

# Formononetin—an Isoflavone Metabolite Found in the Liver of Rats Fed with Soy Protein Isolate

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## ABSTRACT

Soy protein isolate, containing genistein, daidzein and glycitein, was used to study the profiles of soy isoflavone metabolites in Sprague-Dawley rats. After 50 days, rat liver tissues, serum and urine were analyzed by HPLC-MS and HPLC-ECD. HPLC-APCI-MS results showed that daidzein, equol, genistein and *O*-DMA were detected. Interestingly, the aglycone and the conjugated forms of formononetin, which is a methylated derivative of daidzein found in many plants, were also observed in rat liver and serum, but not in urine. The respective concentrations of these two forms of formononetin were  $0.056 \pm 0.022$  and  $0.621 \pm 0.116$  nmol/g (mean  $\pm$  SD) in liver tissue, and were  $0.005 \pm 0.001$  and  $0.035 \pm 0.002$  nmol/mL in rat serum. Formononetin in conjugated forms were 90.8% in liver tissue and 85.7% in serum by calculation. Incubation of daidzein with liver homogenate, microsomes, and catechol 4'-*O*-methyl transferase (COMT) resulted in a 7~10% conversion of daidzein to the aglycone form of formononetin. Formononetin is proposed to be a metabolite of *O*-methylation of daidzein in rat liver and exists mainly in conjugated forms.

Key words: COMT, formononetin, homogenate, isoflavone, soy protein isolate

## INTRODUCTION

Formononetin (7-hydroxy-4'-methoxyisoflavone) is a major phytoestrogen found in alfalfa and clover sprouts, and is also a minor phytoestrogen found in soybean sprouts<sup>(1)</sup>. Formononetin and equol, a secondary metabolite of formononetin, have been reported to be responsible for reproductive dysfunction and infertility in ruminants<sup>(2,3)</sup>. The hormonal effects of formononetin may have a possible influence on breast cancer risk in mice, since it has been reported that formononetin supported mammary gland proliferation, enhanced mammary estrogen receptor expression, and increased circulating prolactin level in an estrogen-deficient murine model<sup>(4)</sup>. However, ER binding of formononetin has much lower affinity than daidzein and equol, which are metabolites of formononetin<sup>(5)</sup>. Equol was proposed to be the major active phytoestrogen found in sheep<sup>(6)</sup> and cattle<sup>(7)</sup> that consumed alfalfa or clover. Aglycone and conjugated forms of formononetin, daidzein, and equol have also been detected in bovine blood plasma and urine from cows fed a diet containing daidzein and formononetin<sup>(8)</sup>. Formononetin was metabolized to daidzein and equol *in vitro*, using a Bovine Rumen fluid system<sup>(6,7)</sup>. An *in vitro* study also showed that human cytochrome isoforms 1A2, 2C9\*1, 2A6, 2D6\*1 and 2C19 catalyzed the transformation of formononetin to daidzein<sup>(9)</sup>.

Schematically, formononetin is biosynthesized by the 4'-*O*-methylation of daidzein<sup>(10)</sup>. However, in a cell-free

extract of *Glycyrrhiza echinata*, isoformononetin was produced from daidzein, and formononetin was produced from 2, 7, 4'-trihydroxyisoflavone<sup>(11)</sup>.

In this study, conjugated forms and aglycones of formononetin were found for the first time in liver tissue of rats fed on diet based on protein containing genistein, daidzein and glycitein. Formononetin was also observed from incubations of rat liver homogenate, microsomes, and COMT with daidzein. These results demonstrated that formononetin might be formed through a 4'-*O*-methylation of daidzein, and conjugated possibly to become a reconstituted precursor of daidzein and equol in rat liver.

