

- Saruta, M., Takahashi, K., Suzuki, T., Torii, A., Kawakami, M., and Sasano, H. (2004). Urocortin 1 in colonic mucosa in patients with ulcerative colitis. *J. Clin. Endocrinol. Metab.* **89**, 5352–5361.
- Sasano, H., Matsuzaki, S., and Suzuki, T. (2001). Estrogen receptor mRNA *in situ* hybridization using microprobe system. *Methods Mol. Biol.* **176**, 317–325.
- Schutze, K., and Lahr, G. (1998). Identification of expressed genes by laser-mediated manipulation of single cells. *Nature Biotechnol.* **16**, 737–742.
- Sgroi, D. C., Teng, S., Robinson, G., Le Vangie, R., Hudson, J. R., Jr., and Elkahloun, A. G. (1999). *In vivo* gene expression profile analysis of human breast cancer progression. *Cancer Res.* **59**, 5656–5661.
- Specht, K., Richter, T., Muller, U., Walch, A., and Hofler, M. W. (2000). Quantitative gene expression analysis in microdissected archival tissue by real-time RT-PCR. *J. Mol. Med.* **78**, B27.
- Suzuki, T., Nakata, T., Miki, Y., Kaneko, C., Moriya, T., Ishida, T., Akinaga, S., Hirakawa, H., Kimura, M., and Sasano, H. (2003). Estrogen sulfotransferase and steroid sulfatase in human breast carcinoma. *Cancer Res.* **63**, 2762–2770.
- Suzuki, T., Sasano, H., Sasaki, H., Fukaya, T., and Nagura, H. (1994). Quantitation of P450 aromatase immunoreactivity in human ovary during the menstrual cycle: Relationship between the enzyme activity and immunointensity. *J. Histochem. Cytochem.* **42**, 1565–1573.
- Suzuki, T., Sasano, H., Sawai, T., Mason, J. I., and Nagura, H. (1992). Immunohistochemistry and *in situ* hybridization of P-45017 alpha (17 alpha-hydroxylase/17,20-lyase). *J. Histochem. Cytochem.* **40**, 903–908.
- Tsuda, H., Sasano, H., Akiyama, F., Kurosumi, M., Hasegawa, T., Osamura, R. Y., and Sakamoto, G. (2002). Evaluation of interobserver agreement in scoring immunohistochemical results of HER-2/neu (c-erbB-2) expression detected by HercepTest, Nichirei polyclonal antibody, CB11 and TAB250 in breast carcinoma. *Pathol. Int.* **52**, 126–134.
- Utsumi, T., Yoshimura, N., Takeuchi, S., Ando, J., Maruta, M., Maeda, K., and Harada, N. (1999). Steroid sulfatase expression is an independent predictor of recurrence in human breast cancer. *Cancer Res.* **59**, 377–381.
- Whetsell, L., Maw, G., Nadon, N., Ringer, D. P., and Schaefer, F. V. (1992). Polymerase chain reaction microanalysis of tumors from stained histological slides. *Oncogene* **17**, 2355–2361.

## [19] Metabolism of Phytoestrogen Conjugates

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### Abstract

Phytoestrogens are plant-derived compounds with physiologic estrogenic effects. They are present in the plant as glycosidic conjugates, some of which contain further chemical modifications (acetate, malonate, and 3-hydroxy-3-methylglutarate esters and 2,3-dihydroxysuccinate ether).

In the gastrointestinal tract, the conjugates undergo hydrolysis catalyzed by enzymes in the intestinal wall and by gut bacteria. On entering the systemic circulation, the phytoestrogens may undergo extensive metabolism to other compounds through reactions involving demethylation, methylation, hydroxylation, chlorination, iodination, and nitration. In addition, all these compounds can undergo conjugation to form  $\beta$ -glucuronides and sulfate esters. This chapter describes the methods of analysis of all these compounds, the sources of or methods to manufacture suitable standards, and the procedures for examining the enzymes that catalyze these reactions.

## Introduction

Phytoestrogens are naturally occurring plant compounds, many of which have weak estrogenic or antiestrogenic activity in mammals. This class includes isoflavonoids, coumestanes, stilbenes, zearalones, and lignans. The soy isoflavones, genistein, daidzein, and glycitein, have been the most commonly studied of this class. Phytoestrogens have been proposed to have beneficial effects in chronic diseases such as atherosclerosis and cardiovascular diseases, hormone-dependent cancers, arthritis, neurodegeneration, and osteoporosis (Middleton, 2000). The mechanisms of action attributed to these disease-prevention effects range from their ability to elicit an estrogen-like response (Jacobs and Lewis, 2002) to tyrosine kinase inhibition (Akiyama *et al.*, 1987) to antioxidant activity (Pietta, 2000). Many of the experiments demonstrating these effects have used phytoestrogens in their aglycone forms; however, phytoestrogens are present in foods and dietary supplements in the form of several types of conjugates as well as aglycones. In addition, in animals, the circulating forms of the phytoestrogens are mostly  $\beta$ -glucuronides and sulfates, with only small amounts (2–5%) as the aglycones. This discrepancy between the chemical forms used in laboratory experiments and those that are ingested and that circulate in the body requires a full appreciation of the metabolism of phytoestrogens during absorption, distribution, and excretion. This involves enzyme systems in the digestive tract, liver, and even at target tissue sites.

The reader is encouraged to read other reviews on the analysis of phytoestrogens (Wang *et al.*, 2002; Wilkinson *et al.*, 2002), particularly those involving mass spectrometry (Prasain *et al.*, 2002, 2004). In many methods used for the analysis of phytoestrogens, for analytical convenience investigators convert the phytoestrogen conjugates to their aglycones. However, this provides an incomplete appreciation of phytoestrogen composition. Recent methodologies provide more accurate profiles of

plasma, urine, feces, and other physiological samples. This chapter describes methods used for (1) the evaluation of phytoestrogen conjugation in foods, (2) the synthesis and biosynthesis of specific phytoestrogen metabolites found *in vivo*, (3) halogenated and nitrated metabolites formed during inflammation, and (4) quantification of these compounds *in vivo*.

### Phytoestrogen Conjugates in Soybeans, Other Foods, and Dietary Supplements

The two major phytoestrogen classes contained in foods are isoflavones and lignans. The predominant isoflavones in soybeans are conjugates of genistein (5, 7, 4'-trihydroxyisoflavone) and daidzein (7, 4'-dihydroxyisoflavone). They are genistein-7-*O*- $\beta$ -D-glycoside (genistin), 6''-*O*-malonylgenistin, daidzein-7-*O*- $\beta$ -D-glycoside (daidzin), and 6''-*O*-malonyldaidzin (Fig. 1) (Barnes *et al.*, 1994; Wang and Murphy, 1994a). Glycitein (7, 4'-dihydroxy-6-methoxyisoflavone) and its conjugates are minor isoflavones in soybean cotyledons, but are major components in dietary supplements and foods made from the soybean hypocotyls (Kudou *et al.*, 1991). Red clover, also used to make dietary supplements, contains 4'-methylated

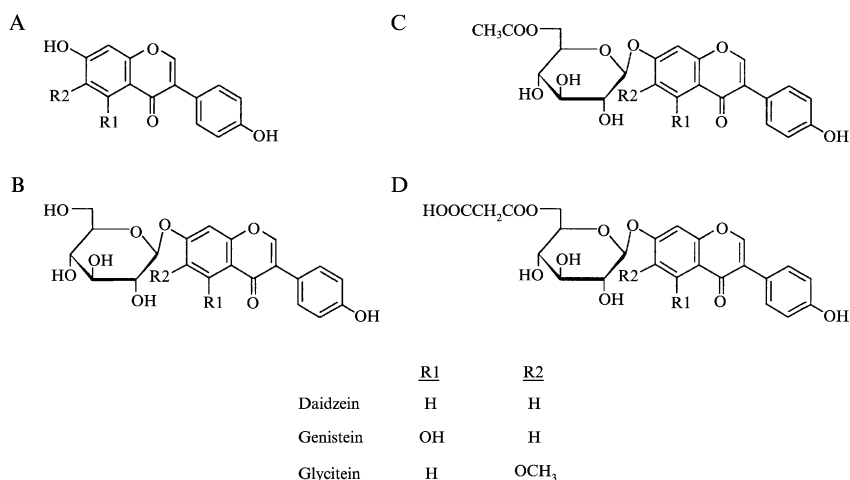


FIG. 1. Chemical structures of isoflavones in soy foods and soy food products. (A) Aglycones, (B)  $\beta$ -glycoside conjugates, (C) acetyl- $\beta$ -glycoside conjugates, and (D) malonyl- $\beta$ -glycoside conjugates.

forms of daidzein (formononetin, Fig. 2A) and genistein (biochanin A, Fig. 2B) (He *et al.*, 1996). In dietary supplements made from kudzu root, C-glycosides of daidzein and genistein are the predominant conjugate forms (Fig. 3) (Prasain *et al.*, 2003b). Another source of isoflavone conjugates exists in soy sauce as ethers of 2,3-dihydroxysuccinic acid with genistein and daidzein (Kinoshita *et al.*, 1997).

Several other phytoestrogens are found in foods, including coumestrol (Fig. 2C), a coumestane in alfalfa and many legumes, zearalenone (Fig. 2D), a fungal estrogen that is a contaminant of stored feed, resveratrol (Fig. 2E), a stilbene of red grapes and red wines, and lignans. The latter are mainly found in the outer layers of cereals and grains, with flaxseed and rye among the most important. The predominant lignans in these foods are matairesinol (Fig. 2F) and secoisolariciresinol (Fig. 2G) with lesser amounts of pinoresinol, lariciresinol, and syringaresinol. Like the isoflavones, lignans exist as glycosides in foods and are converted to enterodiol and enterolactone by gut microflora.

