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Metabolism of the soy isoflavones daidzein, genistein and glycitein in human subjects. Identification of new metabolites having an intact isoflavonoid skeleton

Satu-Maarit Heinonen [a,b,*], Antti Hoikkala [b], Kristiina Wähälä [b], Herman Adlercreutz [a]

[a] *Department of Clinical Chemistry, Biomedicum, Folkhälsan Research Centre, University of Helsinki, P.O. Box 63, FIN–00014 Helsinki, Finland*
[b] *Laboratory of Organic Chemistry, Department of Chemistry, University of Helsinki, P.O. Box 55, FIN-00014 Helsinki, Finland*

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Abstract

Epidemiological studies have associated high soy intake with a lowered risk for certain hormone-dependent diseases. Soy and soy foods are rich sources of isoflavones, which have been shown to possess several biological activities. In this study, the metabolism of soy isoflavones daidzein, genistein and glycitein was investigated in human subjects. The aim was to find and identify urinary phase I metabolites of isoflavones, which have an intact isoflavonoid skeleton, and which might possess some bioactivity. Six volunteers included three soy bars per day into their normal western diet for a 2-week period. Daily urine samples were collected before, and after the supplementation period. Urine samples were hydrolyzed with *Helix pomatia*, extracted with diethyl ether, purified with Sephadex LH-20 chromatography, and analyzed as trimethylsilyl derivatives using gas chromatography–mass spectrometry (GC–MS). The structures of the isoflavone metabolites were identified using authentic reference compounds. The metabolites, for which authentic reference compounds were not available, were identified by the interpretation of mass spectra. Several new isoflavone metabolites were identified, and the presence of previously reported metabolites confirmed. The metabolic pathways of daidzein, genistein and glycitein are presented on the basis of the identification of the metabolites in human urine after soy supplementation.

Keywords: Isoflavone; Daidzein; Genistein; GC–MS; Glycitein; Metabolism; Soy

1. Introduction

Certain isoflavonoids, also known as phytoestrogens, are biologically active plant compounds that occur mainly in legumes, soy being the most important source of isoflavonoids in human diet [1–3]. Epidemiological studies have associated a diet rich in isoflavonoids with a lowered risk for certain diseases, such as breast and prostate cancers, osteoporosis and cardiovascular diseases [4–9]. Laboratory experiments have shown that isoflavonoids possess anti-cancer [10–12], anti-angiogenic [13], hormone-altering [14,15], and both estrogenic and anti-estrogenic activities [16–19].

The principal isoflavonoids of soy are daidzein, genistein and glycitein (Fig. 1), which exist in plants conjugated with sugars, mainly with glucose, but also with 6″-*O*-malonyl- or 6″-*O*-acetylglucose [20,21]. The glycoside conjugates, which are considered biologically inactive before hydrolysis, remain unmodified during various food preparation procedures [22,23]. Thus, in general, soy food when consumed has low levels of aglycones compared to glycosides. However, it has been shown that fermented soy products contain higher amounts of aglycones [24].

Upon ingestion the glycosides are hydrolyzed to free aglycones [25,26], which are subjected to metabolic processes, such as demethylation and reduction, by gut microflora [27–29]. Isoflavone aglycones and their metabolites are absorbed, and transported to liver, where liver microsomes catalyze the hydroxylation and conjugation of isoflavones

Abbreviations: GC-MS, gas chromatography-mass spectrometry; *O*-dma, *O*-desmethylangolensin; HPPA, 2-(4-hydroxyphenyl)-propionic acid; THB, trihydroxybenzene; TMS, trimethylsilyl; COMT, catechol ortho methyl transferase; QSM, quick silylation mixture; rDA, retro Diels-Alder

* Corresponding author. Tel.: +358-9-1912-5454; fax: +358-9-1912-5452.
E-mail address: satu-maarit.heinonen@helsinki.fi (S.-M. Heinonen).

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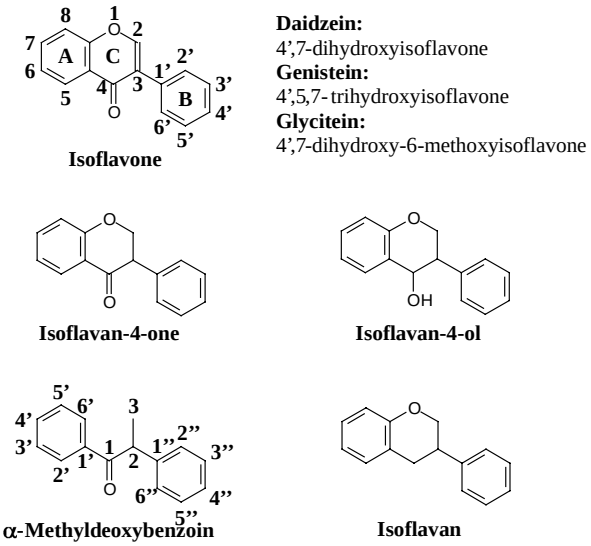


Fig. 1. Structures of isoflavonoids.

to more hydrophilic metabolites, sulfonic or glucuronic acid conjugates, which are excreted in urine. A small part of the absorbed isoflavones enters the enterohepatic circulation [30–34].

One of the metabolites of daidzein, the isoflavan equol (4′,7-dihydroxyisoflavan), has some structural features very similar to the endogenous hormone estradiol, e.g. both being phenolic [35,36]. The estrogenic properties of equol were noticed already in 1940s, when the reason for infertility syndrome among sheep grazing on clover pastures was under investigation [37,38]. Equol is an interesting metabolite because, in humans, only one-third of the population is capable of producing it in higher amounts [39,40]. The production of equol depends on the composition and the enzymatic composition of gut microflora. Recently, this metabolite has gained more interest and it has been suggested that those who produce equol may have particular benefit from soy. The metabolism of isoflavones may play an important role in the effect and the mechanism of action of isoflavones.

The metabolism of daidzein and genistein, the main isoflavones of soy, has been studied to a certain extent. The identification of reduced metabolites of daidzein, e.g. equol, *O*-desmethylangolensin (*O*-dma, 2′,4′,4″-trihydroxy-α-methyldeoxybenzoin), dihydrodaidzein (4′,7-dihydroxyisoflavanone) and *cis*-4-OH-equol (4′,7-dihydroxyisoflavan-4-ol) in human urine after soy supplementation has been confirmed using authentic reference compounds [36,41–43]. Genistein is metabolized to dihydrogenistein (4′,5,7-trihydroxyisoflavanone) and 6′-OH-*O*-dma (2′,4′,6′,4″-tetrahydroxy-α-methyldeoxybenzoin) [29,43]. While equol and *O*-dma are considered as end products of the metabolism of daidzein, the metabolism of genistein has been shown to proceed to 2-(4-hydroxyphenyl)-propanoic acid (HPPA) and trihydroxybenzene (THB), by C-ring fission [29,44]. The oxidative metabolism of soy isoflavones by liver microsomes has been demonstrated in vitro and several hydroxylated metabolites identified in human urine after soy supplementation [32,33]. The metabolites of glycitein, the third soy isoflavone, has not been reported, and it has been suggested that very little metabolism of glycitein does occur.

The aim of this study was to identify phase I metabolites of the soy isoflavones daidzein, genistein and glycitein in human urine collected after a 2 week supplementation period of soy. After soy supplementation several metabolites of soy isoflavones were found in urine samples, the structures of which were characterized using authentic reference compounds, or by the interpretation of mass spectra. Most of these metabolites are presented for the first time in the literature. The metabolic pathways, presenting the probable formation of these metabolites from plant precursor isoflavones, are presented on the basis of the identification of the metabolites in human urine.

2. Materials and methods

2.1. Reagents and standards

All solvents and reagents used were of analytical grade. 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 [45–48]. 3′,4′,7- and 4′,6,7-trihydroxyisoflavones, and orobol (3′,4′,5,7-tetrahydroxyisoflavone) were obtained from Apin Chemicals Ltd. (United Kingdom).

2.2. Synthesis of isoflavan metabolites

3′,4′,7-Trihydroxyisoflavan and 4′,7-dihydroxy-3′-methoxyisoflavan were synthesized from the corresponding isoflavones by a catalytic hydrogenation [49] over palladium on charcoal in 95% ethanol. The products were fully identified and characterized by MS and NMR spectroscopies.