## MATERIALS AND METHODS

### I. Materials

Daidzein (7, 4'-dihydroxyisoflavone), genistein (5, 7, 4'-trihydroxyisoflavone), ( $\pm$ ) equol (7, 4'-isoflavandiol) and formononetin (7-hydroxy-4'-methoxyisoflavone) were purchased from Indofine Chemical Company, Inc. (Somerville, NJ, U.S.A.). DHD (dihydrodaidzein), glycitein (7, 4'-dihydroxy-6-methoxyisoflavone), and *O*-DMA (*O*-desmethylan-golesin) were purchased from Plantech (Reading, U.K.). Ammonium acetate, acetic acid, catechol-*O*-methyl transferase (COMT), S-(5'-Adenosyl)-L-methionine (SAM) and Sulfatase Type H-5 from helix pomatia were purchased from Sigma (St. Louis, MO, U.S.A.). Hexane, ethyl acetate and methanol were purchased from Fisher Scientific (Pittsburg, PA, U.S.A.). Soy protein isolate (SPI+) was a gift from

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Protein Technologies International (St. Louis, MO, U.S.A.).

## II. Animals

Starting on postnatal day 4, pregnant Sprague-Dawley rats were fed an AIN-93G diet formulated with soy protein isolate (SPI+) as the sole source of protein. The nutrient content of this diet was equal to that of the published AIN-93G diet. Soy protein isolate contained 0.72 mg/g genistein, 0.39 mg/g daidzein and 0.07 mg/g glycitein. The offspring were weaned to the same diet as the dam. At 50 days of age, the offspring were euthanized and samples were collected for analysis of cellular components.

## III. Extraction of Isoflavones from Liver Tissue

Aliquots of liver samples (2 g each from 10 female rats) were pulverized under liquid nitrogen, mixed and stored at  $-70^{\circ}\text{C}$  until analysis. Liver tissues were homogenized in 4 volumes of ice cold 1 M ammonium acetate buffer. Homogenates of 20 mg equivalent of liver tissue were brought to a final volume of 2 mL with buffer. For extraction of aglycones, homogenates were acidified with 100  $\mu\text{L}$  of glacial acetic acid defatted with 5 mL of hexane and then centrifuged at 2,800 rpm for 10 min. The hexane extraction was repeated once using the same procedure. The hexane fraction was discarded and the pellet was dried under nitrogen for 15 min. Ethyl acetate (5 mL) was added to the pellet to extract aglycones. The ethyl acetate extraction procedure was repeated twice, then the ethyl acetate fractions were combined and dried under nitrogen. Conjugated isoflavones were determined by incubating liver homogenates with Sulfatase Type H-5 (2000u/200u glucuronidase/sulfatase) in 1 M ammonium acetate buffer, pH 6.0 for 3 hr at  $37^{\circ}\text{C}$ , followed by ethyl acetate extraction of liberated aglycones as described above.

## IV. Extraction of Isoflavones from Serum and Urine

Aliquots of serum samples were pooled (0.5 mL from each rat,  $N = 10$ ) and stored at  $-70^{\circ}\text{C}$  until analysis. Serum (250  $\mu\text{L}$ ) were brought to a final volume of 2 mL with 1 M ammonium acetate buffer, pH 6.0 and acidified with 100  $\mu\text{L}$  of glacial acetic acid. Hexane (5 mL) was added, the samples were then mixed vigorously, centrifuged at 2,800 rpm for 10 min and the hexane fraction was discarded. This step was repeated again and the pellet was dried under nitrogen. The pellets were treated thrice with 5 mL of diethyl ether. The diethyl ether was combined, dried under nitrogen and assayed for aglycones as described below. Serum conjugated isoflavones were studied by incubating 250  $\mu\text{L}$  of serum with Sulfatase Type H-5 (1000u/100u glucuronidase/sulfatase) in 1 M ammonium acetate buffer, pH 6.0 for 3 hr at  $37^{\circ}\text{C}$ . The samples after incubation were then extracted following the same steps as for aglycones as described above. Aliquots of urine samples were pooled (100  $\mu\text{L}$  from each) and stored at  $-20^{\circ}\text{C}$ . Aglycone and

conjugated aglycones in urine samples were extracted following the procedures previously described<sup>(12)</sup>.