The isoflavone content in soybeans varies according to genetics, crop years, and growth location (Wang and Murphy, 1994b). As a consequence, the isoflavone content of soy foods derived from soybeans varies from year

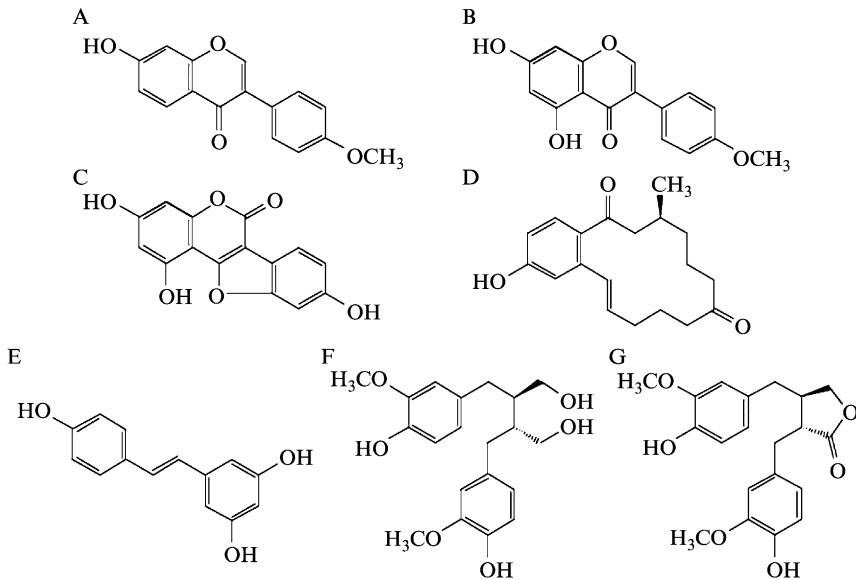


FIG. 2. Chemical structures of other phytoestrogens. (A) Formononetin, (B) biochanin A, (C) coumestrol, (D) zearalenone, (E) resveratrol, (F) matairesinol, and (G) secoisolariciresinol.

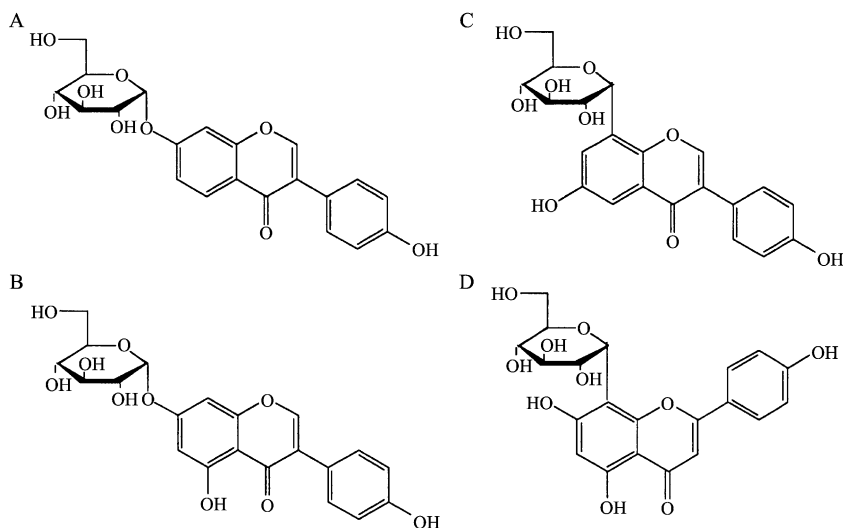


FIG. 3. Different chemistry of O- and C-glycosides of isoflavones. The kudzu root contains daidzin (A), genistin (B), and puerarin (daidzein-8-C-glycoside) (C). Millet contains vitexin (apigenin-8-C-glycoside) (D).

to year. This may also be a factor for other phytoestrogen-containing foods. Those foods that are used in preclinical experiments and clinical trials should therefore be examined carefully for their qualitative and quantitative content of phytoestrogens.

### Phytoestrogen Standards

Many unlabeled phytoestrogens (Figs. 1–3) and their metabolites (Fig. 4) are available from Aldrich-Sigma Chemical Co. (St. Louis, MO), Indofine Chemical Co. (Hillsborough, NJ), LC Laboratories (Woburn, MA), and Plantech (Reading, UK). The  $\beta$ -glycosides of genistein and daidzein are available from Aldrich-Sigma Chemical Co. and LC Laboratories. It should be noted that the 6''-O-malonyl- $\beta$ -glycosides are unstable as soon as they are dissolved in aqueous methanol. Radiolabeled phytoestrogens are not available commercially and may require a custom synthesis (Peterson *et al.*, 1996). However, the British Food Standards Agency funded a project for the preparation of multiply  $^{13}\text{C}$ -labeled phytoestrogens. Investigators should contact Dr. Nigel Botting, Department of Chemistry, St. Andrew's University, Fife, Scotland, regarding the availability of these materials.  $\text{U}^{14}\text{C}$ -labeled genistein is available from the National

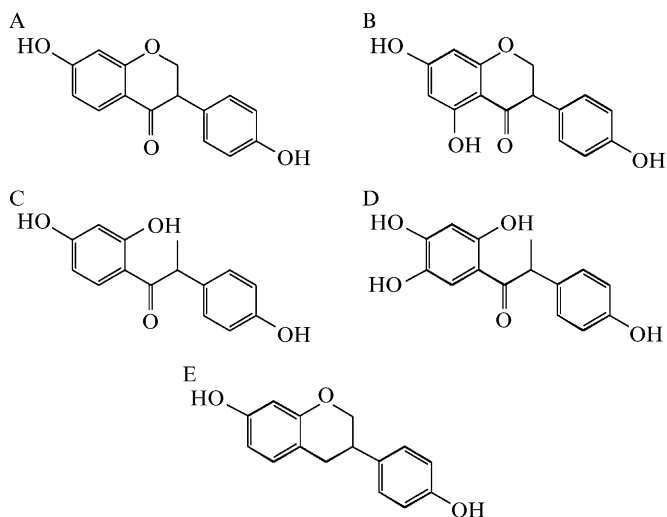


FIG. 4. Chemical structures of bacterial metabolites of isoflavones. (A) dihydrodaidzein, (B) dihydrogenistein, (C) *O*-desmethylangolensin, (D) 6-hydroxy-*O*-desmethylangolensin, and (E) equol.

Cancer Institute Chemical Carcinogen Reference Standard Repository. Another source of radiolabeled phytoestrogens is Dr. Mary Ann Lila at the University of Illinois at Urbana-Champaign, who utilizes plant cell cultures for the biosynthesis of isotopically labeled bioflavonoids (Lila *et al.*, 2005).

All standards should be verified by mass spectrometry (MS) and, when possible, by nuclear magnetic resonance spectrometry (NMR). The expected  $[M-H]^-$  molecular ions of many phytoestrogens are given in Tables I and II. A limited number of phytoestrogens have been studied by  $^1H$ -NMR (Table III).

#### Extraction of Phytoestrogens from Foods

Several methods for the extraction of phytoestrogens, especially isoflavones, have been proposed. Some methods are used to determine the various conjugated forms of phytoestrogens, whereas others are used to determine total phytoestrogens within foods. To determine the individual phytoestrogen composition in foods, extractions are done without a hydrolysis step. To determine total phytoestrogen content, enzymatic hydrolytic with *Aspergillus niger* cellulase is carried out.

TABLE I  
*m/z* VALUES OF MONOISOTOPIC [M-H]<sup>-</sup> MOLECULAR IONS OF ISOFLAVONES AND  
 THEIR METABOLITES

Isoflavone	<i>m/z</i>
Daidzein	253.06
[ <sup>13</sup> C <sub>3</sub> ]Daidzein	256.06
Daidzin	415.11
6''- <i>O</i> -acetyldaidzin	457.12
6''- <i>O</i> -malonyldaidzin	501.11
Daidzein 4'-sulfate	333.01
Daidzein 7-sulfate	333.01
Daidzein 4',7-disulfate	412.97
Daidzein 4'-glucuronide	429.09
Daidzein 7-glucuronide	429.09
Daidzein 4',7-glucuronide	605.12
Daidzein 4'-sulfo-7-glucuronide	509.04
Daidzein 7-sulfo-4'-glucuronide	509.04
3'-Chlorodaidzein	287.02, 289.02
6-Chlorodaidzein	287.02, 289.02
8-Chlorodaidzein	287.02, 289.02
3',6-Dichlorodaidzein	321.98, 323.98, 325.97
3',8-Dichlorodaidzein	321.98, 323.98, 325.97
3'-Nitrodaidzein	298.04
Genistein	269.05
Genistin	431.11
6''- <i>O</i> -Acetylgenistin	473.12
6''- <i>O</i> -Malonylgenistin	517.11
Genistein 4'-sulfate	349.01
Genistein 7-sulfate	349.01
Genistein 4',7-disulfate	429.97
Genistein 4'-glucuronide	445.09
Genistein 5-glucuronide	445.09
Genistein 7-glucuronide	445.09
Genistein 4',7-diglucuronide	621.12
Genistein 4'-sulfo-7-glucuronide	525.04
Genistein 7-sulfo-4'-glucuronide	525.04
[ <sup>13</sup> C <sub>3</sub> ]Genistein	272.05
3'-Chlorogenistein	303.01, 305.01
6-Chlorogenistein	303.01, 305.01
8-Chlorogenistein	303.01, 305.01
3',6-Dichlorogenistein	336.97, 338.97, 340.96
3',8-Dichlorogenistein	336.97, 338.97, 340.96
3'-Nitrogenistein	314.04
Mono-chloro-nitrogenistein	348.00, 350.00
Dichloro-nitrogenistein	381.96, 383.96, 385.97
Glycitein	283.07
Glycitin	445.12
Glycitein 4'-glucuronide	459.10

(continued)

TABLE I (continued)

Isoflavone	<i>m/z</i>
Glycitein 7-glucuronide	459.10
Dihydrodaidzein	255.07
Dihydrodaidzein 7-glucuronide	431.11
Equol	241.09
Equol glucuronide	417.13
<i>O</i> -Desmethylangolensin	257.09
<i>O</i> -Desmethylangolensin glucuronide	433.12