2.3. Urine samples and sample pre-treatment

Six healthy Finnish volunteers, three women and three men, aged 22–25 years, included three soy bars per day into their normal western diet for a 2-week period. Daily intake of isoflavones was calculated to be 48.4, 40.2 and 4.1 mg of daidzein, genistein and glycitein, respectively [43]. The mass spectral analysis confirmed that soy bar did not contain any other isoflavonoids, including the metabolites presented in this paper, in detectable concentrations. Urine sample collection was carried out before and on two subsequent days after the soy supplementation. Urine samples were collected in plastic containers and 1% of ascorbic acid and 0.1% of sodium azide were added as preservatives. The samples were treated according the previously described sample pre-treatment method for urine samples (Fig. 2) [43].

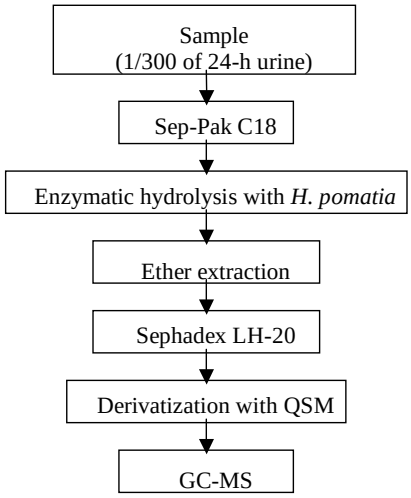


Fig. 2. Sample pre-treatment method for urine samples [43].

2.4. Derivatization and analysis by GC-MS

After pre-treatment, the samples were evaporated to dryness under nitrogen, and silylated with 100 μl of QSM (pyridine:HMDS:TMCS, 9:3:1) for 30 min at room temperature. The mixture was evaporated to dryness and the samples were dissolved in 200 μl of hexane. Deuterated trimethylsilyl (TMS) ethers were used for the structure elucidation of unknown metabolites, for which the reference compounds were not available. The deuterated TMS derivatives were obtained by the same derivatization procedure described above, except that d_9-HMDS and d_3-TMCS were used in the silylation mixture. The analysis of trimethylsilylated urinary extracts was carried out using a Fisons Instrument MD 1000 quadrupole mass spectrometer coupled to a Fisons GC 8000 gas chromatograph as described earlier [43].

2.5. Identification of urinary metabolites of daidzein, genistein and glycitein

After the soy supplementation, the soy isoflavones and their metabolites appeared in the mass spectrometric chromatograms of urine sample extracts. The metabolites could be distinguished from other urinary compounds by comparing the chromatograms of urine samples before the isoflavone challenge to the chromatograms after the isoflavone challenge. The identity of the isoflavones and their metabolites was established with synthetic reference compounds by comparing the retention times and the mass spectra of the metabolite with those of authentic reference compounds.

2.6. Approach for the identification of isoflavone metabolites without synthetic standards

For some of the isolated metabolites, the synthetic reference compounds were not available, so the identification of these was carried out by interpretation of their mass spectra. In the mass spectra of trimethylsilylated isoflavones the most abundant ions are the molecular ion, M^+, and ions formed from the loss of a methyl group, $(M - 15)^+$. Other ions, such as fragments formed via a retro Diels-Alder (rDA) reaction, are not significant. The rDA-fragments, A^+ and B^+, are more abundant in the mass spectra of trimethylsilylated isoflavanones and isoflavans (Figs. 3 and 4), and often they appear as base peaks in the mass spectra [50]. The rDA-fragments provide valuable information on the degree of substitution at each phenolic ring. For example, both dihydrodaidzein and dihydrogenistein (Fig. 3) give the same ring B fragment at *m*/*z* 192, but the A^+ ion of dihydrodaidzein is 88 mass units (mu) lower than A^+ ion of dihydrogenistein that is *m*/*z* 208 instead of 296 indicating one additional trimethylsilylated hydroxy group (TMSO) in the ring A. 3′,4′,7-trihydroxyisoflavan (Fig. 4) gives the same ring A fragment at *m*/*z* 208 as dihydrodaidzein, but the difference between ring B fragments is 88 mu. Thus, 3′,4′,7-trihydroxyisoflavan with a ring B fragment at *m*/*z* 280 has two TMSO groups in the ring B in contrast to dihydrodaidzein, which has just one TMSO group in the ring B (B^+ at *m*/*z* 192).

The fragmentation of *O*-dma (Fig. 5), an α-methyldeoxybenzoin, occurs by α- or *i*-cleavage between carbons having the carbonyl and the methyl group yielding fragments at *m*/*z* 281 and 193. The former fragment, the base peak of the spectrum, is due to the benzoyl fragment from ring A with two TMSO groups. The second fragment is a ring B fragment with one TMSO group attached. The degree of substitution at both phenolic rings can be elucidated by examining the *m*/*z* values of these fragments. For example, the base peak of the 6′-OH-*O*-dma (Fig. 5) is at *m*/*z* 369, that is 88 mu higher than the base peak of *O*-dma, indicating one additional TMSO group in the ring A. In general, the molecular ion of trimethylsilylated α-methyldeoxybenzoins is small in intensity. In the mass spectrum of *O*-dma, a loss of methyl group results in a rather abundant ion at *m*/*z* 459. The peak at *m*/*z* 209 is formed by the McLafferty type elimination of $(CH_3)_2Si = CH_2$ from the ion *m*/*z* 281 [42]. An analogous peak, originating from a similar fragmentation reaction, is observed in the mass spectrum of 6′-OH-*O*-dma at *m*/*z* 297.

When the mass spectra of trimethylsilylated derivatives are investigated, the fragmentation of TMSO groups itself may give information on the position of substituents in phenolic rings. Isoflavones that have a substituent at the C-5 position, such as genistein, for example, easily lose one methyl group to the give stable $(M - 15)^+$ ion (Fig. 6A). Similarly, isoflavones with a TMSO group at C-8 give intense $(M - 15)^+$ ions because of the stabilizing effect of O-1 (Fig. 6B) [51,52]. Isoflavones with *ortho* methoxy-TMSO or TMSO-TMSO substitution give $(M - 30)^+$ and $(M - 88)^+$ ions due to the fragmentation shown in Fig. 6C [51]. The fragments of phenolic rings A and B, formed by rDA reaction or α-cleavage, with similar losses of 15, 30 or 88 mu described above were observed in this study. For example,

