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# Studies of the In Vitro Intestinal Metabolism of Isoflavones Aid in the Identification of Their Urinary Metabolites

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Soy isoflavones have recently gained considerable interest due to their possible health benefits. However, detailed studies on the metabolism of isoflavones are lacking. The aims of the investigation presented here were (1) to study the in vitro intestinal metabolism of isoflavones and their hydroxylated analogues 3′-OH-daidzein, 6-OH-daidzein, 8-OH-daidzein, and 3′-OH-genistein and (2) to characterize the structures of some earlier identified urinary metabolites of soy isoflavones, for which no authentic reference compounds have been available. Isoflavone standards (1−2 mg) were fermented with human fecal flora (16.7%) for 24 h. Metabolites formed during the fermentation were tentatively identified by interpretation of the mass spectra of trimethylsilylated compounds obtained by GC-MS. Compounds having hydroxyl groups at 5-position (i.e., genistein and 3′-OH-genistein) were completely converted to metabolites that could not be detected by the methods used in this study. The metabolism of daidzein and its hydroxylated analogues, 3′-OH-daidzein, 6-OH-daidzein, and 8-OH-daidzein, occurred to a much lesser extent. Minor amounts of reduced metabolites (i.e., isoflavanones and α-methyldeoxybenzoins) of these compounds were tentatively identified in fermentation extracts. The retention times and the mass spectra of reduced isoflavone metabolites, obtained from in vitro fermentations of pure compounds, were utilized to identify unknown urinary metabolites of soy isoflavones. Four novel isoflavone metabolites were identified in human urine collected after soy supplementation: 3′′-OH-*O*-desmethylangolensin, 3′,4′,7-trihydroxyisoflavanone, 4′,7,8-trihydroxyisoflavanone, and 4′,6,7-trihydroxyisoflavanone.

**KEYWORDS: Daidzein; genistein; soy; isoflavone; metabolism**

## INTRODUCTION

Isoflavones are plant compounds that occur mainly in plants belonging to the *Leguminosae* family. In human diet, the most important sources of isoflavones are soy and soy-based foods (*1*, *2*). In recent years, the isoflavones have been under intensive investigation due to their possible role in preventing certain hormone-dependent and other diseases including breast and prostate cancers, osteoporosis, and cardiovascular disease (*3*−*5*). The actual mechanisms by which the isoflavones act have not been fully established. Metabolism may play an important role.

Once ingested, isoflavones that principally occur as glycosides in foods are deconjugated, partly already in mouth and stomach (*6*) and small intestine (*7*), but mainly in the large intestine (*8*). Furthermore, the gut microflora catalyzes reduction and demethylation reactions (*9*−*12*), and so formed free aglycones and their metabolites are absorbed and transported to liver, where they are hydroxylated and conjugated to more water-soluble metabolites (i.e., isoflavone glucuronides and sulfates) which are eventually excreted in urine (*13*−*16*). Recent studies have demonstrated that the conjugation of isoflavone aglycones, especially genistein, takes place already in intestinal cells prior to transport via blood to the liver (*13*).

The in vitro intestinal metabolism of daidzein (4′,7-dihydroxyisoflavone, **Figure 1**) and genistein (4′,5,7-trihydroxyisoflavone), the main isoflavones of soy, has been studied to some extent. In a study on the bioavailability of soybean isoflavones in human subjects, Xu et al. reported that daidzein and genistein completely disappeared from fecal fermentation extracts after 72 and 18 h, respectively (*12*). After 18 h of incubation, some new peaks appeared accompanying the losses of daidzein and genistein, but no attempt was made to elucidate the structures of the metabolites formed during the fermentation. After 72 h of anaerobic fermentation with human fecal flora, Chang et al. identified dihydrodaidzein (4′,7-dihydroxyisoflavanone) and

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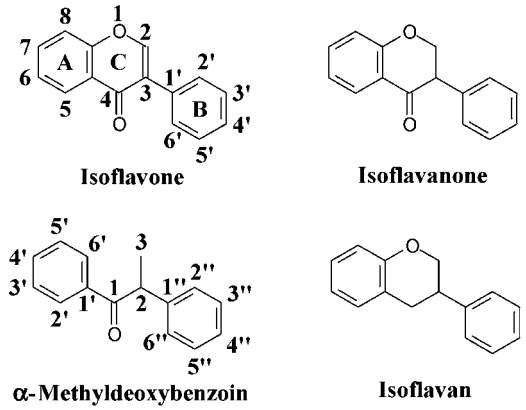


**Figure 1.** Structures of isoflavonoids.

dihydrogenistein (4′,5,7-trihydroxyisoflavanone) as the main metabolites of daidzein and genistein, respectively (*9*). Coldham et al. (*10*) studied the in vitro metabolism of both [$^3$H]-and [$^{13}$C]-labeled genistein with rat and human fecal flora and disclosed that the endproducts of metabolism of genistein are 2-(4-hydroxyphenyl)-propanoic acid (HPPA) and 1,3,5-trihydroxybenzene (THB). Dihydrogenistein and 6′-OH-*O*-desmethylangolensin (6′-OH-*O*-DMA, 2′,4′,4″,6′-tetrahydroxy-α-methyldeoxybenzoin) were also identified in fermentation extracts as intermediate metabolites (*10*, *17*).

It has been well demonstrated both in vitro and in vivo that both daidzein and genistein are metabolized by cytochrome P450s to oxidized metabolites, having the additional hydroxy groups at a vicinal site to the original hydroxy group (*15*, *16*, *18*). We have recently identified small amounts of 3′-OH-daidzein (3′,4′,7-trihydroxyisoflavone), 6-OH-daidzein (4′,6,7-trihydroxyisoflavone), 8-OH-daidzein (4′,7,8-trihydroxyisoflavone), and 3′-OH-genistein, also known as orobol (3′,4′,5,7-tetrahydroxyisoflavone), in human urine after soy supplementation (*19*). In addition to these oxidative metabolites of daidzein and genistein, we have tentatively identified some metabolites having an isoflavanone, α-methyldeoxybenzoin, or isoflavan structure, with additional hydroxy groups in the phenolic rings (*19*, *20*). Because authentic synthetic reference compounds for some of the metabolites were not available, alternative approaches to confirm the structures of the metabolites had to be looked for.