TABLE II  
*m/z* VALUES OF MONOISOTOPIC [M-H]<sup>-</sup> MOLECULAR IONS  
 OF PHYTOESTROGENS

Phytoestrogen	<i>m/z</i>
Coumestrol	267.04
Coumestrol glucuronide	443.07
Resveratrol	227.08
Resveratrol glucuronide	403.11
Resveratrol sulfate	307.04
Zearalenone	317.15
Matairesinol	357.14
Matairesinol glycoside	519.19
Matairesinol glucuronide	533.17
Matairesinol diglucuronide	709.21
Secoisolariciresinol	361.17
Secoisolariciresinol glycoside	523.23
Secoisolariciresinol glucuronide	537.21
Secoisolariciresinol diglucuronide	713.24
Enterodiol	301.15
Enterodiol glucuronide	477.18
Enterolactone	297.12
Enterolactone glucuronide	473.15

### *Acidified Solvent Extraction of Phytoestrogens*

A general procedure that is suitable for the extraction of phytoestrogens and their direct analysis is based on the method described by [Murphy \(1981\)](#) for the extraction of isoflavones from soybeans ([Wang and Murphy, 1994b](#)) and commercial soy foods ([Wang and Murphy, 1994a](#)). Raw soybean seeds (2 g) with their seed coats are ground, mixed with 2 ml 0.1 N HCl and 10 ml of acetonitrile, stirred for 2 h at room temperature, and

TABLE III  
PROTON CHEMICAL SHIFTS OF ISOFLAVONES DETERMINED BY NMR

Compound	2-H	5-H	6-H	8-H	3',5'-H	2',6'-H	C <sub>4'</sub> OH	C <sub>5</sub> OH	C <sub>7</sub> OH
Daidzein <sup>a</sup>	8.30	7.98	6.95	6.84	6.84	7.40	n.m.	-	n.m.
Genistein <sup>a</sup>	8.40	N/A	6.41	6.25	6.81	7.40	n.m.	n.m.	n.m.
Formononetin <sup>a</sup>	8.40	8.00	6.90	6.90	7.00	7.50	-	-	n.m.
Daidzein-4'-sulfate <sup>a</sup>	8.35	7.98	6.95	6.90	7.21	7.46	-	-	n.m.
Daidzein-7-sulfate <sup>a</sup>	8.40	8.03	7.25	7.43	6.80	7.40	n.m.	-	-
Daidzein-4',7-disulfate <sup>a</sup>	8.30	8.02	7.23	7.40	7.16	7.45	-	-	-
Formononetin-7-sulfate <sup>a</sup>	8.40	8.02	7.20	7.39	7.00	7.50	-	-	-
Formononetin-7-glucuronide <sup>a</sup>	8.33	8.04	7.12	7.17	7.06	7.59	-	-	-
Genistein <sup>b,c</sup>	8.30	N/A	6.226	6.38	6.82	7.37	9.57	12.93	10.86
Monochlorogenistein <sup>b,d</sup>	8.416	N/A	6.220	6.380	6.815	7.380	9.648	12.916	n.m.
Dichlorogenistein <sup>b,d</sup>	8.360	N/A	N/A	N/A	6.816	7.379	9.600	13.720	n.m.

<sup>a</sup> Performed at 200 MHz (Clarke *et al.*, 2002).

<sup>b</sup> Performed at 300 MHz (Barnes *et al.*, 1994; Boersma *et al.*, 1999).

<sup>c</sup> Relative to tetramethylsilane (0.00 ppm).

<sup>d</sup> Relative to the DMSO proton resonance at 2.49 ppm.

filtered through Whatman No. 42 filter paper. The filtrate is taken to dryness under vacuum at a temperature below 30°. The dried material is redissolved in 10 ml of 80% aqueous methanol and then filtered through a 0.45- $\mu\text{m}$  filter unit. An aliquot of the filtrate (20  $\mu\text{l}$ ) is analyzed by reversed-phase HPLC. Although this extraction method preserves the isoflavone conjugates, degradation of the 6''-*O*-malonylglycosides in aqueous methanol occurs even at room temperature. Coward *et al.* (1998) have shown that extraction of isoflavones at 4°, where degradation is minimized, is possible if an internal standard such as fluorescein is used. In this latter method, the extract is not concentrated prior to HPLC analysis, but instead analyzed directly. Flavone and *p*-hydroxybenzoic acid have also been used as internal standards (Bednarek *et al.*, 2001; Franke and Custer, 1994).

### *Solvent Extraction of Phytoestrogens*

Isoflavones in solid foods are extracted into 80% aqueous methanol (10 ml/g) by stirring for 1 h at 60°. Other soy products (miso, soy milk, soy paste, and tofu) are freeze-dried and extracted whole. The mixture is centrifuged at 2500g for 10 min and the supernatant is transferred to a round-bottom flask. The pellet is extracted twice more in 5 ml each and centrifuged. Supernatants are combined in the round-bottom flask and evaporated to dryness in a rotary evaporator. The dried extracts are resolubilized in 5 ml of 50% aqueous methanol and then defatted by partitioning the neutral lipids into hexane (4  $\times$  20 ml). The aqueous methanol is taken to dryness in a rotary evaporator, and the resulting dried residue is resolubilized in 10 ml 80% aqueous methanol. An aliquot is centrifuged at 14,000g for 2 min and analyzed by HPLC as described by Coward *et al.* (1993).

While Coward *et al.* (1993) used aqueous methanol, Griffith and Collison (2001) used acetonitrile for isoflavone extraction: soy foods (1 g) are dispersed into a 10-ml volume of acetonitrile followed by the addition of 6 ml double deionized water and 0.5 ml apigenin [2000  $\mu\text{g}/\text{ml}$  in dimethyl sulfoxide (DMSO)]. Samples are shaken to mix and extracted on a rotary mixer for 2 h, after which the sample is recovered and the acetonitrile concentration is adjusted to 50% (v/v) with the addition of deionized water. Samples are centrifuged at 2000g for 10 min to pellet insoluble matter and eliminate foam. An aliquot is filtered through a 0.45- $\mu\text{m}$  PVDF filter and analyzed by HPLC as described (Griffith and Collison, 2001).

### *Extraction Followed by Enzymatic Hydrolysis (Liggins et al., 1998)*

Phytoestrogen-containing materials (2.5 g) are mixed with a minimum of 5 ml of 80% aqueous methanol. If the material under study is particularly enriched in phytoestrogens, then the amount taken for analysis should

be reduced, as phytoestrogens have a limited solubility in aqueous methanol, which would thereby lead to an erroneously low result. Sonication for 10 min solubilizes the phytoestrogen glycosides in the methanol by breaking up cellular material. It is followed by a further hour of soaking in the solvent. Insoluble material is removed by filtration through a double layer of filter paper (Whatman No. 4 and then No. 1), and any adsorbed phytoestrogens are washed through with fresh 80% aqueous methanol (>5 ml). The methanol in the filtrate is evaporated, and 100 Fishman units of cellulase from *A. niger* are added to the sample in 5 ml of 0.1 M sodium acetate buffer, pH 5. Samples are sonicated and subsequently incubated overnight in a shaking water bath at 37°. The hydrolyzed phytoestrogen aglycones usually precipitate because of their reduced aqueous solubility. They are extracted from the aqueous hydrolyzate by the addition of 100% ethyl acetate. Three 2-ml extracts with ethyl acetate are combined; a 1-ml aliquot of the combined extract is pipetted into a separate vial and taken to dryness at 60° under nitrogen prior to chromatographic analysis of the phytoestrogen aglycones.

#### *Extraction of Lignans*

Milder *et al.* (2004) developed an assay to extract lignans from a variety of foods, including flaxseed, broccoli, whole wheat bread, and tea. Previous extraction methods allowed for the quantitation of the major lignans secoisolariciresinol and matairesinol but were lacking for pinoresinol and lariciresinol. To allow for quantification, the lignans need to be extracted from their food matrices. This can be difficult due to their ability to oligomerize with 3-hydroxy-3-methylglutaric acid through ester bonding. Therefore, lignan extractions require the use of alkaline hydrolysis to release the lignan glycosides from the 3-hydroxy-3-methylglutaric acid. There are many unknown lignan glycosides; therefore, lignan extraction methods typically have a hydrolysis step to allow for the release of the glycosides. This results in an estimate of the total lignan content in foods. The following describes the current extraction and analysis methods for lignans from foods.

Alkaline extraction of 1.0 g of dry food is performed with 24.0 ml of methanol/water (70/30, v/v) containing 0.3 M sodium hydroxide in a shaking water bath for 1 h at 150 rpm and 60°. After extraction, the pH is adjusted to 5–6 with 750  $\mu$ l of 100% glacial acetic acid and the extract is centrifuged at 4,500g for 10 min at 10°. An aliquot (1 ml) is transferred to a preweighed test tube. Methanol is evaporated from this aliquot at 60°, under nitrogen, until the residual weight is  $\leq$ 0.30 g. The volume is adjusted to approximately 1.2 ml with 0.05 M sodium acetate buffer, pH 5, and the extract is weighed again to calculate the dilution compared to the original aliquot of 1 ml.

A 1-ml aliquot of the weighed extract or 1 ml of beverage is hydrolyzed by the addition of *Helix pomatia*  $\beta$ -glucuronidase/sulfatase (0.83 mg in 1 ml 0.05 M sodium acetate buffer, pH 5). The samples are incubated overnight at 37°. Samples are extracted twice with 2 ml diethyl ether, and the two organic phases are combined. The diethyl ether is evaporated, and the dried samples are redissolved in 0.3 ml of methanol, mixed, and 0.7 ml water is added. A 240- $\mu$ l aliquot of sample is added to 10  $\mu$ l of internal standard solution containing 50 ng of secoisolariciresinol- $d_8$  and 50 ng of matairesinol- $d_6$  in 30% aqueous methanol. Samples are mixed and transferred to HPLC vials for LC-MRM-MS analysis.

