was relatively unaltered compared to its soymilk. These tofus retained 33% of soybean isoflavones compared to original soybeans.

Tempeh production requires hydration of dry soybean seeds, boiling and washing to remove the seed coats from the cotyledons, cooking the cotyledons in boiling water prior to inoculation with the fermentation organisms for an 18 h fermentation to tempeh. Each step in processing soybeans to tempeh resulted in the loss of isoflavone mass. Losses of 50% occurred during boiling of the dehulled cotyledons. Conversion of the forms of isoflavones was mainly due to conversion of the malonyl forms to the β-glucosides due to the heat processes. Fermentation increased content of aglycons by factor of 5 compared to raw soybeans with the aglycon mass accounting for 50% of isoflavone total compared to raw soybeans which have 5% aglycons as the isoflavone mass. Processing soybeans to tempeh resulted in 76% loss of initial isoflavone content (Wang and Murphy, 1996). Mixed fungi and bacteria species can be used to produce tempeh (Klus *et al.*, 1993). Gyorgy *et al.* (1964) reported production of 4′,6,7-trihydroxyisoflavone in the tempeh they produced. Klus *et al.* (1993) showed that *Brevibacterium epidermidsis*, *Micrococcus luteus* and *Microbacterium aborescens* can convert glycitein and daidzein to 4′,6,7-trihydroxyisoflavone. *Rhizopus* and *Aspergillus*, the more widely used fermentation species, do not possess this ability. Therefore, production of isoflavone metabolites in tempeh is dependent on fermentation organisms selected.

Soy protein isolate production resulted in a 52% loss of isoflavone mass to the alkaline insoluble fraction and acid soluble fraction (Wang and Murphy, 1996). Oil extraction from raw soybean flour did not result in any loss of isoflavones to the oil fraction. Aglucons increased by factor of 5 compared to raw soybeans and accounted for 50% of isoflavone mass in final soy protein isolate. Acetylgenistin appeared to be formed during processing but not to the extent reported in commercial soy protein isolates (Wang and Murphy, 1994a). This is probably due to differences in drying of the final product where industrial products are spray dried and laboratory product was freeze-dried. Wang *et al.* (1998a) has replicated these findings using laboratory-scale production.

Buseman (1996) reported the mass balance of isoflavones during miso fermentation. No net change in isoflavone mass was observed over a 57-day fermentation, the industry standard. The autoclaving cooking step

caused major shift of malonylglucosides to β-glucosides. During the first 10 days of fermentation, hydrolysis of 50% of β-glucosides to aglycons occurred. Thereafter, the β-glucosides and aglycons remained at a steady state. The malonylglucosides and acetylglucosides were continuously hydrolyzed throughout the fermentation.

Buseman (1996) reported on the effects of soy sprout production on isoflavone distribution. The β-glucosides and malonylglucosides remained relatively constant throughout a 168 h germination while the aglucons increase from 10 to 50 $\mu g\ g^{-1}$ (dw). Coumestrol was produced in the soy sprouts as cited previously.

Coward *et al.* (1998) reported effects of baking bread dough and cookies containing soy flour on % distribution of isoflavone forms and baking and frying of texturized vegetable protein (TVP). They did not report individual isoflavone form concentrations but, for example, reported total malonyl-β-glucosides as sum of malonyl-genistin, -daidzin and -glycitin. Baking resulted in 20% loss in malonylglucosides with 15% increase in β-glucoside totals. No differences were observed for acetylglucosides nor aglycons in bread dough. Cookie baking resulted in 100% loss of malonylglucosides with 45% increase in β-glucosides, 20% increase in acetylglucosides and 10% increase in aglycons. Baking TVP resulted in 10% loss in malonylglucosides and 10% increase in β-glucosides. Frying TVP caused 15% loss in malonylglucosides with 7% increase in acetylglucosides and 10% increase in aglycons. These authors apparently reported total mass of each form rather than mole adjusted amounts.

Kinoshita *et al.* (1997) reported presence of tartaric acid 7-O-ethers of genistein, daidzein and 8-hydroxygenistein in Japanese soy sauce during their development of a multivariant pattern recognition profile used to determine ingredient origin of product, i.e. to differentiate between all soybean versus soybean and wheat soy sauces. This profile has allowed them to differentiate soy sauces made from whole soybeans versus defatted soybeans (Kinoshita *et al.*, 1998).

Davies *et al.* (1998) reported the ability of genistein to react with lysine in the Maillard nonenzymatic browning reaction model system. They hypothesized this reaction explained the loss of the biological activity in a colon cancer feeding study using soy protein isolate that was stored for more than 2 years at room temperature. They did not present data on the genistein level in their soy protein isolates, however. Murphy *et al.* (1999) did not observe any loss of any isoflavone form in soy flour or dry soy milk stored at room temperature and at –29°C for 2 years.

Extrusion to produce textured vegetable proteins (TVP) will cause changes in isoflavone isomer distribution but apparently not in total isoflavone mass. In Mahungu *et al.* (1999), soy protein isolate and a 20/80

soy protein isolate/corn meal blend were extruded at three temperatures and three different initial moisture contents. Malonylglucosides were reduced due to the heat processing effect and acetylglucosides were produced. These authors also reported lower isoflavone levels post extrusion. However, these authors extracted their samples with 80% methanol which has been reported to be less efficient in extracting the acetylglucosides compared to 80% acetonitrile (Farmakalidis and Murphy, 1985a). Mahungu *et al.* (1999) reported extensive hydrolysis of glucosides to aglucons with addition of water to the pre-extrusion mixture. According to these authors, the extrusion process was not the cause of hydrolysis of the isoflavone glucosides. Rather the native glucosidases in the soybeans were involved. Their hypothesis is supported by Matsuura and Obata (1993) who demonstrated native soybean glucosidases can hydrolyze isoflavone glucosides. Singletary *et al.* (2000) evaluated extrusion of a water-washed and an ethanol-washed soy protein concentrate. These authors reported a 24% decrease in extractable isoflavones after extrusion. These authors also used 80% methanol to extract their samples which would tend to underestimate the acetylglucoside contribution to the mass balance calculation.

Franke *et al.* (1999) evaluated the effect of heat processing on isoflavones in Hawaiian and Singapore soy foods. Their heat processing involved boiling of the food item in water for 3–60 min depending on the type of food. Cooking resulted in no losses of total isoflavones for tofu and tau kwa (pressed tofu). Tau pok (fried, pressed tofu), foo jook (dried tofu sticks) and intact soybeans were reported to have significant losses of isoflavones during boiling, probably to the boiling water. All these products, except intact soybeans, showed little change in isoflavone distribution as result of boiling. Soybeans, which were boiled for 60 min, showed a 6–8% conversion of malonylglucosides to β-glucosides.

Recently different styles of traditional soy foods have been developed containing no or low fat contents for the US and European marketplace. We have analyzed commercial low and fat-free soy milks and low fat tofus and compared them with the traditional commercial product. The products are shown in Figure 6 and are compared on a dry weight basis so direct comparisons of isoflavone contents can be made. Low and no fat soymilk can be produced in several ways. One production approach involves using traditionally prepared soymilk and skimming of fat in the same manner dairy milk is skimmed. Alternatively, traditional soymilk can have soy protein isolate and/or soy protein concentrate added in order to dilute the total fat percentage when additional water is added during production to adjust the total protein content. Neither of these added ingredients contain fat but add protein. These ingredients may or may not have isoflavones.

