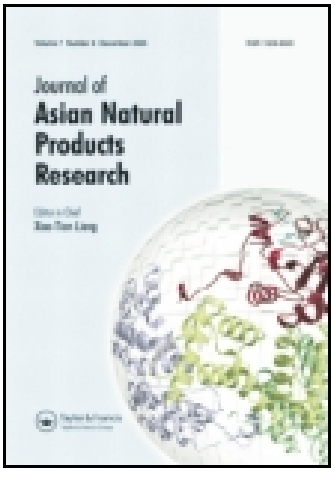

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Metabolic studies of four soy isoflavones in rats by HPLC–HR-MS

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In this paper, the metabolites of four soy isoflavones, daidzein, daidzin, genistein, and genistin, on perfused rat intestine–liver model were investigated by high-performance liquid chromatography coupled with high-resolution mass spectrometer/tandem mass spectrometer. Totally 16 metabolites were detected and identified based on accurate mass, fragmentation patterns, and multiple-stage mass data (MS^n). The metabolic site of dadzein-7-methyl ether (D-7-M) was further confirmed by nuclear magnetic resonance. Methylation, glucuronide conjugation, and sulfate conjugation were the primary metabolic processes. Among them, six metabolites, daidzin-4′,7-diglucoside, genistein-4′-glucoside, D-7-M, dadzein-4′,7-dimethyl ether, genistein-4′-methyl ether, and genistein-7-methyl ether were detected in rats for the first time and not reported in humans. The metabolic pathways of daidzein, daidzin genistein, and genistin in rats were postulated. The biological effects of these metabolites are worthy of further investigation.

Keywords: soy isoflavones; metabolites; HPLC–HR-MS/MS^n

1. Introduction

Soy isoflavones in soybean and soybean-derived products are the major source of phytoestrogens with potent estrogenic activity in the human diet. Genistein and daidzein are the two most active isoflavones in soybeans. Soy isoflavones have been the subject of rigorous investigation during the past two decades. A remarkable amount of epidemiological studies and *in vitro* experiments suggested that soy isoflavones provide important health benefits, including a reduced risk of coronary heart disease and breast, endometrial and cancers and alleviation of menopause-related hot flashes [1–6]. It is still ambiguous, however, what is the whole material basis responsible for the effects in these epidemiological studies? Metabolites play an important role in bioactive effects of soy isoflavones. Several investigators have reported that the conjugates may be biologically active or may be precursors of biologically active compounds for specific target cells. For example, a sulfoconjugate of daidzein, daidzein-4′,7-disulfate (D-4′,7-diS), inhibits sterol sulfatase in hamster liver microsomes [7]. Daidzein and genistein glucuronides have weak estrogenic activity and can activate human natural killer cells [8]. Hence the possible biological activities of the metabolites that to evaluate the health effects of soy isoflavones needs to completely understand their *in vivo* processes including intake, absorption, metabolism and excretion. Among of them metabolites identification is the first step. Soy isoflavones are known to be biotransformed to their corresponding aglycones and sugar moieties by enteral microorganisms [9].

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And then the isoflavones are metabolized as glucuronide, sulfate, and sulfoglucuronide conjugates [10,11]. But it is not the whole metabolism fate of soy isoflavones. With the development of hybride mass spectrometer with high resolution and high sensitivity, the metabolites *in vivo* are easily discovered and identified.

In this report, a high-performance liquid chromatography coupled with a hybrid linear ion-trap and fourier transform ion cyclotron resonance mass spectrometry (HPLC–LTQ-FTICR-MS^n) method was used to discover metabolites of daidzein, genistein, daidzin, and genistin on perfused rat intestine–liver model. The *in situ* single-pass vascularly perfused rat intestine–liver preparation [12] was utilized to produce and collect metabolites of daidzein, genistein, and their respective glycosides. The advantages of this model include that composition of perfusion medium is fully controlled; the effect of enterohepatic circulation is eliminated; the native architecture of the small intestine is preserved allowing the simultaneous examination of intestinal metabolic, absorptive and secretory processes; perfusate was collected continuously beyond sampling time limitation of blood, etc. Also bile duct catheterized rats were used to get metabolites after hepatic metabolism. High-resolution mass spectrometer (HR-MS) and multiple-stage mass spectrometric data of compounds in biological samples were acquired automatically using HPLC–LTQ-FTICR-MS^n via data-dependent scan. The structures of the metabolites were identified on the basis of accurate mass, fragmentation patterns, and MS^n data. As a result, a total of 16 metabolites as shown in Figure 1 were detected and identified in biological samples. Also the metabolic pathways of metabolites were proposed. HPLC–LTQ-FTICR-MS^n had been successfully utilized to discover and identify metabolites of soy isoflavones in rats and the combined method provided a better tool for study and complement up-to-date knowledge of metabolic fate of daidzein, genistein, daidzin, and genistin after oral administration by rats.

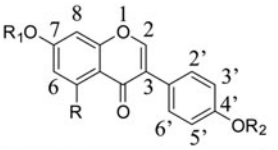
2. Results

The reference substances and bio-samples including processed perfusate, intestinal contents, and bile were analyzed by HPLC–LTQ-FTICR-MS^n. Aglycones of daidzin and genistin were found in bio-samples after administration of daidzin and genistin, respectively, by comparing with the information of standards. Based on multiple-stage mass data of standard and possible metabolic pathway, metabolites were filtered out. Then the structures of the metabolites were elucidated according to the relative mass information, including the proposed elemental composition based on accurate mass, fragmentation patterns, MS^2 and MS^3 spectra collected with data-dependent MS/MS analysis, and possible metabolic pathways. Furthermore, the biotransformation site of one metabolite of dadzein was validated as dadzein-7-methyl ether (D-7-M) by nuclear magnetic resonance (NMR).

Except aglycones metabolized from daidzin and genistin, a total of 14 different metabolites of daidzein, daidzin, genistein, and genistin from three biological samples were detected. Metabolites of daidzein, daidzin, genistein, and genistin detected in three biological samples and related MS data are summarized in Table 1. The representative extract ion current (EIC) chromatograms of daidzein, daidzin, genistein, genistin, and their metabolites are presented in Figures 2 and 3, respectively.

