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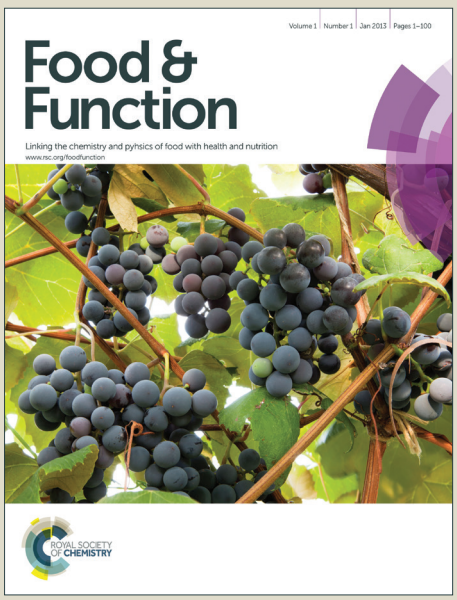

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The intracellular metabolism of isoflavones in endothelial cells

Natalia Toro-Funes[1,2], F.J. Morales-Gutiérrez[1], M. Teresa Veciana-Nogués[1], M. Carmen Vidal-Carou[1], Jeremy P.E. Spencer[2], Ana Rodriguez-Mateos[3*]

[1]Department of Nutrition and Food Science-XaRTA, Faculty of Pharmacy, Campus de l'Alimentació Torribera, University of Barcelona, Avda. Prat de la Riba 171, 08921-Santa Coloma de Gramenet, Barcelona, Spain; [2]Molecular Nutrition Group, School of Chemistry, Food and Pharmacy, University of Reading, Reading, RG2 6AP, UK; [3]Division of Cardiology, Pulmonology, and Vascular Medicine, Medical Faculty, University of Duesseldorf, D-40225 Duesseldorf, Germany

* Author for correspondence

Dr Ana Rodriguez-Mateos; e-mail: ana.rodriguez-mateos@med.uni-duesseldorf.de

Tel:00492118115893

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ABSTRACT

Data from epidemiological and human intervention studies have highlighted potential cardiovascular benefits of soy isoflavone-containing foods. In humans, genistein and daidzein are extensively metabolized after absorption into glucuronides and sulfate metabolites. However, limited data exist on isoflavone cellular metabolism, in particular in endothelial cells. We investigated the uptake and cellular metabolism of genistein, daidzein and its major *in vivo* microbial metabolite, equol, in human endothelial (HUVEC), liver (HepG2) and intestinal epithelial cells (Caco-2 monolayer). Our results indicate that genistein and daidzein are taken up by endothelial cells, and metabolized into methoxy-genistein-glucuronides, methoxy-genistein-sulfates and methoxy-daidzein-glucuronides. In contrast, equol was taken up but not metabolized. In HepG2 and Caco-2 cells, glucuronide and sulfate conjugates of genistein and daidzein and a sulfate conjugate of equol were formed. Our findings suggest that endothelial cell metabolism needs to be taken into account when investigating the cardioprotective mechanisms of action of isoflavones.

Key words: isoflavones; genistein; daidzein; equol; metabolism; endothelial cells, HUVEC; HepG2, Caco-2 monolayer

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INTRODUCTION

Soy isoflavones have recently received much attention because of their potential health benefits, particularly on the prevention of different types of cancer, osteoporosis and cardiovascular diseases (CVD).[1] However, there is also controversy regarding the safety and efficacy of isoflavones and potential adverse effects have also been reported.[2,3] Data from human intervention studies suggest isoflavones may have beneficial effects on prognostically validated surrogate markers of CVD, such as blood pressure, endothelial function, or arterial stiffness.[4-8] Moreover, several studies have shown that isoflavones exert favorable effects on other biomarkers of CVD, such as plasminogen activator inhibitor-1, endothelin 1, VCAM-1 or NO.[9-13] Some studies have shown improvements in plasma lipids and lipoproteins after isoflavone consumption, including lowering blood triglycerides, total and LDL cholesterol levels, increasing HDL cholesterol and the ratio of HDL/LDL cholesterol,[14-16] although some mixed data exist with a recent study and a meta-analysis concluding that there is not enough evidence to support positive effects of isoflavones on blood lipids.[17,18]

The most abundant soy isoflavones in the diet are genistein (Ge) and daidzein (De) (Figure 1).[19] They are ingested mainly in the glucoside form, and undergo extensive hydrolysis by intestinal and bacterial β-glucosidases that release the main aglycones.[20] De is converted into equol (Eq) (Figure 1) due to the action of the intestinal microbiota before absorption.[21] The isoflavone aglycones are converted into glucuronide metabolites by UDT-glucuronosyltransferases (UGT), and to a lesser extent to sulfate esters catalyzed by sulfotransferases (SULT) at either or both 4' or 7 positions on the isoflavone ring by phase II enzymes during transfer across the small intestine and liver.[22] These phase II metabolites are excreted in the bile and are deconjugated in the lower bowel allowing them to be reabsorbed again, creating an enterohepatic

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circulation.[22] Studies showed that the conjugated metabolites of Ge and De are mainly found in human plasma as mono- and diglucuronides, mono- and disulfates, and sulfo-glucuronides.[23] Trace amounts of mono- and dimethoxylated conjugates have also been found in urine.[24,25]

Most of the research investigating the mechanisms of action of flavonoids in the vascular system has tested the bioactivity of flavonoids and their metabolites in human and animal cell models. However, whether flavonoids and metabolites are taken up and metabolized further by cells is at present unclear. In order to assess the potential risks and benefits of soy isoflavones and the mechanisms by which health effects occur, it is important to have a more complete understanding of isoflavone intracellular metabolism. The metabolism of isoflavones has been reported in cell models of the gastrointestinal tract, such as enterocytes and Caco-2 cell monolayers,[26-28] or in hepatic models,[29,30] but little attention has been given to the potential intracellular metabolism of isoflavones in other human cells, such as human umbilical vein endothelial cells (HUVEC), despite being a widely used in vitro model for assessing mechanisms of action of isoflavones in the vascular endothelium.[11,31] Therefore, the aim of this work is to determine the cellular uptake and intracellular metabolism of the isoflavones Ge, De and Eq in endothelial cells, using HepG2 hepatocytes (liver cell model) and Caco-2 cell monolayers (small intestine model) as positive controls.