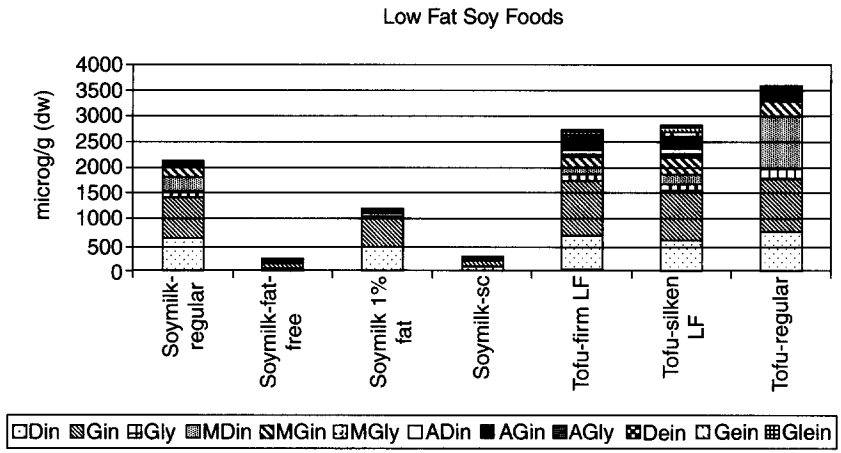

FIG. 6. Isoflavone content of low fat tofu and no and low fat soymilks compared to traditional tofu and soymilk. (Din = daidzin; Gin = genistin; Gly = glycitin; MDin = malonyldaidzin; MGin=malonylgenistin; MGly = malonylglycitin; ADin = acetyldaidzin; AGin = acetylgenistin; AGly = acetylglycitin; Dein = daidzein; Gein = genistein; Glein = glycitein).

The soymilk in Figure 6 labeled as fat-free by the manufacturer listed "soy protein" on the ingredient label but apparently contained no soybeans. Therefore we expect this product must be made using a very low isoflavone containing soy protein isolate or an ethanol-washed soy protein concentrate that would have low isoflavone levels. The 1% fat soymilk lists soybeans as its only source of soy on its ingredient list. The 1% fat soymilk appears to be produced by diluting the soymilk with water to lower the fat content or by skimming the milk to remove the lipid while also removing some of the soy protein. The soymilk identified as "Soymilk-sc" listed soybeans and soy protein concentrate on its ingredient label. This low fat soymilk apparently was produced by adding isoflavone free soy protein concentrate and water to traditional soymilk to dilute the fat content while maintaining the protein content expected for soymilk.

The two low fat (LF) retail tofus shown in Figure 6 were products produced by coagulation of soymilk in the package and are compared to a regular fat content tofu coagulated in the package. Both low fat tofu ingredient labels listed soybeans and soy protein isolate. These tofus were apparently produced by adding additional soy protein, as soy protein isolate, to the soymilk prior to coagulation into tofu to dilute the total fat content while retaining the proper protein content of the final tofu product. The soy protein isolate used in these two tofus apparently contained

modest levels of isoflavones but not as high as the soybeans used to produce the regular fat level tofus.

Soy protein, with its associated isoflavones, can be added to a variety of food products. Soy flour can be added at up to 3% of the formulation recipe to baked goods to improve gluten formation and/or decrease fat absorption (Riaz, 1999a). Soy protein can be used as a protein source in producing coffee whiteners and dairy cream substitutes. Hydrolyzed soy protein can be added in place of monosodium glutamate as a flavor enhancer to a variety of foods (Riaz, 1999b). Figure 7 presents the total isoflavone content in baked products and tuna as well as several non-soy legumes. The data are presented on a log scale to allow comparison of the isoflavone content of the products with added soy to traditional soy foods. Although isoflavones can be quantified in these products, the total isoflavone level is quite low. For example, one serving of doughnuts (60 g) would provide about 5 mg total isoflavones compared to a 100 g serving of tofu with 25–30 mg total isoflavones. In comparison, foods such as navy beans, white beans, chickpeas and peanuts actually contain less isoflavones than doughnuts with added soy flour.

B. FLAX LIGNANS

There are very few reports on the effects of processing on cereal lignans. Nilsson *et al.* (1997) evaluated the lignans in roller milling of rye;

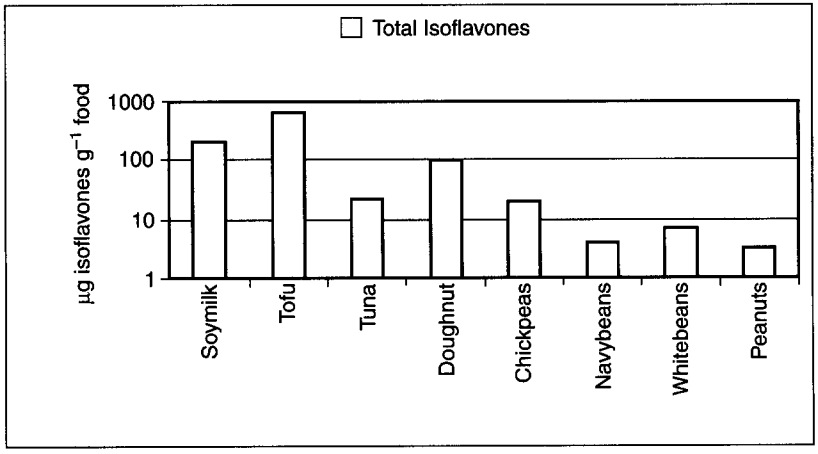

FIG. 7. Total isoflavone content of food naturally containing isoflavones and foods with added isoflavones.

73–87% of the lignans were segregated to the short and bran fractions after milling, closely paralleling the dietary fiber fraction. Very low levels of lignans were detected in any of the flour fractions. The short fraction contained 1200 ng g^{-1} of secoisolariciresinol and 1200 ng g^{-1} matairesinol. The bran fraction contained 1600 ng g^{-1} secoisolariciresinol and 1350 ng g^{-1} matairesinol. Nesbitt and Thompson (1997) reported flax seed could be incorporated into homemade breads from 6 to 13% flax seed to produce quick breads containing 29 to 85 μg/g total lignans.

VIII. BIOAVAILABILITY AND METABOLISM

A. ISOFLAVONES

Isoflavone bioavailability and metabolism have been reviewed recently (Hendrich and Murphy, 2000). Three issues dominate research in this field: relative availability of isoflavone glucosides versus aglucons, role of isoflavone metabolites in exerting biological effects of these compounds, and the role of gut microflora in isoflavone bioavailability.

Isoflavone glucosides, the predominant forms of isoflavones in foods, are not absorbed directly to an appreciable extent, as these compounds have not been found in human plasma or urine to date, using chromatographic systems that were capable of detecting these forms (e.g., Xu *et al.*, 1994; Zhang *et al.*, 1999b). Isoflavone aglucons are neither hydrophobic nor hydrophilic, and given their molecular weights of approximately 250 and their phenolic nature, they are good candidates for rapid absorption, glucuronide and/or sulfate conjugation, and biliary excretion. A recent comparison of human pharmacokinetics of single doses of 50 mg of pure genistein, genistin, daidzein and daidzin showed that the AUCs (areas under curve) in premenopausal women were 4.54 μg $(mL*h)^{-1}$ for genistein v. 4.95 μg $(mL*h)^{-1}$ for genistin, and 2.94 μg $(mL*h)^{-1}$ for daidzein v. 4.52 μg $(mL*h)^{-1}$ for daidzin (Setchell *et al.*, 2001). This suggests greater absorption of the glucosides than of the aglucons, but further studies are needed to confirm this finding and its biological significance.