In this study we investigated the in vitro metabolism of daidzein and genistein and their hydroxylated analogues, 3′-OH-daidzein, 6-OH-daidzein, 8-OH-daidzein, and 3′-OH-genistein in fecal fermentation. The data obtained from these in vitro studies were utilized for the identification of human urinary metabolites of isoflavones after soy supplementation. In this paper, we report the identification of four new isoflavonoid metabolites 3″-OH-*O*-desmethylangolensin, 3′,4′,7-trihydroxyisoflavanone, 4′,7,8-trihydroxyisoflavanone, and 4′,6,7-trihydroxyisoflavanone, in fecal fermentation extracts of the corresponding isoflavone substrate and in human urine collected after soy supplementation.

## MATERIALS AND METHODS

**Reagents.** All reagents were pro analysis (pa) grade or higher. Hydrochloric acid, L(+)- ascorbic acid, chloroform, and diethyl ether were obtained from Merck, Germany. Hexane, heptane, and methanol were from Rathburn Chemicals Ltd., Scotland. *Helix pomatia* juice was purchased from BioSepra SA, France. Hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) were obtained from Pierce, USA, and pyridine, (Super Purity Solvent) from Romil Ltd., Great Britain. Lipidex-5000, was purchased from Packard Bioscience B. V., The Netherlands.

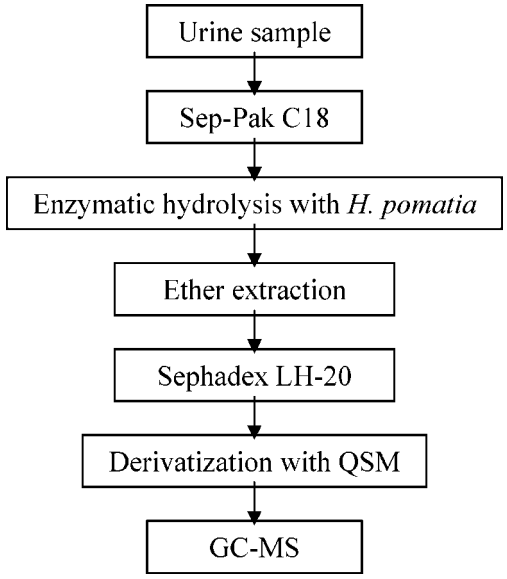


**Figure 2.** Sample pretreatment method for 24-h urine samples (*25*).

**Isoflavone Standards.** Daidzein, genistein, dihydrodaidzein, dihydrogenistein, equol, *O*-DMA, 6′-OH-*O*-DMA, and 4′,7,8-trihydroxyisoflavone were synthesized in the Laboratory of Organic Chemistry, Department of Chemistry, University of Helsinki, Finland (*21*−*23*). 3′,4′,7-Trihydroxyisoflavone, 4′,6,7-trihydroxyisoflavone, and orobol (3′,4′,5,7-tetrahydroxyisoflavone) were obtained from Apin Chemicals Ltd., United Kingdom.

**Incubation with Human Fecal Inoculum.** The incubation method of Karppinen et al. (*24*) was modified as follows: A carbonate−phosphate buffer solution with trace elements was held in an anaerobic chamber for 2 days prior to the fermentation. Feces were collected from three healthy human volunteers who had ingested a western diet, presented no digestive disease, and had not received antibiotics for at least 3 months. Freshly passed feces were immediately taken in an anaerobic chamber, pooled, and homogenized at the same time with an equal weight of culture medium using a Waring Blender. The slurry was diluted to 16.7% (w/w) with culture medium, filtered through a 1-mm sieve, and used immediately as inoculum.

A 1−2 mg quantity of each substrate (daidzein, genistein, 3′-OH-daidzein, 6-OH-daidzein, 8-OH-daidzein and 3′-OH-genistein), corresponding to 2−3.5 μmol, was weighed into 50-mL glass vials, and 10 mL of fecal suspension was added in a 30 °C anaerobic chamber. The vials were sealed with rubber stoppers and shaken in a 37 °C water bath for 24 h. Duplicate incubations were carried out for each substrate. Also, duplicate blanks, containing only culture medium and inoculum, were incubated for 0 and 24 h. The fermentation was stopped by plunging the vials into ice water, after which the vial contents were freeze-dried.

**Sample Pretreatment Method of Fecal Fermentation Samples.** Fecal fermentation samples were weighed (10−20 mg each), 0.5 mL of water was added, and the samples were acidified with 10 μL of 6 M HCl to improve the extraction efficiency. The samples were extracted twice with 2.0 mL of diethyl ether. The extracts were combined and evaporated to dryness under $N_2$ flow. The extracts were applied to a Lipidex 5000 column (0.5- × 5.0-cm) in 2 × 200 μL of MeOH/$H_2O$/$CHCl_3$ (4:1:1, v/v/v). Isoflavones and their metabolites were eluted with 4 mL of the same eluent. The fraction was dried under $N_2$ flow and derivatized with 100 μL of QSM (pyridine/HMDS/TMCS, 9:3:1). The silylating agent was evaporated, and the trimethylsilylated samples were dissolved in 100 μL of hexane prior to analysis by GC-MS.

**Urine Samples.** Urine samples were from our previous soy feeding study (*25*). Six healthy Finnish volunteers (3 women and 3 men) included three soy bars per day into their normal Western diet for a two-week period. Urine samples were collected before and on two subsequent days after the soy supplementation in plastic containers, and 1% of ascorbic acid and 0.1% of sodium azide were added as preservatives. The sample pretreatment method is presented in **Figure 2**.