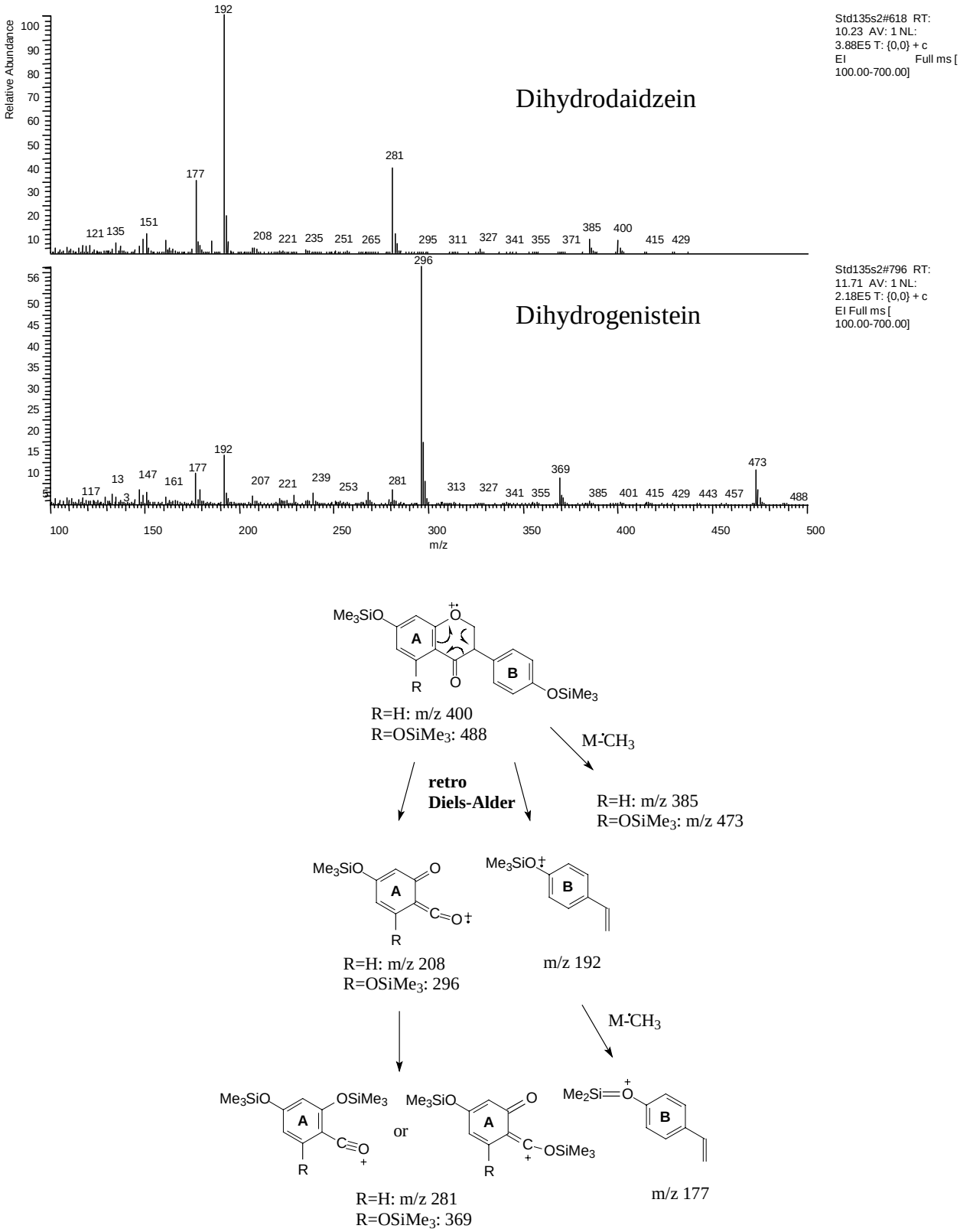
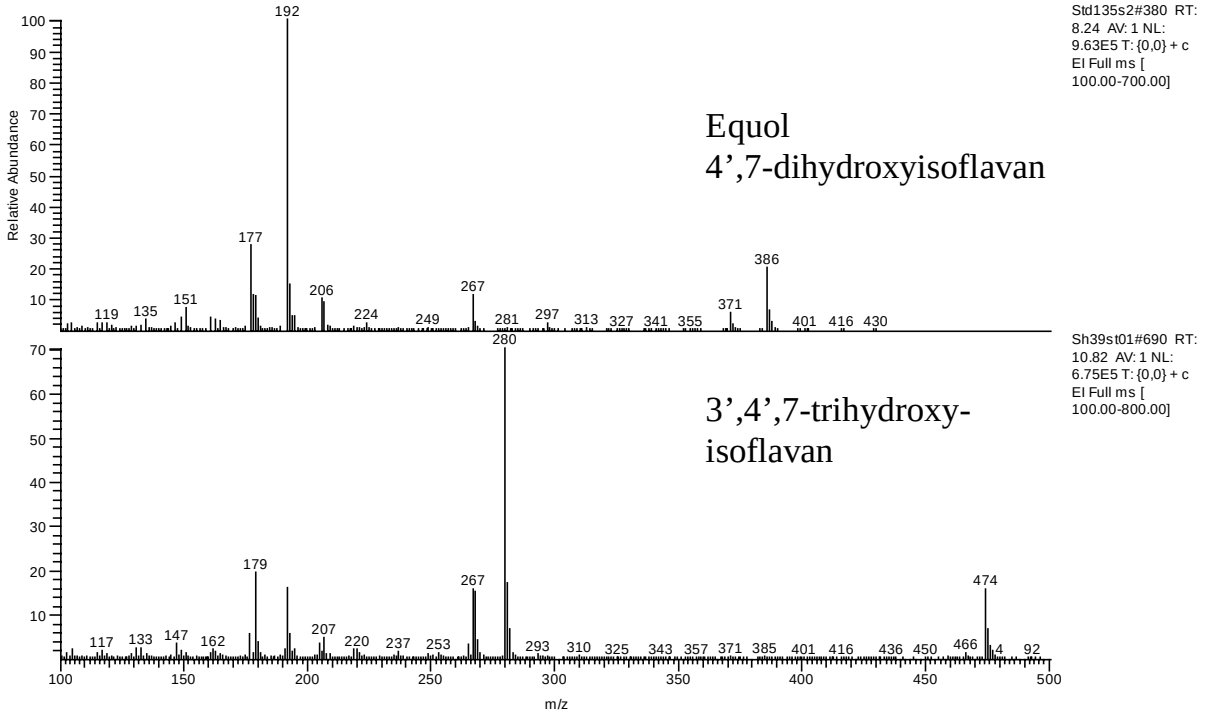


Fig. 3. Electron ionization mass spectra of trimethylsilyl (TMS) ethers of dihydrodaidzein and dihydrogenistein, and the proposed fragmentation pathways.



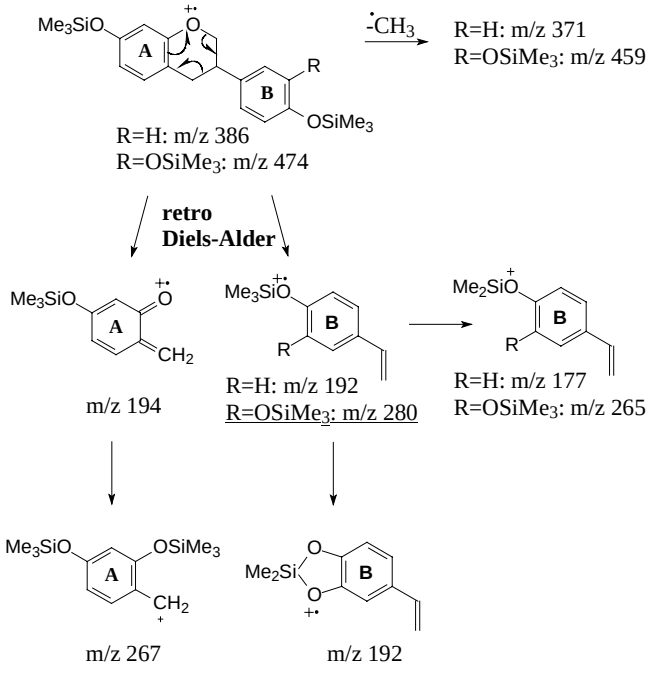
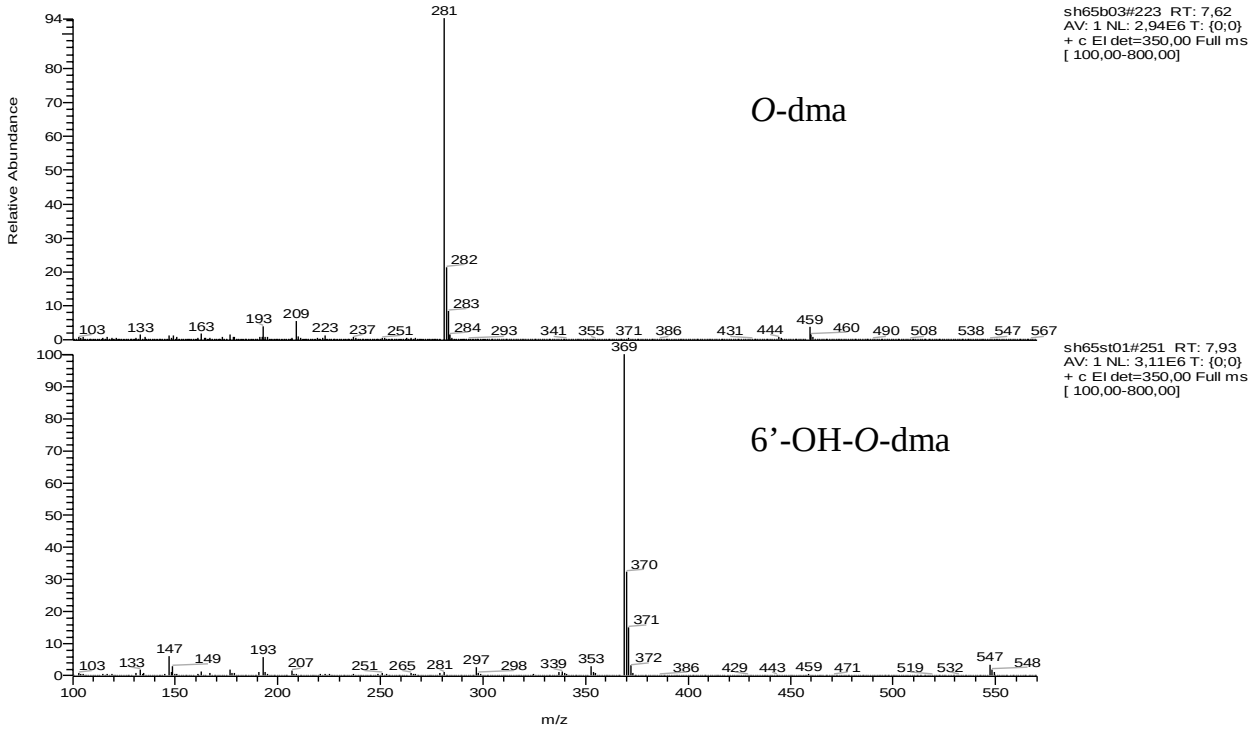


Fig. 4. Electron ionization mass spectra of TMS ethers of equol (4′,7-dihydroxyisoflavan) and 3′,4′,7-trihydroxyisoflavan, and the proposed fragmentation pathways.

in the spectrum of 3′,4′,7-trihydroxyisoflavan (Fig. 4) the peak at *m*/*z* 192 is formed by the loss of tetramethylsilane from the ring B fragment at *m*/*z* 280 or, alternatively, the cleavage of tetramethylsilane from the molecular ion occurs prior rDA reaction.