A significant extent of isoflavone metabolism to conjugates seems to occur immediately upon absorption. When genistein was administered to rats, about 90% of the gavage dose was absorbed and 70% of the administered isoflavone appeared rapidly as glucuronide conjugate in portal blood, due to intestinal mucosal UDP-glucuronosyltransferase (UGT) activity. Humans have significant gut mucosal UGT activity, so it is likely that similar metabolic fates of isoflavones occur in humans as in rats. In rats, most of the glucuronide was immediately excreted in bile, suggesting

the possibility of later enterohepatic recirculation, after gut microfloral β-glucuronidase activity (Sfakianos *et al.*, 1997). But enterohepatic recirculation of isoflavones has not been conclusively demonstrated in humans (only one peak of isoflavone absorption was obvious after a single isoflavone dose was administered (e.g., Xu *et al.*, 1994, 1995)). It is likely that a significant portion (> 70%) of circulating isoflavones in humans are glucuronide conjugates, with minor portions of sulfate and sulfate-glucuronide conjugates. Our laboratory studied effects of daidzein and genistein glucuronides on human natural killer (NK) cell activity *in vitro* (Zhang *et al.*, 1999a). Both glucuronides significantly and moderately stimulated NK activity (by 30%), so these isoflavone metabolites are not inert, but their biological effects *in vivo* remain to be seen.

Gut microbial degradation of isoflavones has been demonstrated (Xu *et al.*, 1995; Hendrich *et al.*, 1998; Zheng 2000). Thus, the current picture of isoflavone metabolism is that, due to rapid biliary excretion of a significant portion of absorbed isoflavones and their subsequent disappearance in the lower gastrointestinal tract, only 5–50% of ingested isoflavones are bioavailable, as reflected in the ultimate proportion of ingested dose that is excreted in urine (Xu *et al.*, 1994; Xu *et al.*, 1999). Urinary excretion of isoflavones is relatively rapid, within 24 h after a single dose (Xu *et al.*, 1994) and 48 h after 3 doses per day (Xu *et al.*, 1995); both experiments were done in women. The pattern of excretion and plasma concentrations in men seems similar to that observed in women (Watanabe *et al.*, 1999). The persistence of biological effects after isoflavone dosing remains to be determined.

Because a significant portion of ingested isoflavones cannot be accounted for by the total of fecal and urinary excretion, further examination of tissue contents of isoflavones has been undertaken using GC/MS methods (Chang *et al.*, 2000). These methods revealed that only a small percentage of ingested dose (< 1% in males, ~5% in females) was recovered in tissues of rats killed within 3 h after feed removal after feeding of genistein-fortified diets (up to 500 mg genistein kg^{-1} feed) for 20 weeks after birth. These results suggest that the missing isoflavones are probably best accounted for by gut microfloral degradation. Flavonoid-degrading organisms have been identified (some *Clostridia* species (Winter *et al.*, 1989)). Because of structural similarities between flavonoids and isoflavones, it is reasonable to presume that isoflavones would also be susceptible to gut microbial degradation, but the major isoflavone-degrading organisms in human gut remain to be determined.

Significant and persistent differences in isoflavone degradation capability of human gut microorganisms, as studied in anaerobic fecal incubations by our laboratory (Zheng, 2000), affect isoflavone bioavailability.

Among 35 Asian women, those who had lesser fecal isoflavone degradation and relatively short gut transit time (40 h) showed threefold greater urinary excretion of genistein (reflecting isoflavone absorption) than did ones who had greater fecal isoflavone degradation and long gut transit time (65 h), after a single soy isoflavone-containing meal was fed. Among 33 Caucasian women, although low and high isoflavone degraders were identified, all Caucasians had relatively long gut transit times (> 80 h), and no difference in isoflavone excretion was noted according to fecal isoflavone degradation phenotype. Thus, gut transit time seems to be a main determinant of isoflavone bioavailability, with better absorption associated with more rapid gut transit! Neither the identity of the isoflavone-degrading organisms nor the explanation for differences in gut transit time among subjects is known. This observation has important health implications, in that lesser isoflavone absorption ought to lessen the health benefits of isoflavones. Better control of human feeding studies of health effects of isoflavones may be achieved by taking fecal isoflavone degradation of subjects into account (Zhang *et al.*, 1999b). When subjects were selected for moderate fecal isoflavone degradation, interindividual variability (5- to 8-fold) in urinary isoflavone excretion (Zhang *et al.*, 1999b) was less than interindividual variability (12- to 14-fold) in excretion seen in a similar study that did not account for isoflavone degradation phenotype (Karr *et al.*, 1997). The health significance of gut microbial metabolism of isoflavones remains to be established. Further characterization of the breakdown products of isoflavones and related compounds (e.g. flavonoids in general) might also be important, because at least one such product, methyl-p-hydroxyphenyllactate, had antiproliferative effects in a tumor cell line (Markaverich *et al.*, 1988).

Isoflavones are not only degraded, but metabolized to other active forms by gut microorganisms. Daidzein is metabolized to another phytoestrogen, equol (Axelson *et al.*, 1982), by human gut microorganisms. Equol is produced by about one-third of human subjects fed isoflavones (Setchell *et al.*, 1984; Lampe *et al.*, 1998). Equol production is seemingly inducible over a period of a few days. Equol was not found in single dosing or single day isoflavone feeding studies (Xu *et al.*, 1994, 1995). But equol was found in human urine after a 4-day soy feeding study (Setchell *et al.*, 1984). The determinants of equol production including the identity of equol-producing microorganisms, as well as equol's health significance remain to be found.

At this point, there seem to be compelling reasons to study in more depth the role of gut microbial metabolism of isoflavones and other phytoestrogens.

B. COUMESTANS

Because these components are seemingly found only in alfalfa, clover and soybean sprouts (see above), there is little practical reason to determine human bioavailability of coumestans. Because coumestrol is a relatively potent estrogen found in some forages and implicated in some cases of ruminant reproductive disruption, Lundh *et al.* (1988) developed an HPLC method to separate formononetin, daidzein, equol and coumestrol in bovine urine and plasma. No coumestrol was detected in bovine blood plasma taken from cows fed a normal silage containing daidzein and formononetin. Franke and Custer (1994b) developed an HPLC method that permitted separation of coumestrol from daidzein, genistein and key isoflavone metabolites. They confirmed that over 24 h after feeding various doses of roasted soybeans, urinary contents of isoflavones and their metabolites, equol and O-desmethylangolensin (ODMA), but not coumestrol, were highly correlated with soybean intake ($r = 0.76$ for equol, $r = 0.98$ for ODMA).

C. LIGNANS

The plant lignans, matairesinol and secoisolariciresinol diglycoside (SDG) are converted into mammalian lignans, enterodiol (ED) and enterolactone (EL), by gut microorganisms. In a randomized cross-over design, women fed 25 g per day of raw flaxseed or flaxseed baked into bread or muffins showed similar urinary excretion of EL, ED and secoisolariciresinol (Seco), the aglycone of SDG (Nesbitt *et al.*, 1999). The three treatments produced similar lignan metabolite patterns with a 4:1:0.2 ratio of ED:EL:Seco. Plasma concentrations of EL + ED after 8 days of consuming 25 g raw flaxseed were about 0.1 μM. These concentrations remained relatively constant when measured at several time points over the 24 h period. In rats, feeding flaxseed (thought to contain 3 μmol SDG g^{-1}) produced fivefold greater urinary contents of ED and EL than did feeding the equivalent amount of purified SDG. This suggests that the flaxseed contained mammalian lignan precursors other than SDG, or that flaxseed was metabolized more efficiently than a bolus dose of SDG. But the flaxseed SDG content was not directly analyzed in this study (Jenab *et al.*, 1999), so the significance of this finding remains uncertain. After 4 subjects were fed 16 g flaxseed for 5 days, ED and 3 monohydroxylated metabolites and EL and 6 monohydroxylated metabolites were identified in urine (Jacobs *et al.*, 1999). These metabolites have not been quantified in humans nor has their biological significance been determined yet.