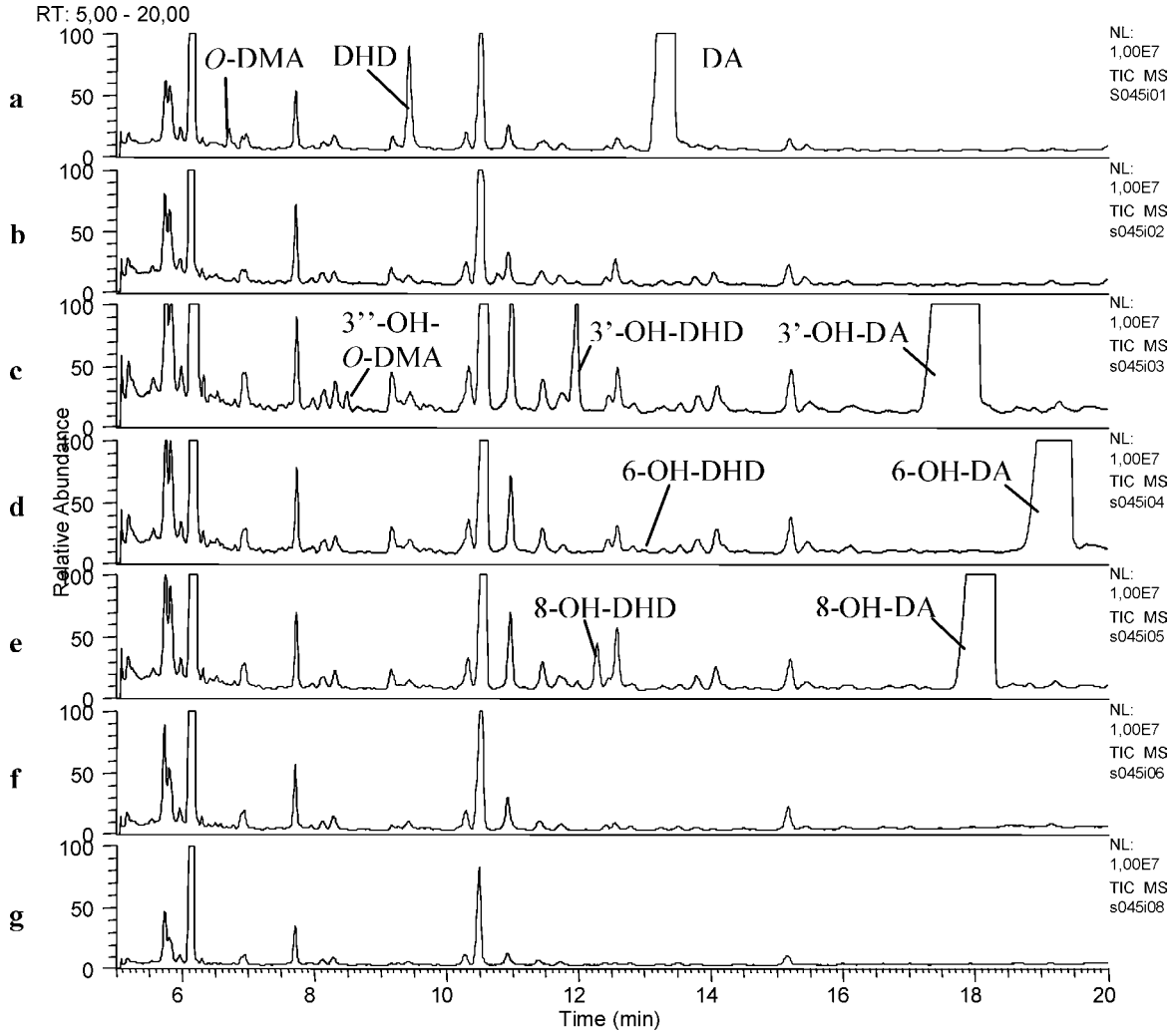


**Figure 3.** Total ion current (TIC) chromatograms of fecal fermentation samples of (**a**) daidzein (DA), (**b**) genistein (GEN), (**c**) 3′,4′,7-trihydroxyisoflavone (3′-OH-DA), (**d**) 4′,6,7-trihydroxyisoflavone (6-OH-DA), (**e**) 4′,7,8-trihydroxyisoflavone (8-OH-DA), (**f**) 3′,4′,5,7-tetrahydroxyisoflavone (i.e., orobol (ORO)), and (**g**) 24-h fermentation blank. Abbreviations: DHD, dihydrodaidzein; *O*-DMA, *O*-desmethylangolensin.

**Analytical Conditions.** GC-MS analyses were carried out with a Fisons Instrument MD 1000 quadrupole mass spectrometer coupled to a Fisons GC 8000 gas chromatograph, equipped with a BP-1 capillary column (12-m × 0.22-mm × 0.25-$\mu$m) from SGE (Scientific Glass Engineering), Australia. The flow rate of helium carrier gas was 1 mL/min. The oven temperature was programmed as follows: 150 °C for 1 min, then at 50 °C/min to 250 °C, then at 250 °C for 15 min, then at 50 °C/min to 290 °C, then at 290 °C for 5 min. The temperatures of the injection port, interface, and ion source were 280 °C, 250 °C, and 200 °C, respectively. Electron-impact ionization with 70 eV electron energy was used, and mass range from 100 to 900 mu was scanned.

## RESULTS AND DISCUSSION

**Fecal Fermentation Samples.** *Daidzein.* In addition to substrate, two minor metabolites were found in fermentation extract of daidzein (**Figure 3a**). These metabolites were identified as *O*-DMA and dihydrodaidzein (DHD) by use of authentic reference compounds (**Figure 4**). The fragmentation of *O*-DMA, eluting at retention time 6.7 min, occurs by α- or *i*-cleavage between the carbonyl and the methine carbon yielding fragments at *m*/*z* 281 and 193 (**Figure 4a**). The former fragment, the base peak of the spectrum, is due to the benzoyl fragment from ring A with two trimethylsilylated hydroxy (TMSO) groups. The second fragment is a ring B fragment with one TMSO group attached. In the mass spectrum of dihydrodaidzein (**Figure 4b**), eluting at the retention time 9.4 min, the molecular ion is at *m*/*z* 400. The base peak of the spectrum is a ring B retro Diels−Alder (rDA) fragment at *m*/*z* 192. The ion at *m*/*z* 281 may be formed by the migration of one additional trimethylsilyl (TMS) group to A-ring rDA fragment.