EXPERIMENTAL

Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from Lonza (Basingstoke, UK) and used at passages 2 or 3. The cells were cultured at 37°C with 5% CO_2 in a humidified atmosphere, and supplemented with endothelial culture medium

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consisted of: 2% foetal bovine serum (10 ml) plus supplements [hEGF (0.5 ml), Hydrocortisone (0.2 ml), GA-1000 (Gentamicin, Amphotericin-B) (0.5 ml), VEGF (0.5 ml), hFGF-B (2 ml), R3-IGF-1 (0.5 ml), ascorbic acid (0.5 ml), heparin (0.5 ml) to 500 ml endothelial cell basal medium without phenol red (Lonza, UK). Foetal bovine serum was heat inactivated by incubation at 56 °C for 30 minutes. Cells were kept at 37°C in a humidified atmosphere containing 5% CO_2. Cells were seeded at a density of 0.5- 1 x 10^6 cells/dish in Petri dishes (diameter 100 mm).

Liver hepatoma cells HepG2 cells (ATCC, Manassas, VA, US) were cultured at 37°C in an atmosphere of 5% CO_2 relative humidity between passages 19-21 in Dulbecco's modified Eagle's medium F-12 with glutamine (500ml), with 10% FBS heat inactivated (50 ml) and 1% of penicillin /streptomycin solution (5ml (PAA, UK). Cells were seeded at a density of 0.5 - 1 x 10^6 cells/dish in Petri dishes (diameter 100 mm).

Human colon adenocarcinoma cells (Caco-2, ECACC Salisbury, Wiltshire, UK) were cultured between passages 15-19 in a humidified atmosphere of 5% CO_2/95% air in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated bovine serum, L-glutamine (2 mM), non-essential amino acids (1%), penicillin (100 U/ml), and streptomycin (100 lg/ml) (all from PAA, UK). Culture medium was changed every 2-3 days and the culture was split approximately every 7 days. For subculturing, the cells were removed enzymatically (0.25% trypsin-EDTA, 1 min, 37°C), split 1:3, and subcultured in 6 well plates (PAA, UK). For transport experiments, 2.5 x10^5 cells were seeded in Transwell-clear, tissue culture treated polyester membrane filter inserts (pore size 0.4 mm, diameter 24 mm, PAA, UK) in 6-wells plates. Cells were allowed to grow and differentiate to confluent monolayers for about 20–22 days. The medium was changed twice a week. The apical and basolateral compartments contained 1.2 and 2 ml of culture medium, respectively. The integrity of

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the monolayers was checked by measuring transepithelial electrical resistance across the layer (TEER) values, using a Millicell-ers epithelial voltohmmeter (Millipore Co., Bedford, MA). Experiments were conducted only in cell monolayers that showed a TEER value between 400 and 1000 Ω per cm^2.

Assessment of cellular uptake/association and metabolism

HUVEC and HepG2 were grown in petri dishes to a confluence of 80-90%. Prior to experiments, old medium was removed and cells were washed with PBS, pH 7.4. Appropriate amounts of Ge, De and Eq (0, 0.1, 1, 10 and 100 μM) were added to 7 ml of the growth medium and cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 2 h. After incubation, 1 mL of the medium was removed and immediately frozen in liquid nitrogen and kept at -80°C afterwards. Cells were washed twice with ice-cold PBS, and 200 μL of ice-cold 0.1M HCl was added. Cells were scraped, the cell homogenates sonicated 3 times for 30 sec, centrifuged at 800 g for 10 min and the supernatant was collected and transferred to -80°C storage.

For Caco-2 cells experiments, the medium was removed and cells were washed with PBS, pH 7.4. Test compounds (conc. as above) were added to the apical side in 1.2 mL of transport buffer consisting in PBS, 1% non-essential amino acids and 1 mM of ascorbic acid. Transport buffer (2 mL) was also added to the basolateral side. Incubation was performed for 2 h at 37°C at a humidified atmosphere of 5% CO_2. Then, the apical and basolateral buffer were collected and immediately stored at -80°C. In addition, cell filters were washed with ice-cold PBS, prior to addition of 500 μL of 0.1 M HCl, and cell homogenate collection, which were again sonicated 3 times for 30 sec and centrifuged at 800 g for 10 min, prior to storage at -80°C.

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Uptake data is expressed as μM in supernatant and cell lysates per petri dish, or pmol/mg protein in cell lysate. Uptake refers to both cytosolic accumulation and membrane/cell-associated. Recoveries were calculated respect to the amount of compound recovered after incubation without cells (control).