2.1 Mass fragmentation of daidzein, daidzin, genistein, and genistin

Daidzein, daidzin, genistein, and genistin were analyzed by HPLC–LTQ-FTICR-MS^n. The combination of FTICR mass



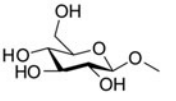
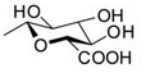
Compound	R	R1	R2	Glc (Glucoside)
daidzein	H	H	H	
daidzin (D-7-Glc)	H	Glc	H	
D-4'-M	H	H	M	
D-4',7-diM	H	M	M	
D-7-Glu	H	Glu	H	Glu (Glucuronide)
D-4'-Glu	H	H	Glu	
D-7-Glu-4'-S	H	Glu	S	
D-7-S-4'-Glu	H	S	Glu	
D-4',7-diGlc	H	Glc	Glc	
genistein	OH	H	H	M (Methyl)
genistin (G-7-Glc)	OH	Glc	H	
G-7-M	M	OH	H	
G-4'-M	OH	H	M	
G-7-Glu	OH	Glu	H	S (Sulfate)
G-4'-Glu	OH	H	Glu	
G-7-Glu-4'-S	OH	Glu	S	
G-7-S-4'-Glu	OH	S	Glu	
G-4'-Glc	OH	H	Glc	

Figure 1. Structures of daidzein, daidzin, genistein, genistin and their metabolites considered in this study. Abbreviations: D-7-M, dadzein-7-methyl ether; D-4′,7-diM, dadzein-4′,7-dimethyl ether; D-7-Glu, daidzein-7-glucuronide; D-4′-Glu, daidzein-4′-glucuronide; D-7-Glu-4′-S, daidzein-7-glucuronide-4′-sulfate; D-4′-Glu-7-S, daidzein-4′-glucuronide-7-sulfate; D-4′,7-diGlc, daidzin-4′,7-diglucoside; G-7-M, genistein-7-methyl ether; G-4′-M, genistein-4′-methyl ether; G-7-Glu, genistein-7-glucuronide; G-4′-Glu, genistein-4′-glucuronide; G-7-Glu-4′-S, genistein-7-glucuronide-4′-sulfate; G-4′-Glu-7-S, genistein-4′-glucuronide-7-sulfate; D-4′-Glc, genistein-4′-glucoside.

analyzer and tandem mass spectrometry is capable of providing useful information such as characteristic fragment ions and fragmentation pattern. And the fragmentation patterns of reference compounds were utilized in correlation to propose the structures of metabolites.

In the full scan of high-resolution mass spectrum, authentic daidzein, daidzin, genistein, and genistin exhibited $[M + H]^+$ ions at *m/z* of 255.06477 ($C_{15}H_{11}O_4$, error −1.63 ppm), 417.11725 ($C_{21}H_{21}O_9$, error −1.85 ppm), 271.05966 ($C_{15}H_{11}O_5$, error −1.62 ppm), and 433.11221 ($C_{21}H_{21}O_{10}$, error −1.65 ppm) with retention time of 18.16 min, 10.24 min, 24.81 min, and 13.38 min, respectively (Table 1).

In the MS^2 spectrum of daidzein, the most abundant fragment ion at *m/z* 199 was formed via loss of 2CO. A fragment ion resulting from retro-Diels–Alder (RDA) fragmentation was found at *m/z* 137 (62) due to loss of C_8H_6O. The product ions at *m/z* 227 (49), 237 (22), and 145 (11) were generated by the loss of CO, H_2O, and $C_6H_6O_2$, respectively. In the MS^3 spectrum, the neutral loss of H_2O from *m/z* 199 was observed and the corresponding base peak at *m/z* 181 was generated [13].

Table 1. Summary of metabolites of daidzein, daidzin, genistein, and genistin detected in three biological samples and their MS data.

Metabolites	R_t	Exact mass	δ (ppm)	Formula	MS^n	Source
Daidzein	18.16	255.06477	−1.63	$C_{15}H_{11}O_4$	MS^2[255]: 199 (100), 137 (62), 227 (49), 237 (22), 255 (13), 145 (11); MS^3[199]: 181 (100)	P, B, I
Daidzin (D-7-Glc)	10.24	417.11725	−1.82	$C_{21}H_{21}O_9$	MS^2[417]: 255 (100); MS^3[255]: 199 (100), 227 (75), 137 (65), 237 (31), 145 (11)	P, B, I
D-7-M	27.15	269.08075	−0.32	$C_{16}H_{12}O_4$	MS^2[269]: 213 (100), 151 (62), 241 (33), 269 (21)	P, B, I
D-4′,7-diM	32.62	283.09616	−1.15	$C_{17}H_{15}O_4$	MS^2[283]: 255 (100); MS^3[255]: 199 (100), 227 (78), 137 (75), 145 (16)	P, B,I
D-7-Glu	10.68	431.09683	−1.03	$C_{21}H_{19}O_{10}$	MS^2[431]: 255 (100); MS^3[255]: 199 (100), 137 (89)	P,B
D-4′-Glu	11.15	431.09650	−1.79	$C_{21}H_{19}O_{10}$	MS^2[431]:255 (100); MS^3[255]: 199 (100), 237 (75), 227 (55),137 (13), 145 (10)	P,B
D-7-Glu-4′-S	7.91	511.05380	−0.57	$C_{21}H_{19}O_{13}S$	MS^2[511]: 355 (100); MS^3[355]: 255 (100)	P,B
D-7-S-4′-Glu	8.87	511.05405	−0.08	$C_{21}H_{19}O_{13}S$	MS^2[511]: 355 (100); MS^3[355]: 255 (100)	P,B
D-4′,7-diGlc	8.82	579.17029	−0.94	$C_{27}H_{31}O_{14}$	MS^2[579]: 255 (100), 417 (22); MS^3[255]: 227 (100), 237 (41), 199 (27)	P
Genistein	24.81	271.05966	−1.62	$C_{15}H_{11}O_5$	MS^2[271]: 153 (100), 215 (80), 243 (72), 253 (37), 149 (20), 145 (14); MS^3[215]: 197 (100)	P, B, I
Genistin (G-7-Glc)	13.38	433.11221	−1.65	$C_{21}H_{21}O_{10}$	MS^2[433]: 271 (100); MS^3[271]: 153 (100), 215 (50), 243 (49), 149 (30), 253 (29), 145 (15), 159 (12)	P, B, I
G-4′-M	33.00	285.07559	−0.56	$C_{16}H_{13}O_5$	MS^2[285]: 269 (100), 271 (11); MS^3[269]: 167 (100), 241 (24), 225 (22)	P, B, I
G-7-M	33.38	285.07553	−0.77	$C_{16}H_{13}O_5$	MS^2[285]: 269 (100), 229 (46), 253 (28), 257 (21), 271 (16), 149 (11); MS^3[269]: 151 (100), 253 (46)	P, B, I
G-4′-Glc	14.08	433.11212	−1.85	$C_{21}H_{21}O_{10}$	MS^2[433]: 271 (100); MS^3[271]: 243 (100), 153 (94), 215 (89), 253 (34), 149 (25)	P, B, I
G-7-Glu	13.98	447.09213	−0.13	$C_{21}H_{19}O_{11}$	MS^2[433]: 271 (100); MS^3[271]: 153 (100), 215 (90), 243 (80), 253 (59), 149 (37), 145 (29), 159 (18)	P, B
G-4′-Glu	14.45	447.09207	−0.26	$C_{21}H_{19}O_{11}$	MS^2[433]: 271 (100); MS^3[271]: 153 (100), 215 (90), 243 (74), 253 (29), 149 (25), 159 (22), 145 (14)	P, B
G-7-Glu-4′-S	10.25	527.04907	0.07	$C_{21}H_{19}O_{14}S$	MS^2[527]: 351 (100); MS^3[351]: 271 (100)	B
G-7-S-4′-Glu	10.93	527.04895	−0.10	$C_{21}H_{19}O_{14}S$	MS^2[527]: 351 (100); MS^3[351]: 271 (100)	B