## V. Incubation of Daidzein with Liver Homogenate, Microsomes and COMT

Two grams of liver was homogenized in 6 mL of 10 mM phosphate buffer containing 1.15% KCl and 10 mM EDTA, pH = 7.4 and centrifuged at 9,000 rpm for 30 min at  $4^{\circ}\text{C}$ . Supernatant was collected and centrifuged at  $100,000 \times g$  for 1 hr at  $4^{\circ}\text{C}$ . The supernatant was collected as cytosol, and microsomes were resuspended with 0.8 mL of 0.1 M phosphate buffer, pH = 7.4. The cytosol and microsomes were stored at  $-80^{\circ}\text{C}$  until analysis. Homogenates, microsomes or cytosol (100  $\mu\text{L}$  of each) was incubated with COMT (180 units), 10 mM  $\text{MgCl}_2$ , 60 mg ascorbic acid, 10 mM SAM, 5  $\mu\text{L}$  of ethanol, 10  $\mu\text{g}$  of daidzein in phosphate buffer with a final volume of 0.5 mL. The samples were incubated at  $37^{\circ}\text{C}$  for 3 hr. After incubation, the samples were vortexed for 30 seconds in 0.5 mL of acetonitrile, then centrifuged at 14,000 rpm for 10 min and aliquots of 200  $\mu\text{L}$  of supernatant were transferred for analysis.

## VI. Identification and Quantification of Isoflavones by HPLC-APCI-MS

A modified HPLC method was used to identify and quantify isoflavones and metabolites from sample extracts. A Supelco Discovery RPamide-C16 column ( $25 \times 4.6\text{mm} \times 5 \mu\text{M}$ ) (Supelco; Supelco Park, Bellefonte, PA, U.S.A.) was used with a mobile phase of solvent A (25% methanol containing 10 mM ammonium acetate, 10% triethylamine (TEA), pH 4.8) and solvent B (95% methanol containing 10 mM ammonium acetate, 10% TEA, pH 4.8) at a flow rate of 1 mL/min. Isoflavones were separated using a linear gradient from 45% solvent B to 65% in 11 min and then to 95% in 5 min. Solvent B was held at 95% B for 5 min and then returned to 45% B in 2.5 min. The column was equilibrated in 45% B for 6.5 min before subsequent runs. Identification and quantification of isoflavones using a MDS Sciex API 100 mass spectrometer with negative single ion monitoring using a heated nebulizer. APCI was applied as described previously<sup>(12)</sup>.

## VII. Identification and Quantification of Equol by HPLC-Coulometric Array Detector (HPLC-ECD)

A modified HPLC method<sup>(13)</sup> was used to identify and quantify the isoflavone equol in sample extracts. Analysis was performed using a 16-channel CoulArray Model 5600 detection system which consisted of two Model 582 pumps, a Model 542 auto injector, and a CoulArray thermostatic chamber (ESA Inc., Chelmsford, MA). A Phenomenex Synergy MAX-RP column ( $25 \times 4.6 \text{ mm} \times 4 \mu\text{M}$ , 80Å) (Phenomenex, Torrance, CA, U.S.A.) was used in a mobile phase of solvent A (20% methanol containing 50 mM sodium acetate, pH 4.8) and solvent B consisting of water-

methanol-acetonitrile (40/40/20, v/v/v) containing 50 mM sodium acetate, (pH 4.8) at a flow rate of 0.8 mL/min. The gradient consisted of an initial condition of 35% B for 5 min, and a linear increase to 65% B in 5 min. The gradient then increased to 100% B from 65% B in 15 min which was held at 100% B for 10 min, and then returned to initial conditions in 5 min. The detector settings were 400, 450, 480, 520, 580, 630, 680, and 730 mV. Equol was monitored at 580 mV with retention time at 19.2 min.