Stability test of Genistein, daidzein and Equol in cell culture medium

Each isoflavone (10 μM) was dissolved in endothelial culture medium phenol red free and kept in 6-well plates at 37°C with 5% CO_2 in a humidified atmosphere. Samples were taken at pre-determined time points (0, 0.4, 1, 6 and 24 h). All the tests were done in triplicate. The amount of each compound remained in endothelial medium was determined and the residue amount of each compound was plotted against time to obtain their stability profiles.

Sample preparation and analysis of isoflavones

Supernatants and cell lysates were evaporated to dryness using a speedvac concentrator (Savant) and dissolved with 0.1 ml of ultra pure water for chromatographic analysis.

Enzymatic treatment: to confirm the identity of isoflavone glucuronides, sulfates and methoxylated compounds as determined by LC-MS, cells were incubated with 0 and 10 μM of each isoflavone as described above and subjected to enzymatic β-glucuronidase/sulfatase hydrolysis. Briefly, 50 μl of β-glucuronidase/sulfatase type H1 from *Helix pomatia* (Sigma G0751) in 0.2 M sodium acetate pH 5 were added to supernatants and cell lysates after incubation with Ge or De and incubated at 37 ºC for 40 min. Then, the same volume of methanol was added to the mixture and centrifugated at 10000 g for 10 min. Resulting supernatant was evaporated to dryness using a

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speedvac concentrator (Savant) and dissolved with 0.1 ml of ultra pure water for chromatographic analysis.

Genistein, daidzein and equol determination

Aglycones and Eq were determined by ultra high pressure liquid chromatography (UHPLC-UV) following the method reported by Toro-Funes *et al*.[32] Briefly, 1 µl was injected in a reversed-phase Acquity UPLC™ EH C18 1.7 µm column (2.1 × 50 mm) (Waters Corp., Milford, MA, USA), which was placed in an oven at a constant temperature (35°C). The UPLC™ system (Waters Acquity System, Milford, MA, USA) consisted of a binary pump and an auto-sampler equipped with a diode array detector set to 262 nm for Ge and De and to 280 nm for Eq. Solvent A was ultra pure water with 0.1% formic acid, and solvent B was acetonitrile with 0.1% formic acid. Linear gradient conditions were as follows: 0 min – 10% B; 1 min – 12% B; 3 min – 22% B; 4 min – 23% B; 5 min – 35% B; 6 min – 50%; 8 min – 50% B; 8.1 min – 10% B at flow of 0.6 mL/min. Vials filled with either standard solutions or samples were kept at 4°C in the auto sampler.

Isoflavone metabolites identification by LC/MS-Orbitrap

Isoflavone metabolites were tentatively identified by liquid chromatography with mass spectrometry detection. An LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) equipped with an ESI source in positive mode was used to acquire mass spectra in profile mode with a setting of 30,000 resolution at m/z 400. Operation parameters were set as follow: source voltage, 3.5 kV; sheath gas, 50 (arbitrary units); auxiliary gas, 20 (arbitrary units); sweep gas, 0 (arbitrary units); and capillary temperature, 275°C. Default values were used for most other acquisition

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parameters (Fourier transform (FT) Automatic gain control (AGC) target 5×10^5 for MS mode and 5×10^4 for MS^n mode). Samples were analysed in full MS mode with the Orbitrap resolution set at 30,000 at m/z 400. The maximum injection time was set to 100 ms with two micro scans for MS mode. The mass range was from 100 to 1,000 m/z. Data analyses were performed using XCalibur software. We used an Accela liquid chromatograph (Thermo Scientific, Hemel Hempstead, UK) equipped with a quaternary pump, a photodiode array detector (PDA) and a thermostated autosampler. An Acquity UPLC™ EH C18 1.7 µm column (2.1 × 50 mm) (Waters corp., Milford, MA, USA) with solvent A (ultra pure water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid) was used. Linear gradient conditions were as follows: 0 min – 10% B; 1 min – 12% B; 3 min – 22% B; 4 min – 23% B; 5 min – 35% B; 6 min – 50%; 8 min – 50% B; 10 min – 10% B at flow of 0.6 ml/min. The injection volume was 2 µl.

Due to the absence of commercial standards, metabolites were tentatively identified from the accurate exact mass data provided by the LC-MS analysis. Deviation from the calculated mass (5 ppm) and the isotopic pattern score were used to confirm the accuracy of possible molecular formulas. Deconjugation experiments with β-glucuronidase/sulfatase were also conducted to confirm the identity of metabolites, as described above. The concentration of Ge, De and Eq was determined using an external calibration curve produced with the use of authentic standards, while their metabolites were quantified as their corresponding aglycone.

RESULTS

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Stability test of isoflavones at 37°C

Stability profiles of Ge, De and Eq compounds in cell medium at 37°C are shown in Figure 2, as the percentage of initial concentration remaining with time. The concentration of Ge, De and Eq decreased by around 5 ± 2% after 2 hour of incubation, and by around 10 ± 2% following 6 hours incubation. After 24 hours, the 78 ± 1%, 75 ± 2% and 82 ± 2% of Ge, De and Eq, respectively, remained stable in endothelial medium.

Identification of isoflavone conjugates in the cell models

The isoflavones and their metabolites identified in HUVEC, HepG2 cells and Caco-2 cell monolayers by LTQ-Orbitrap are shown in Table 1. No Ge, De, Eq or conjugates were detected in any of the control samples (0 μM Ge, De, or Eq) and after incubating cells with 0.1 μM of Ge, De, or Eq. The quantification limits (LOQ) of our method for Ge, De and Eq were 0.1, 0.1 and 0.2 nmol/ml for cell lysates, and 0.02, 0.03 and 0.05 nmol/ml for supernatant samples, respectively.

