Research Article

In vitro and *in vivo* metabolism of the soy isoflavone glycitein

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Glycitein (GLY) is a major isoflavone of soy germ which is used as a functional ingredient to enrich foods with isoflavones as well as a component of soy supplements. Since data on the metabolism of GLY are incomplete, the *in vitro* phase I metabolism of GLY using rat liver microsomes, human liver microsomes (HLM), as well as human fecal flora was investigated. Furthermore, the *in vivo* metabolism has been studied after administration of GLY to Sprague – Dawley rats. The identification of the formed metabolites has been elucidated using HPLC/DAD, HPLC/API-ES MS, GC/MS, as well as reference compounds. With respect to the phase I metabolism, GLY has been converted to ten metabolites by liver microsomes of Aroclor-induced Wistar rats with 8-hydroxy(OH)-GLY as the main metabolite. HLM converted GLY to six metabolites with 8-OH-GLY and 6-OH-daidzein (DAI) being the major products. No sex-related differences were observed in each system. Intestinal metabolism of GLY led to four metabolites with 6-OH-DAI as the main product. The *in vivo* metabolism in rat urine after single-dose administration of GLY resulted in the identification of three oxidative and two bacterial metabolites as well as the demethylation product 6-OH-DAI as the main metabolite.

Keywords: Glycitein / Isoflavones / Metabolism / Soy germ

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1 Introduction

The intake of isoflavones is associated with a broad variety of beneficial properties on human health. Several, although not all epidemiological studies have shown that the intake of soy is inversely correlated with the incidence of hormone-related tumors, like breast and prostate cancer, as well as osteoporosis and coronary heart disease [1, 2]. However, some studies have raised concerns about potential adverse effects from isoflavone intake in infants – as the result of early estrogenic exposure [3, 4] – as well as in adults – as the consequence of an elevated breast cancer risk in woman with occult tumors [5]. The metabolism of the main soy isoflavones daidzein (DAI) and genistein (GEN, Fig. 1) are well investigated [6 – 12]. However, little is known about the metabolism of the third soy isoflavone glycitein (GLY) (Fig. 1). This is surprising, since it makes up to 10% in soy and up to 40% in soy germ [13]. Especially soy germ and soy germ extracts are being used more and more as components of dietary supplements as well as functional ingredients to enrich a wide variety of foods and beverages with isoflavones. The use of these kinds of supplements or foods can lead to a substantial intake of GLY besides GEN and DAI.

Heinonen *et al.* [12] identified dihydro-GLY, 6-methoxy-equol, and 5′-methoxy-*O*-desmethylangolensin (*O*-DMA) as well as 6-hydroxy(OH)-DAI, 6-OH-dihydrodaidzein (DHD), 6-OH-equol, and 5′-OH-*O*-DMA as putative bacterial metabolites of GLY [12]. However, it should be pointed out that an alternative route for the formation of these metabolites is the biotransformation of DAI (that means hydroxylation, reduction, and methylation). Since no tracer methods (radio or stable isotopes) or pure compounds were

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Abbreviations: BSTFA, *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide; **DAI,** daidzein; **DHD,** dihydrodaidzein; **EI,** electron impact; **GEN,** genistein; **GLY,** glycitein; **HLM,** human liver microsomes; **OH-,** hydroxy-; **O-DMA,** *O*-desmethylangolensin; **P450,** cytochrome P450; **RDA,** retro Diels – Alder; **TMS,** trimethylsilyl

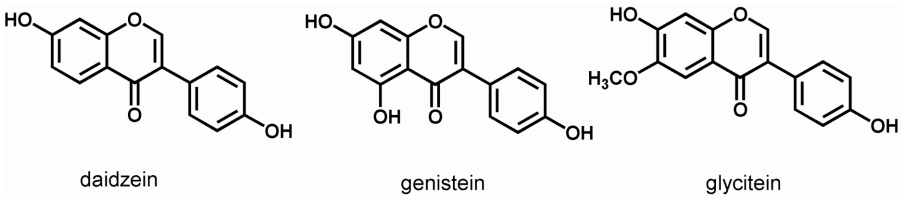


Figure 1. Chemical structures of the main soy isoflavones GEN, DAI, and GLY.

used in the study by Heinonen *et al*. [12] no definitive associations can be made between the parent compounds and the metabolites. Simons *et al*. [14] studied the bacterial metabolism of GLY *in vitro*. They identified dihydro-GLY, 6-OH-DHD, and 5′-methoxy-*O*-DMA. One subject produced DAI and two of them 6-methoxy-equol. However, they were not able to distinguish between "equol-" and "nonequol-producers" in their model system.

Previous studies revealed that small changes in the chemical structure of isoflavones result in marked effects on their biological properties. In terms of the increased use of soy germ and soy germ extracts as mentioned above, a detailed knowledge of the biotransformation of GLY seems essential for a safety assessment of this isoflavone. Therefore, in the present study the *in vitro* phase I metabolism as well as the intestinal metabolism of GLY was investigated. Furthermore, the *in vivo* biotransformation was assessed in urine and feces of male Sprague–Dawley rats after single-dose administration of pure GLY. The identification of the formed metabolites has been elucidated using HPLC with DAD, HPLC/API-ES MS, GC/MS as well as with the help of reference compounds.

2 Materials and methods

2.1 Chemicals

GLY was purchased from LC Laboratories (Woburn, MA, USA), 6-OH-DAI, 5-OH-GLY, 5,6-dihydroxy-DAI, *O*-DMA, and DHD from Plantech (Reading, UK). The purity was >99% according to GC/MS analysis. Pooled human male and female liver microsomes (HLM) were obtained from BD Biosciences (Heidelberg, Germany), pooled liver microsomes from Aroclor-induced male and female Wistar rats were prepared as described recently [10]. BHI dehydrated medium was purchased by Merck (Darmstadt, Germany). Ascorbic acid, NADH, $NADP^+$, tyrosinase, *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA), DMSO, isocitrate and isocitrate dehydrogenase as well as β-glucuronidase and sulfatase (both from *Helix pomatia*) were obtained from Sigma–Aldrich Chemical Co. (Deisenhofen, Germany). All other chemicals were of the highest grade available.

2.2 Incubation with microsomes and extraction

Incubations with microsomes and the extraction of the metabolites were carried out as described earlier [10, 12]. Briefly, standard incubations with rat liver microsomes contained 2 mg and those with human microsomes 4 mg microsomal protein, respectively. Furthermore, the mixtures contained 25 μM GLY (from 1.25 mM stock solutions in DMSO) and a NADPH-generating system (3 mM $MgCl_2$, 1 mM $NADP^+$, 8 mM D,L-isocitrate, and 0.5 U isocitrate dehydrogenase) in a final volume of 2 mL 0.05 M potassium phosphate buffer (pH = 7.4).