### VIII. Microplate Assay for Oxygen Radical Absorbance Capacity (ORAC)<sup>(14)</sup>

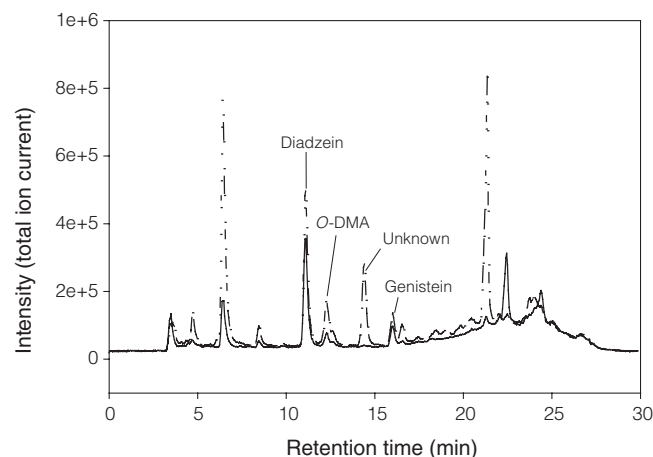
A stock fluorescein solution was prepared by dissolving 0.0225 g of fluorescein in 50 mL of 75 mM phosphate buffer (pH 7.0). Then 50  $\mu$ L of stock solution was diluted in 10 mL of phosphate buffer. Diluted solution (320  $\mu$ L) was diluted in 20 mL of phosphate buffer before use. Stock solutions of 500  $\mu$ M Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and AAPH (2, 2'-azobis(2-amidino-propane) dihydrochloride (6 mM) were prepared freshly and all solutions were stored at 4°C before use.

Aliquots of 40  $\mu$ L of samples or 40  $\mu$ L of Trolox calibration solutions (6.25, 12.5, 25, 50  $\mu$ M) in phosphate buffer (75 mM, pH 7.0) were added to a mixture solution of 400  $\mu$ L of 14  $\mu$ M fluorescein and 100  $\mu$ L of 6.3 mM AAPH. The mixtures were then analyzed by FLUOstar Optima microplate reader at 37°C. Fluorescence filters with excitation wavelength of 485 nm and an emission wavelength of 520 nm were used.

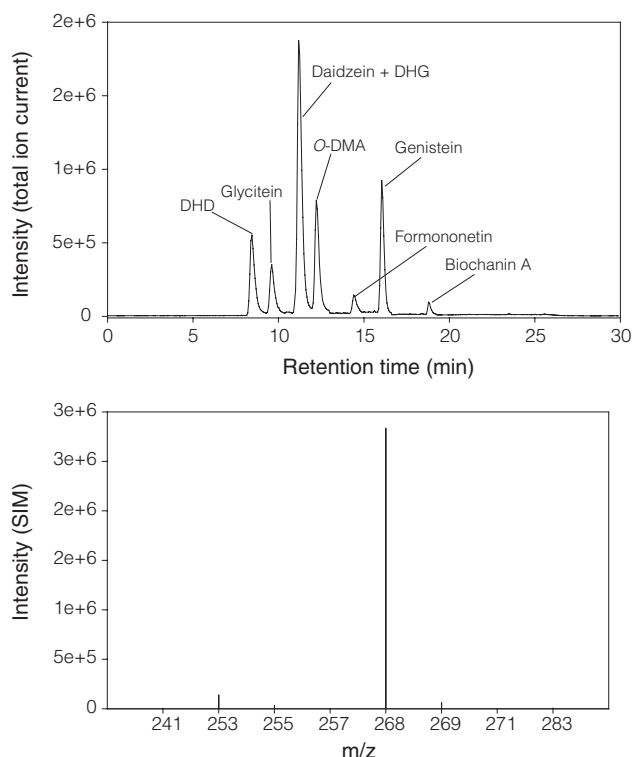
## RESULTS AND DISCUSSION

Soy isoflavone metabolites were studied extensively in biological fluids such as urine and serum. Genistein metabolites, mainly the conjugated forms, were observed in rat tissues<sup>(15,16)</sup>. In female DA/Han rat model, daidzein and its conjugates were observed in several tissues. Equol was also suggested to be present in bile, plasma and tissue samples, but was not able to be identified and quantified reliably<sup>(17)</sup>. In our study, results from extraction of total aglycones from incubation of pooled rat liver tissue homogenates with glucuronidase/sulfatase showed that daidzein, *O*-DMA, genistein, and a significant metabolite which eluted at 14.40 min were observed (Figure 1). This metabolite was identified as formononetin ( $m/z = 268$ ) using a standard solution containing a mixture of dihydrodaidzein ( $m/z = 255$ ), glycitein ( $m/z = 283$ ), daidzein ( $m/z = 253$ ), *O*-DMA ( $m/z = 257$ ), formononetin ( $m/z = 268$ ), genistein ( $m/z = 269$ ), and biochanin A ( $m/z = 283$ ) analyzed by LC/MS. The retention times for these isoflavone metabolites were 8.46, 9.78, 11.10, 12.24, 14.40, 16.02 and 18.77 min, respectively (Figure 2). The concentrations of total formononetin were 0.621 nmol/g and 0.035 nmol/mL in rat liver and serum, respectively. Urinary formononetin was not detected in this study (Table 1). In the urine of men and women that consumed a soy diet containing