These metabolites are products of hepatic microsomal metabolism that catalyzes aliphatic and aromatic hydroxylations. The induction of hepatic cytochromes P450 by Arochlor pretreatment of rats increases the aromatic oxidation of the lignans. Human hepatic microsomes produced mostly aliphatic hydroxylated lignan metabolites (Jacobs and Metzler 1999). In rats dosed with ED, EL or flaxseed, the parent lignans were the predominant urinary metabolites. Lesser amounts of 3 aromatic and 2 aliphatic hydroxylated metabolites of ED and 6 aromatic and 5 aliphatic hydroxylated metabolites of EL were formed (Niemeyer *et al.*, 2000). These metabolites were also found in bile. The sum of the hydroxylated metabolites were similar to the amounts of ED or EL recovered from biological fluids, suggesting that these hydroxylated forms contribute to the biological activity of the lignans.

IX. PHYSIOLOGICAL EFFECTS

A. EFFECTS OF PHYTOESTROGENS ON ESTROGEN RECEPTORS, ERα AND ERβ

Estrogens are known to exert their effects on the estrogen receptor (ER) by functioning as a ligand-activated transcriptional regulators (Tsai and O'Malley, 1994). The effects of estrogens have been attributed to a single ER, ERα, until recently. A second ER, called ERβ (Kuiper *et al.*, 1996; Ogawa *et al.*, 1998), has increased the complexity in understanding the effects of estrogens. The two ERs, ERα and ERβ, have overlapping but distinct tissue distributions and different ligand binding activities (Kuiper *et al.*, 1997). ER knockout studies suggest the two receptors have different biological roles (Korach, 1994; Krege *et al.*, 1998). Kuiper *et al.* (1997) has reported that genistein binds to ERβ with 30 times greater affinity than it binds to ERα. Coumestrol and daidzein bind with three- to sevenfold greater affinity to ERβ than to ERα (Kuiper *et al.*, 1998). Relative affinities of phytoestrogens for either ER showed coumestrol > genistein > daidzein, each differing by about an order of magnitude, but this depended on the assay used. With a solid phase competition assay using mammalian nuclear extracts, coumestrol and genistein had similar affinity for ERβ. With a solubilized receptor binding assay using insect cell extracts, genistein and daidzein had similar binding, and both had two orders of magnitude less affinity for ERα than did coumestrol (Kuiper *et al.*, 1998). In a human embryonic kidney cell line genetically engineered to contain ER response elements, transactivation of the ER response elements was twofold greater

for genistein than for both coumestrol and daidzein, and responses were similar for ERs α and β (Kuiper *et al.*, 1998). Pike *et al.* (1999) suggests that when raloxifene, an estrogen antagonist, is in the ERβ binding site, a portion of the ligand protrudes from the binding site and prevents assembly of the final transcriptional element thus acting as an antagonist. In contrast, genistein is completely contained within the receptor binding site, similar to the natural ligand, 17-β-estradiol, but causes a conformational change in the ER similar to ER antagonists. Genistein was called a "partial agonist" by Pike *et al.* (1999). Makela *et al.* (1999) has shown that ERα and ERβ are expressed at 40-fold difference in carotid arteries after endothelial denudation in rats. Genistein was shown to bind with 20 fold higher affinity to ERβ than ERα in the carotid endothelial cells whereas 17-β-estradiol showed no difference in affinity. At < 10 μM both 17-β-estradiol and genistein had equal vascular protective effects through inhibitory activity for replication and migration of smooth muscle cells. At these same doses 17-β-estradiol caused a dose-dependent response in ovariectomized rat uteri while genistein was ineffective.

B. ESTROGEN-RELATED EFFECTS *IN VITRO*

In the RUCA-1 (rat endometrial adenocarcinoma) cell line, competitive binding of phytoestrogens to ER showed the relative pattern of coumestrol > genistein > daidzein. In doses about 100-fold greater than 17β-estradiol, all three phytoestrogens induced expression of complement C3 in the RUCA-1 cells (Hopert *et al.*, 1998).

In the MCF-7 mammary tumor cell line, increasing concentrations of coumestrol or genistein increased DNA synthesis in the presence of estradiol at phytoestrogen concentrations of 0.1–10 μM; such concentrations represent the maximal likely concentrations of genistein achievable via dietary exposures. Both phytoestrogens, in the same concentration range, increased MCF-7 cell DNA synthesis in the presence of the antiestrogen tamoxifen (Wang and Kurzer, 1998).

These studies suggest that the estrogen-like effects of the phytoestrogens cause potentially deleterious effects in stimulating estrogen-dependent neoplastic cell growth. This remains to be determined *in vivo*. However, sex steroid binding proteins (SSBPs) from human plasma (stripped of endogenous sex steroids) showed no displacement of estradiol from the SSBPs by daidzein or coumestrol, and only 0.01% displacement of estradiol from the SSBPs by genistein (Milligan *et al.*, 1998). Thus, displacement of estradiol from SSBPs is unlikely to be involved in any estrogen-like effects of the phytoestrogens.

C. ESTROGEN-RELATED EFFECTS *IN VIVO*

A Tier I screening battery for estrogen active compounds (EAC) was applied to coumestrol. Ovariectomized 8 week old female Sprague Dawley rats were given coumestrol by intraperitoneal injection for 4 days. Coumestrol at 0.5–2.5 mg kg^{-1} per day significantly increased uterine weight, and 0.1–2.5 mg coumestrol kg^{-1} increased uterine cell proliferation. Coumestrol at 1.0–2.5 mg $(kg/d)^{-1}$ significantly increased follicle stimulating hormone. Only 2.5 mg coumestrol $(kg/d)^{-1}$ given for 15 days affected 10 week old male Sprague Dawley rats, decreasing dihydrotestosterone and increasing prolactin significantly. Thyroid hormone (T_4) was decreased significantly in the male rats when 1.0–2.5 mg coumestrol $(kg/d)^{-1}$ was given (O'Connor *et al.*, 2000). These data suggest that whereas coumestrol is estrogenic, the doses required for such effects could not be obtained from the human food supply. For example, 0.5 mg coumestrol $(kg/d)^{-1}$ would equal about 30 mg coumestrol for a 60 kg woman. Factoring in the surface area difference between rats and humans of sevenfold, the human equivalent dose to 0.5 mg kg^{-1} in rats would be 0.07 mg kg^{-1}, or 4 mg per 60 kg person.

Male rats exposed to 100 μg of coumestrol (approximately 10 mg kg^{-1} body weight/d) for 5 days after birth showed no adverse effects on testes weight, sperm counts, or reproductive hormones. Follicle stimulating hormone β was significantly greater in coumestrol treated rats than in controls, when rats were examined at 60 days of age (Awoniyi *et al.*, 1997). Even at this relatively extreme dose, coumestrol exerted little effect on male reproductive potential. In three-week-old female Sprague Dawley rats fed coumestrol (0.01–0.1% by weight of diet) for 90 h, uterine weight doubled. A dose of 0.005% coumestrol was as effective in increasing uterine weight over 8 days as 0.01% coumestrol was in 4 days (Whitten *et al.*, 1992). Induction of uterine cytosolic progestin receptors paralleled the increases in uterine weights, which is a key effect of estrogens. A chow diet increased uterine weight by 47% after 180 h of feeding (rats were of age comparable to the coumestrol study) compared with a standard semi-purified diet, the American Institute of Nutrition (AIN) diet (Whitten *et al.*, 1992). This may have been due to soy isoflavones in the chow, but the chow phytoestrogen content was not analyzed. Feeding 0.005% coumestrol would be the human equivalent of 25 mg coumestrol per person per day (500 g typical daily human food intake × 0.00005 = 25 mg). Female Sprague Dawley rats fed 0.01% coumestrol for 38 days after weaning showed earlier vaginal opening and onset of estrus than did controls, but by about 6 months of age the coumestrol treated rats had less regular estrus cycles than controls (Whitten and Naftolin 1992).