2.3 Incubation with tyrosinase

Isoflavones (125 μM; from 10 mM stock solutions in DMSO) were incubated with tyrosinase (500 U) and NADH (5 mg) in 4 mL 0.1 M Tris-HCL buffer (pH = 7.4) for 30 min at 37°C. The samples were extracted with 3 × 3 mL ethyl acetate and the combined organic extracts evaporated to dryness. HPLC and GC analyses were carried out as described in Sections 2.8, 2.9 and 2.10.

2.4 Human fecal incubations

Incubations were carried out according to Chang and Nair [15]. BHI medium (3.7 g/100 mL) was supplemented with vitamin K (20 μL/100 mL) and heme (0.5 mg/100 mL). Cystine hydrochloride (50 mg/100 mL) and resazurin (0.4 mL/100 mL) were used as reducing agent and O_2 indicator, respectively. The pH of the medium was adjusted to pH = 7. Fresh human feces of two female volunteers (age 25 and 27, healthy, nonsmoking, no oral antibiotic, and contraceptive use for the last 6 months) (1 g) were suspended in BHI medium (10 mL). One milliliter of these suspensions was diluted with 25 mL BHI-medium, 100 μL of a 4 mg/mL stock solution in DMSO of GLY was added and the mixture incubated for 72 h at 37°C under anaerobic conditions. Control incubations were carried out with DAI instead of GLY, medium and feces alone and medium with GLY alone under the same conditions described. The incubations were stopped by the addition of 2.5 mL of H_3PO_4 (85%), extracted with 3 × 8 mL ice-cold ethyl acetate, and the combined organic extracts were evaporated to dryness.

Further sample clean-up and preparation for HPLC and GC analysis was carried out as described in Sections 2.7–2.10.

2.5 Biotransformation of GLY by rats

Eight male Sprague–Dawley rats were obtained at 5 wk of age (about 160 g) from Charles-River (Wilmington, MA) and housed at 25°C with a 12 h light/dark cycle with free access to food and water. The experiment was approved by the State Veterinary Office and the experiments complied with its guidelines for the care and use of laboratory animals. Rats were maintained on an isoflavone-free diet (Altromin, Lage, Germany) for 2 wk and housed individually in metabolism cages for 24 h before dosing. After weighing (about 245 g) and 6 h of fasting six rats were dosed by gavage with 4 mg GLY suspended in 1.5 mL corn oil, the remaining two rats with 1.5 mL corn oil only. Urine and feces samples were collected for 24 h after dosing. Samples were stored at −80°C and in the case of urine 0.1% sodium azide was added to retard bacterial growth.

2.6 Clean-up of urine samples

Frozen urine samples were thawed, vortex-mixed, and centrifuged at 3000 × *g* for 10 min. Aliquots of 2 mL rat urine were mixed with 0.2 mL sodium acetate buffer (1.5 M, pH = 5) and applied to a preconditioned RP-18 cartridge (see above). The methanol extract was evaporated to dryness under a stream of nitrogen, the residue was dissolved in 3 mL of 0.15 M sodium acetate buffer (pH = 5), and incubated with 5000 U β-glucuronidase and 150 U sulfatase (both from *Helix pomatia*) for 12 h at 37°C. The hydrolyzed samples were extracted with 3 × 2 mL ethyl acetate and the combined organic phases evaporated to dryness. Preparation for GC analysis was carried out as described in Section 2.9.

2.7 Clean-up of feces samples

Frozen feces samples were warmed up to room temperature and homogenized using a Stomacher (Seward, Thetford, UK). Two grams of blended feces was mixed with 20 mL of 80% methanol and extracted by sonication for 60 min at 60°C resulting in very fine particles. After centrifugation the supernatant was extracted three times with 5 mL hexane to remove fat. After evaporating methanol 10 mL sodium acetate buffer (0.15 M; pH = 5) was added and the mixture was incubated with 5000 U β-glucuronidase and 150 U sulfatase (both from *Helix pomatia*) for 12 h at 37°C. The samples were extracted with 3 × 8 mL ethyl acetate and the combined organic extracts evaporated to dryness. Preparation for GC analysis was carried out as described in Section 2.9.

2.8 HPLC analysis

HPLC separation of the bacterial metabolites was carried out on a Prontosil (250 mm × 4.6 mm id, particle size 3 µm) RP column (Bischoff, Leonberg, Germany). The solvent system consisted of 0.1% formic acid in water (pH = 3) (A) and ACN (B) with the following linear gradient: from 15 to 35% B in 50 min, from 35 to 50% in 10 min, and from 50 to 60% in 30 min. The flow rate was 0.9 mL/min and the eluent was recorded with a diode array detector at 260 nm. Observed peaks were scanned between 190 and 400 nm.

Oxidative metabolites were analyzed by LC using a Prontosil (150 nm × 4.0 mm id, particle size 3 µm) RP column (Bischoff). Elution was effected using a linear gradient from 15 to 35% B in 22 min, from 35 to 50% in 5 min, and from 50 to 60% B in 13 min. The flow rate was 1.2 mL/min and the eluent was recorded with a diode array detector at 260 nm. Observed peaks were scanned between 190 and 400 nm.

2.9 GC/MS analysis

GC/MS was carried out on an Agilent Technologies system (gas chromatograph model HP6890 connected to a quadrupol mass detector model 5973; Agilent Technologies, Waldbronn, Germany). The metabolites were isolated by HPLC and freeze-dried. For GC/MS analysis, the dry residues were derivatized with BSTFA for 2 h at room temperature. Chromatographic separation of the trimethylsilyl (TMS) derivatives was achieved on a nonpolar capillary column (MDN-5S, 30 m × 0.25 mm id, 0.25 µM film thickness, Supelco, Munich, Germany) using a helium carrier gas flow of 1.2 mL/min and a linear temperature gradient (60°C for 2 min then 30°C/min to 250°C, hold for 10 min, then 1°C/min to 275°C and hold for 5 min). The injector port temperature was set to 250°C. The injection volume was 1 µL in the splitless mode. Mass spectra were obtained by electron impact (EI) ionization at 70 eV and an ion source temperature of 230°C. Full scan spectra (mass range 50–750 amu) were recorded at a rate of two spectra *per* second. Furthermore, the SIM mode was used for the detection of GLY and its metabolites.

2.10 LC/MS analysis

HPLC/MS analysis was performed on an HP 1100 series HPLC (Agilent Technologies) equipped with an autoinjector, quaternary HPLC pump, column heater, UV detector, and HP Chem Station for data collection and handling. The HPLC was interfaced to an HP series 1100 mass selective detector equipped with an API-ES chamber. For the analysis of the formed metabolites conditions in the positive mode were the following: capillary voltage 3.5 kV; fragmentor voltage 150 V; nebulizing pressure 50 psi; drying gas temperature 350°C; drying gas flow 12.5 L/min. Data

were collected using both the scan mode and SIM mode. Spectra were scanned over a mass range of *m*/*z* 100–600 at 0.98 s *per* cycle. For LC, the same conditions as described for the bacterial metabolites were used.