42-100 mg of genistein and 32-77 mg of daidzein, urinary formononetin was also not detected<sup>(18)</sup>. Interestingly, aglycone and conjugated forms of formononetin were observed in the urine of humans consuming a soy diet containing mainly genistein, daidzein, and glycitein; however, quantification results were not available<sup>(19)</sup>. In a BALB/c mice model, formononetin was neither found in blood plasma or



**Figure 1.** HPLC-MS analysis of aglycones (solid line) and total aglycones (dotted line) from rat liver extracts using single ion monitoring (SIM) for identification and quantification<sup>(24)</sup>. Daidzein,  $m/z = 253$ , RT = 11.10 min, *O*-DMA,  $m/z = 257$ , RT = 12.24 min, unknown peak,  $m/z = 268$ , RT = 14.40 min, Genistein,  $m/z = 269$ , RT = 16.02 min. The unknown peak was fatherly identified as formononetin (please see Figure 2).



**Figure 2.** HPLC-MS analysis of aglycone standards using single ion monitoring (SIM) for identification and quantification. Standard formononetin eluted at 14.40 min ( $m/z = 268$ ).

**Table 1.** Soy isoflavones found in rat liver, serum and urine

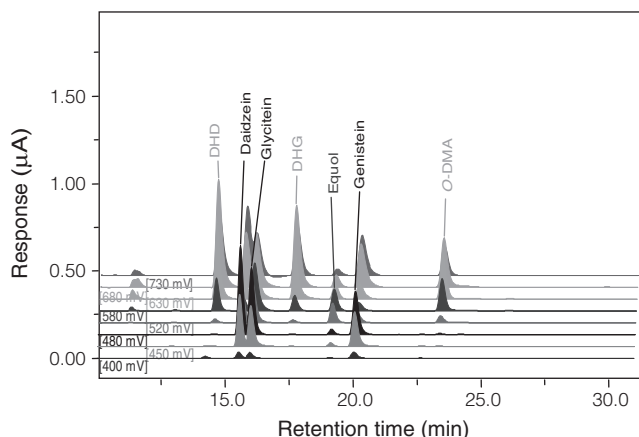
Isoflavone	Aglycone	Liver (nmol/g) <sup>a</sup>	Serum (nmol/mL)	Urine (nmol/mL)
Genistein	Aglycone	1.078 ± 0.252	0.017 ± 0.002	16.530 ± 3.100
	Total aglycone	1.822 ± 1.051	0.173 ± 0.007	35.600 ± 1.600
	% <sup>b</sup>	40.1	90.2	53.6
Daidzein	Aglycone	0.902 ± 0.016	0.107 ± 0.028	42.660 ± 6.380
	Total aglycone	1.374 ± 0.039	0.229 ± 0.036	54.960 ± 2.660
	%	34.4	53.3	22.4
O-DMA	Aglycone	0.678 ± 0.143	0.008 ± 0.003	5.290 ± 0.830
	Total aglycone	1.698 ± 0.217	0.249 ± 0.05	14.000 ± 0.890
	%	60.1	96.8	62.2
Formononetin	Aglycone	0.056 ± 0.022	0.005 ± 0.001	ND <sup>d</sup>
	Total aglycone	0.621 ± 0.116	0.035 ± 0.002	ND <sup>d</sup>
	%	90.8	85.7	
Equol <sup>c</sup>	Aglycone	7.335 ± 0.165	0.017 ± 0.003	32.330 ± 2.230
	Total aglycone	13.983 ± 1.34	1.432 ± 0.043	98.470 ± 5.240
	%	47.5	98.8	67.2

<sup>a</sup>nmol isoflavone/ g liver tissue (mean ± SD).