The identity of glucuronides, sulfates, methoxy-glucuronides and methoxy-sulfates of isoflavones were further confirmed by treating samples with β-glucuronidase/sulfatase enzymes, which resulted in the appearance of their aglycones or methoxylated forms. Figure 3 and Figure 4 show representative chromatograms of HUVEC supernatants after incubation with Ge and De, before (Figures 3a and 4a, respectively) and after enzymatic hydrolysis (Figures 3b and 4b, respectively). Enzymatic hydrolysis led to the formation of methoxylated forms of Ge and De, which corresponded to $[M-H]^+$ with *m/z* 301.0706 and *m/z* 285.0761, respectively. This confirmed that the methylation occur in the aglycone, not in the glucuronide or sulfate moiety. The increase in mass of 30 units between the glucuronide or sulfate metabolites and the methoxylated conjugates (Table

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1) corresponds to the addition of a methoxy group, which can be explained by a hydroxylation step leading to the formation of a catechol moiety, followed by methylation by COMT, as it has been previously demonstrated. [24,25]

Uptake and metabolism of isoflavones in HUVEC cells

When 1, 10 or 100 µM of Ge was incubated with HUVEC cells for 2 hours, Ge and its methoxy-glucuronide and methoxy-sulfate metabolites were detected in both supernatant and cells (Table 2), indicating that Ge is taken up and metabolized by endothelial cells. The amount of Ge (and metabolites) associated with cells represented 6-7% of the total initial Ge. The percentage of total conjugated Ge in the supernatant and cell lysate was around 14-18%, with 82-86% remaining in the unconjugated form. Total methoxy-genistein-glucuronide and methoxy-genistein-sulfate represented 25 and 55% of the metabolized aglycone, respectively (Table 2).

When De was incubated with HUVEC cells, De and a methoxy-glucuronide metabolite were detected in the supernatant and cell lysate (Table 2), indicating that De is also taken up and metabolized by endothelial cells. Around 5-8% of De and its metabolite were found associated to cells at 10 and 100 µM. Neither De nor its metabolites were found associated to cells at the lower concentrations tested, probably because concentrations were lower than the limit of detection of our method. The methoxy-glucuronide metabolite represented around 10-15% of the initial De.

Some examples of tentative chemical structures of the isoflavone metabolites identified in HUVEC cells are shown in Figure 5. The methoxy group could be mostly likely in position 3' or 4';[24] whereas the glucuronide or sulfate moiety could be in position 3', 4', 5 or 7 for genistein and 3',4' and 7 for daidzein.

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No Eq metabolites were detected in the supernatant or cell lysate at any concentration after the incubation of Eq in HUVEC. Around 90% of Eq was found in the supernatant, with around 8-12% of Eq found in the cell lysate at 1, 10 and 100 µM (Table 2).

Uptake and metabolism of isoflavones in HepG2

Incubation of 1, 10, and 100 µM of Ge with HepG2 cells for 2 hours led to the detection of a genistein-glucuronide and a genistein-sulfate in supernatant and cells (Table 3). Around 50% of the initial Ge was metabolized at 1 and 10 µM, and around 20% at 100 µM. Practically all Ge associated to cells was metabolized, being the glucuronide conjugates the most abundant metabolites representing 60-70% of the total, and genistein-sulfate representing 30-40% (Table 3). At 1 µM, the aglycone was not detected in the supernatant neither in the cell lysate.

When incubation of HepG2 cells was carried out with De for 2 hours, De and daidzein-glucuronide and daidzein-sulfate metabolites were detected in both supernatant and cells. However, daidzein-sulfate could not be quantified because the level of the metabolite was lower than the limit of quantification (Table 3). At 1 µM, the aglycone De was not detected in the supernatant or cell lysate, and only the glucuronide metabolite was found in the supernatant (~ 7% of total De).

After the incubation of Eq in HepG2 cells, Eq and equol-sulfate were detected in the supernatant and/or cells. However, the sulfate metabolite of equol could only be quantified in the supernatant at the highest concentration. Around 90% of Eq was found in the supernatant, and 3-13% associated to cells (Table 3).

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Uptake, transport and metabolism of isoflavones in the Caco-2 monolayer model

After incubation with Ge for 2 hours, Ge-glucuronide and Ge-sulfate metabolites were detected in the apical side, basolateral side and cells. Ge-sulfate could not be quantified because the level of the metabolite present was lower than the limit of quantification. The glucuronide metabolite of Ge was observed to be associated with Caco-2 cells at all concentrations tested (Table 4). In addition, at the highest concentration Ge was also detected in the cell lysate. The glucuronide conjugate was excreted to the apical side (~30-40%) and to the basolateral side (~40-50%). Significant amounts of Ge were also detected in the basolateral side, suggesting that both the aglycone and glucuronide transverse the monolayer. At 1 and 10 μM, around 80% of the initial Ge was glucuronidated, whereas at 100 μM only 12% was found as Ge-glucuronide (Table 4).

Incubation of Caco-2 cell monolayers with De led to the detection of De, a glucuronide and a sulfate metabolite in the cell lysate (Table 4). De-sulfate was detected only at the higher concentration tested and could not be quantified because levels were lower than the limit of quantification. When De was incubated at 1 and 10 μM, around 50% of the glucuronide conjugate of De was found in the apical side, and at the higher concentration tested, around 35%. Around 15% of the aglycone and 40% of its metabolite were found in the basolateral side. De-glucuronide represented around 50% of the initial De at 1 and 10 μM, and 10% at the higher concentration tested (Table 4).