Coumestrol is clearly estrogenic, and a potential disruptor of normal estrogen function in standard female animal model systems. In males, coumestrol in supranutritional doses exerts no ill effects on reproduction, based on limited data. Because the median content of coumestrol in foods is nil (0.0 $\mu g\ g^{-1}$, see Section VIII above), it would be nearly impossible for a person to consume enough coumestrol to experience estrogenic effects.

Because lignans are more widely dispersed in foods than coumestrol, they may have greater potential to alter estrogen activities *in vivo*. Eight-week-old female Sprague Dawley rats were fed 2.5–10% flaxseed or 0.75–3 mg secoisolariciresinol (SDG, a major flaxseed lignan) for 4 weeks. Increased estrus cycle length, and increased acyclicity or cycle irregularity were significantly and strongly correlated ($r = 0.77$ and 0.90, respectively) with SDG intake (either purified or from flaxseed) (Orcheson *et al.*, 1998). This indicates an antiestrogenic effect of lignans in doses potentially achievable by humans (e.g., 5% flaxseed = 500 g human food intake × 0.05 = 25 g flaxseed or about 2 tablespoons per day), but the result remains to be extended to humans. Lignans inhibit binding of testosterone to human sex hormone binding globulin (Schottner *et al.*, 1997; Schottner and Spiteller, 1998), but the IC_{50} for SDG (one of the more potent inhibitors) was 230 μM, a concentration far greater than likely to occur in humans (e.g. enterolactone concentration of 30 nM in plasma of humans on habitual diets (Rowland *et al.*, 2000)). The limited data so far suggest anti-estrogenic potential for lignans in ovulating women, which in turn suggests estrogen-like effects after menopause. These effects remain to be proven.

D. ANTIOXIDANT EFFECTS

Antioxidant effects of isoflavones have been described previously (Kurzer and Xu, 1997). Genistein, daidzein, equol and coumestrol were compared by ESR spectroscopy for ability to reduce galvinoxyl radicals; coumestrol and equol reduced fivefold more radicals than did daidzein or genistein, but reduction was very modest, only 0.1 radicals quenched per molecule of coumestrol. When incorporated into human plasma *in vitro*, genistein and daidzein showed only half the ferric reducing ability of equol or coumestrol, which reduced one ion per molecule (Mitchell *et al.*, 1998). In the Trolox equivalent antioxidant assay, all four phytoestrogens showed similar efficacy. Daidzein and genistein required greater concentrations than coumestrol and equol to inhibit ascorbate/ADP/Fe^{2+}-induced lipid peroxidation of rat liver microsomes depleted of vitamin E (600–1100 μM concentrations were needed, nearly 1000 times greater amounts than typically found in people consuming isoflavones (see Section XI). In all of these *in vitro* antioxidant assays (Mitchell *et al.*, 1998), the isoflavonoids

were much less effective than Trolox, vitamin C or the flavonoid quercetin. From these predictive assays, antioxidant activities do not seem likely to contribute significantly to the biological effects of the isoflavones.

Antioxidant effects of the lignans, SDG, enterodiol (ED) and enterolactone (EL), have also been evaluated *in vitro* (Kitts *et al.*, 1999). Oxidation of a linoleic acid emulsion was significantly inhibited by all three compounds (10 and 100 μM), but less so by ED than by the other compounds. All three compounds also had significiant hydroxyl radical scavenging activity in a deoxy-D-ribose/Fe/EDTA/ascorbate reaction mix. But SDG was 5–10 fold less effective than either ED or EL. Enterodiol and enterolactone, the mammalian metabolites of SDG, were effective antioxidants in lipid and aqueous systems, suggesting that these compounds might be physiologically important antioxidants. But plasma concentrations of ED and EL were only 0.1 mM after feeding of 25 g flaxseed to women for 8 days (Nesbitt *et al.*, 1999), two orders of magnitude less than the effective antioxidant concentrations observed by Kitts *et al.* (1999). These lignans could theoretically reach concentrations of > 100 μM in the colon, due to dietary exposure and gut microbial metabolism. Therefore, antioxidant effects with respect to colonic mucosa should be considered in evaluating mechanisms of colon cancer protection by lignans.

E. POTENTIAL TOXICITY OF PHYTOESTROGENS

Toxicity must be considered particularly when assessing dietary components that may have effects in pharmacological doses. Nutritional doses are of less concern in that at least some populations have been exposed to such doses for thousands of years without apparent harm. Such observations provide weak evidence of lack of toxicity. There is little toxicity data on phytoestrogens. For example, Zhang *et al.* (1999a) showed that doses of 10 μM genistein but not similar doses of genistein glucuronide inhibited human natural killer cell activity *in vitro* after a 4.5 h incubation. Cultured human peripheral blood lymphocytes exposed to 50 μM coumestrol or 25 μM genistein showed chromosomal abnormalities by various cytogenetic analyses (Kulling *et al.*, 1999). Daidzein at 100 μM showed no such cytogenetic toxicity. In a separate study, enterolactone, enterodiol and secoisolariciresinol were evaluated for genotoxicity in Chinese hamster V79 fibroblasts, using assays including mitotic arrest, micronucleus formation, microtubule assembly and mutation. At 100 μM in cell culture, and 200 μM in cell-free systems, none of the lignans were genotoxic (Kulling *et al.*, 1998). These concentrations were much greater than would be seen in blood plasma, but perhaps higher concentrations should also be assayed, given calculated potential concentrations in the

colonic lumen. There is little reason to suspect that the phytoestrogens as dietary components pose risks for toxicity. But as these components begin to be used as dietary supplements and are incorporated into new foods, toxicity assessment should continue.

X. HEALTH EFFECTS

The potential health-protective effects of soy phytoestrogens is an active area of research. However, one of the difficulties in interpreting many of the clinical trials is the lack of identity of the type of soy protein fed and/or analysis for isoflavone or other phytoestrogens associated with the soy or other plant protein. Unfortunately in many citations, clinical researchers report feeding "soy protein" without recognizing the wide variation and number of commercial soy protein products available. The difference between soy flours, soy protein concentrates, soy protein isolates and texturized vegetable protein and the different phytoestrogen levels among and within these commercial products has made interpreting the literature to associate certain components with physiological effects difficult (Table I). Similarly, the ingredient processors, until recently, did not appreciate the commercial value of estimating the phytochemical concentrations of their plant protein products. With the approval of the health claim for soy protein and cardiovascular health, there has been greater attention to these details.