In the mass spectra of isoflavanones and isoflavans having a TMSO group in ring B at the 4′-position, the migration of one TMS group from ring B to ring A rDA-fragment was observed. The reaction may involve a long-lived ion–molecule complex [53]. For example, in the mass spectrum of



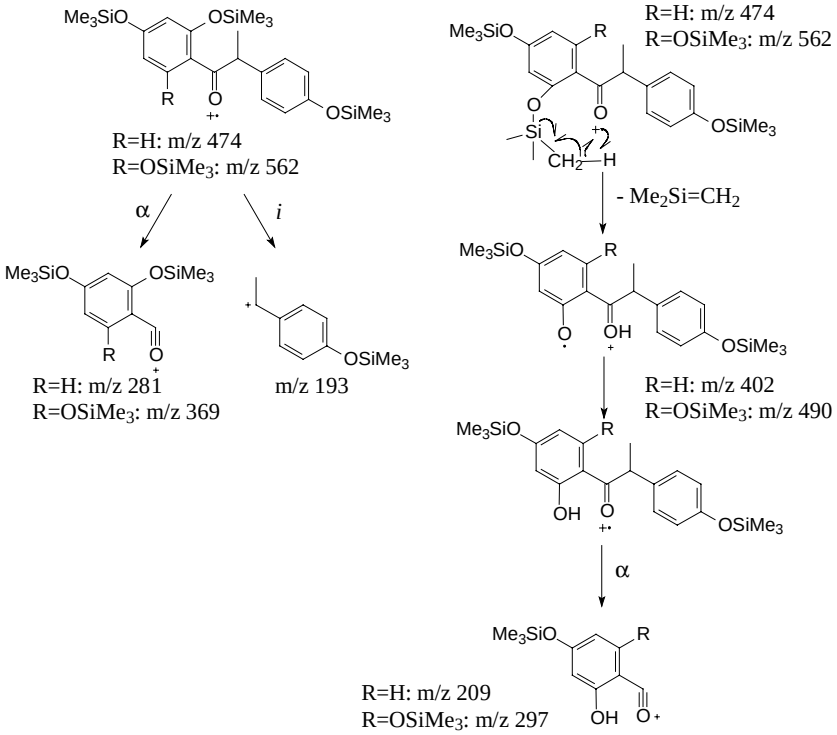


Fig. 5. Electron ionization mass spectra of TMS ethers of *O*-dma and 6′-OH-*O*dma, and the proposed fragmentation pathways.

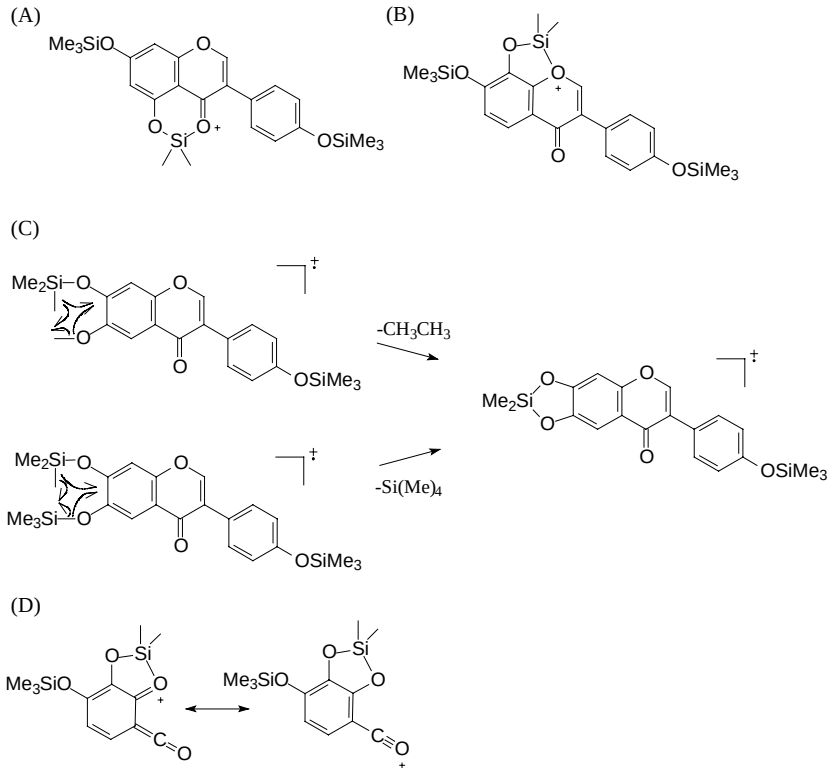


Fig. 6. Fragmentation of TMS groups and formation of stable $(M - 15)^+$, $(M - 30)^+$ and $(M - 88)^+$-ions.

dihydrodaidzein the ion at *m*/*z* 281 corresponds to ring A rDA-fragment plus one additional TMS group. Two possible structures of the ion, for which reasonable mechanisms of formation can be written, are presented in Fig. 3. The latter structure has been suggested earlier by Joannou et al. [39].

The use of deuterated TMS derivatives aids the interpretation of mass spectra of unknown urinary compounds, and the proposed structures of the molecular and, especially, the fragment ions can be confirmed. As the result of derivatization process, every free hydroxy group in the molecule is converted to the TMS ether. In deuterated trimethylsilyl derivatives, every hydrogen atom in the methyl groups are replaced by deuteriums. This means that the mass difference between deuterated and non-deuterated derivatives is nine mass units for each TMS group present in a molecule. By comparing the mass spectra of deuterated and non-deuterated compounds, it is easy to calculate the number of TMS groups present in the molecule, and thus establish the number of original hydroxy groups in the compound, or as in the case of 3′,4′,7-trihydroxyisoflavan, it is possible to confirm that the fragment at *m*/*z* 192 is not a ring B fragment similar to those of dihydrodaidzein or dihydrogenistein (Fig. 3), occurring at the same *m*/*z* value, but has two methyl groups originating from the silyl group.

Thus, the information obtained from the rDA-fragmentation for isoflavanones and isoflavans, α-cleavage of α-methyldeoxybenzoins, and fragmentation of TMS groups with certain substitution patterns, allows the tentative identification of many of the isolated unknown urinary isoflavonoids, presented in more detail in the Results section.

3. Results

Daidzein, genistein and glycitein were detected in all urine samples collected after the soy supplementation. In addition to these ingested isoflavones, several other compounds having an isoflavonoid structure were identified. The total ion chromatograms of urine samples of one subject before and after the soy supplementation are shown in Fig. 7. Equol, the reduced metabolite of daidzein, was found in urine samples of three volunteers. The total ion chromatograms of a urine sample extract of one of these so-called equol-producers are shown in Fig. 8. The mass spectral data of metabolites shown in Figs. 7 and 8 are listed in Tables 1–4.