*Genistein.* The total ion current chromatogram of fermentation extract of genistein (**Figure 3b**) did not differ from the 24 h blank (**Figure 3g**). Neither the substrate nor metabolites of genistein could be detected.

*3′-OH-Daidzein.* Two metabolites were tentatively identified in the fermentation extract of 3′-OH-daidzein (**Figure 3c**). The metabolite at the retention time 8.5 min was tentatively identified as 3′′-OH-*O*-DMA (**Figure 5a**). The base peak of the spectrum is at *m*/*z* 281. The other ions are very low in intensity. The molecular ion at *m*/*z* 562 is absent from the spectrum, but an ion at *m*/*z* 547, formed from the molecular ion by the loss of one methyl radical, is present. The base peak is formed from the molecular ion by α- and *i*-cleavages yielding two fragments of equal size, 281 amu. The metabolite eluting at the retention time 11.9 min was an isomer of dihydrogenistein having a molecular ion at *m*/*z* 488 and the base peak at 280 *m*/*z* (**Figure 5b**). The compound was tentatively identified as 3′,4′,7-trihydroxyisoflavanone. The loss of one methyl group from the molecular ion gives the ion at *m*/*z* 473. The even mass base peak at *m*/*z* 280 is an rDA fragment of a compound having two TMSO groups in ring B. The abundance of the ion at *m*/*z* 281

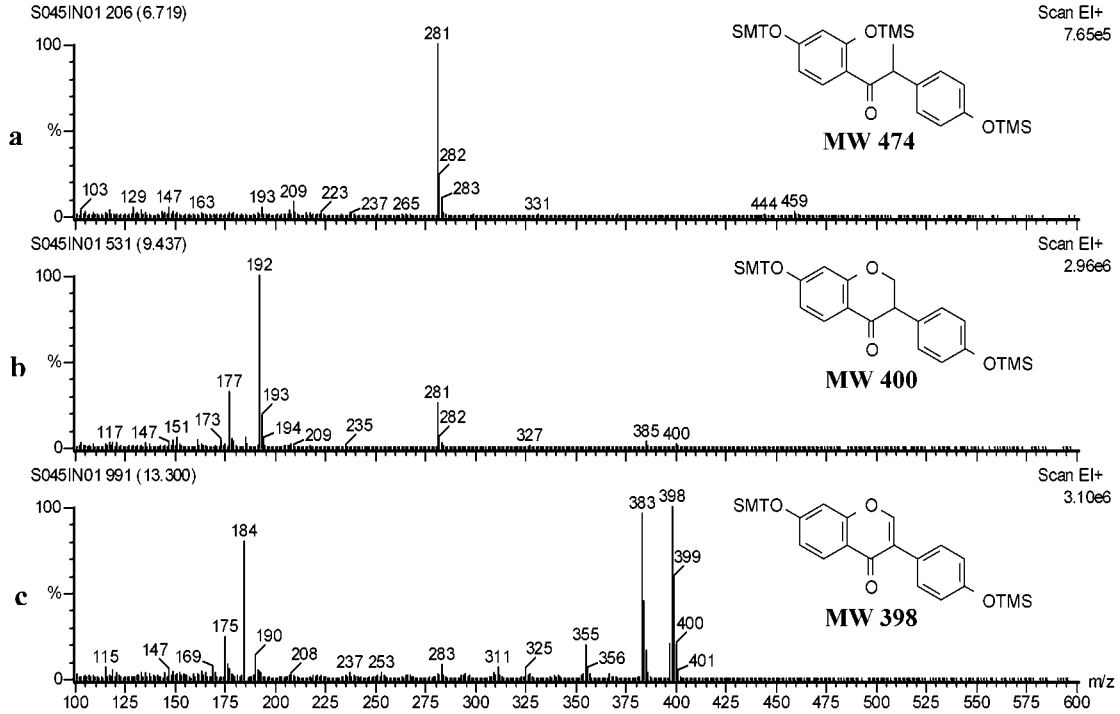


**Figure 4.** Mass spectra of compounds identified in fecal fermentation extracts of daidzein: (**a)** *O*-DMA, (**b)** dihydrodaidzein, and (**c)** daidzein.