Notes: P, perfusate; I, intestine content; B, bile. Abbreviations: D-7-M, dadzein-7-methyl ether; D-4′,7-diM, dadzein-4′,7-dimethyl ether; D-7-Glu, daidzein-7-glucuronide; D-4′-Glu, daidzein-4′-glucuronide; D-7-Glu-4′-S, daidzein-7-glucuronide-4′-sulfate; D-4′-Glu-7-S, daidzein-4′-glucuronide-7-sulfate; D-4′,7-diGlc, daidzin-4′,7-diglucoside; G-7-M, genistein-7-methyl ether; G-4′-M, genistein-4′-methyl ether; G-7-Glu, genistein-7-glucuronide; G-4′-Glu, genistein-4′-glucuronide; G-7-Glu-4′-S, genistein-7-glucuronide-4′-sulfate; G-4′-Glu-7-S, genistein-4′-glucuronide-7-sulfate; D-4′-Glc, genistein-4′-glucoside.

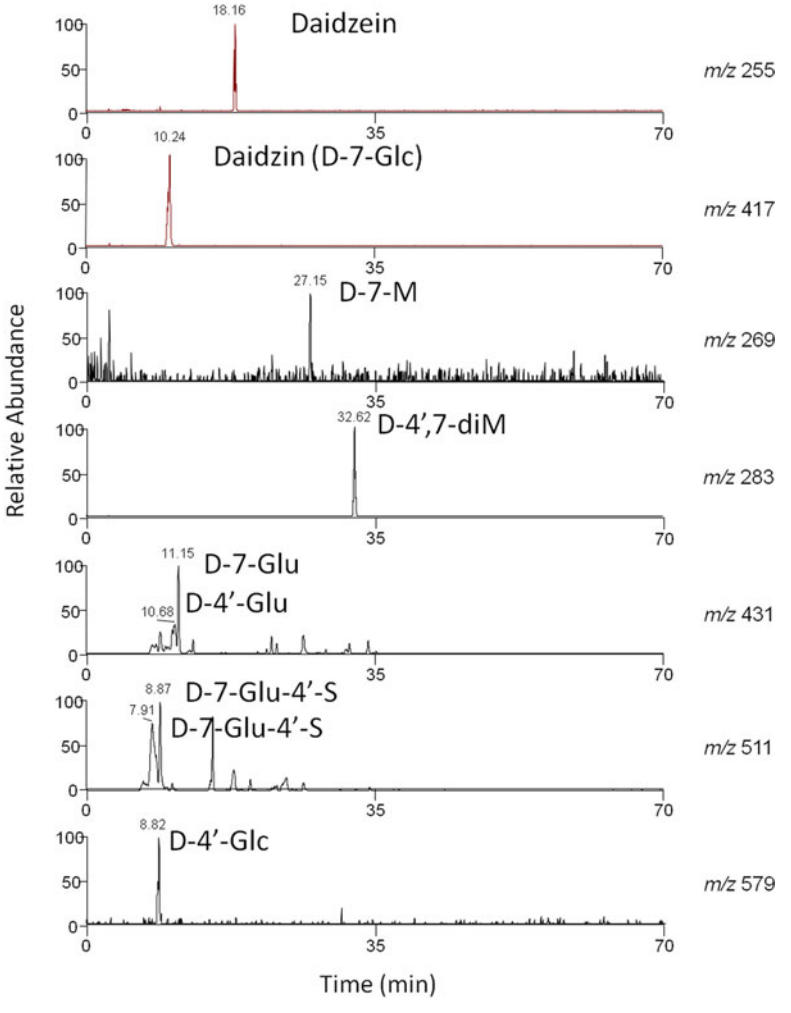


Figure 2. Representative EIC chromatograms of daidzein, daidzin, and their metabolites. See abbreviations in the caption of Figure 1.

In the MS^2 of genistein, the most abundant fragment ion at *m/z* 153 was formed via RDA fragmentation by loss of C_8H_6O. The product ions at *m/z* 215 (80), 243 (72), 253 (37), 149 (20), and 145 (14) were generated via loss of 2CO, CO, H_2O, $C_7H_6O_2$, and $C_6H_6O_3$, respectively. In the MS^3 spectrum, the neutral losses of H_2O from *m/z* 215 were observed and the base peak at *m/z* 197 was generated [13].

Both daidzin and genistin produced quasi-molecular ion at *m/z* of 255 and 271, respectively, via loss of the glycosidic moiety in the MS^2 spectrum. The MS^3 ions at *m/z* 199 (100), 227 (75), 137 (65), 237 (31), 145 (11) of daidzin were same as the MS^2 ions of daidzein due to the loss of 2CO, CO, C_8H_6O, H_2O, and $C_6H_6O_2$. The MS^3 ions at *m/z* 153 (100), 215 (50), 243 (49), 149 (30), 253 (29), and 145 (15) of genistin were same as the MS^2 ions of genistein due to the loss of C_8H_6O, 2CO, CO, $C_7H_6O_2$, H_2O, and $C_6H_6O_3$. The fragmentation pathways of daidzein and genistein are proposed in Figure 4 [13].