The metabolites of soy isoflavones, reported below in more details, were identified in variable amounts in urine samples of all volunteers and in all sampling points after the soy supplementation, except the metabolites having an isoflavan structure that were found in urine samples of equol-producers only. No significant inter-individual variation was found in excretion pattern of the metabolites within the two groups (non-equol and equol-producers). Also no remarkable differences in urinary profiles, except reduced levels of both soy isoflavones and the metabolites, were found when analyzing the samples collected on first or on second day after the soy supplementation. However,

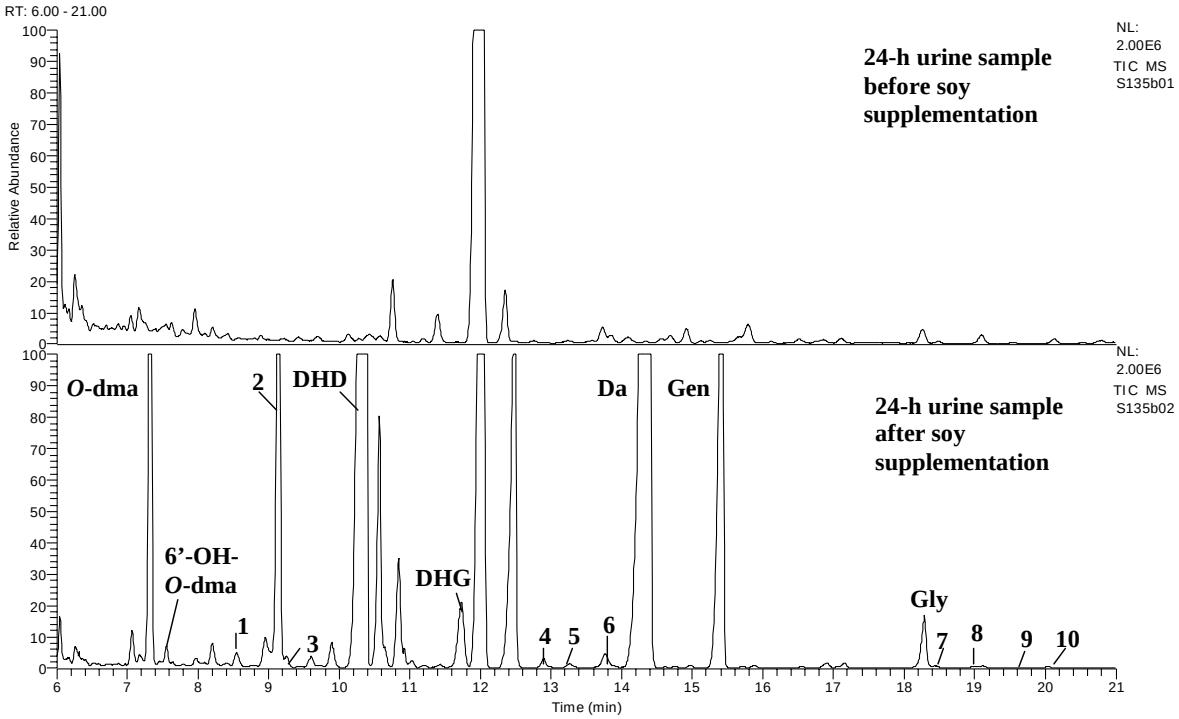


Fig. 7. Total ion chromatograms of urine sample extracts of one person before and after soy supplementation. The metabolites identified in the urine extracts are: 5′-OMe-*O*-dma (**1**); 5′-OH-*O*-dma (**2**); 3″-OH-*O*-dma (**3**); 3′,4′,7-trihydroxyisoflavanone (**4**); 4′,7,8-trihydroxyisoflavanone (**5**); 4′,6,7-trihydroxyisoflavanone (**6**); 3′,4′,7-trihydroxyisoflavone (**7**); 4′,7,8-trihydroxyisoflavone (**8**); 3′,4′,5,7-tetrahydroxyisoflavanone (**9**); and 4′,6,7-trihydroxyisoflavanone (**10**).

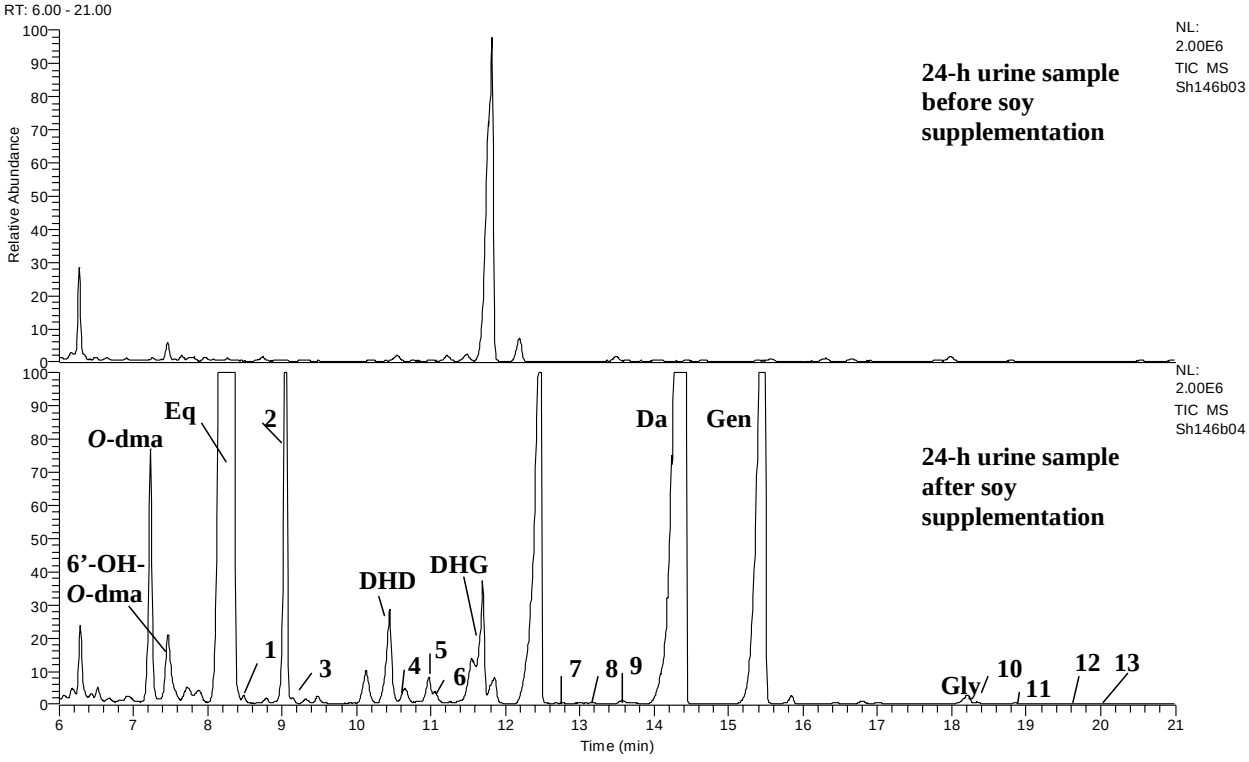


Fig. 8. Total ion chromatograms of urine sample extracts of so-called equol-producer before and after soy supplementation. The metabolites that were identified are: 5′-OMe-*O*-dma (**1**); 5′-OH-*O*-dma (**2**); 3″-OH-*O*-dma (**3**); 3′-OMe-equol (**4**); 6-OMe-equol (**5**); 3′,4′,7-trihydroxyisoflavan (**5**); 3′,4′,7-trihydroxyisoflavanone (**7**); 4′,7,8-trihydroxyisoflavanone (**8**); 4′,6,7-trihydroxyisoflavanone (**9**); 3′,4′,7-trihydroxyisoflavone (**10**); 4′,7,8-trihydroxyisoflavone (**11**); 3′,4′,5,7-tetrahydroxyisoflavanone (**12**); and 4′,6,7-trihydroxyisoflavanone (**13**).

Table 1
Mass spectral data of trimethylsilyl derivatives of isoflavones and metabolites having an isoflavone structure and identified in human urine

Compound		t_R (min)	Ion (abundance)
Daidzein	a	14.25	175 (11), 184 (38), 190 (5), 283 (4), 311 (3), 325 (3), 355 (9), 383 (70), 398 (100)
	b	13.86	181 (14), 190 (34), 199 (8), 290 (3), 318 (3), 334 (3), 370 (8), 398 (57), 416 (100)
Genistein	a	15.38	192 (1), 228 (22), 370 (1), 383 (2), 399 (11), 414 (4), 441 (1), 471 (100), 486 (1)
	b	14.99	198 (1), 239 (24), 385 (1), 396 (1), 415 (8), 432 (4), 459 (1), 495 (100), 513 (1)
Glycitein	a	18.29	175 (9), 184 (23), 191 (41), 199 (15), 208 (9), 238 (2), 253 (5), 325 (6), 355 (12), 383 (13), 398 (62), 413 (31), 428 (100)
	b	17.72	181 (12), 189 (18), 197 (19), 205 (13), 214 (12), 247 (3), 259 (4), 331 (6), 367 (9), 395 (14), 413 (63), 428 (20), 446 (100)
4′,3′,7-Trihydroxyisoflavone	a	18.45	175 (6), 190 (8), 253 (3), 383 (7), 398 (5), 413 (3), 455 (1), 471 (85), 486 (100)
	b	18.09	178 (5), 196 (7), 259 (3), 395 (7), 413 (4), 431 (3), 476 (1), 495 (74), 513 (100)
4′,6,7-Trihydroxyisoflavone	a	20.04	175 (4), 253 (3), 355 (6), 383 (8), 398 (3), 443 (2), 471 (68), 486 (100)
	b	19.45	181 (3), 259 (2), 367 (5), 395 (7), 413 (3), 467 (2), 495 (53), 513 (100)
4′,7,8-Trihydroxyisoflavone	a	19.12	175 (4), 208 (1), 253 (3), 281 (2), 355 (6), 383 (9), 398 (6), 413 (1), 443 (2), 471 (63), 486 (100)
	b	18.53	181 (4), 214 (1), 259 (2), 296 (<1), 367 (5), 395 (7), 413 (4), 431 (1), 467 (2), 495 (48), 513 (100)
3′,4′,5,7-Tetrahydroxyisoflavone	a	19.62	175 (1), 399 (5), 471 (12), 487 (5), 502 (2), 559 (100), 574 (5)
	b	19.23	178 (1), 412 (5), 492 (11), 512 (4), 528 (2), 592 (100), 610 (5)

Data of both (a) trimethylsilyl and (b) deuterated trimethylsilyl derivatives are presented.

because no quantitative analyses were carried out in this study, any definitive conclusions of the variation cannot be provided.