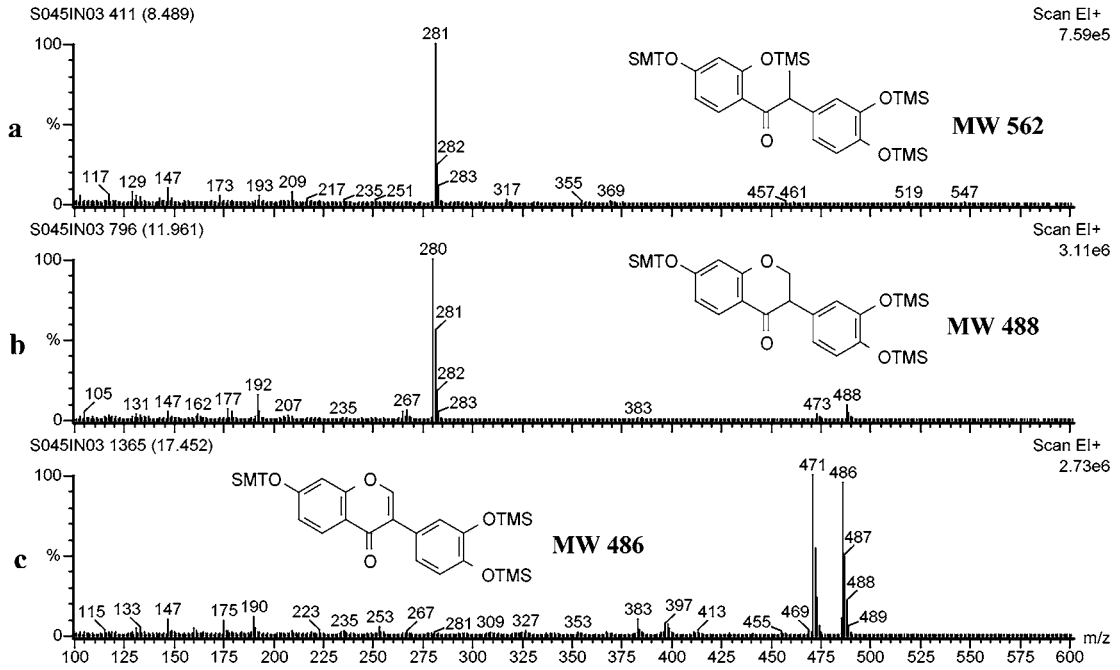


**Figure 5.** Mass spectra of metabolites identified in fecal fermentation extracts of 3′-OH-daidzein: (**a)** 3′′-OH-*O*-DMA, (**b)** 3′,4′,7-trihydroxyisoflavanone and (**c)** 3′-OH-daidzein.

(37%) is much higher than expected for the isotope peak of ring B rDA fragment, for which the calculated abundance would be about 26%. The ion may also be formed in a reaction where a TMS group migrates from the ring B rDA fragment to the ring A rDA fragment. A rather abundant ion at *m*/*z* 192 is formed from the ring B rDA fragment by the loss of tetramethylsilane. This kind of fragmentation is only possible for compounds having two vicinal hydroxy groups, so it can be assumed that this metabolite has 3′,4′-dihydroxysubstitution in the ring B.

*6-OH-Daidzein.* Only one metabolite, at the retention time 12.9 min, was detected in the fermentation extract of 6-OH-daidzein (**Figure 3d**). The compound had a molecular ion at *m*/*z* 488 and the base peak of the spectrum at *m*/*z* 296 (**Figure 6a**). The metabolite was tentatively identified as 4′,6,7-trihydroxyisoflavanone. The base peak of the spectrum is due to a ring A rDA fragment. The ring B rDA fragment occurs at *m*/*z* 192, from which the loss of one methyl radical yields the ion at *m*/*z* 177.

*8-OH-Daidzein.* In addition to the substrate, one metabolite was identified in the fermentation extract of 8-OH-daidzein (**Figure 3e**). The metabolite was tentatively identified as 4′,7,8-trihydroxyisoflavanone by interpretation of the mass spectrum (**Figure 7a**). The molecular ion and the base peak of the

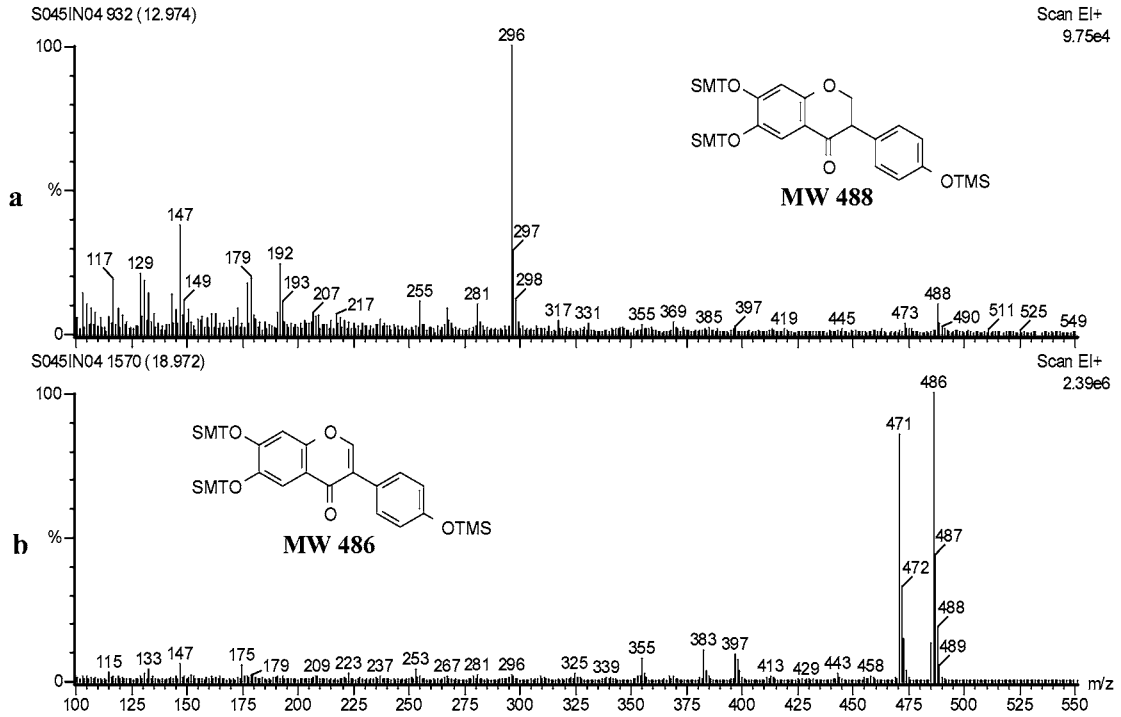


**Figure 6.** Mass spectra of metabolites identified in fecal fermentation extracts of 6-OH-daidzein: (**a)** 4′,6,7-trihydroxyisoflavanone and (**b)** 6-OH-daidzein.