### Reversed-Phase HPLC Analysis of Phytoestrogens in Foods

Phytoestrogens are readily separated by reversed-phase HPLC on  $C_8$  or  $C_{18}$  columns. The most commonly used methods involve a gradient of acetonitrile in a background of either acetic acid, formic acid, or trifluoroacetic acid (0.1–1.0%) (Fig. 5B). However, because of the complexity of phytoestrogen forms in the plant material (aglycones,  $\beta$ -glycosides, 6''-O-acetylglycosides, and 6''-O-malonylglycosides), there is considerable advantage in using a neutral buffer. This brings out differences in hydrophobicity based on changes in the charge state. For example, the malonate group at pH 7 is negatively charged, whereas it is unprotonated at an acidic pH. The 6''-O-malonylglycosides elute earlier than the  $\beta$ -glycosides (Fig. 5A). As a result, the major classes are better separated (Wang *et al.*, 2002).

By virtue of their phenolic groups and some heterocyclic ring systems, intact phytoestrogens have a strong molar absorbance. However, reduction and oxidation of the heterocyclic ring that typically occur during metabolism substantially reduce molar absorbance. Therefore, while phytoestrogens can be detected readily by their UV absorbance in foods containing them in moderate concentrations, the HPLC-UV analysis approach is inadequate for most studies of physiological fluids (serum, plasma, and urine). In the latter case, LC-MS provides both the sensitivity and the specificity for measurement of both phytoestrogens and their metabolites.

For example, lignan samples (50  $\mu$ l) are separated on a 150  $\times$  3.0-mm i.d.  $C_{18}$  column at a flow rate of 0.4 ml/min at 40°. The mobile phases consist of water (A) and methanol (B). The gradient is 0–0.5 min, 30% B; 0.5–12 min, linear gradient from 30 to 50% B; 12–15 min, isocratic at 50% B; 15–15.2 min, linear return to 30% B; and 15.2–19 min, isocratic at 30% B to equilibrate. The divert valve allows flow into the mass spectrometer from 8 to 19 min. A Micromass Quatro mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source is operated in

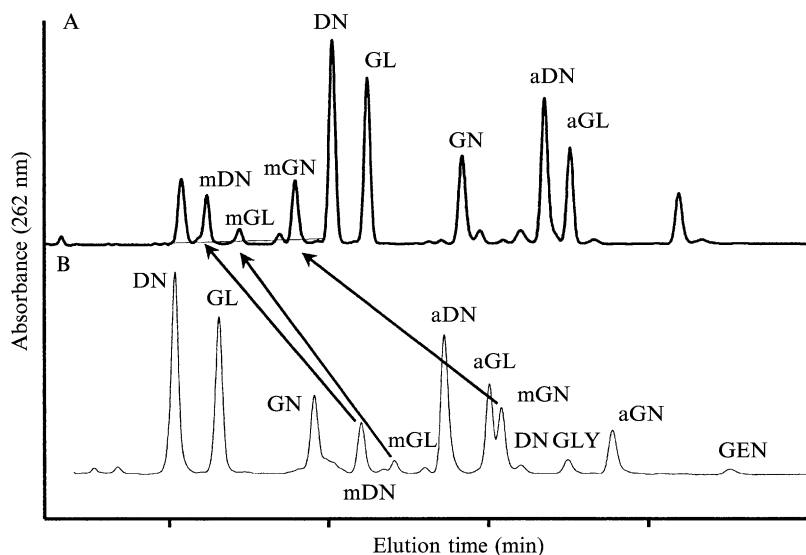


FIG. 5. Reversed-phase, gradient elution LC analysis of isoflavones and their conjugates in soy foods. Isoflavones were eluted with a 0–50% linear gradient of acetonitrile. In A, the background solvent is 10 mM ammonium acetate, whereas in B, it is 0.1% trifluoroacetic acid. The peaks are DN, daidzin; GL, glycitin; GN, genistin; mDN, malonyldaidzin; mGL, malonylglycitin; mGEN, malonylgenistin; and, aDN, acetyldaidzin; aGL, acetylglycitin; mGN, malonylgenistin; GEN, genistein. The mobility of the malonyl glycosides is much higher in A than in B.

the negative mode. For quantification, parent ion/product ion pairs are monitored using LC-MRM-MS analysis.

## Gastrointestinal Tract Hydrolysis

### *Deconjugation by Intestinal Lactase Phlorizin Hydrolase (LPH)*

Lactase phlorizin hydrolase is a membrane-spanning enzyme on the luminal side of the brush border of the small intestine. LPH is responsible for the hydrolysis of lactose, the main carbohydrate in mammalian milk. Structure studies have shown a second active site that is capable of hydrolyzing  $\beta$ -glycosylceramide (another component of milk) and phlorizin, a dihydrochalcone glycoside (Day *et al.*, 2000). Structural similarities among dihydrochalcones, flavonoids, and isoflavonoids led to the discovery that LPH has a role in the hydrolysis of the  $\beta$ -glycosides of these compounds (Day *et al.*, 2000; Wilkinson *et al.*, 2003).

*LPH Enzyme Assay.* Phytoestrogen glycosides (100  $\mu\text{M}$ ) are incubated in a volume of 0.1 ml with LPH (final concentration 1  $\mu\text{g}/\text{ml}$ ) in phosphate buffer, pH 6, for 20 min at 37°. The reaction is terminated by the addition of 0.15 ml acetonitrile–water (30/70, v/v) followed by centrifugation at 13,600g for 10 min at 4°. The supernatant is filtered through 0.22- $\mu\text{m}$  PTFE filter units and analyzed by reversed-phase HPLC. For kinetic studies, various concentrations of phytoestrogen are added to LPH (final concentration 1  $\mu\text{g}/\text{ml}$ ) and phosphate buffer in a volume of 0.2 ml. Samples are incubated at 37° for 5–30 min during the linear phase of the reaction (depending on the substrate and its concentration). Reactions are terminated by the addition of 0.3 ml acetonitrile–water and analyzed by reversed-phase HPLC (Day *et al.*, 2000). The amount of substrate converted is calculated from standard curves of peak areas produced by injecting known amounts of various phytoestrogens directly onto the column.

HPLC analysis is carried out using a 250  $\times$  4.6-mm i.d. column packed with Prodigy 5  $\mu\text{m}$  ODS-3 reversed-phase silica (Phenomenex, Torrance, CA). A gradient sequence using solvents A (water–tetrahydrofuran–trifluoroacetic acid, 98:2:0.1, v/v) and B (acetonitrile) is run at a flow rate of 1 ml/min with 17% B for 2 min, increasing to 25% over 5 min, 35% B for 8 min, 50% B for 5 min, and then to 100% B over 5 min. A column clean-up step maintains B at 100% for 5 min followed by a reequilibration at 17% B (15 min). A diode array method is used to monitor the eluant (at 270 and 370 nm). The appropriate phytoestrogen glycoside for each enzyme kinetic analysis is used as an external standard at concentrations ranging from 0 to 250  $\mu\text{M}$  (400  $\mu\text{M}$  for daidzin). The product in each of the reactions is confirmed by coelution of peak with standard compounds and by matching UV spectra.

*Metabolism in the Cannulated Everted Sac Model* (Wilkinson *et al.*, 2003). Cannulated everted sacs of rat proximal jejunum are prepared from segments (5 cm) of male Wistar rat small intestine. The everted jejunal sacs, ligated at one end, are tied onto a 1-ml tapered disposable syringe precharged with 0.5 ml Krebs bicarbonate buffer (pH 7.2–7.4). The sacs are filled with Krebs buffer by depressing the plunger and can be emptied with minimal contamination of the serosal solutions by the reverse operation at the end of the experiment (Gee *et al.*, 1998). Daidzein and daidzin are dissolved in ethanol containing 2–3% (v/v) DMSO and then diluted to 1, 10, and 100  $\mu\text{M}$  final concentrations using Krebs bicarbonate buffer (pH 7.3). Sacs are suspended in organ baths containing 8 ml Krebs buffer with or without isoflavone and incubated for 15 min at 37° with continuous aeration using oxygen:carbon dioxide (95:5). After incubation, the sacs are rinsed thoroughly using physiological saline (0.9% NaCl). The serosal

solutions are then withdrawn from the sacs, transferred to Eppendorf tubes, and immediately stored at  $-20^{\circ}$ . The mucosal solutions are also collected at the end of the incubation period and stored as described earlier. Samples are analyzed for aglycones by ELISA and HPLC.

To assess the role of the glucose transporters, incubations are carried out with daidzin or daidzein ( $100 \mu\text{M}$ ) in the absence or presence of  $250 \mu\text{M}$  *N*-(*n*-butyl)-deoxygalactonojirimycin (NB-DGJ), an inhibitor of LPH. A control incubation is carried out in Krebs buffer with inhibitor present but no isoflavonoid. Mucosal and serosal fluids are collected, frozen, and analyzed by ELISA and HPLC. Analysis of samples by ELISA is performed according to Creeke *et al.* (1998) using a fresh polyclonal antidaidzein serum.

Mucosal samples from the control (no isoflavones) and test daidzein and daidzin ( $10$  and  $100 \mu\text{M}$ ) incubations are prepared for HPLC analysis by purification using a polyamide column. Briefly, a  $0.3\text{-ml}$  sample is added to a preconditioned column and the column is eluted with  $100\%$  methanol followed by methanol/ammonium hydroxide ( $99.5:0.5\%$ , v/v) to isolate neutral and acidic fractions, respectively. Both fractions are evaporated to dryness and reconstituted in methanol ( $1.0$  and  $0.2 \text{ ml}$ ).