A. BONE

Osteoporosis is a worldwide problem related to aging characterized by loss of bone mass and deterioration of bone microarchitecture which may lead to fracture. Twice as many women as men develop osteopenia and the fracture rate distribution is similar. Estrogen plays a critical role in bone health as observed in the large bone loss that occurs in women immediately prior to and after menopause as estrogen levels drop (Riggs *et al.*, 1998). Additionally, the ability of conjugated equine estrogens, alone or in combination with progestins, to prevent bone loss supports the role of estrogenic chemicals in maintaining bone mass and density (Komulainen *et al.*, 1999). However, 67% of women discontinue estrogen replacement therapy within 5 years (Coope and Marsh, 1992; Groeneveld *et al.*, 1998). Research suggests the treatment with estrogens for less than 10 years, if started at menopause, has little effect on bone health at 70 (Cauley *et al.* 1995). Dietary soy might provide an alternative to protect bone health based on several observations in the literature. Isoflavones are estrogenic

(Farmakalidis *et al.*, 1984; Farmakalidis *et al.*, 1985b; Song *et al.*, 1999). Ipriflavone, a synthetic isoflavone, has demonstrated ability to reduce bone loss in peri- and post-menopausal women (Valente *et al.*, 1994; Brandi, 1992; Civitelli *et al.*, 1997). Asians who consume more soy have lower rates of hip fracture (Ho *et al.*, 1993; Ross *et al.*, 1991). There are a number of animal studies that support the hypothesis that isoflavones can improve bone mineral density in the ovariectomized rodent model, the US Food and Drug Administrations approved model for osteoporosis study (Thompson *et al.*, 1998). Blair *et al.* (1996), Ishimi *et al.* (1999), Fanti *et al.* (1998), Draper *et al.* (1997) and Ishida *et al.* (1998) have all observed bone sparing effects in rodents administered isoflavones. Dietary soy protein, instead of casein, has reduced loss of bone mineral density in rodents (Kalu *et al.*, 1988; Arjmandi *et al.*, 1996; Harrison *et al.*, 1998; Omi *et al.* 1994). However, these studies did not quantify isoflavone levels in these diets. Arjmandi *et al.* (1998) reported rats fed soy protein with isoflavones had high bone mineral density compared to rats fed soy protein with isoflavones removed. In contrast to these rodent studies, two reports with non-human primates, the cynomologus monkey, failed to show a bone sparing effect of dietary soy protein with or without isoflavones over 23 months (Jayo *et al.*, 1996) or 7 months with isoflavone rich soy protein (Lees *et al.*, 1998). In ovariectomized Wistar rats, osteopenia was allowed to develop before isoflavone feeding (as Novasoy™, a soy isoflavone concentrate at 20, 40 or 80 mg total isoflavones kg^{-1} body weight). The isoflavones caused no improvement in bone mineral density as assessed by DEXA (X-ray bone densitometer) compared with ovariectomized controls. But after 84 days of feeding 40–80 mg isoflavones kg^{-1}, bone turnover was reduced significantly compared with the ovariectomized controls, as measured by urinary deoxypyridinoline and plasma osteocalcin (Picherit *et al.*, 2001). Given the doses needed for these modest effects (human equivalent of about 5 mg isoflavones kg^{-1} body weight, taking into account rat to human surface area differences), dietary isoflavones would not be likely to be of much help to reverse osteoporosis. Their role in primary prevention of osteoporosis seems more promising.

Messina *et al.* (2001) have recently reviewed the effects of soy protein and/or isoflavones in humans. However, as these authors state, most of the work is still in abstract form. But most of the studies do show a consistent trend that soy consumption is related to slower rates of bone turnover in women. Alekel *et al.* (2000) and Potter *et al.* (1998) reported improved spinal lumbar bone mineral density in peri- and post-menopausal women, respectively, consuming soy isoflavones with intact soy protein. However, both these studies also reported no change in bone mineral density at other bone sites.

One confounding variable in evaluating the soy phytoestrogens' ability to prevent bone loss is the effect of dietary soy protein on calcium loss in comparison to animal proteins. A number of reports show animal proteins, such as casein, whey, chicken, eggs, beef, and fish, result in greater losses of urinary calcium in comparison to diets based on soy proteins (Anderson *et al.*, 1987; Watkins *et al.*, 1985; Breslau *et al.*, 1986). Messina *et al.* (2000) suggest daily consumption of 6–9 g soy protein will have minimal effects of calcium balance, but that two or more servings per day or in the range of > 20 g per day may provide favorable effects on retarding calcium loss.

There may be other variables in habits that will affect the ability to discern effects of phytoestrogens on bone. One study measured an association between phytoestrogen excretion (isoflavones and lignans) and bone loss over ten years in postmenopausal women consuming typical diets in the Netherlands (Kardinaal *et al.*, 1998). A composite urine sample was analyzed for isoflavones and enterolactone. After adjustment for BMI, age, years post-menopause, intake of calcium and dietary fiber, equol excretion was significantly positively associated with bone loss over the first five years of the study period; greater enterolactone excretion was significantly associated with increased bone loss over ten years. Although these associations between greater phytoestrogen intake and greater bone loss were relatively weak, they suggest additional issues to consider in study design, i.e., the possibility of a confounding variable of increased whole grain intake or some lifestyle factor(s) that increase enterolactone production.

Although there is too little evidence to support a health claim for soy and bone health nor can soy phytoestrogens be recommended as a substitute for conventional estrogen replacement therapy, these foods can be recommended for women who do not wish to use estrogen therapy. From the few reports in the literature to date, it appears humans may need to consume 60–90 mg of isoflavones per day or 2–3 servings of traditional soy foods. There may be an opportunity for the food industry to create foods rich in isoflavones to allow consumers to be less challenged in incorporating these foods into the diet.

B. CANCER

1. Soy and isoflavones

Messina and Bennink (1998) and Messina (1999) recently reviewed the roles of soy in colon and breast cancer, respectively. The phytoestrogens are only one class among several soy components linked with cancer

prevention and control (e.g. saponins, Bowman-Birk protease inhibitor, sphingolipids, phytates). Epidemiological and intervention studies not measuring isoflavones specifically cannot be interpreted as supportive of a role of phytoestrogens in cancer protective effects. Even when the isoflavones are measured, other soy components might still be partly responsible for observed effects. It is yet uncertain as how well the soy phytoestrogens serve as biomarkers of soy intake, but these compounds do seem to provide a unique chemical "signature" for soy. Few *in vivo* carcinogenesis studies on purified isoflavones have been performed. A multitude of *in vitro* studies showed that genistein (or daidzein, but in far fewer studies) altered cancer cell proliferation. Such studies generally did not show effects of nutritionally relevant concentrations of these components, 0.01–5 μM according to bioavailability studies (e.g. Xu *et al.*, 1994), or of significant metabolites (e.g. Zhang *et al.*, 1999b). As reviewed by Adlercreutz (1998), isoflavone intake as reflected in urinary excretion is associated with lesser risks of breast cancer, lower mortality from prostate cancer and lesser incidence of prostate cancer.

In addition to prevention of cancer, isoflavones have been investigated for their ability to treat cancer. In C57Bl/6 mice transplanted with MB49 bladder cancer cells, genistein (50 mg kg^{-1} body weight given intraperitoneally), isoflavone concentrate (1700 mg total isoflavones kg^{-1} diet), or 20% soy protein isolate (400 mg total isoflavones kg^{-1} diet) significantly inhibited tumor cell growth (Zhou *et al.*, 1998). This inhibition was accompanied by significant decreases in tumor cell proliferation and tumor vascular density, and increased apoptotic index. B16BL6 melanoma cell metastases in C57Bl/6 mouse lung were inhibited in number by 900 μmol total isoflavones kg^{-1} diet, and inhibited in tumor median cross-sectional diameter by 225, 450 or 900 μmol isoflavones kg^{-1} diet (Li *et al.*, 1999a). Total isoflavones at 900 μmol kg^{-1} diet would approximate a human dietary intake of about 120 mg isoflavones per day. In the study by Zhou *et al.*, the minimum effective isoflavone dose would be the human equivalent of about 200 mg total isoflavones per day. Both studies of transplanted tumors suggest pharmacological, rather than nutritional applications of soy phytoestrogens.