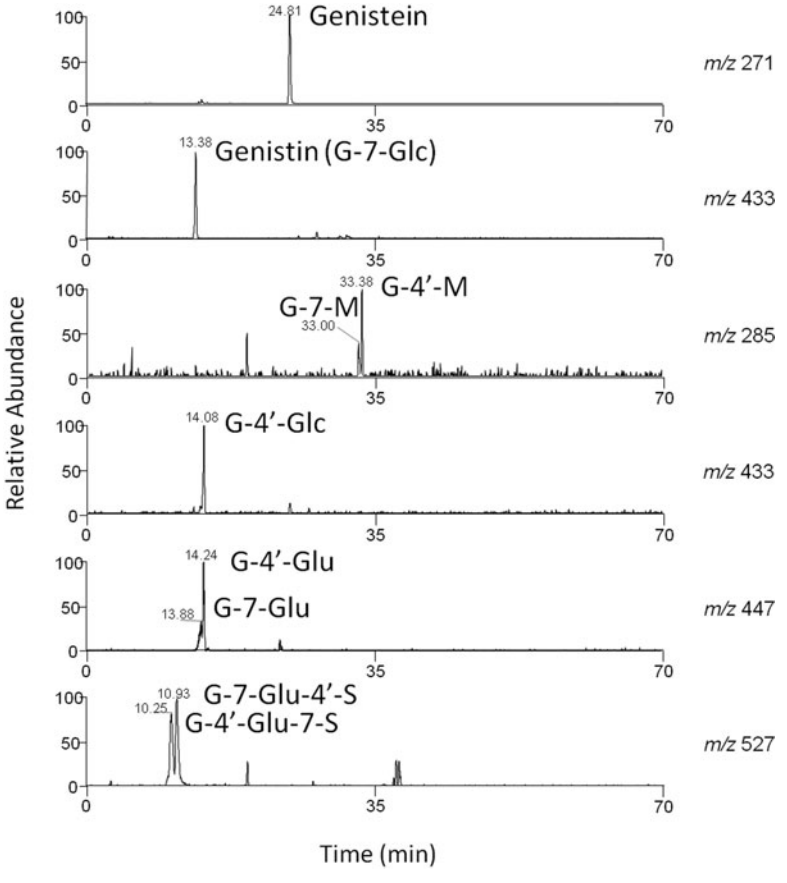


Figure 3. Representative EIC chromatograms of genistein, genistin, and their metabolites. See abbreviations in the caption of Figure 1.

2.2 *Identification of metabolites of daidzein and daidzin*

Totally eight different metabolites of daidzein and daidzin were detected in perfusate, intestine, and bile samples (Figure 1). The structures of metabolites were identified and tentatively characterized on the basis of the proposed formulas of the precursor ions, the fragmentation rules, and MS^n data (Table 1).

D-7-M ($t_R = 27.1$ min) exhibited the quasi-molecular ion at *m/z* 269.08075 ($C_{16}H_{13}O_4$, error -0.32 ppm), 14 Da higher than that of daidzein, in intestinal contents of perfused rat intestine–liver preparation after administration of daidzein in duodenum, suggesting a mono-methyl metabolite of daidzein. In the MS^2 spectrum, the product ions at *m/z* 213 (100), 151 (62), and 241 (33) were generated by the loss of 2CO, C_8H_6O, and CO, respectively. The fragment ions at *m/z* 213 and 151 showing 14 Da higher than those of daidzein at *m/z* of 199 and 137 indicated a methyl group attached to the C-7 hydroxyl group. To further verify the methylated position on the metabolite formed from daidzein, an authentic compound with same retention time, MS^2, and MS^3 as D-7-M was synthesized, and the NMR spectral data of daidzein and the authentic compound were obtained (Table 2). The ^1H NMR data of the authentic compound exhibited three protons of methyl at δ 3.89 (3H, s, MeO-7), not

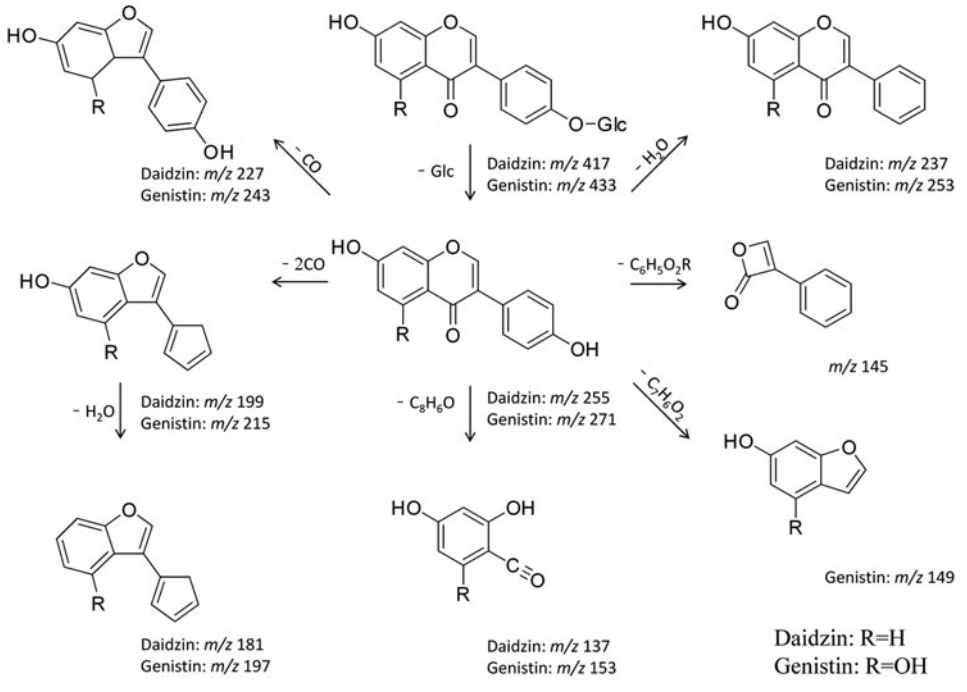


Figure 4. Fragmentation of daidzein, daidzin, genistein, and genistin.

found in the ^{1}H NMR spectrum of daidzein, one proton of hydroxyl group at δ 9.52 (1H, s, 4′-OH), and eight protons at δ 8.35 (1H, s, 2-H), 8.01 (1H, d, 5-H), 7.12 (1H, d, 6-H), 7.05 (1H, dd, 8-H), 7.39 (2H, d, 2′-H and 6′-H), and 6.80 (2H, d, 3′-H and 5′-H). While two signals for hydroxyl groups at δ 10.74 (1H, s) and 9.49 (1H, s) were found in the ^{1}H NMR spectrum of daidzein. These results confirmed that a methyl group was transferred to the C-7 hydroxyl group of daidzein to get D-7-M.