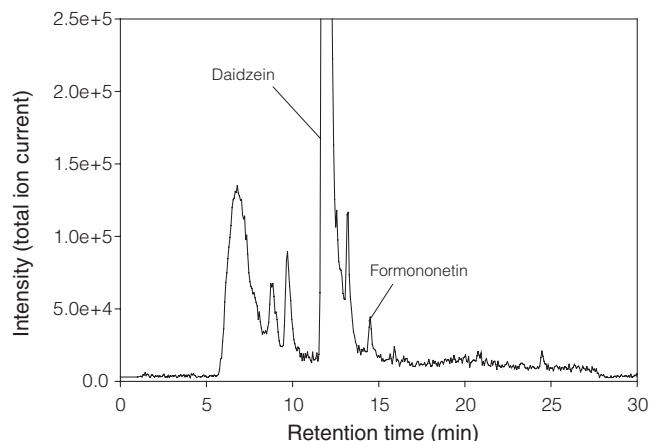
<sup>b</sup>% of conjugates = (total aglycone - aglycone)/total aglycone.

<sup>c</sup>Detected using HPLC-ECD.

<sup>d</sup>Not detected.



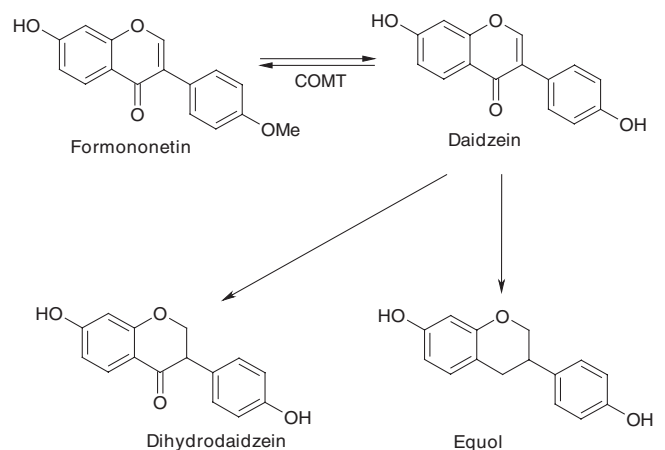
**Figure 3.** Multi-channel chromatogram of an isoflavone standard mixture (20 ng each) analyzed using HPLC with coulometric array detectors (HPLC-ECD).



**Figure 4.** HPLC-MS analysis of extracts following incubation for 90 min of daidzein with COMT in the presence of SAM. Chromatogram produced using single ion monitoring (SIM) for identification of daidzein,  $m/z = 253$ , RT = 11.10 min; and formononetin,  $m/z = 268$ , RT = 14.40 min.

mammary gland of the ovariectomized mice treated with 100 mg/kg formononetin orally<sup>(20)</sup>. These results suggested that formononetin might be transformed to other metabolite(s) or sequestered in other murine tissues<sup>(30)</sup>. When formononetin was given intraruminally in the sheep and cow, it was concluded that formononetin was responsible for estrogenic activity in the ovine and bovine, and equol (~5% of total formononetin) was the main excretory product found in the urine<sup>(21)</sup>. Although the demethylation of formononetin occurred in sheep liver microsomes<sup>(22)</sup>, and in *in vitro* incubations of human CYP450 isoforms with formononetin<sup>(8)</sup>, this metabolism is more likely to be a microbial action in the gastrointestinal tract of sheep and cow<sup>(6)</sup> and in human<sup>(23)</sup>. However, the gastrointestinal tract rate is lower than liver capacity<sup>(9)</sup>.

Most soy foods contain undetectable levels of formononetin except miso (32.8 µg/100 g) and soybean sprouts (186.7 µg/100 g)<sup>(1)</sup>. In this study, formononetin was not a