2. *Lignans*

Decreasing lignan intake (and excretion) is associated with an increasing prostate cancer risk in Finnish men observed within the past decade (Adlercreutz, 1998). Lignan intake is associated with decreased breast and prostate cancer risk, as occurs respectively in vegetarian women v.

omnivores in the US, and Finnish men who eat more whole grains. But the Japanese population, with a relatively low risk of breast cancer, has very low lignan intake and excretion.

Experiments on lignan inhibition of carcinogenesis include work related to colon and breast cancer. *In vitro*, colon cancer cell proliferation (LS174T, Caco-2, HCT-15, and T84 lines), measured by BrdU uptake, was inhibited in the presence or absence of 17β-estradiol by enterodiol or enterolactone (100 μM) (Sung *et al.*, 1998). Such concentrations of mammalian lignans in the colonic lumen are theoretically achievable after consuming 10 g flaxseed. Although colonic contents of lignans after flaxseed feeding have not been directly measured, this observation of inhibition of colon cancer cell proliferation *in vitro* supports epidemiological observations that diets high in whole grains are colon cancer protective. In male Sprague Dawley rats initiated with azoxymethane (AOM, 15 mg kg^{-1} body weight) prior to lignan feeding, flaxseed fed at 2.5 or 5% by weight of diet or secoisolariciresinol diglucoside (SD, 1.5 mg per day by gavage) for 100 days significantly decreased aberrant crypt multiplicity (crypts per focus). But total numbers of crypts (distal colon only) were only suppressed significantly by SD, and total numbers of aberrant crypt foci (proximal colon only) were only significantly suppressed by feeding 2.5% defatted flaxseed. Urinary lignans were significantly related to dietary lignan content, so the lack of strong effect of lignans on colon carcinogenesis may reflect a high degree of interindividual variability in response to AOM.

Dietary supplementation for 2 weeks with SD (73–293 μmol kg^{-1} diet, equivalent to 2.5–10% flaxseed) inhibited C57Bl/6 mouse lung metastases from the B16BL6 melanoma cell line (Li *et al.*, 1999b), as did isoflavones (Li *et al.*, 1999a).

Gavage of SD (1.5 mg per rat) for 20 weeks significantly inhibited dimethylbenz(a)anthracene (DMBA, 5 mg per rat)-initiated mammary carcinogenesis in Sprague Dawley rats (Thompson *et al.*, 1996). When Sprague Dawley rats were initiated with N-methyl-N-nitrosourea (MNU, 50 mg kg^{-1} body weight intraperitoneally), a low dose of SD (equivalent to 2.5% flaxseed by weight of diet) increased mammary tumor multiplicity (Rickard *et al.*, 1999). SD equivalent to 5% flaxseed significantly inhibited tumor multiplicity after 16 weeks of gavage. Neither dose of flaxseed (2.5 or 5%) significantly altered tumorigenesis. Flaxseed is also a rich source of n-3 fatty acids, which were demonstrated to have cancer protective effects in numerous studies (Karmali and Doshi, 1987). The study of effects of SD and flaxseed on MNU carcinogenesis illustrates the challenges of sorting out anticancer effects of complex mixtures such as foods.

3. Comparisons among phytoestrogens with respect to anticancer mechanisms

Adlercreutz *et al.* (1992) reviewed anticancer activities of phytoestrogens. Phytoestrogens may be biomarkers of dietary habits that reduce risk of some cancers, but anticarcinogenic mechanisms of action of nutritionally relevant phytoestrogen intakes remain to be proven. An example of such a mechanistic approach is a study of the ability of enterolactone, daidzein, genistein and coumestrol to induce NADPH:quinone reductase (QR) in a human colon cancer cell line, Colo205 (Wang *et al.*, 1998). Induction of QR detoxifies a variety of carcinogens. Genistein and enterolactone induced Colo205 cell QR in concentrations of 0.1–10 μM, by a maximum of about 6–8-fold. Coumestrol (1.0–10 μM) induced Colo205 cell QR by about twofold. Daidzein had no QR inducing activity. Enterolactone and genistein at 1.0–10 μM were also moderately cytotoxic to Colo205 cells. This is one of a few studies to demonstrate an effect of phytoestrogens in nutritionally relevant concentrations, and may therefore be a model for future studies in this field. However, induction of QR in colon cancer cells might actually protect cancer cells, acting as one mechanism of their resistance to some anticancer therapies. The choice of *in vitro* or other model system for characterizing phytoestrogens must be considered carefully with respect to what health effects are desired.

C. CARDIOVASCULAR

The connection between soy protein, isoflavones and improved cardiovascular health was highlighted by Anderson *et al.* (1995) meta analysis of 38 controlled clinical trials. Currently, FDA has approved a health claim for soy protein describing the relationship between soy protein consumption and reduced risk of heart disease as part of a diet low in saturated fat and cholesterol (Dotzel, 1999). The health claim must include 25 g soy protein per day to meet the claim, typically as 4 servings per day. In evaluating the 43 human intervention feeding studies submitted by the petitioner, the FDA identified 27 studies that met their criteria of reliability and accuracy of methods, estimates of intakes of saturated fat and cholesterol, information on soy protein and control used, measured endpoints and study design characteristics (Schultz, 1998). Of these 27 studies, 14 trials were given particular weight due to subject representation of the US population, reported intakes of saturated fats and cholesterol and avoidance of small sample size and other experimental design problems. For a more detailed analysis of the petition review, the reader should see Schultz (1998). The petitioner originally asked that

isoflavones be included in the health claim. However, the FDA "tentatively concluded that the evidence is not sufficient to establish that the presence or absence of isoflavones accounts for or is related to the effect on blood lipids" (Schultz, 1998). Since this review, 14 human studies have been published on feeding isoflavones and some aspect of cardiovascular health. Of these 14 studies, 6 studies have described feeding soy protein isolate with measured isoflavones and have observed some degree of plasma cholesterol lowering as either total cholesterol and/or low density lipoprotein cholesterol (Potter *et al.*, 1998; Washburn *et al.*, 1999; Crouse *et al.*, 1999; Baum *et al.*, 1999; Merz-Demlow *et al.*, 2000; Teixeira *et al.*, 2000). Three of the studies with humans fed isoflavones without associated soy proteins observed no change in serum cholesterol status (Nestel *et al.*, 1997; Hodgson *et al.*, 1998; Samman *et al.*, 1999). Goodman-Guren and Kirtz-Silverstein (2001) reported dietary isoflavone intake was associated with cardiovascular risk factors. Their study evaluated free living women with usual dietary intake of isoflavones from their regular diet and found a positive correlation between isoflavone intake and HDL-cholesterol. The remaining studies did not evaluate serum cholesterol or did not report isoflavone concentrations in the diets of the human subjects. There have also been a number of studies published evaluating soy isoflavones with and without soy protein in a variety of experimental animal models (nonhuman primates, rats, rabbits, hamsters, gerbils, LDL receptor deficient mice) and cholesterol metabolism since the FDA approval of the soy protein health claim. However, the FDA will evaluate the health claim based on the results in human studies to make definitive conclusions with regard to isoflavones. Clearly, there is mounting evidence that the cholesterol lowering in humans is associated with the consumption of soy protein with isoflavones and/or other ethanol extractable components such as but not limited to saponins, phospholipids and phenolic acids. Modification of the health claim will await the completion of additional studies with humans.