Dadzein-4′,7-dimethyl ether (D-4′,7-diM, t_R = 32.35 min) gave an $[M + H]^+$ ion at *m/z* 283.09660 ($C_{17}H_{15}O_4$, error −1.15 ppm), 28 Da higher than that of daidzein, in perfusate and intestinal contents of perfused rat intestine–liver preparation after administration of daidzein in duodenum which indicated two

Table 2. ^{1}H NMR spectral data for daidzein and D-7-M.[a]

Proton	Daidzein	D-7-M
CH_3	–	3.89 (3H, s)
7-OH	10.74 (1H, s)	–
4′-OH	9.49 (1H, s)	9.52 (1H, s)
2	8.34 (1H, s)	8.35 (1H, s)
5	7.95 (1H, d, J = 9.0 Hz)	8.01 (1H, d, J = 8.5 Hz)
6	6.92 (1H, dd, J = 9.0, 2.0 Hz)	7.05 (1H, dd, J = 8.5, 2.5 Hz)
8	6.85 (1H, d, J = 2.0 Hz)	7.12 (1H, d, J = 2.5 Hz)
2′,6′	7.36 (2H, d, J = 8.0 Hz)	7.39 (2H, d, J = 8.5 Hz)
3′,5′	6.77 (2H, d, J = 8.0 Hz)	6.80 (2H, d, J = 8.5 Hz)

Note: d, doublet; dd, doublet of doublets; s, singlet.
[a] The reported data were obtained in DMSO-d_6.

methyl groups substituted on daidzein. In the MS^2, the loss of $2CH_3$ formed the characteristic ion at *m/z* 255. Then the MS^3 of *m/z* 255 presented the specific fragment ions at *m/z* 199 (100), 227 (78), 137 (75), and 145 (16) via loss of 2CO, CO, C_8H_6O, and $C_6H_6O_2$. According to the possible metabolic path, methyl groups were attached to 7-OH and 4′-OH of daidzein, respectively, to get D-4′,7-diM.

Two glucuronide conjugates of daidzein, daidzein-7-glucuronide (D-7-Glu) and daidzein-4′-glucuronide (D-4′-Glu), were detected both in perfusate of perfused rat intestine–liver preparation and bile samples of rat after duodenum or oral administration of daidzein and daidzin, respectively. D-7-Glu and D-4′-Glu exhibited protonated ions at *m/z* 431.09683 ($C_{21}H_{19}O_{10}$, error −1.03 ppm) and 431.09650 ($C_{21}H_{19}O_{10}$, error −1.79 ppm), respectively, 176 Da higher than that of daidzein. Both MS^2 gave the base peak of *m/z* 255 via loss of a glucuronide moiety (176 Da) from *m/z* 431. In the MS^3 spectrum of D-7-Glu, the *m/z* 255 ion produced specific ions at *m/z* 199 (100) and 137 (89). In the MS^3 of D-4′-Glu, the *m/z* 255 ion produced specific ions at *m/z* 199 (100), 237 (75), 227 (55), 137 (13), and 145 (10) due to the loss of 2CO, H_2O, CO, C_8H_6O, and $C_6H_6O_2$. According to the report [14], the glucuronide of daidzein whose conjugation site was assigned to the 7-hydroxyl group was eluted earlier than the metabolite whose conjugation group was assigned to the 4′-hydroxyl group. We proposed that the metabolite with earlier retention time should be D-7-Glu ($t_R = 10.68$ min) and the later one should be D-4′-Glu ($t_R = 11.15$ min).

Also two isomers, daidzein-7-glucuronide-4′-sulfate (D-7-Glu-4′-S) and daidzein-4′-glucuronide-7-sulfate (D-4′-Glu-7-S), were detected in bile samples of rat after oral administration of daidzein and daidzin. These two compounds showed $[M + H]^+$ at *m/z* 511.05380 ($C_{21}H_{19}O_{13}S$, error −0.57 ppm) and 511.05405 ($C_{21}H_{19}O_{13}S$, error −0.08 ppm), respectively. The MS^2 of both quasi-molecular ions at *m/z* 511 presented the base peak at *m/z* 335 via loss of 176 Da. The subsequent MS^3 of *m/z* 335 generated the fragment ion at *m/z* 255 by loss of 80 Da. Based on the above data, both compounds were sulfoglucuronides of daidzein. According to the report [14], the daidzein sulfates whose conjugation site was assigned to the 4′-hydroxyl group were eluted earlier than the metabolite whose conjugation group was assigned to the 7-hydroxyl group. So we proposed that the earlier metabolite should be D-7-Glu-4′-S ($t_R = 7.91$ min) and the later one should be D-4′-Glu-7-S ($t_R = 8.87$ min).

In the full scan of FTICR mass spectrum, a trace glycoside conjugate of daidzin, daidzin-4′,7-diglucoside (D-4′,7-diGlc), was found in perfusate of perfused rat intestine–liver preparation at 9.48 min and gave a protonated ion at *m/z* 579.17029 ($C_{27}H_{31}O_{14}$, error −0.94 ppm) after administration of daidzin in duodenum. In the MS^2, two fragment ions were observed. The higher product ion at *m/z* 255 was formed by the loss of two glucose moieties from the precursor ion. The lower fragment ion at *m/z* 417 was generated by cleaving one glycoside. In the MS^3, the neutral losses of CO, H_2O, and 2CO from *m/z* 255 were observed and the corresponding fragment ions at *m/z* 227 (100), 237 (41), and 199 (27) were generated, respectively. Based on the possible metabolic path, we postulated that one glycoside was attached to 7-OH and 4′-OH of daidzein, respectively, to get D-4′,7-diGlc.

Daidzein was found in perfusate, intestinal contents, and bile samples after administration of daidzin which was identified by comparison with standard daidzein.

The proposed metabolic pathways of daidzein and daidzin in rats are shown in Figure 5.