No Eq conjugates were detected in the apical side, basolateral side or cell lysate at any concentration after the incubation of Eq in Caco-2 cell monolayers (Table 4). Around 20% of Eq was transported to the basolateral side and 8-15% was found associated to cells.

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DISCUSSION

In this work, the uptake and intracellular metabolism of the isoflavones Ge, De and its microbial metabolite Eq in endothelial cells were investigated, and compared with cell models of liver and small intestine cells. We showed that Ge is metabolized into methoxy-genistein-glucuronide and methoxy-genistein-sulfate, and De is converted into methoxy-daidzein-glucuronide in HUVEC. This indicates that, beside the liver and the small intestine, endothelial cells can also metabolize isoflavones by the action of UDT-glucuronosyltransferases (UGT), sulfotransferases (SULT), and catechol methyl transferases (COMT). The isoflavone metabolites were found in the cell lysate and in the cell medium, suggesting that Ge and De are taken up by endothelial cells, methoxylated, glucuronidated, and sulfated before export to the medium. We cannot discard though that additional conjugation and/or deconjugation reactions might have occurred in the cell medium; however we did not see any of these reactions occurring in the cell medium when testing the stability of Ge, De and Eq, suggesting that the cell medium alone has no glucuronidase/sulfatase/UGT, SULT or COMT activity (Figure 2).

In vivo, glucuronidation and sulfation seem to be the major metabolic pathways in the metabolism of isoflavones, since glucuronides, sulfates, and sulfoglucuronides of Ge and De, and also freely circulating aglycones have been shown to be the major human plasma and urinary metabolites of isoflavones.[23] However, little evidence exists regarding the formation of methoxylated isoflavone metabolites. Ge, De or Eq cannot be subjected to methylation by COMT, as they do not have catechol moieties. However, it has been demonstrated in human liver microsomes that Ge and De are converted to hydroxylated metabolites by cytochrome P450 enzymes.[24] Because some of these aromatic hydroxylated products of Ge and De contain a catechol moiety, these

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346 metabolites could undergo further biotransformation by COMT in HUVEC, leading to
347 the formation of Ge and De methoxylated sulfate and methoxylated glucuronide
348 derivatives. Treatment of samples with glucuronidase and sulfatase led to the formation
349 of methoxylated metabolites of Ge and De (Figure 3 and 4), which confirms that the
350 methoxy group is attached to the aglycone and not to the glucuronide or sulfate moiety,
351 supporting the action of COMT in the cells. Indeed, COMT has been reported to be
352 present in HUVEC cells,[33] and we have recently identified the formation of *O*-methyl-
353 glucuronide and *O*-methyl-sulfates derivatives of (−)-epicatechin in HUVEC.[34] Kulling
354 *et al.* and Heinonen *et al.* have identified mono- and dimethoxylated conjugates of Ge
355 and De in trace amounts in human urine samples after soy supplementation,[24,25] which
356 suggests that methylation of isoflavones does occur *in vitro* but *in vivo* only to a very
357 minor extent. As the metabolism of isoflavones in HUVEC cells *in vitro* is not the same
358 than the observed in human *in vivo*, caution should be taken when reaching conclusions
359 on the investigations of mechanisms of action of isoflavones *in vitro*.

360 It is presently unknown whether the major *in vivo* conjugates of isoflavones are taken
361 up and further metabolized by endothelial cells. In our previous work, no uptake or
362 metabolism of the major *in vivo* metabolites of (−)-epicatechin were observed after
363 incubation with HUVEC cells[34]. In agreement with this, it has been reported that
364 glucuronide conjugates of epicatechin and quercetin were unable to enter dermal
365 fibroblasts and cortical neurons.[35-37] This suggests that the higher polarity of
366 glucuronides and sulfates may limit their capacity to enter the cells and thus, being
367 metabolized further. Further work is needed in order to confirm that this is the case for
368 genistein and daidzein conjugates.

369 When the concentration of isoflavones in the three cell models investigated here was
370 normalized per mg of protein, the uptake of genistein and daidzein in HUVECs was

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found to be higher than in HepG2 and Caco-2 monolayer (Table 2-4), which is in agreement with our previous work with (−)-epicatechin[34], suggesting that endothelial cell metabolism may be of relevance *in vivo*.

Beside investigations on hepatic and small intestine cell models, some evidence exist regarding the intracellular metabolism of other flavonoids, such as flavanols, flavonols, and flavanones, in dermal fibroblasts, central nervous system and cancer cells,[35-41] however very few reports on endothelial metabolism of flavonoids exist. We have recently shown that the flavonoid (−)-epicatechin was taken up and metabolized into 3'-*O*-methyl-(−)-epicatechin-7-β-D-glucuronide and 3'-*O*-methyl-(−)-epicatechin-7-sulfate conjugates after 1 hour of incubation,[34] which is in agreement with the data presented here for isoflavones, where formation of methoxy-glucuronides and methoxy-sulfates took place in HUVEC cells. Anthocyanins, another subclass of flavonoids, have also been shown to be taken up and metabolized into methylated conjugates after incubation with EA.hy926 endothelial cells.[42] To our knowledge, this is the first study to address the uptake and intracellular metabolism of isoflavones in endothelial cells (HUVEC).