2.11 Approach for the elucidation of the oxidative metabolite structures

2.11.1 Use of reference compounds

6-OH-DAI and 5-OH-GLY were commercially available and employed as reference compounds for cochromatography and comparison of mass spectra.

2.11.2 Use of tyrosinase

The enzyme tyrosinase was used to generate catechol metabolites of GLY. GLY was converted to one product, 3′-OH-GLY. No A-ring catechols could be detected in accordance to the findings with GEN and DAI [10].

2.11.3 HPLC/MS and GC/MS analyses

MS can be used to determine the molecular weight of the metabolites and to clarify the distribution of hydroxyl groups between the A- and B-rings of the isoflavone molecule. GC/MS with EI ionization (70 eV) of TMS derivatives of the metabolites gave mass spectra with an intense molecular ion or $[M^+ - 15]$ ion (loss of CH_3 radical) as depicted in Fig. 2. EI mass spectra can thus be used to determine the molecular weight of the metabolites. The other fragment ions are of rather low intensity and were not used to obtain information about the chemical structure of the metabolites; they are formed through loss of a TMSO group and breakdown of the A- and B-rings.

Valuable information about the location of the hydroxyl groups can be obtained by HPLC/API-ES MS as already shown for GEN and DAI by Kulling *et al*. [10]: A base peak ion as well as a fragment ion derived from the molecular ion by retro Diels–Alder (RDA) reaction is displayed; the fragment ion can be used to determine the number of hydroxyl groups in the A-ring of the molecule. However, for GLY RDA reactions play a minor role in its fragmentation pattern compared to DAI and GEN. Main fragment ions are generated by the loss of a CH_3-radical and of CO. Representative mass spectra of GLY compared with DAI and the proposed fragmentation pattern of GLY are depicted in Figs. 3 and 4.

3 Results

3.1 Formation and identification of microsomal metabolites of GLY after incubation with Aroclor-induced rat liver microsomes

GLY (Fig. 1) was incubated with hepatic microsomes of Aroclor-treated female (cytochrome P450 (P450) (total): 1.4 nmol/mg protein) as well as male Wistar rats (P450

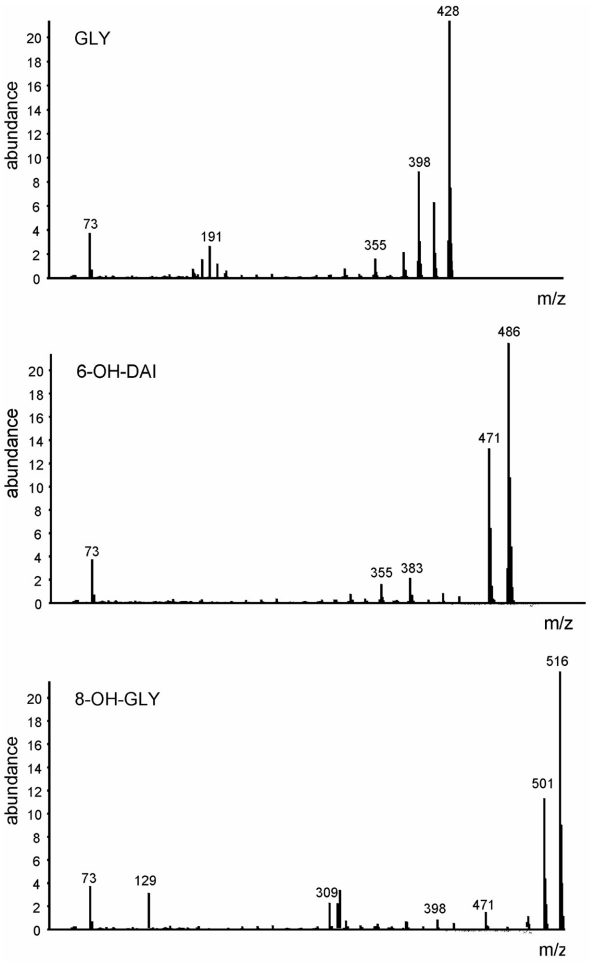


Figure 2. Representative EI mass spectra of TMS derivatives of GLY and 6-OH-DAI as well as 8-OH-GLY.

(total): 1.7 nmol/mg protein). Aroclor 1254 induces P450 isoenzymes, especially of the 1A but also of the 2B subfamily [16, 17]. Therefore, the use of Aroclor-induced microsomes increases both the variety of the formed metabolites and the yield, thus facilitating the elucidation of the structures.

The complete organic extract of each incubation was analyzed by RP HPLC with DAD. GLY was extensively metabolized. Eighty percent of the applied concentration of GLY is converted to ten metabolites. Nine metabolites were clearly detectable by HPLC analysis (Fig. 5), one could only be identified by GC/MS (peak 11, Table 1). The chemical structures of all of the numbered peaks are listed together with the analytical data in Table 1. These products were not observed when NADPH was omitted or heat-inactivated microsomes were used. No sex differences were observed between female and male microsomes (data not shown).

Furthermore, the extracted metabolites were analyzed by HPLC/API-ES MS and by GC/MS after trimethylsilylation with BSTFA. The mass spectra indicated the formation of

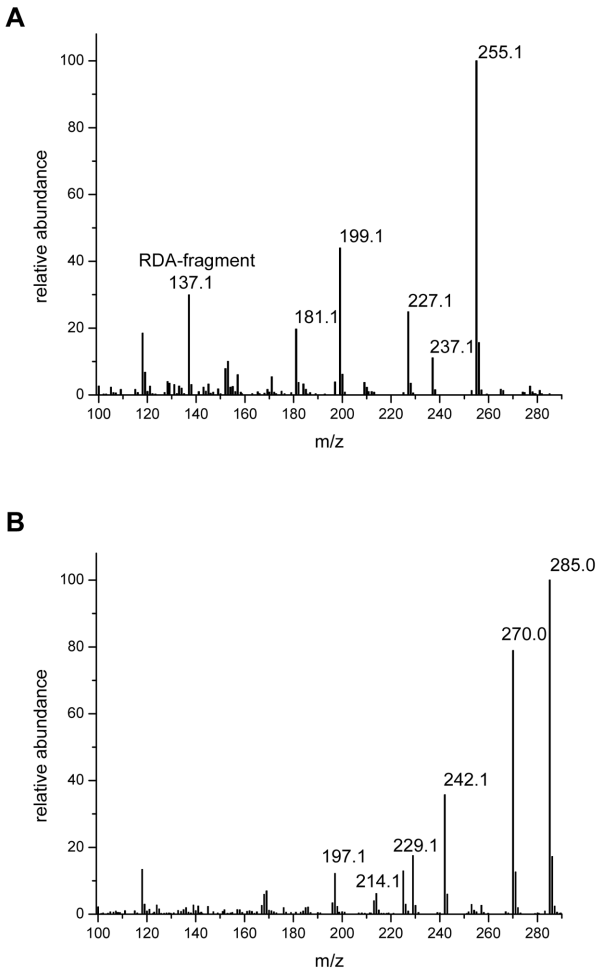


Figure 3. API-ES mass spectra of DAI (A) and GLY (B).

four monohydroxylated and three monohydroxylated demethylated products, two dihydroxylated metabolites as well as the demethylation product 6-OH-DAI. Two minor metabolites (peak 7 and 11) and one major metabolite (peak 5) have not been identified yet. Identification has been elucidated as described in Section 2. The chromatographic and MS data, the UV maxima as well as the assigned chemical structures are summarized in Table 1.