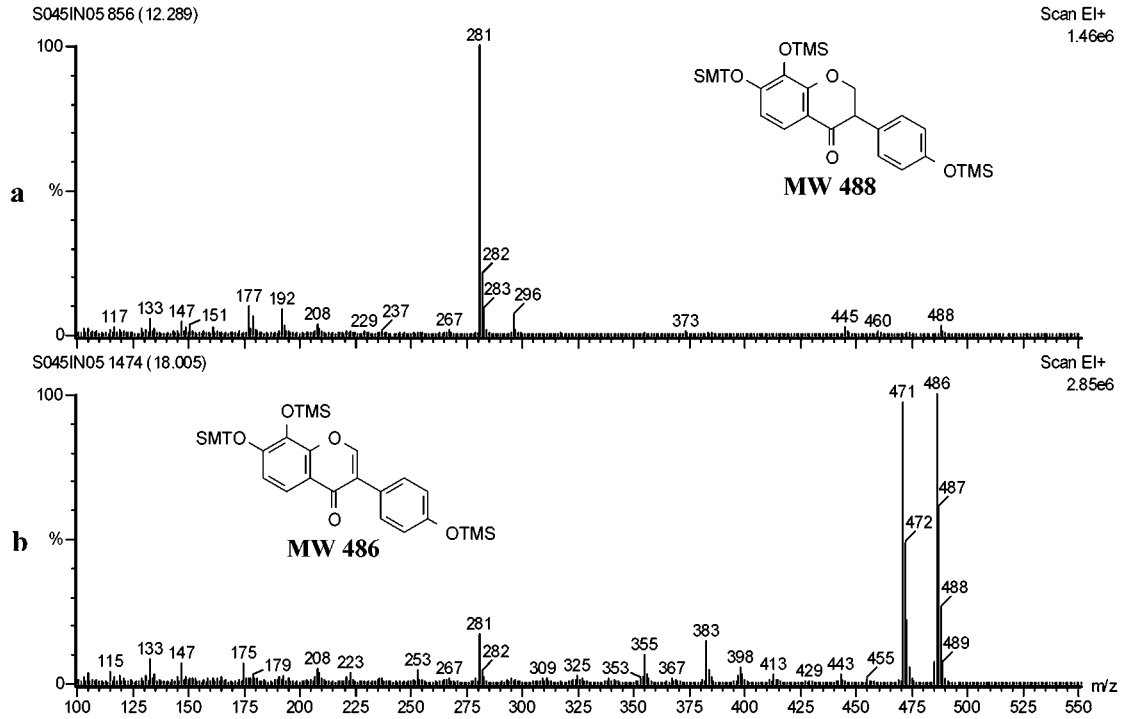


**Figure 7.** Mass spectra of metabolites identified in fecal fermentation extracts of 8-OH-daidzein: (**a)** 4′,7,8-trihydroxyisoflavanone and (**b)** 8-OH-daidzein.

spectrum occur at *m*/*z* 488 and 281, respectively. The loss of methyl radical from the molecular ion yields the fragment at *m*/*z* 473. The ion at *m*/*z* 445 is formed by the loss of carbon monoxide from the ion at *m*/*z* 473. The rDA fragment of the ring A at *m*/*z* 296 is low in intensity compared to the intensity of the corresponding ion in the mass spectrum of dihydrogenistein and 4′,6,7-trihydroxyisoflavanone. The base peak of the spectrum, at *m*/*z* 281, is a ring A fragment from which one methyl radical is lost from the TMS group at C8 position to yield a more stable cation. The ring B rDA fragment is at *m*/*z* 192.

*3′-OH-Genistein.* The total ion current chromatogram of orobol, 3′-OH-genistein (**Figure 3f**), was similar to those of genistein and 24 h blank: No substrate nor metabolites were found.

**Identification of Urinary Isoflavonoid Metabolites.** The retention times and mass spectra of unknown urinary metabolites were compared with those of metabolites identified in in vitro fermentation extracts. A typical chromatogram of urine sample extract of one person (nonequol producer) and metabolites identified in it are presented in **Figure 8**. *O*-DMA and dihydrodaidzein (DHD), at retention times 6.7 and 9.5 min, respectively,

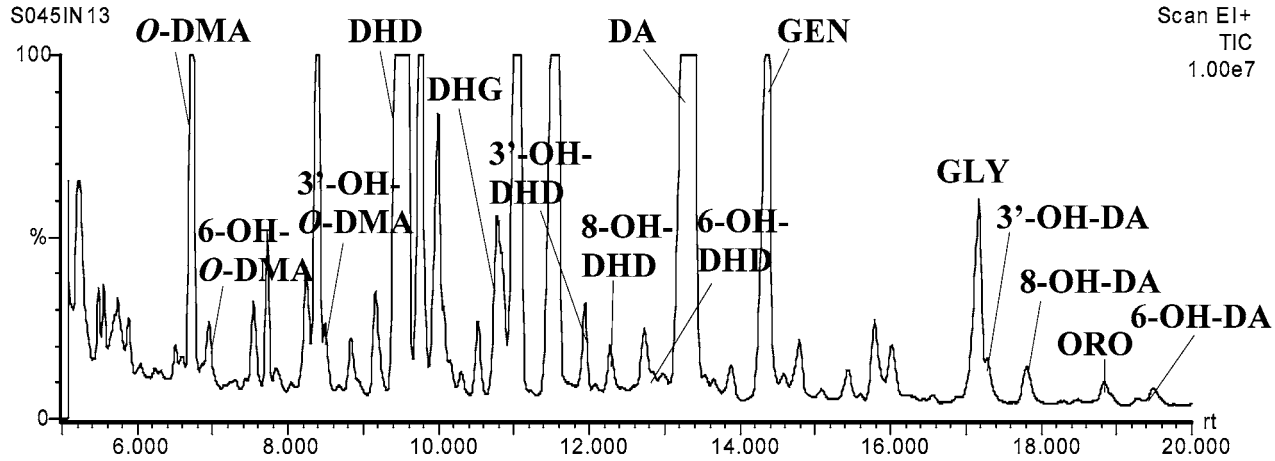


**Figure 8.** TIC chromatogram of human urine sample and metabolites, the structures of which were established with in vitro metabolites identified in this study. Abbreviations: see **Figure 3**; dihydrogenistein, DHG; glycitein, GLY.

were present in the urine of all six participants in rather high amounts. Dihydrogenistein (DHG) and 6′-OH-*O*-DMA, which could not be detected in the fermentation extract of genistein, were also found in all urine samples at retention times 10.8 and 6.9 min, respectively. New isoflavonoid metabolites were identified as 3′′-OH-*O*-DMA (8.5 min), 3′,4′,7-trihydroxyisoflavanone (3′,-OH-DHD, 11.9 min), 4′,7,8-trihydroxyisoflavanone (8-OH-DHD, 12.3 min), and 4′,6,7-trihydroxyisoflavanone (6-OH-DHD, 12.9 min). The metabolites were detected in all urine samples at low or moderate levels. In general, no significant inter-individual differences in excretion patterns of the new metabolites were observed. However, because no quantitative analyses were carried out in this study, any conclusions regarding the variation cannot be drawn.