In the present work, different cell types led to different uptake and metabolism of isoflavones. The metabolism of tumour/transformed cell lines such as HepG2 and Caco-2 might not be exactly similar to that of primary hepatocytes and colonocytes, although they still represent a fair model for a comparative study to primary HUVEC cells. In the HepG2 cell model, glucuronide and sulfate conjugates of Ge and De were detected in both the supernatant and cell lysate, in agreement with previous work, where the 7-*O*-glucuronide conjugate was the major metabolite of De and Ge in hepatocytes, together with sulfate conjugates.[29,30] In the Caco-2 monolayer, we observed that Ge and De were taken up by cells, glucuronidated (and, to a smaller extent, sulfated) and excreted to the

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apical side (~30-40%) and to the basolateral side (~40-50%) (Table 3). These results are also in agreement with previously reported data in Caco-2 cell monolayers.[26,27]

The microbial metabolite of daidzein, equol, was found to be associated to HUVEC, HepG2 and Caco-2 cells at significant concentrations (Tables 2, 3 and 4). In HepG2 cells, a sulfate metabolite of Eq was excreted to the medium, suggesting that sulfation is the main metabolic pathway of equol in hepatocyte cell model. This is in accordance to an earlier study in which Eq was mainly sulfated (~95% of initial Eq) in HepG2 cells after 4 days incubation.[43] However, in our case conjugated equol represented around 4% of the initial Eq, and was only found at the higher concentrations tested, which may be due to the shorter incubation time with HepG2 cells (2 h vs 4 days). In contrast, Schwen *et al.* reported glucuronidation as the primary pathway for the metabolism of Eq in human hepatocytes (~73% of initial Eq), with lesser sulfation metabolism (~22% of initial Eq).[44] We did not find any Eq conjugates being formed in HUVEC and Caco-2 cell models. No data available about the metabolism of Eq in HUVEC was found in the literature. In Caco-2 monolayers, Eq was shown to be metabolized into phase II conjugates, determined using enzymatic treatment with glucuronidase and sulfatase, so it is unknown whether the metabolites formed were glucuronide or sulfate conjugates.[45] Differences in the analytical methodology and incubation times may explain the differences observed between studies, an it is possible that the limit of detection of our method was not low enough to detect glucuronide conjugates, although the recoveries obtained respect to the initial Eq were around 100% (Tables 2-4). In humans, Eq circulates in plasma and is excreted in urine predominantly as a glucuronide conjugate, and to a lesser extent as a sulfate.[21,25] Thus, according to the work presented here, equol *in vitro* metabolism does not reflect human *in vivo* metabolism.

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Few reports have been published describing the biological activity of isoflavone conjugates, such as the weak estrogenic effect and the activation of human natural killer cells of Ge and De glucuronides,[46] the inhibitory effect of daidzein-4',7-disulfate on the sterol sulfatase in hamster liver microsomes,[47] the stimulatory effect of daidzein-7-glucuronide-4'-sulfate on the growth of MCF-7 cells,[48] and the hypotensive and vasodilator effects of De sulfates in rats.[49] Rimbach *et al.* reported that sulfation of Ge decreased its antioxidant activity, anti-aggregatory effect and its impact on monocyte and endothelial function *in vitro*.[50] Although the mechanisms by which isoflavones and their metabolites mediate their observed effects have not been fully established, methylation of Ge and De could have an important role in the metabolism of isoflavones in some target cells. Further studies are needed to understand the cellular bioactivity of isoflavones and their metabolites.

A limitation of this work is that the major isoflavone metabolites were quantified as their corresponding aglycone, due to the lack of authentic glucuronidated, sulfated and methoxylated standards. Enzymatic treatment with glucuronidase/sulfatase was not used for quantification as methoxylated compounds cannot be quantified with this method without authentic standards, and there are also limitations concerning quantification of glucuronides and sulfates, such as the inability to differentiate between glucuronide and sulfate concentrations, or batch to batch variations in the activity and specificity of the enzymes. In addition, the limits of quantification of the method were not low enough to quantify all the metabolites identified in the cell samples, in particular the sulfate conjugates of Ge and De. We cannot discard that additional metabolites present in the samples below our limits of detection were formed and not accounted for here.

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CONCLUSION

Our data shows that the isoflavones Ge and De are taken up by endothelial cells and are metabolized by phase II enzymes into their methoxylated, glucuronide and sulfate conjugates. However, the microbial metabolite equol is taken up by endothelial cells but is not metabolized. These findings suggest that endothelial cell metabolism needs to be taken into account when investigating the mechanisms of action of isoflavones in the cardiovascular system. Further work in this area is warranted, as the uptake and metabolism of flavonoids in human cells will likely determine their biological actions.

ACKNOWLEDGEMENTS

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Figure Legends

Figure 1: Chemical structures of isoflavones.

Figure 2: Stability profiles of genistein, daidzein and equol after 0.4, 1, 6 and 24 h incubation with endothelial basal medium at 37 ºC with 5% CO_2 in a humidified atmosphere (10 µM). Stability is expressed as percentage of initial concentration remaining with time as determined by UHPLC-UV (n=3).

Figure 3: Representative UHPLC-UV chromatograms of HUVEC supernatant a) incubated with genistein for 2 h and (b) after enzymatic hydrolysis with β-glucuronidase/sulfatase.

Figure 4: Representative UHPLC-UV chromatograms of supernatant of HUVEC incubated with daidzein for 2 h (a) and after enzymatic hydrolysis (b).

Figure 5: Examples of tentative chemical structures of genistein and daidzein metabolites detected in endothelial cells: a) 3'-*O*-methyl-genistein-4'-sulfate; b) 3'-*O*-methyl-genistein-7-glucuronide; c) 3'-*O*-methyl-daidzein-7-glucuronide.

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Table 1: List of the compounds identified by an LTQ Orbitrap Velos mass spectrometer in supernatant and cell lysates of HUVEC, HepG2 and Caco-2 monolayer after incubation with genistein, daidzein or equol for 2 hours.