In addition, serosal solutions from incubations in the absence and presence of the isoflavonoids ( $100 \mu\text{M}$ ) are prepared with or without  $\beta$ -glucuronidase ( $25 \text{ U}$ ) treatment as follows. Duplicate aliquots of serosal fluid ( $50$  or  $100 \mu\text{l}$ ) are spiked with genistein ( $250 \text{ ng}$ ,  $5 \mu\text{l}$ ) as an internal standard. An equivalent volume of Krebs buffer is added to one subsample followed by an equal volume of  $0.8 \text{ mM}$  ascorbic acid in methanol.  $\beta$ -Glucuronidase ( $25 \text{ U}/25 \mu\text{l}$ ) is added to the second aliquot, and the sample is incubated at  $37^{\circ}$  for  $2 \text{ h}$ . An equal volume of the methanol/ascorbic acid solution is again added. Both subsamples are evaporated and reconstituted in methanol ( $0.2 \text{ ml}$ ) before HPLC analysis.

Mucosal and serosal samples are analyzed by HPLC as described (Day *et al.*, 2000). Peak identification is confirmed by coelution of peaks with authentic standard compounds by matching UV spectra and by positive ion electrospray LC/MS, such as on a Micromass Quattro II mass spectrometer equipped with a Z-spray ion source. Samples are introduced using a Hewlett Packard 1050 HPLC equipped with a diode array detector. Eluent flow ( $1 \text{ ml}/\text{min}$ ) is split between the diode array detector and the mass spectrometer ion source. Selected ion-monitoring measurements are performed for  $m/z$  255.07 (daidzein  $[\text{M}+\text{H}]^{+}$ ), 271.06 (genistein  $[\text{M}+\text{H}]^{+}$ ), 417.12 and 439.10 (daidzin  $[\text{M}+\text{H}]^{+}$ ,  $[\text{M}+\text{Na}]^{+}$ ), 431.10 and 453.08 (daidzein-7-*O*- $\beta$ -glucuronide  $[\text{M}+\text{H}]^{+}$ ,  $[\text{M}+\text{Na}]^{+}$ ). Diode array spectra are scanned from  $190$  and  $450 \text{ nm}$ , with an interval of  $2 \text{ nm}$ . Instrument control, data acquisition, and processing are performed using a Microssoft MassLynx version 3.4 system.

## Metabolism of Phytoestrogens in the Liver

*Microsomal Hydroxylation of Isoflavones (Fig. 6)*

Rat liver microsomes (2 mg protein) are incubated with 50 nmol of isoflavone dissolved in 40  $\mu$ l DMSO and a NADPH-generating system (3 mM MgCl<sub>2</sub>, 1 mM NADP<sup>+</sup>, 8 mM D,L-isocitrate, and 0.5 U of isocitrate dehydrogenase) in 2 ml of 0.05 M potassium phosphate buffer, pH 7.4 (Kulling *et al.*, 2000, 2001). Microsomal reactions are preincubated for 2 min at 37° in a shaking water bath. Reactions are initiated with addition of the NADPH-generating system at 37° and terminated after 60 min by extracting with 4  $\times$  2 ml of ice-cold ethyl acetate. The organic solvent layers are pooled and dried under reduced pressure at room temperature. Dried residues are resolubilized in 0.2 ml of methanol and analyzed by HPLC. Controls consist of either heat-inactivated microsomes or reaction mixtures without the NADPH-generating system.

Hu *et al.* (2003) used a similar protocol with the addition of monoclonal antibodies to different cytochrome P450s (CYP450s) to elucidate the CYP450 responsible for the hydroxylation and demethylation of isoflavones.

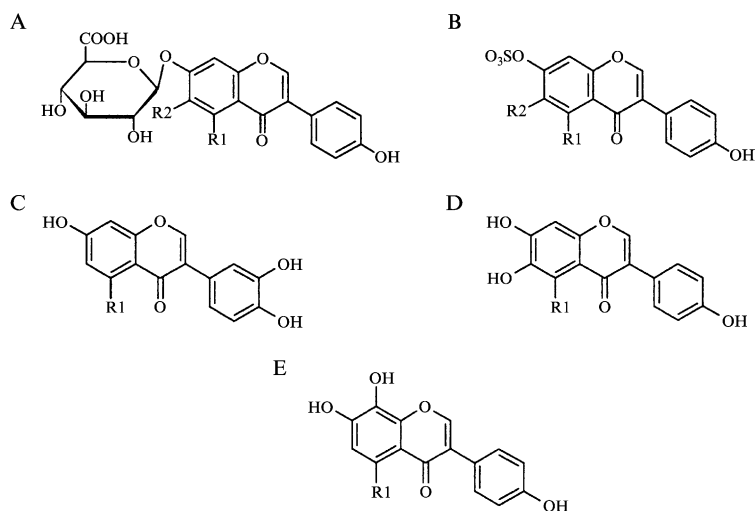


FIG. 6. Chemical structures of isoflavone metabolites in physiological fluids. (A)  $\beta$ -Glucuronides, (B) sulfate esters, and (C–E) hydroxylated metabolites. Individual isoflavones are daidzein (R1, R2 = H), glycitein (R1 = H, R2 = OCH<sub>3</sub>), and genistein (R1 = OH, R2 = H).

### *Glucuronidation of Phytoestrogens (Fig. 6)*

*In Vitro Glucuronidation of Phytoestrogens.* The *in vitro* method described by Doerge *et al.* (2000) is well suited for phytoestrogens. Enzyme studies using purified UDP-glucuronosyltransferase (UGT) consist of reaction mixtures containing the following: 0.08 to 0.32 mg/ml UGT, 0 to 400  $\mu$ M (final concentration) daidzein or genistein, and 5 to 10 mM MgCl<sub>2</sub>. An inhibitor of  $\beta$ -glucuronidase,  $\gamma$ -saccharolactone (10 mM), is added to the incubation to prevent hydrolysis of  $\beta$ -glucuronides. The reactions are initiated by the addition of 0.08 to 3 mM uridine-5'-diphospho- $\beta$ -D-glucuronic acid ester (UDPGA) in 0.05 M Tris-HCl, pH 7.4–7.5 (for UGT isozymes), or 0.1 M phosphate buffer, pH 8.0 [for bovine hepatic UGT or microsomes prepared from liver, kidney, and colon samples (0.1–0.25 mg/ml)], in a final volume of 125  $\mu$ l for 2 h at 37°. The reactions are terminated with an equal volume of methanol followed by brief vortexing and centrifugation at 10,000g for 10 min. Products are analyzed by reversed-phase LC with UV detection (260 nm for genistein) on a 5- $\mu$ m Prodigy ODS-3 4.6  $\times$  230-mm column (Phenomenex Co., Torrance, CA). The solvent system consists of solvents A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The gradient starts at 95% A and 5% B for 3 min followed by a linear gradient to 50% A and 50% B over 15 min and isocratic elution at 50% A and 50% B for 5 min. The flow rate is 1.0 ml/min.

*In Vivo Glucuronide Conjugation.* Conjugation *in vivo* can be carried out using the everted intestinal sac preparation (Sfakianos *et al.*, 1997; Wilkinson *et al.*, 2003) or the bile duct cannulation model (Sfakianos *et al.*, 1997). In the latter, the common bile duct of anesthetized rats is exposed and occluded distally with surgical silk. A polyethylene (PE-10) cannula is introduced into the common bile duct through a small opening made with eye scissors and tied in place with surgical silk. Rats are particularly useful because they do not have a gallbladder and therefore bile can be collected quantitatively. The phytoestrogen aglycone is dissolved in a small volume of DMSO and diluted with physiological saline. It is introduced into either the femoral vein with a bevel-edged PE-10 cannula or the portal vein with a 27-gauge needle inserted into a PE-10 cannula. The veins are exposed, temporarily occluded with surgical silk, and the tubing inserted into the vein. The tubing is held in place with surgical cement. The phytoestrogen is slowly infused into the vein; bile is collected simultaneously. During the experiment the body temperature of the rats is maintained at 38° with a heating pad.

*Analysis of Biliary Phytoestrogen Glucuronides.* Verification of the identity of phytoestrogen  $\beta$ -glucuronides in bile can be carried out by treating the bile sample with  $\beta$ -glucuronidase (in 100 mM ammonium

acetate buffer, pH 5.0) and carrying out reversed-phase HPLC analysis of the products. The putative  $\beta$ -glucuronide peak will disappear and there will be a new peak eluting with the same retention time as the expected isoflavone aglycone.

An alternative method is to carry out LC-MS analysis. Bile extracts are separated by reversed-phase HPLC on a  $15 \times 0.21$ -cm i.d. Brownlee Aquapore C<sub>8</sub> column using a linear 0–50% gradient (5%/min) of acetonitrile in 10 mM ammonium acetate at a flow rate of 0.2 ml/min. The column eluate is split 1:1, and one stream is passed into the IonSpray interface of a PE-Sciex (Concord, ON, Canada) API III triple quadrupole mass spectrometer operating in the negative ion mode, with an orifice potential of –60 V. In the MS-MS mode, daughter ion spectra are obtained by selecting parent ions in the first quadrupole, which are then collided with argon/10% nitrogen gas in the second quadrupole and analyzed in the third quadrupole.

When the expected molecular  $[M-H]^-$  ion  $m/z$  445 for genistein  $\beta$ -glucuronide is searched for, a complex chromatographic peak is observed (Fig. 7A) (Sfakianos *et al.*, 1997). However, by repeating the analysis and carrying out tandem mass spectrometry on the  $m/z$  445 ion, the correct  $\beta$ -glucuronide peak can be discerned with the expected  $m/z$  269 aglycone ion (Fig. 7B). The other  $m/z$  445 peak produces  $m/z$  80 and 97 daughter ions, suggesting that it is a phosphate or sulfate conjugate.