In this study, we investigated the in vitro intestinal metabolism of the soy isoflavones daidzein and genistein and their hydroxylated analogues 3′-OH-daidzein, 6-OH-daidzein, 8-OH-daidzein, and 3′-OH-genistein that have been reported to be the oxidative metabolites of daidzein and genistein (*15*, *16*). During 24 h fecal fermentation, daidzein and its hydroxylated analogues were converted to some extent to reduced metabolites, and the corresponding isoflavanones and α-methyldeoxybenzoins were tentatively identified, namely *O*-DMA, 3′′-OH-*O*-DMA, dihydrodaidzein, 3′,4′,7-trihydroxyisoflavanone, 4′,7,8-trihydroxyisoflavanone, and 4′,6,7-trihydroxyisoflavanone (**Figure 8**). It should be noted that the isoflavan equol was not formed during the fecal fermentation of daidzein, suggesting that the donors were not so-called equol producers. The capacity to convert daidzein to equol varies between individuals depending on the composition of the gut microflora, and approximately only one-third of the human population is capable of producing high levels of equol (*26*, *27*).

Genistein and 3′-OH-genistein, which both have a hydroxy substituent at the 5-position, were completely metabolized and converted to compounds that could not be determined by the methods used in this study. Results are in accordance with the findings of Xu et al. (*12*), who reported that no genistein was present in the fecal fermentation extracts of genistein after 18 h of fermentation. They suggested that genistein, as well as flavonoids possessing a hydroxy group at the 5-position, are particularly susceptible to C-ring cleavage by intestinal microflora (*8*, *12*). Most probably, genistein was converted to HPPA and THB, the end-products of intestinal metabolism of genistein, reported by Coldham et al. (*10*, *17*). In the present study, no attempt was made to identify these metabolites of small molecular weight in the fermentation extracts.

We have recently found several new isoflavonoid metabolites, isoflavones, isoflavanones, α-methyldeoxybenzoins, and isoflavans in human urine after soy supplementation (*19*, *20*). Most of the metabolites have been identified using synthetic reference compounds, but for some of the metabolites, these have not been available, and the identification has been based on interpretation of mass spectra of trimethylsilylated derivatives. The studies of in vitro metabolism of isoflavones with human fecal inoculum aided in the elucidation of the structures and confirmed the identity of these metabolites because it was possible to compare the chromatographic and mass spectral characteristics of unknown urinary compounds to those of the metabolites formed in vitro from well characterized substrates. Fermentation of daidzein with fecal inoculum resulted in the formation of reduced metabolites. Other metabolites formed by other metabolic reactions were not observed. Therefore, it was assumed that other isoflavone substrates would also be metabolized mainly to analogous reduced metabolites having the original substitution pattern of the phenolic rings, which made the tentative identification of the in vitro metabolites and thus also the identification of urinary metabolites easier and more secure.

It is possible, or even likely, that in human subjects, the isoflavone aglycones are first converted to reduced metabolites by gut microflora prior to the oxidative metabolism by liver microsomes, as suggested by Kulling et al. (*18*). However, a small fraction of oxidized isoflavone metabolites may enter the enterohepatic circulation, be excreted in bile, and thus get involved with reductive reactions of the gut microflora. Our study shows that the reductive metabolism of the oxidized metabolites of isoflavones does occur to a certain extent in vitro. The formation of hydroxylated metabolites of daidzein and genistein seems to occur to a lesser extent than the formation of reduced metabolites because the levels of hydroxylated analogues of daidzein, for example, are notably lower in urine than the levels of its reduced metabolites (i.e., dihydrodaidzein, and *O*-DMA) or equol to those of high equol producers. The levels of the new metabolites identified in this study were low in urine when compared to the levels of other previously identified metabolites or the levels of ingested soy isoflavones, daidzein, and genistein.

The gut microflora may play an important role in the mechanisms of action of isoflavones because the intestinal metabolism of isoflavones largely determines the levels of circulating isoflavones and their metabolites (*12*). The more active microflora are, the higher levels of isoflavonoid metabolites and the

lower urinary levels of ingested isoflavones are. Whether one is capable of producing high levels of equol, an isoflavan metabolite of daidzein, depends on the composition and capability of the intestinal microflora. More work is still needed to find out the true end-products of the metabolism of isoflavones and to develop the methods to quantify the levels of circulating isoflavonoids and their metabolites, to understand the role of isoflavones in prevention of the diseases.

## ABBREVIATIONS USED

amu, atomic mass unit; DA, daidzein; DHD, dihydrodaidzein; DHG, dihydrogenistein; GEN, genistein; GLY, glycitein; HMDS, hexamethyldisilazane; HPPA, (4-hydroxyphenyl)-propanoic acid; *O*-DMA, *O*-desmethylangolensin; rDA, retro Diels−Alder; THB, 1,3,5-trihydroxybenzene; TMCS, trimethylchlorosilane; TMS, trimethylsilyl

## LITERATURE CITED


(1) Axelson, M.; Sjövall, J.; Gustafsson, B. E.; Setchell, K. D. R. Soya − a dietary source of the nonsteroidal oestrogen in man and animals. *J. Endocrin.* **1984**, *102*, 49−56.

(2) Wang, H.; Murphy, P. A. Isoflavone content in commercial soybean foods. *J. Agric. Food Chem.* **1994**, *42*, 1666−1673.

(3) Clarkson, T. B. Soy, soy phytoestrogens, and cardiovascular disease. *J. Nutr.* **2002**, *132*, 566S−569S.

(4) Messina, M. J. Legumes and soybeans: overview of their nutritional profiles and health effects. *Am. J. Clin. Nutr.* **1999**, *70*, 439S−450S.