Compound	Cell model	$[M+H]^+$	Acc. Mass	M.F.
Genistein	HUVEC / Hep-G2 / Caco-2	271	271.0602	$C_{15}H_{10}O_5$
Genistein-glucuronide	Hep-G2 / Caco-2	447	447.0919	$C_{21}H_{18}O_{11}$
Genistein-sulfate	Hep-G2 / Caco-2	351	351.0176	$C_{15}H_{10}O_8S$
Methoxy-genistein-glucuronide	HUVEC	477	477.1028	$C_{22}H_{20}O_{12}$
Methoxy-genistein-sulfate	HUVEC	381	366.0275	$C_{16}H_{12}O_9S$
Daidzein	HUVEC / Hep-G2 / Caco-2	255	255.0655	$C_{15}H_{10}O_4$
Daidzein-sulfate	Hep-G2 / Caco-2	335	335.0229	$C_{15}H_{10}O_7S$
Daidzein-glucuronide	Hep-G2 / Caco-2	431	431.0973	$C_{21}H_{18}O_{10}$
Methoxy-**d**aidzein-glucuronide	HUVEC	461	461.1078	$C_{22}H_{20}O_{11}$
Equol	HUVEC / Hep-G2 / Caco-2	243	243.1018	$C_{15}H_{14}O_3$
Equol-sulfate	Hep-G2	323	323.0584	$C_{15}H_{14}O_6S$

Abbreviations: Acc. mass, accurate mass; MF, molecular formula.

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Table 2: Uptake and metabolism of genistein, daidzein and equol in HUVEC. Results are given in µM±SEM (n=3). The concentration of compound in cell lysate is also expressed as pmol/mg protein (in brackets). The recoveries were calculated respect to the amount of compound recovered after incubation without cells (control).

	1 µM		10 µM		100 µM	
	Supernatant	Cell lysate (pmol/mg protein)	Supernatant	Cell lysate (pmol/mg protein)	Supernatant	Cell lysate (pmol/mg protein)
Genistein	0.83 ± 0.05	0.05 ± 0.01 (60 ± 3)	8.34 ± 0.09	0.48 ± 0.05 (571 ± 58)	78.95 ± 4.85	0.61 ± 0.03 (726 ± 35)
Ge-Gluc	nd	nd	nd	nd	nd	nd
Ge-S	nd	nd	nd	nd	nd	nd
M-Ge-Gluc	0.05 ± 0.01	nd	0.46 ± 0.02	0.08 ± 0.01 (95 ± 2)	4.97 ± 0.39	1.62 ± 0.09 (1928 ± 106)
M-Ge-S	0.09 ± 0.01	0.02 ± 0.01 (24 ± 1)	0.68 ± 0.06	0.21 ± 0.02 (250 ± 19)	7.85 ± 0.75	3.47 ± 0.25 (4131 ± 297)
Total	0.97 ± 0.09	0.07 ± 0.02 (84 ± 3)	0.95 ± 0.09	0.79 ± 0.06 (916 ± 57)	91.77 ± 8.32	5.70 ± 0.52 (6785 ± 421)
% Recovery	99%	7%	97%	7%	94%	6%
Daidzein	0.81 ± 0.04	nd	7.93 ± 0.56	0.38 ± 0.02 (452 ± 22)	78.98 ± 6.05	1.46 ± 0.09 (1738 ± 104)
De-Gluc	nd	nd	nd	nd	nd	nd
M-De-Gluc	0.08 ± 0.01	nd	0.84 ± 0.07	0.51 ± 0.04 (607 ± 48)	10.61 ± 1.01	4.44 ± 0.55 (5286 ± 598)
De-S	nd	nd	nd	nd	nd	nd
Total	0.89 ± 0.04	0	8.77 ± 0.58	0.89 ± 0.05 (1059 ± 56)	89.59 ± 8.89	5.90 ± 0.84 (7024 ± 609)
% Recovery	91%	0%	89%	10%	92%	6%
Equol	0.89 ± 0.07	0.12 ± 0.02 (143 ± 7)	9.14 ± 0.84	0.08 ± 0.01 (95 ± 4)	89.15 ± 3.85	10.84 ± 1.33 (12905 ± 1311)
Eq-S	nd	nd	nd	nd	nd	nd
Total	0.89 ± 0.07	0.12 ± 0.02 (143 ± 7)	9.14 ± 0.84	0.08 ± 0.01 (95 ± 4)	89.15 ± 3.85	10.84 ± 1.33 (12905 ± 1311)
% Recovery	95%	13%	96%	8%	95%	12%

Abbreviations: Ge-Gluc, genistein-glucuronide; Ge-S, genistein-sulfate; M-Ge-Gluc, methoxy-genistein-glucuronide; M-Ge-S, methoxy-genistein-sulfate; De-Gluc, daidzein-glucuronide; M-De-Gluc, methoxy-daidzein-glucuronide; De-S, daidzein-sulfate; Eq-S, equol-sulfate; nd, not detected.

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Table 3: Uptake and metabolism of genistein, daidzein, and equol (μM) in HepG2 cells. Results are given in μM±SEM (n=3). The concentration of compound in cell lysate is also expressed as pmol/mg protein (in brackets). The recoveries were calculated respect to the amount of compound recovered after incubation without cells (control).