The mechanism for the cholesterol lowering ability of soy protein and isoflavones is the topic of considerable debate and many hypotheses (Anthony, 2000). Some of the suggested mechanisms whereby soy protein and/or isoflavones affect atherosclerosis and cardiovascular disease include improvement of plasma lipid and lipoprotein profiles, mediated effects on blood pressure (Washburn *et al.*, 1999; Crouse *et al.*, 1999), effects on vascular and endothelial cell functions (Honore *et al.*, 1997; Nestel *et al.*, 1997), platelet aggregatation, activation and serotonin storage (Helmeste and Tang, 1995; Schoene and Guidry, 1999; Williams and Clarkson, 1998), LDL oxidation state (Tikkanen *et al.*, 1998; Jenkins *et al.*, 2000; Samman *et al.*, 1999), smooth muscle cell proliferation, estrogen receptor β (ERβ)-

mediated effects (Makela *et al.*, 1999), and LDL receptor interactions (Lovati *et al.*, 1998; Manzoni *et al.*, 1998). Additionally, the long-term effects of soy protein with isoflavones consumption on plasma lipids have yet to be evaluated. The short-term studies clearly seem to show a cholesterol lowering effect. But what happens on longer-term diets? US consumers will want to know the minimal dose required to obtain the desired outcomes. Finally, will soy consumption have an effect on human morbidity and mortality?

Greater serum concentrations of the mammalian lignan enterolactone were associated with lower risk of acute coronary events in a case control study of Finnish men (Vanharanta *et al.*, 1999). It remains to be seen whether phytoestrogenic effects underlie both this finding and the many findings of protection against cardiovascular disease risk associated with soy and soy phytoestrogens.

XI. STATUS AND CONCLUSIONS

Progress has been made in identifying and analyzing phytoestrogens in foods. Developing recommendations for their intake as dietary components must be based on sound data showing their ability to reduce chronic disease risk. Ideally, knowledge of mechanisms of disease risk reduction of the phytoestrogens will also underpin such recommendations. This work will require better models for human osteoporosis than currently exist, and/or at great cost, several very well-designed long-term (multi-year) human intervention trials. This work will require careful attention to carcinogenesis models that more realistically simulate the natural history of common human neoplasms. Fortunately, some genetically modified animal models are beginning to address this concern, e.g. TRAMP mouse, a prostate adenocarcinoma model (Greenberg *et al.*, 1995). Attention to more than cholesterol-lowering effects is needed to address cardiovascular disease risk reduction. Phytoestrogens should be studied in models emphasizing atherosclerotic lesions that mimic human lesions and factors that precipitate acute coronary events. More attention needs to be paid to factors that alter human bioavailability of phytoestrogens. This means turning our attention to gut microbial ecology, and to create beneficial gut microbial environments, i.e. environments that maximize phytoestogen bioavailability and efficacy. Quantifying phytoestrogens in foods and biological fluids, and doing so more rapidly and economically will also be important. Above all, emphasizing nutritionally relevant doses in mechanistic studies and animal models will be key to relevant recommendations for the human diet, which seems to have much health protective potential. The phytoestrogens are promising candidates to fulfill that potential.

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PLATE 1 Co-field flow PEF treatment chambers for benchtop and pilot plant-scale PEF system using stainless steel electrodes (top), a PEF treatment chamber for pilot plant-scale PEF system using stainless steel electrodes (middle), a PEF treatment chamber for pilot plant-scale PEF system using boron carbide electrodes (bottom).

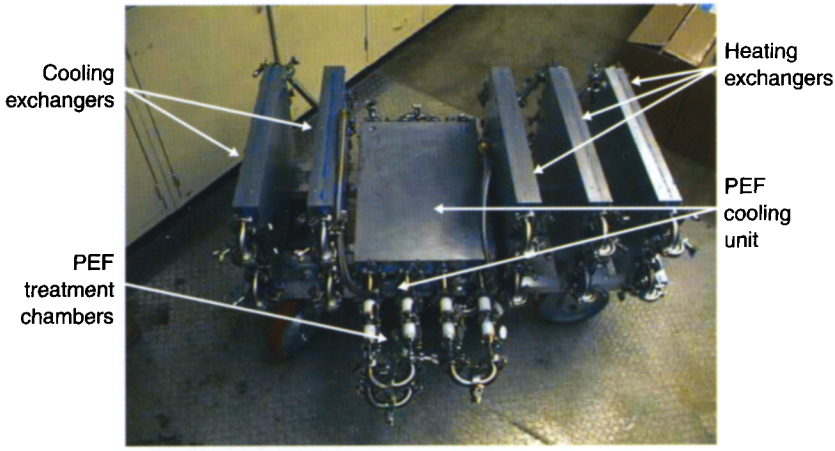

PLATE 2 PEF treatment chambers connected to PEF cooling unit, heating exchangers and cooling exchangers (Streaker, 1999).

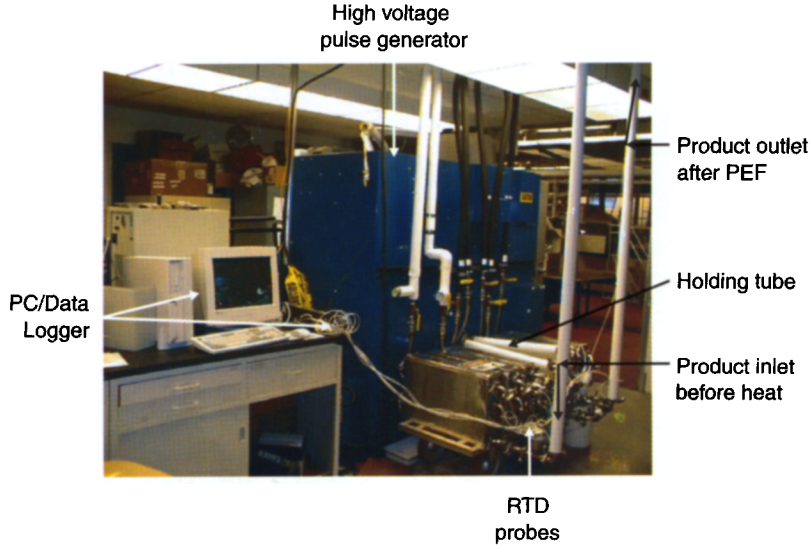

PLATE 3 PEF treatment unit connected to a high voltage pulse generator, heat exchangers, and a data logging system (Streaker, 1999).

PLATE 4 *Chaomai-ke* (buckwheat shell) dish in Yulin, Shaanxi Prov., China.

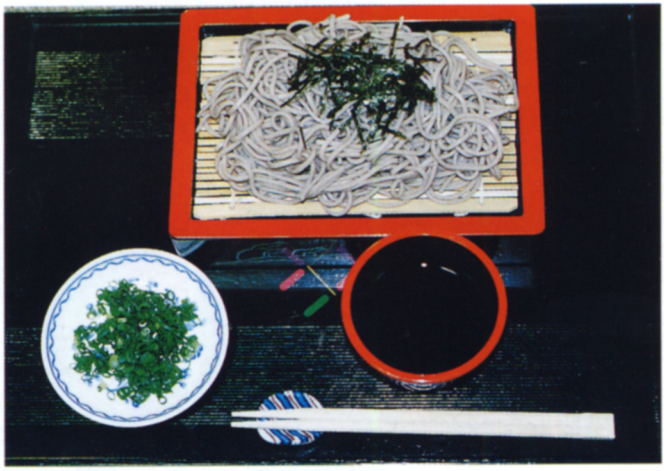

PLATE 5 Traditional buckwheat noodles dish (*Zaru-soba*) in Japan. *Zaru-soba* means buckwheat noodles on a bamboo plain basket (*zaru*).

PLATE 6 Buckwheat groats dish in Slovenia.

PLATE 7 Buckwheat pasta dish (*called pizzoccheri*) in Italy.