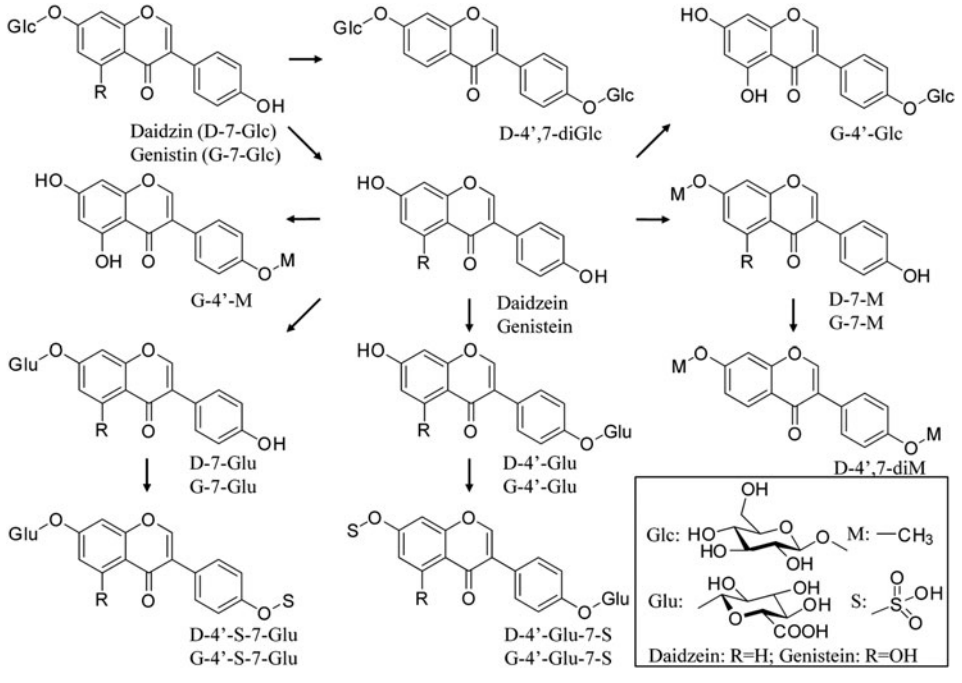


Figure 5. Proposed metabolic pathways of daidzein, daidzin, genistein, and genistin in rats.

2.3 *Identification of metabolites of genistein and genistin*

Totally eight metabolites of genistein and genistin were detected in perfusate, intestine, and bile samples (Figure 1). The structures of metabolites were identified and tentatively characterized on the basis of the proposed formulas of the precursor ions, the fragmentation rules, and the MS^n data (Table 1).

Genistein-4′-methyl ether (G-4′-M, $t_R = 33.00$ min) and genistein-7-methyl ether (G-7-M, $t_R = 33.38$ min) were also isomers detected in rat intestinal contents of perfused rat intestine–liver preparation after administration of genistein in duodenum. The isomers showed $[M + H]^+$ at *m/z* 285.07559 ($C_{16}H_{13}O_5$, error −0.56 ppm) and 285.07553 ($C_{16}H_{13}O_5$, error −0.77 ppm), respectively. The MS^2 of G-4′-M presented the fragment ions at *m/z* 269 (100) and 271 (11) via loss of CH_4 and CH_2. The MS^2 of G-7-M displayed product ions at *m/z* 269 (100), 229 (46), 253 (28), 257 (21), 271 (16), and 149 (11) via loss of CH_4, 2CO, CH_4O, CO, CH_2, and $C_8H_8O_2$, respectively. In the MS^3, the isomer with earlier retention time yielded the fragment ions at *m/z* 167 (100), 241 (24), and 225 (22) due to the loss of C_7H_2O, CO, and CO_2 from *m/z* 269, respectively. The MS^3 spectrum of later isomer eluted displayed fragment ions at *m/z* 151 (100) and 253 (46) derived from loss of $C_7H_2O_2$ and CH_4 from *m/z* 269, respectively. According to the above data, both the compounds were mono-methyl metabolite of genistein. For the later isomer, the characteristic ion at *m/z* 229 (46) in MS^2 which was 14 Da higher than the MS^2 ion of protonated genistein at *m/z* 215 indicated that the methyl was attached to 7-OH or 5-OH. In consideration of the chemical inertia of the 5-hydroxyl group which formed a hydrogen bond with the ketone carbonyl group in the structure of flavone, the glycosidation on the 5-hydroxyl group is impossible. So we proposed that the metabolites with reten-

tion times of 33.00 and 33.38 min were G-4′-M and G-7-M, respectively.

The metabolite with the quasi-molecular ion at *m/z* 433.11212 ($C_{21}H_{21}O_{10}$, error −1.85 ppm) was discovered at 14.08 min in the chromatogram of intestinal contents of perfused rat intestine–liver preparation after administration of genistein in duodenum. In the MS^2, the base peak of *m/z* 271 was generated by loss of a glycoside from the quasi-molecular ion at *m/z* 433. In the MS^3, the characteristic fragment ions at *m/z* 243 (100), 153 (94), 215 (89), 253 (34), and 149 (25) were observed due to the neutral losses of CO, C_8H_6O, 2CO, H_2O, and $C_7H_6O_2$ from the fragment ion at *m/z* 271, respectively. Based on the possible metabolic path and earlier retention time than genistin, we speculated that the glycosylation occurred at 4′-OH to get genistein-4′-glucoside (G-4′-Glc).

Genistein-7-glucuronide (G-7-Glu, $t_R = 13.84$ min) and Genistein-4′-glucuronide (G-4′-Glu, $t_R = 14.29$ min) were screened both in perfusate of perfused rat intestine–liver preparation and bile samples after duodenum or oral administration of genistein and genistin. The isomers with $[M + H]^+$ ions at *m/z* of 447.09213 ($C_{21}H_{19}O_{11}$, error −0.13 ppm) and 447.09207 ($C_{21}H_{19}O_{11}$, error −0.26 ppm) showed similar fragmentation rules. Both the MS^2 fragmentations yielded the base peak ions at *m/z* 271 from the quasi-molecular ions at *m/z* 447 due to the loss of a glucuronide moiety (176 Da). The typical fragment ions at *m/z* 153 (100), 215 (90), 243 (80), 253 (59), 149 (37), 145 (29), and 159 (18) in MS^3 of G-7-Glu from the ion at *m/z* 271 were observed. Similar typical fragment ions at *m/z* 153 (100), 215 (90), 243 (74), 253 (29), 149 (25), 159 (22), and 145 (14) in MS^3 spectrum of G-4′-Glu from ion at *m/z* 271 were observed. Due to chemical inertia of the 5-hydroxyl group, the metabolites were tentatively identified as G-7-Glu and G-4′-Glu, glucuronide of genistein, according to the retention time [14].