**Scheme 1**



component of SPI (data from PTI), and thus was unlikely to be absorbed from the diet by rats. Formononetin might have been transformed from daidzein as the result of a 4'-*O*-methylation process in rat liver by catechol-*O*-methyl transferase (COMT) (scheme 1). Suzuki and colleagues<sup>(24)</sup> reported that *O*-methylation of 4-hydroxyestrone occurred in an incubation with rat liver cytosolic fraction in an *in vitro* study<sup>(24)</sup>. *O*-methylation of tea catechins was shown to occur in the presence of a rat liver homogenate and S-adenosyl-L-methionine (SAM) *in vitro*<sup>(25)</sup>. Catechol-*O*-methyltransferase (COMT), which catalyzes the transfer of the methyl group from SAM to a phenolic group of catechol, also has been shown to be present in rat liver cytosol<sup>(26)</sup>. These reports supported the concept that 4'-*O*-methylation of daidzein to produce formononetin could be a possible biotransformation mechanism in rat liver. It was also showed that low but measurable activity of COMT with various hydroxylated isoflavone metabolites<sup>(27)</sup>. To confirm this hypothesis, further studies with incubation of formononetin with rat liver cytosol, homogenate, or COMT in the presence of SAM were processed. Results showed that only 7~10% of the daidzein was transformed to formononetin in the systems containing liver homogenate, microsomes, and with COMT (Figure 4). These results showed that formononetin (MRM transition: Q1 = 268, Q3 = 253), a 4'-*O*-methylation product of daidzein transformed by COMT, existed in liver tissue and serum of rats fed with soy protein isolate (SPI) which primarily contains daidzein, genistein, and glycitein but no formononetin.

It is interesting that 90.8% of the total formononetin aglycones are conjugated in rat liver. The conjugated forms of genistein, daidzein, *O*-DMA and equol are only 40.1, 34.3, 60.1 and 47.5% respectively. In rat serum, the conjugated genistein, daidzein, *O*-DMA, formononetin and equol are 90.2, 53.3, 96.8, 85.7, and 98.8% respectively. The results also showed that these soy isoflavone metabolites were bioconcentrated in rat liver (Table 1). Results from this study showed that conjugation of formononetin can indeed occur in rat liver and be detected in serum. Glucuronidation of formononetin was reported to occur in sheep and cow liver microsomes and was recovered after incubation of its conjugated form with  $\beta$ -glucuronidase<sup>(22)</sup>. Conjugation of formononetin was also found in the tissue homogenates of gastrointestinal tract, including rumen, reticulum, omasum and small intestine of sheep and cow<sup>(28)</sup>. It was reported that the relative binding affinity (RBA) of formononetin at human ER $\alpha$  is <0.01 compared with those of 17 $\beta$ -estradiol, genistein and daidzein (100, 87 and 0.5, respectively)<sup>(29)</sup>. Another study also reported that RBA of 17 $\beta$ -estradiol, genistein, equol, daidzein and formononetin at rat ER $\alpha$  were 100, 0.45, 0.15, 0.023 and 0.0013, respectively<sup>(5)</sup>.

Equol, a non-steroidal estrogen transformed from the phytoestrogen daidzein, was also able to be identified and quantified by HPLC-ECD (Figure 2). Equol is apparently the major compound found in rat liver and serum (70.0% and 67.6% of total isoflavones), as well as in urine (48.5%

**Table 2.** Oxygen radical absorbance capacity (ORAC<sup>®</sup>) analysis<sup>a</sup> for soy isoflavones

Soy isoflavone	$\mu\text{mol TE}^b/\mu\text{mol ORAC}^{\text{®}}$
Genistein	0.039
Daidzein	0.035
Equol	0.019
<i>O</i> -DMA	0.044
DHD	0.034
DHG	0.035
Biochanin A	0.015
Formononetin	0.001

<sup>a</sup>Please see reference 14 for ORAC methodology.

<sup>b</sup>TE: trolox equivalent.

of total isoflavones) (Table 1). Equol was found to be a weak estrogen in mice<sup>(3)</sup>. RBA of equol to rat ER $\alpha$  was about 33.3% of genistein and >100% of formononetin<sup>(3)</sup>. Equol is also proposed to be an important nonsteroidal estrogen for clinical studies<sup>(30)</sup>. The RBA studies of formononetin for rat and human ER $\alpha$  and the phenomenon that conjugated forms of formononetin were mainly found in rat liver imply that formononetin might not be important in rat liver in binding to estrogen receptors. Using the *in vitro* ORAC assay<sup>(14)</sup>, formononetin did not have similar antioxidant capacity as other soy isoflavones (Table 2). These results showed that formononetin is not considered to have antioxidant activity in rat liver. The fact that the partition coefficient of serum/liver of formononetin is 0.056, compared to other isoflavones which are all more than 0.100, suggests that formononetin is a transformed metabolite of possible excess daidzein and may be sequestered in rat liver.

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