3.1. Isoflavones

Three oxidized metabolites of daidzein and one of genistein were identified by comparison with authentic reference compounds. The metabolites eluting at retention times 18.5, 19.1, 20.0 and 19.6 were identified as 3′,4′,7-, 4′,7,8- and 4′,6,7-trihydroxyisoflavones and 3′,4′,5,7-tetrahydroxyisoflavone, respectively. The mass spectra of these metabolites are listed in Table 1.

3.2. Isoflavanones

In addition to previously identified dihydrodaidzein and dihydrogenistein, four new metabolites having an

Table 2
Mass spectral data of isoflavanone metabolites identified in human urine

Compound		t_R (min)	M^+	Ion (abundance)
4′,7-Dihydroxyisoflavanone (dihydrodaidzein)	a	10.18	400	177 (27), 192 (100), 208 (1), 209 (1), 235 (1), 251 (<1), 281 (26), 327 (1), 385 (4), 400 (3)
	b	9.86	418	183 (25), 201 (100), 217 (1), 218 (1), 244 (1), 266 (<1), 299 (31), 336 (1), 400 (2), 418 (3)
4′,5,7-Trihydroxyisoflavanone (dihydrogenistein)	a	11.62	488	177 (14), 192 (20), 229 (5), 239 (4), 268 (5), 281 (6), 296 (100), 340 (3), 369 (9), 415 (1), 473 (12), 488 (<1)
	b	11.40	515	183 (21), 201 (47), 233 (3), 240 (7), 286 (6), 296 (5), 314 (100), 352 (3), 396 (12), 434 (2), 497 (13), 515 (<1)
4′,7-Dihydroxy-6-methoxyisoflavanone (dihydroglycitein)[a]	a	13.02	430	177 (38), 192 (95), 208 (28), 238 (100), 311 (20), 415 (4), 430 (16)
	b	12.76	448	183 (35), 201 (91), 214 (25), 247 (100), 329 (19), 430 (2), 448 (15)
3′,4′,7-Trihydroxyisoflavanone[a]	a	12.88	488	192 (16), 280 (100), 281 (37), 473 (3), 488 (9)
	b	12.65	515	198 (14), 298 (100), 299 (38), 497 (2), 515 (10)
4′,6,7-Trihydroxyisoflavanone[a]	a	13.57	488	177 (12), 192 (18), 281 (9), 296 (100), 473 (3), 488 (11)
	b	13.33	515	183 (12), 201 (18), 296 (6), 314 (100), 497 (2), 515 (9)
4′,7,8-Trihydroxyisoflavanone[a]	a	13.18	488	177 (11), 179(9), 192 (11), 208(4), 267 (2), 281 (100), 296 (7), 445 (3), 473 (2), 488 (3)
	b	12.89	515	183 (10), 188 (7), 201 (7), 214 (3), 285 (5), 296 (100), 314 (11), 469 (2), 487 (1), 515 (4)

Data of both (a) trimethylsilyl and (b) deuterated trimethylsilyl derivatives are presented.
[a] Authentic reference compound was not available, identification based on the interpretation of mass spectrum.

Table 3
Mass spectral data of isoflavan metabolites authentically identified in human urine with reference compounds

Compound		t_R (min)	M^+	Ion (abundance)
4′,7-Dihydroxyisoflavan (equol)	a	8.09	386	177 (25), 192 (100), 206 (8), 207 (8), 267 (10), 371 (5), 386 (16)
	b	7.74	404	183 (23), 201(100), 215 (8), 216 (8), 285 (10), 386 (2), 404 (16)
4′,7-Dihydroxy-3′-methoxyisoflavan	a	10.52	416	179 (12), 192 (73), 207 (21), 222 (100), 267 (3), 401 (4), 416 (21)
	b	10.24	434	185 (14), 198 (74), 213(16), 231 (100), 285 (4), 418 (4), 434 (23)
4′,7-Dihydroxy-6-methoxyisoflavan	a	10.64	416	177 (47), 179 (26), 186 (13), 192 (100), 237 (22), 297 (5), 386 (10), 401 (<1), 416 (74)
	b	10.32	434	183 (44), 188 (30),192 (28), 201 (100), 246 (22), 315 (4), 401 (7), 418 (<1), 434 (77)
3′,4′,7-Trihydroxyisoflavan	a	10.85	474	179 (30), 192 (24), 267 (26), 268 (23), 280 (100), 444 (1), 474 (22)
	b	10.52	501	185 (21), 198 (21), 285 (19), 286 (23), 298 (100), 473 (8), 501 (25)

The metabolites were found only in urine samples of three volunteers. Data of both (a) trimethylsilyl and (b) deuterated trimethylsilyl derivatives are presented.

isoflavanone structure were found in urine samples after soy supplementation. The authentic reference compounds were not available so a tentative identification of these metabolites is presented. The mass spectral data are presented in Table 2.

The mass spectral analysis of the metabolite eluting at the retention time 12.8 min shows a base ion at *m*/*z* 280 and the molecular ion at *m*/*z* 488. According to the molecular ion the metabolite is an isomer of dihydrogenistein, having three TMSO groups attached to the isoflavanone skeleton. The compound was tentatively identified as 3′,4′,7-trihydroxyisoflavanone by interpretation of the mass spectrum of non-deuterated and deuterated TMS derivatives as follows. The loss of one methyl group from the molecular ion gives the ion at *m*/*z* 473, and the corresponding fragment appears in the spectrum of the deuterated derivative at *m*/*z* 497. The even mass base peak at *m*/*z* 280 (*m*/*z* 298 in the mass spectrum of the deuterated derivative) is an rDA-fragment of a compound having two TMSO substituents in ring B. The abundance of the ion at *m*/*z* 281 (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 the silyl group migrates from the ring B rDA-fragment to the ring A rDA-fragment. In the mass spectrum of the deuterated derivative analogous peaks with the 280/281 peak pair appear at *m*/*z* 298 and 299, respectively. A rather abundant ion at *m*/*z* 192 is formed from the ring B rDA-fragment by loss of tetramethylsilane. On the basis of the ion at *m*/*z* 196 in the mass spectrum of the deuterated derivative it can be seen that the fragment indeed contains only two methyl groups originating from the silyl group. This kind of fragmentation is only possible for compounds having two vicinal hydroxy groups, so it can be assumed that this metabolite has the 3′,4′-dihydroxysubstitution in the ring B.

The molecular ions of compounds at the retention times 13.2 and 13.6 min were also at *m*/*z* 488. The former compound had a base peak at *m*/*z* 281 and other abundant fragments at *m*/*z* 473, 296 and 192. The metabolite was tentatively identified as 4′,7,8-trihydroxyisoflavanone. The ion at *m*/*z* 473 is formed by the loss of one methyl group from one of the TMS groups. An rDA rearrangement yields fragments of rings A and B at *m*/*z* 296 and 192, respectively. The

Table 4
Mass spectral data of metabolites having α-methyldeoxybenzoin structure identified in human urine