HPLC peaks 3 and 5 represent two major metabolites and account for more than 50% on the basis of the peak area at $\lambda = 260$ nm. The GC/MS analysis of both peaks shows molecular ions at *m/z* 516 indicating monohydroxylated GLY derivatives. The API-ES mass spectra display quasi-molecular ions at *m/z* 301, implying a monohydroxylation, too. In the case of peak 3 a diagnostic fragment ion at *m/z* 168, which was detected by MS^2 analysis (data not shown) of the parent molecular ion $[M + H]^+$, indicates a hydroxylated A-ring and an unchanged B-ring. Possible positions for hydroxylation in the A-ring are C-5 and C-8. 5-OH-GLY was available as a reference substance and is not identical with peak 3. Furthermore, the UV spectrum of peak 3 is almost identical with that of 6,8-dihydroxy-DAI, a metabolite which is formed in the microsomal metabolism of 6-OH-DAI and which has already been identified by Kulling *et al*. [10]. It is well known that a methoxy group instead of a hydroxy group does not change the UV spectrum. Therefore, peak 3 is identified as 8-OH-GLY.

The structure of peak 5 could not be identified explicitly: theoretically, a hydroxylation is possible at the aromatic C-5 and C-8 positions of the A-ring, at the aromatic C-2′ and C-3′ positions in the B-ring as well as at the aliphatic C-2 position of the central C-ring. Since the hydroxylation at C-8, C-3′, and C-2 can be allocated to other metabolites,

Table 1. Chromatographic and spectroscopic data of the oxidative metabolites of GLY

HPLC peak	t_R in HPLC [min]	UV maxima (nm)	$[M + H]^+$, LC/MS	t_R in GC [min]	M^+, GC/MS TMS derivative	Chemical structure
1	10.5	263, (290, 320)	317	43.2	*604*, 590	8,3′-Dihydroxy-GLY
2	12.1	220, 322	301	27.0	444, *429*	2-OH-GLY (keto-form)
				27.3	516, *501*	2-OH-GLY (enol-form)
3	14.6	263, 320	301	37.5	516, *501*	8-OH-GLY
4	14.9	258, 288, 324	287	38.4	*574*, 559	6,3′-Dihydroxy-DAI
5	17.3	262, (320)	301	33.6	516, *411*	Monohydroxylated GLY derivative (2′-OH-GLY?)
6	17.6	260, (292), 320	301	37.3	*516*, 501	3′-OH-GLY
7	19.2	260, 215	287	–	–	Degrades during GC/MS analysis hydroxylated, demethylated GLY derivative
8	20.2	257, 322	271	33.2	*486*, 471	6-OH-DAI
9	22.6	271, 215	287	29.5	574, *559*	5,6-Dihydroxy-DAI
10	23.1	257, 320	285	31.2	*428*, 413	GLY
11	–	–	–	39.0	604, *499*	Dihydroxylated GLY derivative

Peak numbers correspond to Fig. 5. The base peak in the GC/MS mass spectra of the trimethylsilylated metabolites is marked in italics.

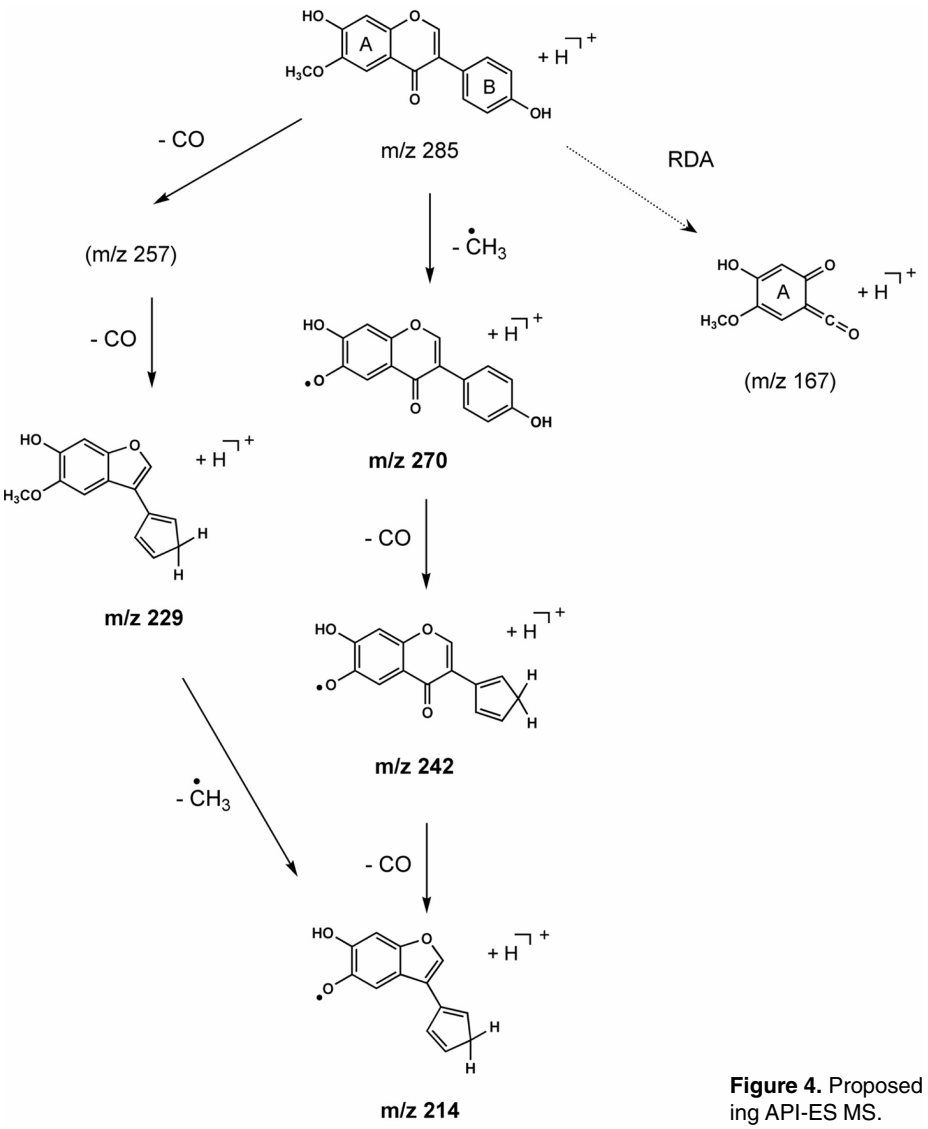


Figure 4. Proposed fragmentation pathways of GLY during API-ES MS.

only C-5 and C-2′ remain free for possible modification. The reference substance 5-OH-GLY is not identical with peak 5. Neither do the retention times, nor the mass spectra from the corresponding GC and HPLC analysis agree. Therefore, the data obtained point to the peak 5 being 2′-OH-GLY, although a hydroxylation in a meta position to the existing OH group at C-4′ seems to be rather unusual.