*Isolation of Biliary Phytoestrogen  $\beta$ -Glucuronides.* Purification of phytoestrogen conjugates from bile is carried out by chromatography using Sephadex LH-20 (Coward *et al.*, 1996; Sfakianos *et al.*, 1997). Pooled bile obtained after infusions with genistein is diluted with 50% aqueous ethanol and chromatographed on a  $15 \times 3$ -cm i.d. Sephadex LH-20 column pre-equilibrated with 50% aqueous methanol. Fractions containing  $\beta$ -glucuronides are passed over a  $5 \times 1.5$ -cm i.d. diethylaminoethyl-Sephadex (DEAE-Sephadex) column equilibrated with 70% aqueous methanol. Excess reagents are removed by washing with 70% aqueous methanol–0.2 M acetic acid. The  $\beta$ -glucuronide is eluted with 0.3 M LiCl in 70% aqueous methanol. The eluate is dried to remove the methanol; ammonium acetate (150 mM), pH 5, and triethylammonium sulfate (0.5 M) are added and the mixture is passed over several Sep-Pak C<sub>18</sub> cartridges to adsorb the  $\beta$ -glucuronide. LiCl is removed by washing the cartridges with water. The  $\beta$ -glucuronide is eluted from the cartridges with several column volumes of methanol. Because the phytoestrogen glucuronides derived this way are heavily contaminated with the bile salt taurocholate (as determined by negative ion electrospray mass spectrometry), the partially purified material is treated with cholyglycine hydrolase to convert taurocholate to cholic acid and taurine. These can be removed by rechromatography of

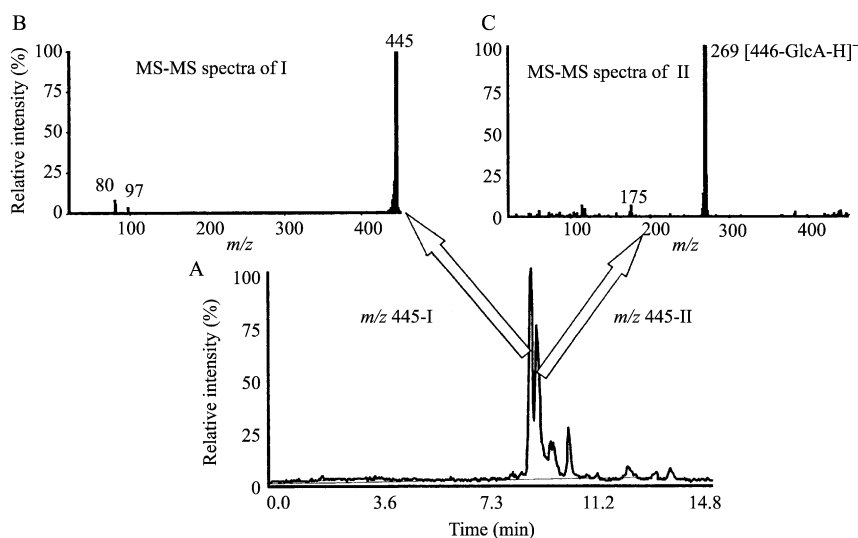


FIG. 7. LC-ESI-MS of genistein metabolites in rat bile. In A, the selected ion chromatogram for  $m/z$  445 (the molecular  $[M-H]^-$  ion for genistein 7-*O*- $\beta$ -glucuronide) contains several peaks. Daughter ion spectra (B, C) of the two major peaks are quite distinct. The mass spectrum of the later eluting peak corresponded to genistein 7-*O*- $\beta$ -glucuronide.

the partially purified material on the Sephadex LH-20 column. Fractions containing the phytoestrogen glucuronide are finally purified by preparative reversed-phase HPLC. Depending on the gender of the animal, sulfate esters of the phytoestrogen may also be present. Bile from female rats contains only  $\beta$ -glucuronides of the phytoestrogens (Coward *et al.*, 1996).

#### *Sulfation of Phytoestrogens (Fig. 6)*

The method described by Doerge *et al.* (2000) is the preferred method. Individual sulfotransferases, SULT 1A1\*2, 1A2\*1, 1A3, and 1E, have been studied. The enzyme is diluted to 220 (1A1\*2), 180 (1A2\*1), 400 (1A3), or 100 (1E) ng/ml in a prechilled solution containing 5 mM phosphate buffer, pH 6.5, 1.5 mg/ml bovine serum albumin, and 10 mM dithiothreitol (DTT). To start the reaction, 100  $\mu$ l of diluted SULT is mixed with 50  $\mu$ l of 25 mM phosphate buffer, pH 6.5, containing 25 mM DTT, 1.28  $\mu$ M adenosine-3'-phosphate 5'-phosphosulfate, and various concentrations of phytoestrogens (0–400  $\mu$ M) in a final volume of 200  $\mu$ l. The mixtures are incubated at 37° for 2 h and analyzed by LC-UV (as described for

the UGT enzyme). Reactions containing SULT 2A1 and 1E also contain 0.25 mM MgCl<sub>2</sub>.

In a similar manner for the phytoestrogen  $\beta$ -glucuronides, identification of phytoestrogen sulfate esters can be carried out by reversed-phase HPLC and/or LC-ESI-MS. The expected molecular ion for the sulfate ester is 80 Da above that of the aglycone. For genistein-7-sulfate it is  $m/z$  349 in the negative ion mode. Electrospray ionization is preferred over heated nebulizer chemical ionization, which circumvents the effects of heating in this ionization process.

### Metabolism of Isoflavones in Mammary Cell Lines

While Akiyama *et al.* (1987) demonstrated that biochanin A inhibits PTK activity 30-fold less effectively than genistein in cell-free systems, Peterson and Barnes (1991) reported that biochanin A and genistein inhibited breast cancer cell growth approximately equally in cell culture. Peterson *et al.* (1996) later showed that the metabolism of these compounds by human breast cancer MCF-7 cells was the reason for the discrepancy between cell-free and cell culture experiments. MCF-7 cells are able to rapidly demethylate biochanin A to genistein and to slowly sulfate genistein to genistein-7-sulfate (Peterson *et al.*, 1996, 1998). The following studies detail the methodologies employed to evaluate this type of metabolism.

### *Metabolism Studies of Isoflavones in Cells from the Mammary*

Mammary cells (transformed human breast cancer MCF-7 cell line or cultured normal human mammary epithelial cells) are plated in 6-well plates and grown to 70% confluence. [4-<sup>14</sup>C]Biochanin A or [4-<sup>14</sup>C]genistein is added to the cells at a final concentration of 1  $\mu$ g/ml in 100% DMSO [0.5% (v/v) final (DMSO) concentration] (0.083 and 0.087  $\mu$ Ci/ml for biochanin A and genistein, respectively). Blank wells containing media and DMSO but no cells are used as controls. After incubation for the indicated times, media are collected and the cells are scraped and collected in 2 ml modified proteinase K buffer (25 mM EDTA, 137 mM NaCl, and 10 mM Tris-HCl, pH 7.6). Cell lysates are sonicated and incubated with proteinase K (0.1 mg/ml) for 4 h at 37°. Aliquots (100  $\mu$ l) of media and lysates are sampled for radioactivity analysis by scintillation counting. The remainder of the samples are passed through Sep-Pak C<sub>18</sub> cartridges to collect the [4-<sup>14</sup>C]isoflavones and their metabolites. The cartridges are washed with 15 ml of distilled water to remove hydrophilic materials. Hydrophobic compounds are eluted with 10 ml of 80% aqueous methanol and dried at room temperature with air. Samples are resuspended in 100  $\mu$ l

80% aqueous methanol and analyzed by reversed-phase HPLC. Samples are injected onto a 30 cm  $\times$  4.6 mm i.d. Aquapore C<sub>8</sub> column, which is eluted at a flow rate of 1 ml/min with a mobile phase consisting of a linear gradient of 0–45% acetonitrile (at 4.5%/min) in 0.1% (v/v) aqueous trifluoroacetic acid. Eluted substances are detected by their absorbance at 262 nm. The column eluate is collected in 30-s fractions and counted to determine the location of radioactive peaks using liquid scintillation counting.

Media extracts or individual radioactive metabolite peaks are analyzed by reversed-phase HPLC-MS as described earlier for the analysis of biliary isoflavone metabolites. In the case of mammary tumor cells, the principal metabolites are sulfate esters of phytoestrogens. Identification of the site of sulfation is possible by <sup>1</sup>H-NMR. The sulfate esters are isolated by preparative HPLC and dried thoroughly. When they are dissolved in d<sub>6</sub>-DMSO, protons on the ring hydroxyl groups remain. These appear as singlets with marked downfield chemical shifts in the <sup>1</sup>H-NMR spectrum. The one that disappears from the spectrum readily allows identification of the site of sulfation. The amount of sulfotransferase activity varies considerably among the tumor cells, being the highest in ZR-75-1 cells (Peterson *et al.*, 1998). The cells also exhibit marked demethylation of biochanin A.

### Metabolism of Phytoestrogens by Products of Inflammatory Cells

Phytoestrogens described earlier all contain phenyl ring systems. This makes them capable of undergoing chemical reaction with highly reactive products of activated inflammatory cells. The latter generate hypohalogenic acids (HOCl, HOBr, and HOI) and peroxynitrite (OONO<sup>-</sup>) once stimulated. These proinflammatory oxidants react with phenyl groups containing hydroxyl substituents. Proteins from sites of inflammation contain 3-chlorotyrosine and 3-nitrotyrosine in patients with atherosclerotic lesions. Genistein and daidzein both undergo chlorination and nitration when added to the medium of stimulated inflammatory cells (Boersma *et al.*, 1999, 2003; D'Alessandro *et al.*, 2003). Several isoflavones are converted to similar iodo derivatives when incubated with thyroid peroxidase, H<sub>2</sub>O<sub>2</sub>, and sodium iodide (Divi *et al.*, 1997) as are many other polyphenols (Divi and Doerge, 1996) (Fig. 8).