Compound		t_R (min)	Ion (abundance)
2′,4′,4″-Trihydroxy-α-methyldeoxybenzoin (*O*-dma)	a	7.32	193 (4), 209 (6), 223 (1), 237 (1), 281 (100), 459 (3), 474 (<1)
	b	7.02	202 (4), 219 (5), 235 (1), 252 (<1), 299 (100), 483 (2), 501 (<1)
2′,4′,6′,4″-Tetrahydroxy-α-methyldeoxybenzoin (6′-OH-*O*-dma)	a	7.59	147 (8), 193 (7), 297 (3), 353 (3), 369 (100), 459 (1), 547 (3), 562 (<1)
	b	7.33	162 (7), 202 (6), 316 (2), 376 (2), 396 (100), 480 (<1), 580 (2), 598 (<1)
2′,4′,4″-Trihydroxy-5′-methoxy-α-methyldeoxybenzoin (5′-Ome-*O*-dma)[a]	a	8.20	504 (<1), 489 (1), 311 (100), 281 (13), 209 (1), 193 (9)
	b	7.98	531 (<1), 513 (2), 329 (100), 296 (25), 216 (2), 202 (7)
2′,4′,5′,4″-Tetrahydroxy-α-methyldeoxybenzoin (5′-OH-*O*-dma)[a]	a	9.18	147 (4), 193 (8), 281 (8), 369 (100), 547 (2), 562 (1)
	b	8.97	162 (3), 202 (6), 296 (8), 396 (100), 580 (1), 598 (1)
2′,4′,3″,4″-Tetrahydroxy-α-methyldeoxybenzoin (3″-OH-*O*-dma)[a]	a	9.32	147 (4), 193 (4), 209 (7), 223 (3), 281 (100), 547 (1), 562 (<1)
	b	9.05	162 (2), 199 (3), 219 (5), 232 (2), 299 (100), 580 (1), 598 (<1)

Data of both (a) trimethylsilyl and (b) deuterated trimethylsilyl derivatives are presented.
[a] Authentic reference compound was not available, identification based on the interpretation of mass spectrum.

positions of two hydroxy groups at ring A can be deduced by the base peak at *m*/*z* 281, which is formed by loss of one methyl group from a TMSO group at 8-position to yield the stable ion analogous to that shown in Fig. 6D. Fragments corresponding to those described above were found also in the mass spectrum of the deuterated derivative of the metabolite.

The metabolite eluting at the retention time 13.6 min was tentatively identified as 4′,6,7-trihydroxyisoflavanone. The molecular ion and the base peak of the metabolite are at *m*/*z* 488 and 296, respectively. The base peak of the spectrum is an rDA-fragment of ring A suggesting that the compound has two TMSO groups at ring A. A TMSO substituent at 5-position would result in an abundant fragment at *m*/*z* 473, as in the spectrum of dihydrogenistein, and the substituent at 8-position would yield the abundant fragment at *m*/*z* 281, as in the spectrum of tentatively identified 4′,7,8-trihydroxyisoflavanone, so most probably this compound has the hydroxy substituents at positions 6 and 7.

The minor metabolite at the retention time 13.0 min had a molecular ion at *m*/*z* 430 and a base peak at *m*/*z* 238. The metabolite was tentatively identified as 4′,7-dihydroxy-6-methoxyisoflavanone, dihydroglycitein, by interpretation of the mass spectrum of the TMS derivative. The base peak at *m*/*z* 238 is a ring A rDA-fragment having one methoxy and one TMSO group. The ion at *m*/*z* 208 is formed from the ring A rDA-fragment by the loss of ethane. The fragment can be formed only if the two substituents, methoxy and TMSO group, are attached at adjacent carbon atoms, so it is suggested that the compound has the 7-hydroxy-6-methoxy-substitution similar to the substitution pattern of ring A of glycitein. Other ions formed from ring A rDA-fragment are at *m*/*z* 311 and 281. The former ion is formed by the migration of the TMS group from ring B rDA-fragment to ring A rDA-fragment as shown in Fig. 6D. The ion at *m*/*z* 281 results from the loss of ethane as in formation of the ion at *m*/*z* 208. The ring B rDA-fragment is at *m*/*z* 192.

3.3. Isoflavans

Metabolites having an isoflavan structure were detected only in urine samples of the so-called equol-producers. The structures of three new isoflavan metabolites were determined by comparison with reference compounds.

The metabolite at retention time 10.9 min gave a molecular ion at *m*/*z* 474 and the base peak at *m*/*z* 280. The base peak originates from the ring B rDA-fragment of a compound having two hydroxy groups attached to ring B. The retention time and the mass spectrum of the urinary compound were identical to those of a synthetic 3′,4′,7-trihydroxyisoflavan. The mass spectral data are listed in Table 3.

Two minor metabolites with a molecular ion at *m*/*z* 416 eluted at the retention times 10.5 and 10.6 min. Using authentic reference compounds these metabolites were identified as 4′,7-dihydroxy-3′-methoxyisoflavan and 4′,7-dihydroxy-6-methoxyisoflavan, respectively. The mass spectra of both deuterated and non-deuterated TMS derivatives of the compounds are listed in Table 3.

3.4. α-Methyldeoxybenzoins

Three new metabolites with a α-methyldeoxybenzoin structure were found. The first metabolite, eluting at the retention time of 8.5 min, was identified as 5′-OMe-*O*-dma by the interpretation of mass spectra of non-deuterated and deuterated TMS derivatives. The molecular ion of the spectrum was at *m*/*z* 504, from which loss of one methyl radical yielded a more abundant fragment at *m*/*z* 489. As typical for α-methyldeoxybenzoins, the spectrum had only one intensive peak at *m*/*z* 311 (the base peak, *m*/*z* 329 in the spectrum of deuterated derivative), originating from the ring A fragment having one methoxy and two TMSO substituents. The loss of ethane yields the fragment at *m*/*z* 281, which can be confirmed by the presence of ion at *m*/*z* 296 in the spectrum of deuterated derivative. The ring B fragment appears at *m*/*z* 193.

Two isomers of 6′-OH-*O*-dma, having a molecular ion at *m*/*z* 562, eluted at the retention times 9.2 and 9.3 min. The metabolites were tentatively identified as 5′-OH-*O*-dma and 3″-OH-*O*-dma, respectively. The mass spectrum of 5′-OH-*O*-dma is almost identical to that of 6′-OH-*O*-dma. The base peak of the spectrum is at *m*/*z* 369 suggesting that the metabolite has three TMSO groups attached to ring A. The loss of tetramethylsilane from the ring A fragment ion yields a fragment ion at *m*/*z* 281. On the basis of this fragment,it can be assumed that the ring A fragment has two adjacent hydroxy groups. The ring B fragment is at *m*/*z* 193.

In the spectrum of 3′-OH-*O*-dma, the base peak at *m*/*z* 281 is formed by the α-cleavage of the bond between carbon atoms C1 and C2 to yield two fragments of equal size. Other fragment ions are very low in intensity. The ion at *m*/*z* 193 is formed from ring B fragment by loss of tetramethylsilane. It can thus be assumed that in this compound the ring B has two vicinal hydroxy groups that most probably are at the 3″ and 4″ positions. The ion at *m*/*z* 209 is formed by McLafferty-type elimination of $(CH_3)_2Si = CH_2$ from the ring A fragment ion.

4. Discussion

There is a growing interest in the proposed health effects of soy and soy isoflavones. Until recently, the studies have focused on two main soy isoflavones, daidzein and genistein, which have been tested as aglycones or as glucoside conjugates in several cell lines and animal models [12,16,54–56]. Very little attention has been paid to the metabolism of isoflavones, and the possible biological activities of the metabolites themselves. However, the estrogenic activity of equol, a metabolite of daidzein, has been demonstrated [37,38] and the metabolites of soy isoflavones are potent antioxidants [57–61]. Also phase II metabolites,