The GC/MS analysis of peak 1 shows a molecular ion at *m*/*z* 604 and the HPLC/MS analysis a quasi-molecular ion at *m*/*z* 317 indicating a dihydroxylated GLY-metabolite. It is the only product formed after incubation of 8-OH-GLY (peak 3) with tyrosinase and thus, we assign peak 1 to be 8,3′-di-OH-GLY. The HPLC/MS analysis of peak 2 gives a quasi-molecular ion at *m*/*z* 301, indicating a monohydroxylated GLY derivative. The GC/MS analysis after trimethylsilylation results in two peaks with molecular ions at *m*/*z* 516 and 444, respectively. If the hydroxylation takes place in the aliphatic C-2 position of the C-ring, keto-enol tautomers can be formed and could explain these two GC peaks and the corresponding mass spectra (Table 1). In agreement with the microsomal metabolism of GEN [10] the third expected peak with a molecular ion *m*/*z* 516 (second enol tautomer) could not be detected. It is therefore proposed that HPLC peak 2 is 2-OH-GLY. Peak 4 is a hydroxylated, demethylated GLY derivative according to GC/MS and HPLC/MS analyses. This compound also appeared in the *in vitro* microsomal metabolism of DAI and has already been identified by Kulling *et al.* [10]. Therefore, peak 4 is assigned to be 6,3′-dihydroxy-DAI. Peak 6 is formed in the *in vitro* incubation of GLY with tyrosinase (see above) and is therefore proposed to be 3′-OH-GLY. Regarding peak 7 the isolated HPLC peak was degraded during GC/MS analysis. The HPLC/MS analysis shows a molecular ion at *m*/*z* 287 indicating a hydroxylated deme-

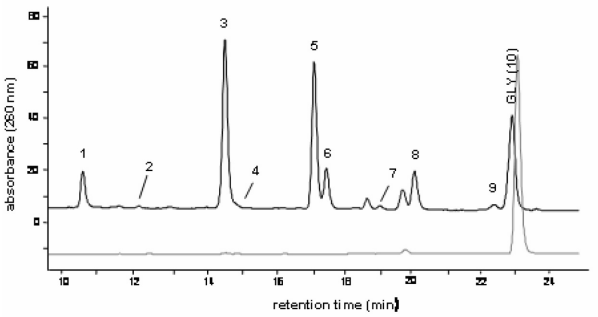


Figure 5. Representative HPLC chromatograms of GLY metabolites formed by the incubation of GLY with liver microsomes of Aroclor-induced female Wistar rats (upper trace) as well as of a control incubation of GLY with heat-inactivated microsomes (lower trace). For peak numbers refer to Table 1.

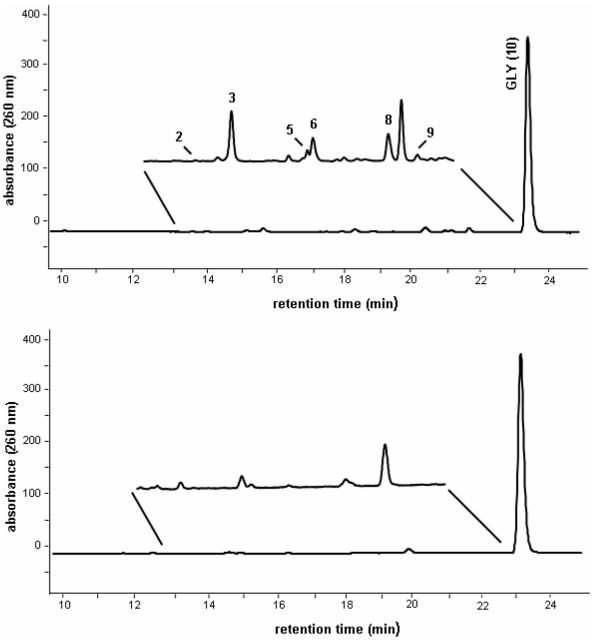


Figure 6. Representative HPLC chromatograms of the incubation of GLY with pooled female human liver microsomes (upper figure) as well as of a control incubation of GLY with heat-inactivated microsomes (lower figure). For peak numbers refer to Table 1.

thylated GLY derivative. However, due to the lack of reference compounds no conclusion can be drawn about the positions of the additional hydroxyl groups. The mass spectral data of peak 8 indicate a demethylated GLY derivative and it has been identified by cochromatography with the reference compound as 6-OH-DAI. Peak 9 is a hydroxylated, demethylated GLY derivative according to GC/MS and HPLC/MS analyses. This compound also appeared in the *in vitro* microsomal metabolism of GEN and has already been identified by Kulling *et al.* [10]. Therefore, peak 9 is identified as 5,6-dihydroxy-DAI. Peak 11 was only detected by GC/MS analysis and the molecular ion at *m*/*z* 604 indicates a dihydroxylated GLY derivative. It is also formed in the metabolism of peak 5 with tyrosinase and is therefore the 3′- or 5′-hydroxylated derivative of peak 5.

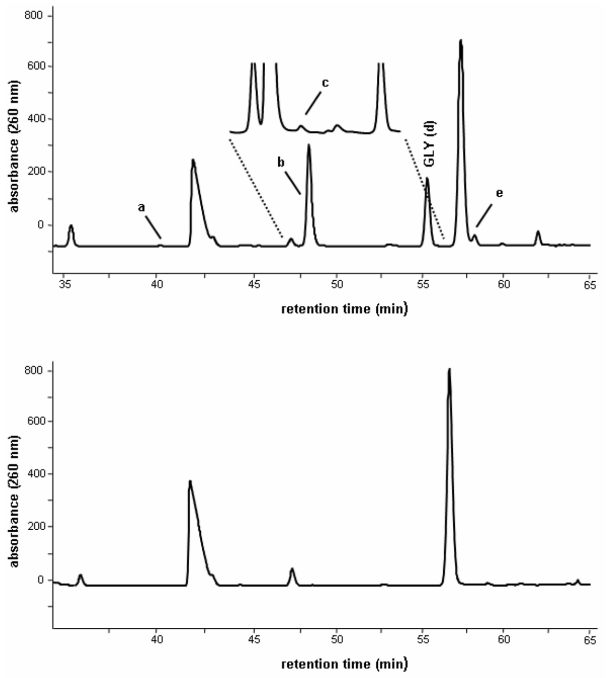


Figure 7. Representative HPLC chromatograms of GLY metabolites formed by the incubation of GLY with human intestinal microflora (upper figure) as well as of a control incubation with BHI medium and feces alone (lower figure). For peak numbers refer to Table 2.