Two metabolites with quasi-molecular ions at *m/z* 527.04907 ($C_{21}H_{19}O_{14}S$, error 0.07 ppm) and 527.04895 ($C_{21}H_{19}O_{14}S$, error −0.10 ppm) were found at 10.25 and 10.93 min in the chromatogram of rat bile after oral administration of genistein or genistin. Both peaks produced the base peak at *m/z* 351 in the MS^2 via loss of a glucuronide moiety (176 Da). Then subsequent MS^3 of *m/z* 351 generated the base peak at *m/z* 255 by loss of SO_3 (80 Da). Therefore, these two metabolites were tentatively identified as sulfoglucuronides of genistein. Based on the report [14], genistein sulfate whose sulfate conjugation site was assigned to the 4′-hydroxyl group was eluted earlier than the metabolite whose sulfate conjugation group was assigned to the 7-hydroxyl group. So we proposed that the earlier metabolite should be genistein-7-glucuronide-4′-sulfate (G-7-Glu-4′-S) and the later one should be genistein-4′-glucuronide-7-sulfate′.

Aglycone of genistin was found in perfusate, intestinal contents, and bile (0–36 h) after duodenum and oral administration of genistin and was identified by comparison with standard daidzein.

The proposed metabolic pathways of genistein and genistin in rats are shown in Figure 5.

3. Discussion

The aim of this study was to identify the metabolites of daidzein, daidzin genistein, and genistin in rats based on HPLC–LTQ-FTICR-MS^n analysis incorporating spectral tree data mining method. Although previous reports investigated metabolites and conversion pathway of soy isoflavones (daidzin, daidzein, genistein, etc.) in biological samples of humans [15,16], rodents, or their intestine bacteria [17,18], the application of HPLC combined with high resolution and multiple stage mass spectrometer for investigation of metabolites of soy isoflavones in multiple biosamples (rat perfusate, intestinal contents,

and bile) presented in this manuscript provided a better tool for study and complement up-to-date knowledge in biotransformation of daidzein, genistein, daidzin, and genistin after oral administration by rats.

Most of the isoflavone glycosides are hydrolyzed to their aglycones by enteral microorganisms [17,18]. After administration of daidzin or genistin in duodenum, daidzein or genistein, the aglycone of daidzin or genistin, was found in intestinal contents and perfusate. And intact aglycones were still detected in bile at relative low level after oral administration of daidzein and genistein. Several studies have shown that the main metabolites of isoflavone are 7-glucuronides followed by 4′-glucuronides and that there are also small proportions of sulfate esters. In perfusate and bile samples, two glucuronide conjugates of daidzein and genistein were found. In addition, two sulfoglucuronides of daidzein and genistein in bile were detected after oral administration of daidzein, daidzin, genistein, and genistin to rats, respectively. However, there was no mono- or di-sulfate conjugates detected in perfusate and bile for both daidzein and genistein. In addition, only one mono glycoside of daidzin, D-4′,7-diGlc, was filtered in perfusate after administration of daidzin in duodenum. And one mono glycoside of genistein, G-4′-Glc, was detected in perfusate after administration of genistein in duodenum. In addition, except phase II metabolites, methylation metabolites of daidzein (D-7-M and D-4′,7-diM) and genistein (G-4′-M and G-7-M) were found in perfusate or intestinal contents after administration of daidzein in duodenum. To the best of our knowledge, D-4′,7-diGlc, G-4′-Glc, D-7-M, D-4′,7-diM, G-4′-M, and G-7-M were detected as metabolites of isoflavone in rats for the first time.

Conjugated metabolites of soy isoflavones were identified and their pharmacokinetic data have been estimated. However, whether the newly detected metabolites, D-4′,7-diGlc, G-4′-Glc, D-7-M, D-4′,7-diM, G-4′-M, and G-7-M, are present in humans after oral administration of soy isoflavones needs to be investigated further. Based on the corresponding pharmacodynamic effects and pharmacokinetic data of the newly detected metabolites of soy isoflavones, the pharmacological effects of soy isoflavones could be further investigated.

4. Experimental

4.1 Materials and reagents

Daidzein, daidzin, genistein, and genistin with purity more than 98.0% were supplied by Shanghai Xinran Biotechnology Co., Ltd (Shanghai, China). Dextran T40, bovine serum albumin, glucose, dexamethasone injection (2 mg/ml), and norepinephrine injection (2 mg/ml) were supplied by Beijing Double-Crane Pharmaceutical Co., Ltd (Beijing, China), Bovogen Biologicals Pty Ltd (East Keilor, VIC, Australia), Sinopharm Chemical Reagent Beijing Co., Ltd (Beijing, China), Tianjin Pharmaceutical Group Jiaozuo Co., Ltd (Tianjin, China), and Shanghai Harvest Pharmaceutical Co., Ltd (Shanghai, China), respectively. Urethane was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Acetonitrile of mass grade was provided by Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). Acetic acid of HPLC grade was purchased from Mallinckrodt Baker, Inc., and the other chemicals used were all analytical reagent grade. Deionized water was purified using a water purification system (Millipore, Billerica, MA, USA).

4.2 Synthesis of D-7-M

A total of 100 mg of daidzein (0.39 mmol) in 4 ml of *N*-dimethyl formamide were added to 50 mg of anhydrous K_2CO_3 (0.36 mmol) and 20 μl dimethyl sulfate (0.21 mmol). The mixture was stirred for 5 h [19]. After

cooling the reaction mixture, the mixture was purified on a Sephadex LH-20 (Pharmacia Fine Chemicals, Inc., Piscataway, NJ, USA) column with methanol. The structure of the synthesized compound was confirmed by MS and ^{1}H NMR techniques. The purity of chemically synthesized D-7-M was more than 98.5%, determined by HPLC–UV.

4.3 Animals

Male Wistar rats (220 ± 20 g) were used and supplied by Vital River Laboratories (Beijing, China). The rats were housed under standard conditions of temperature, humidity, and light, and had free access to standard rodent diet and water before the experiment. Animal experiments were conducted according to the Animal Care and Welfare Committee of the Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China).