(5) Setchell, K. D. R. Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. *Am. J. Clin. Nutr.* **1998**, *68* (Suppl), 1333S−1346S.

(6) Allred, C. D.; Ju, Y. H.; Allred, K. F.; Chang, J.; Helferich, W. G. Dietary genistin stimulates growth of estrogen-dependent breast cancer tumors similar to that observed with genistein. *Carcinogenesis* **2001**, *22*, 1667−1673.

(7) Day, A. J.; DuPont, M. S.; Ridley, S.; Rhodes, M.; Rhodes, M. J.; Morgan, M. R.; Williamson, G. Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver beta-glucosidase activity. *FEBS Lett.* **1998**, *436*, 71−75.

(8) Griffiths, L. A.; Barrow, A. Metabolism of flavonoid compounds in germ-free rats. *The Biochem. J.* **1972**, *130*, 1161−1162.

(9) Chang, Y. C.; Nair, M. G. Metabolism of daidzein and genistein by intestinal bacteria. *J. Nat. Prod.* **1995**, *58*, 1892−1896.

(10) Coldham, N. G.; Darby, C.; Hows, M.; King, L. J.; Zhang, A. Q.; Sauer, M. J. Comparative metabolism of genistin by human and rat gut microflora: detection and identification of the end-products of metabolism. *Xenobiotica* **2002**, *32*, 45−62.

(11) Hur, H.; Rafii, F. Biotransformation of the isoflavonoids biochanin A, formononetin, and glycitein by Eubacterium limosum. *FEMS Microbiol. Lett.* **2000**, *192*, 21−25.

(12) Xu, X.; Harris, K. S.; Wang, H.-J.; Murphy, P. A.; Hendrich, S. Bioavalability of soybean isoflavones depends upon gut microflora in women. *J. Nutr.* **1995**, *125*, 2307−2315.

(13) Doerge, D. R.; Chang, H. C.; Churchwell, M. I.; Holder, C. L. Analysis of soy isoflavone conjugation in vitro and in human blood using liquid chromatography−mass spectrometry. *Drug Metab. Dispos.* **2000**, *28*, 298−307.

(14) Adlercreutz, H.; van der Wildt, J.; Kinzel, J.; Attalla, H.; Wähälä, K.; Mäkelä, T.; Hase, T.; Fotsis, T. Lignan and isoflavonoid conjugates in human urine. *J. Steroid Biochem. Mol. Biol.* **1995**, *52*, 97−103.

(15) Kulling, S. E.; Honig, D. M.; Metzler, M. Oxidative metabolism of the soy isoflavones daidzein and genistein in humans in vitro and in vivo. *J. Agric. Food Chem.* **2001**, *49*, 3024−3033.

(16) Kulling, S. E.; Honig, D. M.; Simat, T. J.; Metzler, M. Oxidative in vitro metabolism of the soy phytoestrogens daidzein and genistein. *J. Agric. Food Chem.* **2000**, *48*, 4963−4972.

(17) Coldham, N. G.; Howells, L. C.; Santi, A.; Montesissa, C.; Langlais, C.; King, L. J.; Macpherson, D. D.; Sauer, M. J. Biotransformation of genistein in the rat: elucidation of metabolite structure byproduct ion mass fragmentology. *J. Steroid Biochem. Mol. Biol.* **1999**, *70*, 169−184.

(18) Kulling, S.; Lehmann, L.; Metzler, M. Oxidative metabolism and genotoxic potential of major isoflavone phytoestrogens. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2002**, *777*, 211.

(19) Heinonen, S. M.; Hoikkala, A.; Wähälä, K.; Adlercreutz, H. Identification of Isoflavone Metabolites by Gas Chromatography−Mass Spectrometry. *J. Nutr.* **2002**, *132*, 579S.

(20) Heinonen, S. M.; Hoikkala, A.; Wähälä, K.; Adlercreutz, H. Metabolism of the soy isoflavones daidzein, genistein, and glycitein in human subjects. Identification of new metabolites having an intact isoflavonoid skeleton. *J. Steroid Biochem. Mol. Biol.* **2003**, *87*, 285−299.

(21) Salakka, A.; Wähälä, K. Synthesis of α-methyldeoxybenzoins. *J. Chem. Soc., Perkin Trans I* **1999**, 2601.

(22) Wähälä, K.; Salakka, A.; Adlercreutz, H. Synthesis of novel mammalian metabolites of the isoflavonoid phytoestrogens daidzein and genistein. *Proc. Soc. Exp. Biol. Med.* **1998**, *217*, 293−299.

(23) Wähälä, K.; Hase, T.; Adlercreutz, H. Synthesis and labeling of isoflavone phytoestrogens, including daidzein and genistein. *Proc. Soc. Exp. Biol. Med.* **1995**, *208*, 27−32.

(24) Karppinen, S.; Liukkonen, K.; Aura, A.-M.; Forssell, P.; Poutanen, K. In vitro fermentation of polysaccharides of rye, wheat, and oat brans and inulin by human fecal bacteria. *J. Sci. Food Agric.* **2000**, *80*, 1469−1476.

(25) Heinonen, S.; Wähälä, K.; Adlercreutz, H. Identification of isoflavone metabolites dihydrodaidzein, dihydrogenistein, 6′-OH-*O*-dma, and *cis*-4-OH-equol in human urine by gas chromatography-mass spectroscopy using authentic reference compounds. *Anal. Biochem.* **1999**, *274*, 211−219.

(26) Kelly, G. E.; Nelson, C.; Waring, M. A.; Joannou, G. E.; Reeder, A. Y. Metabolites of dietary (soya) isoflavones in human urine. *Clin. Chim. Acta* **1993**, *223*, 9−22.

(27) Rowland, I. R.; Wiseman, H.; Sanders, T. A.; Adlercreutz, H.; Bowey, E. A. Interindividual variation in metabolism of soy isoflavones and lignans: influence of habitual diet on equol production by the gut microflora. *Nutr. Cancer* **2000**, *36*, 27−32.


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