3.2 Human microsomal metabolites of GLY

Pooled female and male HLM were used to get information about the metabolites formed *in vitro* by humans in comparison to rats. Both preparations contained 405 pmol P450 *per* mg protein. In Fig. 6, a representative HPLC chromatogram is depicted. For peak numbers refer to Table 1. Again there have been no sex-related differences detectable. Less than 4% of the used concentration of GLY is converted to six metabolites.

Main metabolites are 8-OH-GLY and 6-OH-DAI. Further metabolites are peak 5 (assumed as 2′-OH-GLY), 3′-OH-GLY, 2-OH-GLY as well as 5,6-dihydroxy-DAI. The last two products are only found in very small amounts.

3.3 Human fecal incubations with GLY

GLY was incubated with human fecal flora of two female volunteers in order to receive information about the intestinal metabolism of GLY. Incubations with DAI – used as positive control (see Section 2) – revealed that both female volunteers are equol-producers (data not shown).

Table 2. Chromatographic and spectroscopic data of the bacterial metabolites of GLY

HPLC-peak	t_R in HPLC (min)	UV maxima (nm)	t_R in GC(min)	M^+, GC/MS TMS derivative	Chemical structure
a	40.2	279, 344	26.3	488, *296*	6-OH-DHD
b	48.8	255, 323	36.4	*486*, 471	6-OH-DAI
c	50.4	222, 295	22.3	474	6-OH-equol
d	55.7	257, 320	31.2	*428*, 413	GLY
e	58.5	279, 349	17.1	562, 547, *369*	5′-OH-*O*-DMA

Peak numbers correspond to Fig. 7. The base peak in the GC/MS mass spectra of the trimethylsilylated metabolites is marked in italics.

GLY was extensively metabolized. Both volunteers converted about 70% of the used concentration to four metabolites which were clearly detectable by HPLC analysis. These products were not observed when GLY or fecal inoculation were omitted. A representative HPLC profile of the metabolites of GLY is depicted in Fig. 7. Furthermore, the extracted metabolites were analyzed by GC/MS after trimethylsilylation with BSTFA. The chemical structures of all of the numbered peaks are listed together with the analytical data in Table 2.

Peak b represents the main metabolite of incubation of GLY with human fecal flora. It is assigned to be the demethylation product 6-OH-DAI since it coelutes with the reference compound and has already been identified in the oxidative biotransformation of GLY. It accounts for more than 60% on the basis of the peak area at $\lambda = 260$ nm assuming similar ε values of 6-OH-DAI and the methylated form GLY. This seems a reasonable assumption since the effects of conjugation on the UV-Vis spectra are not significant. GC/MS analysis of peak e shows a molecular ion at *m*/*z* 369 indicating a monohydroxylated α-methyldeoxybenzoin derivative. It is also formed in the microsomal metabolism of *O*-DMA. Since the chemical composition of GLY possesses an additional methoxy-group at C-6 compared to DAI peak e is tentatively identified as 5′-OH-*O*-DMA.

Peaks a and c are formed only in trace amounts by human fecal incubations. In the GC/MS analysis, peak a shows a molecular ion at *m*/*z* 488 and a fragment ion at *m*/*z* 296 derived by a RDA reaction, indicating a hydroxylated isoflavan-4-one. It is also formed during the microsomal metabolism of DHD. Therefore, peak a is assigned to be 6-OH-DHD. Peak c is proposed to be 6-OH-equol since it coelutes with this compound formed during the *in vitro* microsomal metabolism of equol which has already been identified by Rüfer *et al.* [18].

3.4 Biotransformation of GLY by rats

The parent compound GLY was detected in all urine and feces samples collected after administration of pure GLY by gavage. Additionally, several other compounds having an isoflavonoid structure were identified. In Fig. 8, a representative GC profile of rat urine is depicted. Three oxidative (8-OH-GLY, 3′-OH-GLY, 5,6-dihydroxy-DAI) and two bacterial metabolites (6-OH-DHD and 6-OH-equol) as well as the demethylation product 6-OH-DAI can be identified through comparison with the already obtained reference compounds of the oxidative and bacterial metabolism. In feces only two metabolites can clearly be detected, namely 6-OH-DAI and 6-OH-DHD. In both cases 6-OH-DAI represents the main metabolite and it accounts for up to 0.2% in urine and up to 1% in feces on the basis of the peak areas assuming similar ionization efficiencies. No significant interindividual variations were found in the excretion pattern of the metabolites. However, since no quantitative analysis was carried out due to the lack of reference compounds, no definite conclusions can be drawn.