4.4 Perfused rat intestine–liver preparation

A perfuser consisting of a peristaltic pump, oxygen, temperature chamber, and recirculating pump was used. Perfusate consisted of 3% dextran T40, 1% bovine serum albumin, 3 mg/ml glucose in Krebs-Henseleit Bicarbonate solution buffered to pH 7.4. Perfusate was oxygenated in rotating reservoirs with 95% O_2 and 5% CO_2. Before perfusion, 40 μl of dexamethasone injection and 20 μl of norepinephrine injection were added for each 100 ml perfusate.

A previously described surgical procedure [20,21] was modified in this study. To get enough metabolites, two male Wistar rats were used for each compound. The rats were fasted overnight with free access to tap water before the perfusion experiment. The rats were anesthetized with i.p. injection of 20% urethane (0.6 ml/100 g) and placed under a tunable heating light to maintain a body temperature of 37°C. Upon verification of the loss of pain reflex, the abdomen was opened by a 3–4 cm midline longitudinal incision. The bile duct, gastric artery, gastro duodenal artery, and splenic blood vessels perfusing the stomach and spleen were ligated in this order. The celiac artery and the pyloric vein were ligated. Immediately after the aorta was tied off near the superior mesenteric artery, a cut was made in the superior mesenteric artery. A teflon catheter with diameter of 1 mm was inserted into the artery. The catheter was secured with 3-0 surgical silk (Shanghai Medical Suture Needle Factory Co., Ltd, Shanghai, China). Perfusion into the superior mesenteric artery with blank perfusate was immediately initiated at a flow rate of 4 to 5 ml/min. The thoracic cavity was opened and a cut was made in the right atrium of the heart. A teflon catheter from an i.v. placement unit was inserted into the opening of the right atrium till the hepatic vein and secured in place. The catheter was connected to tygon tubing allowing the exit of perfusate from the liver. Then the proximal part of the colon was ligated and an open ligature was positioned around the duodenum. Henceforth, the perfusion flow rate was then adjusted to approximately 7 ml/min. The experiment started after a preperfusion to wash out the residual blood in tissues using 30 ml of perfusate.

4.5 Bile duct catheterization preparation

Also two rats for each compound were used to collect bile samples. The rats were anesthetized with i.p. injection of 20% urethane (0.6 ml/100 g) and placed under a tunable heating light to maintain a body temperature of 37°C. Upon verification of the absence of a pain reflex, a midline abdominal incision of 3–4 cm was made. Then the intestine was moved away to expose the hepatic artery and bile duct. Following isolation, a polyethylene catheter (0.2 mm i.d.) was inserted about 5 mm

inside and firmly ligated. The abdominal cavity was closed using surgical wire and the catheter was passed under the skin of the lower abdomen and out through a puncture made in the tail. The skin of the abdomen was then closed using surgical wire. The bile duct cannula was secured with a harness from the tail of the rat to the roof of the metabolism cage. During the whole period of the experiment, rats were kept on a homoeothermic board with light anaesthesia and were supplemented with water containing 2% glucose and 0.9% NaCl.

4.6 Drug administration and sample collection

For rats accepting intestine–liver perfused preparation, in order to collect enough metabolite, a dosage of 10 mg suspended daidzein, daidzin, genistein, and genistin solved in 1 ml saline, respectively, was administrated by an injector at the proximal part of the duodenum after exsanguinations of tissues, and then the open ligature positioned around the duodenum was ligated. Perfusions were continued at a constant flow rate (7.0 ml/min). About 25 and 50 ml samples were taken before (blank sample) and after (metabolite sample) intra-duodenum administration of compound at 120 min, respectively. Intestinal content between the ligated duodenum and ligated colon was collected.

For rats accepting bile duct catheterization preparation, the same dosage of daidzein, daidzin, genistein, and genistin was administrated by oral gavage, respectively. Bile was collected 2 h and 36 h, respectively, before and after dosing.

4.7 Preparation of samples

Perfusate, intestinal contents, and bile samples were concentrated to dryness under reduced pressure at −20°C, and then the residues were extracted by 5 ml methanol for three times. The methanol was pooled and evaporated to dryness under N_2 flow at 40°C. The residues were reconstituted in 2 ml of methanol and 1.5 ml supernatant was transferred after 5 min of vortex and centrifugation at 13,000 *g* for 10 min at 4°C. Finally, a 3 μl aliquot of supernatant was injected to HPLC–HR-MS/MS^n for analysis.

4.8 Instrumentation and analytical conditions

HPLC–HR-MS/MS^n was performed using a Surveyor LC system coupled to a hybrid LTQ-FTICR mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). LC separation was performed on Kinetex C_{18} reversed phase column (4.6 × 100 mm, 2.6 μm, Phenomenex (Macclesfield, UK)) and the column temperature was maintained at 35°C. Purified water with the addition of 0.1% formic acid was used as solvent A and acetonitrile containing 0.1% formic acid as solvent B. The gradient profile was as follows: 0–5 min 10% B, 5–38 min, a linear increase to 50% B, by a linear increase to 95% B at 50 min to wash the column. The column was then returned to 10% B at 52 min and allowed to re-equilibrate (18 min) before the next sample was analyzed. Analyses were performed at a flow rate of 0.6 ml/min with a splitter after the analytical column to introduce 200 μl/min eluent to the ESI source.

The positive ion ESI-MS and tandem MS were used for the detection of analytes under the following mass spectrometric conditions. The spray voltage was set at 3.5 kV, the temperature of the heated capillary was set at 250°C, and the capillary voltage was set at 30 V. The sheath gas (N_2) flow rate was 35 (arbitrary units), the auxiliary gas flow rate was set at 10 (arbitrary units), the sweep gas flow rate was set at 3 (arbitrary units), and tube lens offset voltage was set at 120 V.

Full-scan experiments were performed in the range of *m/z* 100–1500 at a resolving power of 50,000 with data-

dependent MS^2 analysis triggered by the two most abundant ions from full-scan mass analysis, followed by MS^3 analysis of the most abundant product ions. Upon data-dependent analyses, high accuracy mass-to-charge and multiple-stage mass spectrometry of compounds in biological samples could be acquired automatically. Data acquisition was performed with Xcalibur version 2.0 software (Thermo Fisher Scientific, San Jose, CA, USA).

4.9 NMR spectrometry

^{1}H NMR spectra were measured on a Bruker AV-500 instrument (500 MHz, 11.74 T) (Karlsruhe, Germany). Samples were recorded in DMSO-d_6. Chemical shifts are given in δ (ppm) scale relative to solvent peaks as references.

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Note

1. These authors contributed equally to this work.

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