### *Synthesis of Chlorinated and Nitrated Phytoestrogens*

Solutions of phosphate-buffered saline with 1 mM diethylenetriamine pentaacetic acid (DTPA) with 50  $\mu$ M of each isoflavone are reacted with HOCl (0 to 500  $\mu$ M). Aliquots of a stock solution of 10 mM HOCl are

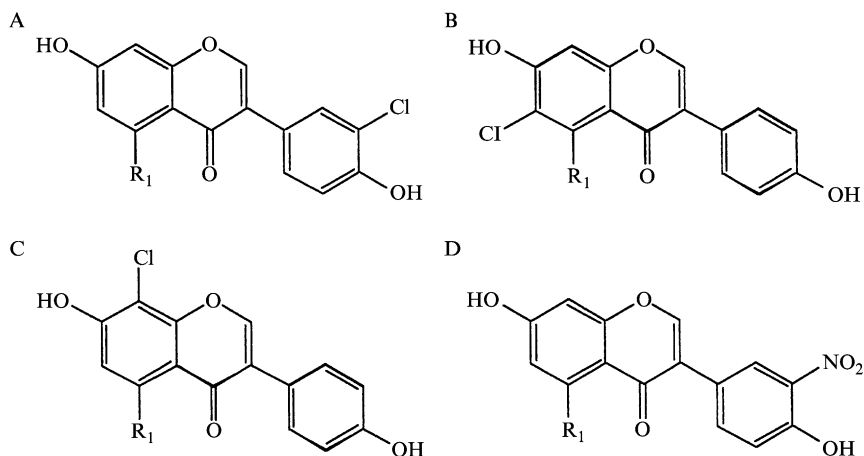


FIG. 8. Sites of chlorination and nitration in isoflavones. For daidzein  $R_1 = H$ , whereas for genistein  $R_1 = OH$ . In A, chlorination is in the 3' position; in B, it is in the 6 position, and in C it is in the 8 position. Nitration occurs in the 3' position (D).

added to the reaction mixture with continuous mixing to achieve the nominal concentration. Decomposed HOCl is made by reacting the acid with an equimolar solution of glutathione for 20 min (Winterbourn and Brennan, 1997). This mixture is added, while vortexing, to each isoflavone reaction mixture. Samples are extracted by adding 200  $\mu\text{l}$  of the reaction mixture to 800  $\mu\text{l}$  of water followed by 2 ml of diethyl ether. The samples are vortexed and then centrifuged at 3000g, whereupon the ethereal, upper layer is removed and the ether is evaporated under  $N_2$ . Prior to injection, 150  $\mu\text{l}$  80% methanol is added to redissolve the dried residues. HPLC analysis is carried out on a 4.6 mm  $\times$  25 cm C<sub>8</sub> Aquapore reversed-phase column with a linear 0–50% gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. Isoflavones and their chlorinated derivatives are detected by their absorbance at 262 nm.

Analysis of chlorinated isoflavones by LC-MS reveals that addition of one chlorine atom increases the observed  $m/z$  by 34 or 36. Indeed, the relative amount of the chlorine isotopomers is a recognizable characteristic of a chlorinated compound. Also, several chlorinated isomers of each isoflavone are formed (Prasain *et al.*, 2003a). The MS-MS spectrum of 3'-chlorogenistein is quite distinct from that of 6- or 8-chlorodaidzein. Dichloro derivatives of isoflavones (Boersma *et al.*, 1999) and quercetin (Binsack *et al.*, 2001) have been reported; these have an increase in  $m/z$  of

68, 70, and 72. Specific isomers of chlorodaidzein (3', 6-, and 8-monochlorodaidzein, and 3',6- and 3',8-dichlorodaidzein) have been synthesized by Dr. Nigel Botting, University of St. Andrew's, Fife, Scotland.

To prepare nitrated isoflavones, buffered solutions of 200 mM sodium phosphate (pH 7.4) with 1 mM DTPA and 50  $\mu\text{M}$  of each isoflavone are reacted with  $\text{OONO}^-$  (0 to 500  $\mu\text{M}$ ). Aliquots of a 10 mM  $\text{OONO}^-$  stock solution are added to the reaction mixture with continuous mixing to obtain the final concentration. Decomposed  $\text{OONO}^-$  is prepared by adding the  $\text{OONO}^-$  to the buffer solution (pH 7.4) and allowing this to react for 10 min. The isoflavones are then added to the decomposed reaction mixture with continuous mixing. Genistein can also be nitrated by adding 500  $\mu\text{M}$  tetranitromethane to 50  $\mu\text{M}$  genistein in a buffer solution composed of a 1:1 mixture of sodium bicarbonate (50 mM, pH 9)/50% ethanol with 1 mM DTPA. To carry out reduction of nitrated groups,  $\text{Na}_2\text{S}_2\text{O}_4$  (60  $\mu\text{M}$ ) is added to the nitrated isoflavones. Following each reaction, samples are extracted as described previously and subjected to HPLC and MS analysis. Changes in the UV absorption of the isoflavones are monitored at 262 nm. Mixing both HOCl and sodium nitrite with genistein resulted in a monochloronitrogenistein and a dichloronitrogenistein (Boersma *et al.*, 1999). Nitration causes an increase in  $m/z$  of 45, whereas for chloronitration it is 79 and 81. For dichloronitration, the increase is 113, 115, and 117.

#### *Formation of Chlorinated and Nitrated Isoflavones by Inflammatory Cells*

Either the human leukemia cell line (HL-60) or isolated polymorphonuclear cells (PMNs) can be used for this experiment. When using HL-60 cells, the cells are cultured in the presence of 1.3% DMSO for 1 week in order for them to differentiate into neutrophil-like cells (Boersma *et al.*, 2003), whereas PMNs are isolated using a Histopaque dual-phase gradient (D'Alessandro *et al.*, 2003). Cells are suspended in Krebs-Henseleit buffer (K-H: 118 mM NaCl, 27.3 mM  $\text{NaHCO}_3$ , 4.8 mM KCl, 1.75 mM  $\text{CaCl}_2$ , 1.0 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4$ , 11.1 mM glucose, pH 7.4) at  $1 \times 10^6$  cells/ml and activated with 10  $\mu\text{M}$  phorbol 12-myristate-13-acetate (PMA) either in the presence or in the absence of 10  $\mu\text{M}$  genistein/daidzein and 50  $\mu\text{M}$   $\text{NaNO}_2$ . Reaction occurs for 60 min at 37° followed by termination using 5 U/ml catalase and a 10-min incubation on ice. The cells are centrifuged at 800g at 4° and the supernatant (~950  $\mu\text{l}$ ) is extracted with diethyl ether (2 ml). The samples are vortexed and centrifuged at 2000g, whereupon the ethereal top layer is removed to a 13  $\times$  100-mm glass tube. Ether extraction

is repeated until a total volume of 6 ml of ether has been added. Ether extracts are combined and dried to completion under air. The dried extracts are redissolved in 100  $\mu$ l 80% aqueous methanol and analyzed by liquid chromatography–multiple reaction monitoring–mass spectrometry (LC-MRM-MS) (Boersma *et al.*, 2003; D'Alessandro *et al.*, 2003).

#### *Analysis of Chlorinated and Nitrated Isoflavones*

Reaction mixtures are analyzed by LC–MS as described earlier except that the mobile phase is isocratic, composed of 40% acetonitrile in 10 mM  $\text{NH}_4\text{OAc}$  at a flow rate of 1.0 ml/min. To obtain quantitative data, specific parent ion product ion combinations are used in LC-MRM-MS analysis. After termination of the reaction, another phytoestrogen is used as the internal standard. For example, in the case of experiments examining the reaction of genistein, daidzein is added as the internal standard. A series of samples prepared with several known concentrations of the varying phytoestrogen and a single concentration of the internal standard are analyzed to generate an area response–concentration curve. These typically give correlation coefficients of 0.98 or better for a five-point curve (D'Alessandro *et al.*, 2003). Typically, concentrations as low as 5 nM can be measured, although newer triple quadrupole mass spectrometers are capable of quantitatively detecting as little as 10–50 fmol.

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#### References

- Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., and Fukami, Y. (1987). Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* **262**, 5592–5595.
- Barnes, S., Kirk, M., and Coward, L. (1994). Isoflavones and their conjugates in soy foods: Extraction conditions and analysis by HPLC-mass spectrometry. *J. Agric. Food Chem.* **42**, 2466–2474.
- Bednarek, P., Franski, R., Kerhoas, L., Einhorn, J., Wojtaszek, P., and Stobiecki, M. (2001). Profiling changes in metabolism of isoflavonoids and their conjugates in *Lupinus albus* treated with biotic elicitor. *Phytochemistry* **56**, 77–85.