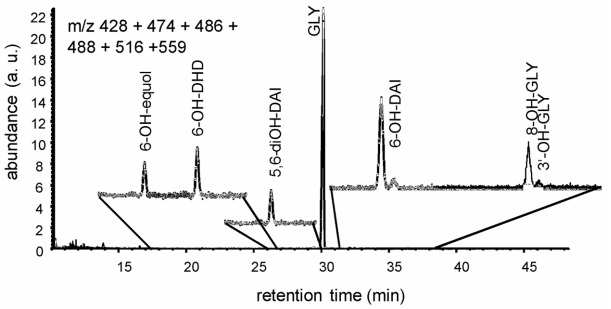
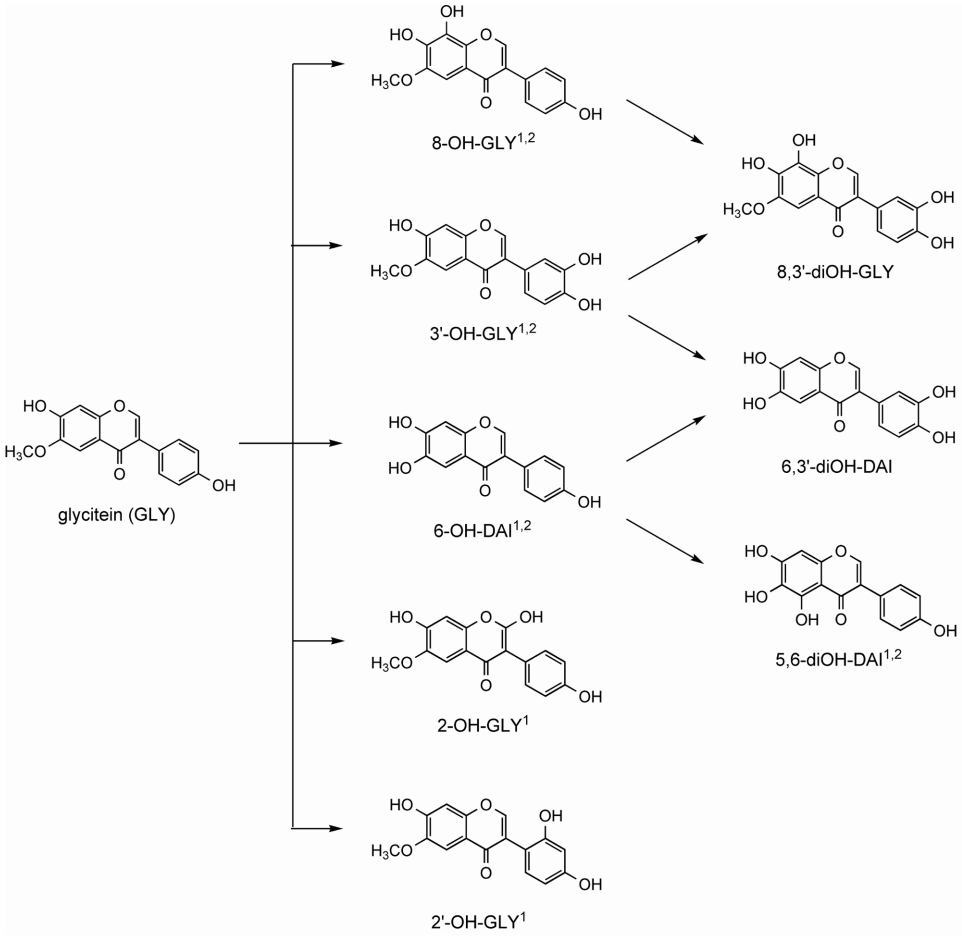


Figure 8. GC selected ion chromatogram of 24 h urine after administration of 4 mg GLY to male Sprague Dawley rats by gavage.

4 Discussion

In the present study, we have investigated the *in vitro* phase I metabolism of GLY using Aroclor-induced female and male rat liver microsomes, female and male HLM as well as human fecal flora. Furthermore, the *in vivo* biotransformation was assessed in urine and feces of male Sprague – Dawley rats after single-dose administration of pure GLY. The formed metabolites were separated by HPLC and GC and identified with the help of their API-ES and EI mass spectra and by comparison with reference compounds. Our results show that GLY is metabolized by P450 enzymes as well as



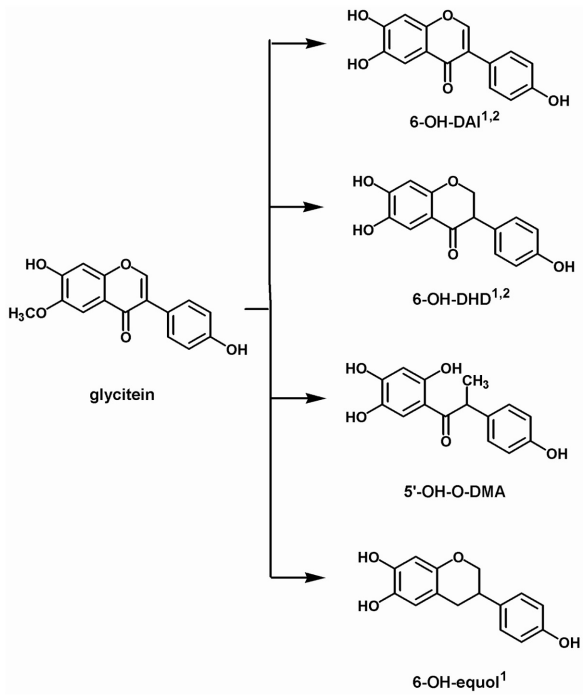


Figure 9. (upper figure) Oxidative metabolites of GLY formed after incubation of GLY with Aroclor-induced rat liver microsomes *in vitro*. Metabolites also found after incubation of GLY with HLM as well as in rat urine are labeled with 1 and 2, respectively. (lower figure) Bacterial metabolism of GLY *in vitro*. Metabolites also found in rat urine and feces are labeled with 1 and 2, respectively.

by the intestinal microflora. A complete scheme of the identified metabolites is shown in Fig. 9.

With respect to the oxidative phase I metabolism female and male HLM and rat liver microsomes have been used and no sex-related differences have been observed. There are no other studies investigating sex-related differences with respect to the oxidative metabolism of isoflavones. Only two groups studied the oxidative biotransformation of GEN and DAI *in vivo* after administration of soy products to female and male volunteers [11, 12]. Both groups do not report on significant differences between the two sexes. However, in animal studies and in some human studies profound sex differences regarding the bacterial metabolism have been observed. For instance, Coldham and Sauer [19] report on different main metabolites in rat feces following an oral dose of [^{14}C] GEN: female rats excrete predominantly dihydrogenistein, male rats 4-hydroxyphenyl-2-propionic acid. Wiseman *et al.* [20] detected higher *O*-DMA concentration in plasma and urine of male volunteers compared to female study participants after 10 wk of soy consumption. In other studies no sex differences have been stated [12, 14, 21]. However, we were not able to confirm these results *in vitro* or in rats since only female feces samples for the *in vitro* fecal incubations and male rats were used. Thus, further studies are needed to clarify the situation.

There are only two studies in the literature investigating the biotransformation of GLY *in vitro* and *in vivo* [12, 14]. Heinonen *et al.* [12] identified dihydro-GLY, 6-methoxyequol and 5′-methoxy-*O*-DMA as well as 6-OH-DAI, 6-OH-DHD, 6-OH-equol, and 5′-OH-*O*-DMA as putative bacterial metabolites of GLY. Simons *et al.* [14] identified dihydro-GLY, 6-OH-DHD and 5′-methoxy-*O*-DMA as well as DAI in one and 6-methoxy-equol in two subjects. In the *in vitro* fecal incubations as well as in rat urine and feces after administration of pure GLY we were only able to detect 6-OH-DAI, 6-OH-DHD, 6-OH-equol, and 5′-OH-*O*-DMA. No bacterial metabolites of the methylated isoflavone GLY were observed. However, it should be emphasized that an alternative route for the formation of these metabolites is the biotransformation of DAI (that means hydroxylation, reduction, methylation). Since no tracer methods (radio or stable isotopes) or pure compounds were used in the study of Heinonen *et al.* [12] no definitive associations can be made whether these compounds were formed in the metabolism of DAI or GLY. We observed that BHI medium already contains little amounts of GEN and DAI due to its bovine origin. Therefore, it seems questionable if DAI is formed in the metabolism of GLY as observed by Simons *et al.* [14].