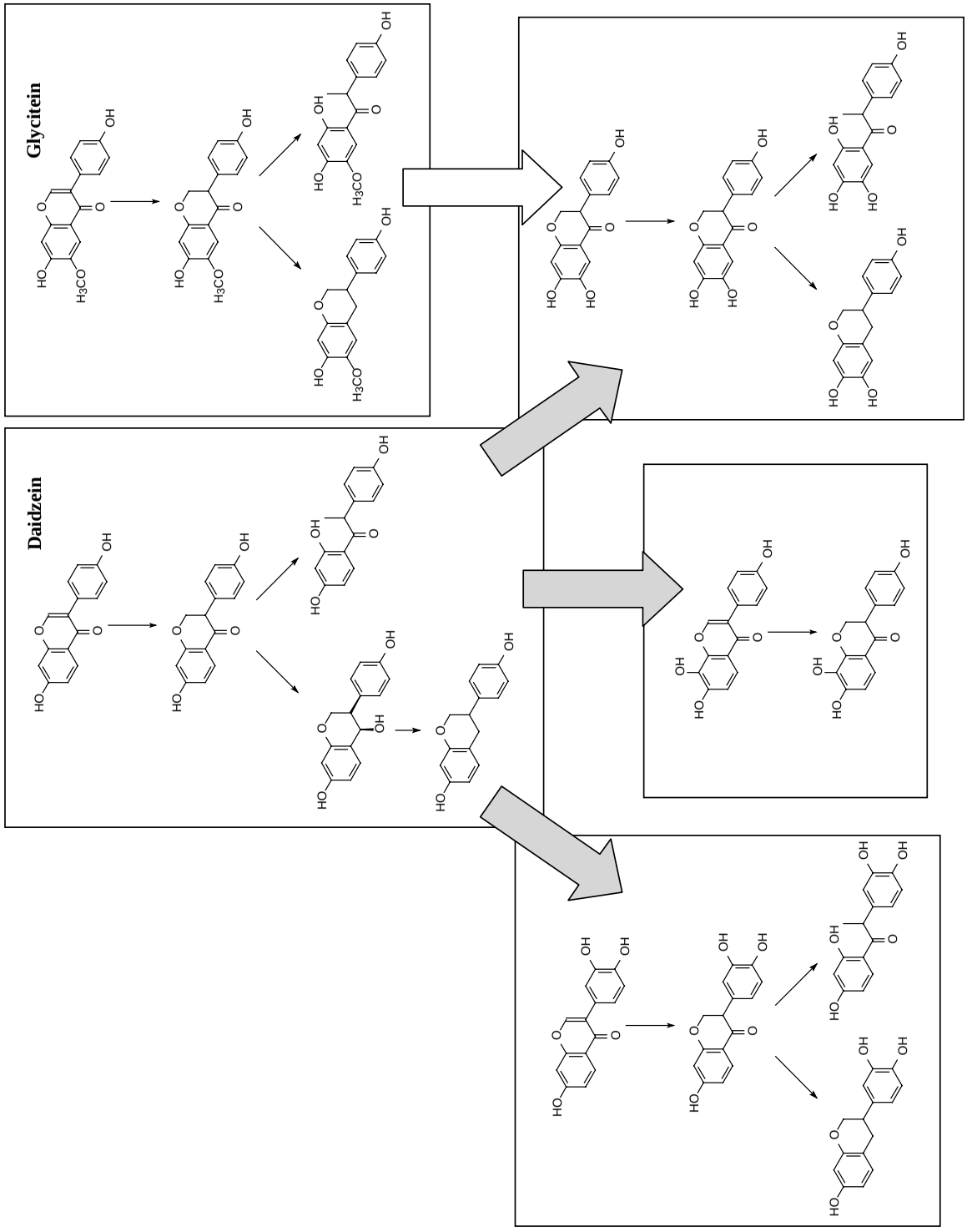


Fig. 9. Proposed metabolic pathway of soy isoflavones daidzein and glycitein.

sulfate and glucuronic acid conjugates of isoflavones, have been under investigation, and some biological activities have been reported [62].

In the present study, he phase I metabolism of isoflavones was studied by identifying isoflavone metabolites in human urine, collected after soy supplementation. Several new metabolites of isoflavones, listed in Tables 1–4, were identified either using authentic reference compounds or by the interpretation of mass spectra. The presence of previously reported reduced metabolites of daidzein and genistein, dihydrodaidzein, equol, *O*-dma and *cis*-4-OH-equol, dihydrogenistein and 6′-OH-*O*-dma was confirmed. We report for the first time the identification of reduced metabolites of glycitein, the third soy isoflavone, the metabolism of which has not been previously established in humans. The levels of glycitein metabolites, dihydroglycitein, 5′-OMe-*O*-dma and 6-OMe-equol, in soy urine samples were low, but the identification of these metabolites in human urine suggest that, like daidzein and genistein, also glycitein is converted to reduced metabolites by gut microflora.

The formation of hydroxylated metabolites of daidzein, genistein and glycitein has been demonstrated by Kulling et al. [33,63] and they have also identified a number of hydroxylated metabolites in human urine after soy supplementation. In our study, we were able to identify three hydroxylated metabolites of daidzein, 3′,4′,7-, 4′,6,7- and 4′,7,8-trihydroxyisoflavones, and one of genistein, 3′,4′,5,7-tetrahydroxyisoflavone. It is suggested that these four compounds can be considered as the main oxidized metabolites of daidzein and genistein, since the levels of other previously reported hydroxylated metabolites, tentatively identified in urine samples of this study too, are notably lower. Whether there is individual variation in producing these oxidized metabolites remains to be studied. It should be noted that an alternative route for the formation of 4′,6,7-trihydroxyisoflavone is the demethylation of glycitein. It has been seen when analyzing the urine samples collected after red clover supplementation that the levels of 4′,6,7-trihydroxyisoflavone, and other metabolites having the same substitution pattern, are notably lower than in the urine samples collected after soy supplementation (Heinonen et al., submitted for publication), which can be explained by the lower levels of glycitein in red clover as compared to those in soy.

Our results suggest that oxidative metabolic pathways also exist for isoflavone metabolites formed by gut microflora. Additionally, but probably to lesser extent, liver metabolites that undergo enterohepatic circulation may be subjected to reductive reactions by gut microflora. Several new metabolites having isoflavanone, isoflavan and α-methyldeoxybenzoin structures with additional hydroxy groups at 3′-, 6- or 8-positions were identified in our study. The levels of these metabolites were low in urine. Three of the six participants were so-called equol-producers, and equol as well as other metabolites having an isoflavan structure were detected in urine of these persons only.

It can be concluded that the metabolism of isoflavones in humans is diverse; many metabolic products are found after isoflavone supplementation. The metabolic pathways of daidzein, genistein and glycitein are proposed on the basis of the identification of the metabolites in human urine after soy supplementation (Figs. 9 and 10). Even though no quantitative analysis of isoflavonoids was carried out in this study, some tentative information on amounts of metabolites can be obtained by comparing the heights of the peaks in total ion current chromatograms (Figs. 7 and 8). The main phase I metabolic reaction of soy isoflavone aglycones seems to be reduction. The metabolites formed by other reactions are

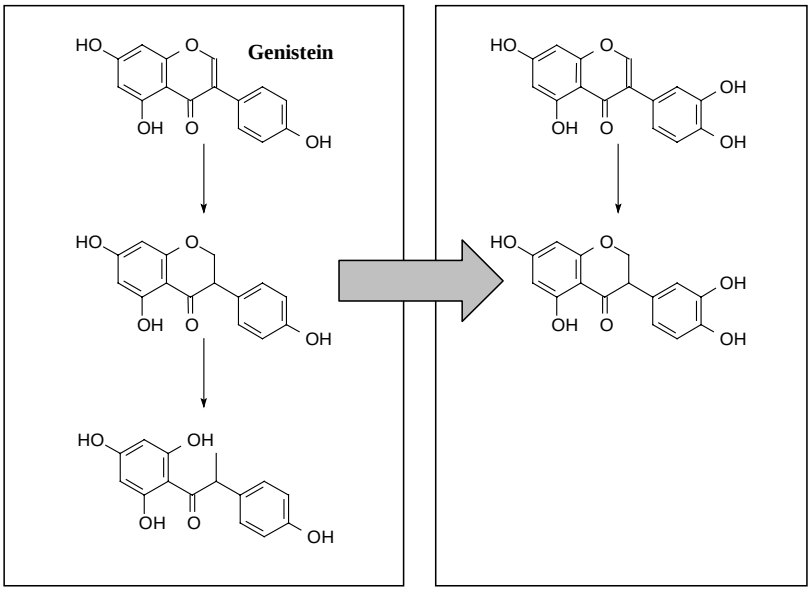


Fig. 10. Proposed metabolic pathway of genistein. Note that no isoflavan metabolites have been identified for genistein or orobol.

less abundant. For example, the hydroxylated metabolites account only for a minor percentage of total isoflavone metabolites. Some demethylation of glycitein does occur [28]. The extent of demethylation seems to be dependent on the position of the methoxy group, since biochanin A and formononetin that have the methoxy groups at ring B are almost completely demethylated to genistein and daidzein, respectively. Kulling et al. [33] have demonstrated that methylation of oxidized metabolites of isoflavones does occur in vitro and in vivo to a minor extent [33]. The identification of 4′,7-dihydroxy-3′-methoxyisoflavan, 3′-OMe-equol, in human urine after soy supplementation suggests that, as well as isoflavones, also isoflavone metabolites that have vicinal hydroxy groups may be methylated by catecol ortho methyl transferase (COMT). It should be noted, however, that because no tracer methods (radio, or more usually in humans, stable isotopes) were used in this study, no definitive associations between the parent compounds and the metabolites presented in the Figs. 9 and 10 can be provided.

The analysis of metabolic products, formed by C-ring fission or conjugation with sulphonic or glucuronidic acids, was not possible using the GC–MS method presented here. Further studies are thus needed to find out the true end products of metabolism of isoflavones, as well as the actual amounts and types of circulating isoflavone conjugates.

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