- Binsack, R., Boersma, B. J., Patel, R. P., Kirk, M. C., White, C. R., Darley-USmar, V. M., Barnes, S., Zhou, F., and Parks, D. A. (2001). Enhanced antioxidant activity following chlorination of quercetin by hypochlorous acid. *Alcohol. Clin. Exp. Res.* **25**, 434–443.
- Boersma, B. J., Patel, R. P., Kirk, M., Jackson, P. L., Muccio, D., Darley-USmar, V. M., and Barnes, S. (1999). Chlorination and nitration of soy isoflavones. *Arch. Biochem. Biophys.* **368**, 265–275.
- Boersma, B. J., D' Alessandro, T., Benton, M. R., Kirk, M., Wilson, L. S., Prasain, J., Botting, N. P., Barnes, S., Darley-USmar, V. M., and Patel, R. P. (2003). Neutrophil myeloperoxidase chlorinates and nitrates soy isoflavones and enhances their antioxidant properties. *Free Radic. Biol. Chem.* **35**, 1317–1330.
- Clarke, D. B., Lloyd, A. S., Botting, N. P., Oldfield, M. F., Needs, P. W., and Wiseman, H. (2002). Measurement of intact sulfate and glucuronide phytoestrogen conjugates in human urine using isotope dilution liquid chromatography-tandem mass spectrometry with [<sup>13</sup>C<sub>3</sub>] isoflavone internal standards. *Anal. Biochem.* **309**, 158–172.
- Coward, L., Barnes, N. C., Setchell, K. D. R., and Barnes, S. (1993). Genistein, daidzein, and their  $\beta$ -glycoside conjugates: Antitumor isoflavones in soybean foods from American and Asian diets. *J. Agric. Food Chem.* **41**, 1961–1967.
- Coward, L., Kirk, M., Albin, N., and Barnes, S. (1996). Analysis of plasma isoflavones by reversed-phase HPLC-multiple reaction ion monitoring-mass spectrometry. *Clin. Chem. Acta* **247**, 121–142.
- Coward, L., Smith, M., Kirk, M., and Barnes, S. (1998). Chemical modification of isoflavones in soyfoods during cooking and processing. *Am. J. Clin. Nutr.* **68**, 1486S–1491S.
- Creeke, P. I., Wilkinson, A. P., Lee, H. A., Morgan, M. R. A., Price, K. R., and Rhodes, M. J. C. (1998). Development of ELISAs for the measurement of the dietary phytoestrogens daidzein and equol in human plasma. *Food Agric. Immunol.* **10**, 325–333.
- D' Alessandro, T., Prasain, J., Benton, M. R., Botting, N., Moore, R., Darley-USmar, V., Patel, R., and Barnes, S. (2003). Polyphenols, inflammatory response, and cancer prevention: Chlorination of isoflavones by human neutrophils. *J. Nutr.* **133**, 3773S–3777S.
- Day, A. J., Cañada, F. J., Díaz, J. C., Kroon, P. A., Mclauchlan, R., Faulds, C. B., Plumb, G. W., Morgan, M. R. A., and Williamson, G. (2000). Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. *FEBS Lett.* **468**, 166–170.
- Divi, R. L., Chang, H. C., and Doerge, D. R. (1997). Anti-thyroid isoflavones from soybean: Isolation, characterization, and mechanisms of action. *Biochem. Pharmacol.* **54**, 1087–1096.
- Divi, R. L., and Doerge, D. R. (1996). Inhibition of thyroid peroxidase by dietary flavonoids. *Chem. Res. Toxicol.* **9**, 16–23.
- Doerge, D. R., Chang, H. C., Churchwell, M. I., and Holder, C. L. (2000). Analysis of soy isoflavone conjugation *in vitro* and in human blood using liquid chromatography-mass spectrometry. *Drug Metab. Dispos.* **28**, 298–307.
- Franke, A. A., and Custer, L. J. (1994). High-performance liquid-chromatographic assay of isoflavonoids and coumestrol from human urine. *J. Chromatogr. B.* **662**, 47–60.
- Gee, J. M., DuPont, M. S., Rhodes, M. J. C., and Johnson, I. T. (1998). Quercetin glycosides interact with the intestinal glucose transport pathway. *Free Radic. Biol. Chem.* **25**, 19–25.
- Griffith, A. P., and Collison, M. W. (2001). Improved methods for the extraction and analysis of isoflavones from soy-containing foods and nutritional supplements by reversed-phase high-performance liquid chromatography and liquid chromatography-mass spectrometry. *J. Chromatogr. A.* **913**, 397–413.

- He, X.-G., Lin, L.-Z., and Lian, L.-Z. (1996). Analysis of flavonoids from red clover by liquid chromatography-electrospray mass spectrometry. *J. Chromatogr. A* **755**, 127–132.
- Hu, M., Krausz, K., Chen, J., Ge, X., Li, J., Gelboin, H. L., and Gonzalez, F. J. (2003). Identification of CYP1A2 as the main isoform for the phase I hydroxylated metabolism of genistein and a pro-drug converting enzyme of methylated isoflavones. *Drug Metab. Dispos.* **31**, 924–931.
- Jacobs, M. N., and Lewis, D. F. (2002). Steroid hormone receptors and dietary ligands: A selected review. *Proc. Nutr. Soc.* **61**, 105–122.
- Kinoshita, E., Ozawa, Y., and Aishima, T. (1997). Novel tartaric acid isoflavone derivatives that play a key role in differentiating Japanese soy sauces. *J. Agric. Food Chem.* **45**, 3753–3759.
- Kudou, S., Fleury, Y., Welti, D., Magnolato, D., Uchida, T., Kitamura, K., and Okubo, K. (1991). Malonyl isoflavone glycosides in soybean seeds (*Glycine max* MERRILL). *Agric. Biol. Chem.* **55**, 2227–2233.
- Kulling, S. E., Honig, D. M., and Metzler, M. (2001). Oxidative metabolism of the soy isoflavones daidzein and genistein in humans *in vitro* and *in vivo*. *J. Agric. Food Chem.* **49**, 3024–3033.
- Kulling, S. E., Honig, D. M., Simat, T. J., and Metzler, M. (2000). Oxidative *in vitro* metabolism of the soy phytoestrogens daidzein and genistein. *J. Agric. Food Chem.* **48**, 4963–4972.
- Liggins, J., Bluck, L. J. C., Coward, W. A., and Bingham, S. A. (1998). Extraction and quantification of daidzein and genistein in food. *Anal. Biochem.* **264**, 1–7.
- Lila, M. A., Yousef, G. G., Jiang, Y., and Weaver, C. M. (2005). Sorting out bioactivity in flavonoid mixtures. *J. Nutr.* **135**, 1231–1235.
- Middleton, E., Jr., Kandaswami, C., and Theoharides, T. C. (2000). The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* **52**, 673–751.
- Milder, I. E. J., Arts, I. C. W., Venema, D. P., Lasaroms, J. J. P., Wahala, K., and Hollman, P. C. H. (2004). Optimization of liquid chromatography-tandem mass spectrometry method for quantification of the plant lignans secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol in foods. *J. Agric. Food Chem.* **52**, 4643–4651.
- Murphy, P. A. (1981). Separation of genistin, daidzin, and their aglycones and coumestrol by gradient high-performance liquid chromatography. *J. Chromatogr.* **211**, 166–169.
- Peterson, G., and Barnes, S. (1991). Genistein inhibition of the growth of human breast cancer cells: Independence from estrogen receptors and multi-drug resistance gene product. *Biochem. Biophys. Res. Commun.* **179**, 661–667.
- Peterson, T. G., Coward, L., Kirk, M., Falany, C. N., and Barnes, S. (1996). The role of metabolism in mammary epithelial cell growth inhibition by the isoflavones genistein and biochanin A. *Carcinogenesis* **17**, 1861–1869.
- Peterson, T. G., Ji, G.-P., Kirk, M., Coward, L., Falany, C. N., and Barnes, S. (1998). Metabolism of the isoflavones genistein and biochanin A in human breast cancer cell lines. *Am. J. Clin. Nutr.* **68**, 1505–1511.
- Pietta, P. G. (2000). Flavonoids as antioxidants. *J. Nat. Prod.* **63**, 1035–1042.
- Prasain, J. K., Boersma, B. J., Kirk, M., Wilson, L., Patel, R., Darley-USmar, V. M., Botting, N., and Barnes, S. (2003a). ESI-tandem mass spectrometric analysis of chlorinated and nitrated isoflavones. *J. Mass Spectrom.* **38**, 764–771.
- Prasain, J. K., Jones, K., Kirk, M., Wilson, L., Smith-Johnson, M., Weaver, C., and Barnes, S. (2003b). Profiling and quantification of isoflavonoids in kudzu dietary supplements by high-performance liquid chromatography and electrospray ionization tandem mass spectrometry. *J. Agric. Food Chem.* **51**, 4213–4218.

- Prasain, J. K., Wang, C.-C., and Barnes, S. (2002). Mass spectrometry in the analysis of phytoestrogens in biological samples. In "Phytoestrogens and Health" (G. S. Gilani and J. J. Anderson, eds.), pp. 147–177. AOCS Press, Champaign, IL.
- Prasain, J. K., Wang, C. C., and Barnes, S. (2004). Mass spectrometric methods for determination of flavonoids in biological samples. *Free Radic. Biol. Med.* **37**, 1324–1350.
- Sfakianos, J., Coward, L., Kirk, M., and Barnes, S. (1997). Intestinal uptake and biliary excretion of the isoflavone genistein in rats. *J. Nutr.* **127**, 1260–1268.
- Wang, C.-C., Prasain, J. K., and Barnes, S. (2002). Review of the methods used in the determination of phytoestrogens. *J. Chromatogr. B.* **777**, 3–28.
- Wang, H.-J., and Murphy, P. A. (1994a). Isoflavone content in commercial soybean foods. *J. Agric. Food Chem.* **42**, 1666–1673.
- Wang, H.-J., and Murphy, P. A. (1994b). Isoflavone composition of American and Japanese soybeans in Iowa: Effects of variety, crop year, and location. *J. Agric. Food Chem.* **42**, 1674–1677.
- Wilkinson, A. P., Gee, J. M., DuPont, M. S., Needs, P. W., Mellon, F. A., Williamson, G., and Johnson, I. T. (2003). Hydrolysis by lactase phlorizin hydrolase is the first step in the uptake of daidzein glycosides by rat small intestine *in vitro*. *Xenobiotica* **33**, 255–264.
- Wilkinson, A. P., Wahala, K., and Williamson, G. (2002). Identification and quantification of polyphenol phytoestrogens in foods and human biological fluids. *J. Chromatogr. B.* **777**, 93–109.
- Winterbourn, C. C., and Brennan, S. O. (1997). Characterization of the oxidation products of the reaction between reduced glutathione and hypochlorous acid. *Biochem. J.* **326**, 87–92.

## [20] Sulfation and Glucuronidation of Phenols: Implications in Coenzyme Q Metabolism

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### Abstract

Phase II conjugation of phenolic compounds constitutes an important mechanism through which exogenous or endogenous toxins are detoxified and excreted. Species differences in the rates of glucuronidation or sulfation can lead to significant variation in the metabolism of this class of compounds. Conjugation of the hydroxyl groups of phenols can occur with glucuronate or sulfate. Quinone metabolism, deactivation, and detoxification are also affected by the same conjugatory systems as phenols; however, reduction of quinones to hydroquinols seems to be a prerequisite. This work reviews current knowledge on phenol conjugation and its implications on hydroquinone metabolism with special consideration for coenzyme Q metabolism.