GEN and DAI undergo extensive biotransformation in rats and in humans. King [22] could show that in rats urinary excretion of DAI over a 48-h postdose period was 17% and of its main metabolite equol 5%, 12%, and 42% of GEN and its main metabolite *p*-ethylphenol, respectively, of the administered amount of GEN were excreted *via* urine over 48 h. This is in contrast to GLY: 12% is excreted unchanged *via* urine; only 0.2% is found as the main metabolite 6-OH-DAI in urine. Furthermore, GLY, which contains an OCH_3-group in the C-6 position, is metabolized to a lesser extent than biochanin A and formononetin, the 4′-methyl-ether of GEN and DAI, respectively, found mainly in red clover in humans. Setchell *et al*. [23] report on high plasma concentrations of the demethylation products GEN and DAI and low concentrations of biochanin A and formononetin after administration of a food supplement based on red clover. DAI and GEN accounted for more than 95% of the total isoflavones measured. However, after administration of GLY-7-O-β-D-glucoside biotransformation was negligible [23]. Furthermore, Tsunoda *et al*. [24] and Heinonen *et al*. [25] detected only little amounts of the parent compounds biochanin A and formononetin and high amounts of DAI and GEN in human urine after ingestion of red clover isoflavones.

In conclusion, this is the first study to show that GLY is a substrate for P450 enzymes as well as human intestinal microflora *in vitro* and *in vivo*. However, only small amounts of the oxidative and bacterial metabolites of GLY were detected in rat urine and feces. Thus, there is evidence to suggest that GLY is – in contrast to biochanin A and Formononetin – a rather stable molecule. Therefore, a closer look at the biological activities of GLY is essential.

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5 References

[1] Adlercreutz, H., Goldin, B. R., Gorbach, S. L., Höckerstedt, K., *et al.*, Soybean phytoestrogen intake and cancer risk, *J. Nutr.* 1995, *125*, 75S – 77S.

[2] Thompson, L. U., in: Eisenbrand, G., Dayan, A. D., Elias, P. S., Grunow, W., Schlatter, J. (Eds.), *Carcinogenic and Anticarcinogenic Factors in Food*, Wiley-VCH Verlag, Weinheim 2000, pp. 348 – 358.

[3] Irvine, C., Fitzpatrick, M., Robertson, L., Woodhams, D., The potential adverse effects of soybean isoflavones in infants feeding, *N. Z. Med. J.* 1995, *108*, 208 – 209.

[4] Shecchan, D. M., Herbal medicines, phytoestrogens and toxicity: risk:benefit considerations, *Exp. Biol. Med*. 1998, *217*, 379 – 385.

[5] Bouker, K. B., Hilakivi-Clarke, L., Genistein: Does it prevent or promote breast cancer?, *Environ. Health Perspect.* 2000, *108*, 701 – 709.

[6] 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.

[7] Joannou, G. E., Kelly, G. E., Reeder, A. Y., Waring, M., Nelson, C., A urinary profile study of dietary phytoestrogens. The identification and mode of metabolism of new isoflavonoids, *J. Steroid Biochem. Mol. Biol.* 1995, *54*, 167 – 184.

[8] Kurzer, M. S., Xu, X., Dietary phytoestrogens, *Annu. Rev. Nutr.* 1997, *17*, 353 – 381.

[9] Coldham, N. G., Howells, L. C., Santi, A., Montesissa, C., *et al.*, Biotransformation of genistein in the rat: Elucidation of metabolite structure by product ion mass fragmentology, *J. Steroid Biochem. Mol. Biol.* 1999, *70*, 169 – 184.

[10] 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.

[11] Kulling, S. E., Honig, D., Metzler, M., Oxidative metabolism of the soy isoflavones daidzein and genistein *in vitro* and *in vivo*, *J. Agric. Food Chem.* 2001, *49*, 3024 – 3033.

[12] Heinonen, S. M., Hoikkala, A., Wahala, 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.

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

[14] Simons, A. L., Renouf, M., Hendrich, S., Murphy, P. A., Metabolism of glycitein (7,4′-dihydroxy-6-methoxy-isoflavone) by human gut microflora, *J. Agric. Food Chem.* 2005, *53*, 8519 – 8525.

[15] Chang, Y. C., Nair, M. G., Metabolism of daidzein and genistein by intestinal bacteria, *J. Nat. Prod.* 1995, *58*, 1892 – 1896.

[16] Burke, M. D., Thompson, S., Elcombe, C. R., Halpert, J., *et al.*, Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: A series of substrates to distinguish between different induced cytochromes P-450, *Biochem. Pharmacol.* 1985, *34*, 3337 – 3345.

[17] Correia, M. A., in: d. Montellano P. R. O. (Ed.), *Cytochrome 450: Structure, Mechanism, and Biochemistry*, Plenum Press, New York 1995, pp. 607 – 630.

[18] Rüfer, C. E., Glatt, H. R., Kulling, S. E., Structural elucidation of hydroxylated metabolite of the isoflavan equol by gas chromatography-mass spectrometry and high-performance liquid chromatography-mass spectrometry, *Drug Metab. Disp.* 2006, *34*, 51 – 60.

[19] Coldham, N. G., Sauer, M. J., Pharmacokinetics of [^{14}C]genistein in the rat: Gender-related differences, potential mechanisms of biological action, and implications for human health, *Toxicol. Appl. Pharmacol.* 2000, *164*, 206 – 215.

[20] Wiseman, H., Casey, K., Bowey, E. A., Duffy, R., *et al.*, Influence of 10 wk of soy consumption on plasma concentrations and excretion of isoflavonoids and on gut microflora metabolism in healthy adults, *Am. J. Clin. Nutr.* 2004, *80*, 692 – 699.

[21] Setchell, K. D. R., Phytoestrogens: The biochemistry, physiology, and implications for human health of soy isoflavones, *Am. J. Clin. Nutr.* 1998, *68*, 1333 – 1346.

[22] King, R. A., Daidzein conjugates are more bioavailable than Genistein conjugates in rats, *Am. J. Clin. Nutr.* 1998, *68*, 1496S – 1499S.

[23] Setchell, K. D. R., Brown, N. M., Desai, P., Zimmer-Nechemias, L., *et al.*, Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements, *J. Nutr.* 2001, *131*, 1362S – 1375S.

[24] Tsunoda, N., Pomeroy, S., Nestel, P., Absorption in humans of isoflavones from soy and red clover is similar, *J. Nutr.* 2002, *132*, 2199 – 2201.

[25] Heinonen, S. M., Wahala, K., Adlercreutz, H., Identification of urinary metabolites of the red clover isoflavones formononetin and biochanin A in human subjects, *J. Agric. Food Chem.* 2004, *52*, 6802 – 6809.