	1 μM		10 μM		100 μM	
	Supernatant	Cell lysate (pmol/mg protein)	Supernatant	Cell lysate (pmol/mg protein)	Supernatant	Cell lysate (pmol/mg protein)
Genistein	nd	nd	2.32 ± 0.25	nd	81.77 ± 6.11	0.91 ± 0.02 (276 ± 6)
Ge-Gluc	0.28 ± 0.01	0.07 ± 0.01 (21 ± 3)	2.14 ± 0.19	0.47 ± 0.02 (142 ± 6)	8.54 ± 0.77	3.88 ± 0.20 (1176 ± 60)
Ge-S	0.11 ± 0.01	0.04 ± 0.01 (12 ± 1)	1.66 ± 0.14	0.16 ± 0.01 (48 ± 3)	4.03 ± 0.32	1.22 ± 0.04 (370 ± 12)
M-Ge-Gluc	nd	nd	nd	nd	nd	nd
M-Ge-S	nd	nd	nd	nd	nd	nd
Total	0.39 ± 0.01	0.11 ± 0.01 (33 ± 3)	6.12 ± 0.74	0.63 ± 0.03 (190 ± 5)	94.34 ± 9.28	6.01 ± 0.37 (1822 ± 71)
% Recovery	40%	11%	62%	6%	96%	6%
Daidzein	nd	nd	5.09 ± 0.03	nd	78.63 ± 4.39	1.18 ± 0.03 (358 ± 9)
De-Gluc	0.07 ± 0.01	nd	4.15 ± 0.02	0.63 ± 0.03 (191 ± 9)	14.03 ± 1.22	4.45 ± 0.23 (1348 ± 69)
M-De-Gluc	nd	nd	nd	nd	nd	nd
De-S	nd	nd	nd	nd	nq	nq
Total	0.07 ± 0.01	0	9.24 ± 0.06	0.63 ± 0.03 (191 ± 9)	92.66 ± 7.73	5.63 ± 0.27 (1702 ± 669
% Recovery	7%	0%	94%	6%	94%	6%
Equol	0.93 ± 0.06	0.07 ± 0.01 (21 ± 1)	8.72 ± 0.07	1.26 ± 0.09 (382 ± 27)	96.71 ± 3.47	3.28 ± 0.12 (994 ± 32)
Eq-S	nd	nd	nq	nd	4.11 ± 0.25	nd
Total	0.93 ± 0.06	0.07 ± 0.01 (21 ± 1)	8.72 ± 0.07	1.26 ± 0.09 (382 ± 27)	100.88 ± 5.26	3.28 ± 0.12 (994 ± 32)
% Recovery	99%	7%	93%	14%	108%	3%

Abbreviations: Ge-Gluc, genistein-glucuronide; Ge-S, genistein-sulfate; M-Ge-Gluc, methoxy-genistein-glucuronide; M-Ge-S, methoxy-genistein-sulfate; De-Gluc, daidzein-glucuronide; M-De-Gluc, methoxy-daidzein-glucuronide; De-S, daidzein-sulfate; Eq-S, equol-sulfate; nd, not detected.

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Table 4: Uptake and metabolism of genistein, daidzein, and equol (μM) in Caco-2 monolayers. Results are given in μM±SEM (n=3). The concentration of compound in cell lysate is also expressed as pmol/mg protein (in brackets). The recoveries were calculated respect to the amount of compound recovered after incubation without cells (control).

	1 μM			10 μM			100 μM		
	Apical	Basolateral	Cell lysate (pmol/mg protein)	Apical	Basolateral	Cell lysate (pmol/mg protein)	Apical	Basolateral	Cell lysate (pmol/mg protein)
Genistein	0.18 ± 0.01	nd	nd	2.13 ± 0.11	0.41 ± 0.02	nd	66.26 ± 2.33	14.05 ± 0.98	6.12 ± 0.04 (4451 ± 29)
Ge-Gluc	0.34 ± 0.02	0.43 ± 0.03	0.03 ± 0.01 (22 ± 1)	3.49 ± 0.22	4.48 ± 0.03	0.39 ± 0.01 (284 ± 8)	3.87 ± 0.25	4.65 ± 0.03	3.77 ± 0.02 (2742 ± 15)
Ge-S	nd	nd	nd	nd	nd	nd	nd	nd	nd
M-Ge-Gluc	nd	nd	nd	nd	nd	nd	nd	nd	nd
M-Ge-S	nd	nd	nd	nd	nd	nd	nq	nq	nq
Total	0.52 ± 0.02	0.43 ± 0.03	0.03 ± 0.01 (22 ± 1)	5.62 ± 0.38	4.48 ± 0.06	0.39 ± 0.01 (284 ± 8)	70.13 ± 3.60	18.70 ± 1.38	9.89 ± 0.07 (7193 ± 22)
% Recovery	53%	44%	3%	57%	50%	4%	71%	19%	10%
Daidzein	0.23 ± 0.01	0.11 ± 0.01	nd	3.19 ± 0.23	1.87 ± 0.07	0.41 ± 0.02 (298 ± 12)	73.87 ± 4.52	10.49 ± 0.85	2.07 ± 0.01 (1505 ± 7)
De-Gluc	0.34 ± 0.02	0.23 ± 0.01	0.03 ± 0.01 (22 ± 1)	2.16 ± 0.15	1.59 ± 0.08	0.64 ± 0.04 (465 ± 30)	3.58 ± 0.26	4.31 ± 0.02	2.46 ± 0.01 (1789 ± 8)
M-De-Gluc	nd	nd	nd	nd	nd	nd	nd	nd	nd
De-S	nd	nd	nd	nd	nd	nd	nq	nq	nq
Total	0.57 ± 0.02	0.34 ± 0.01	0.03 ± 0.01 (22 ± 1)	5.35 ± 0.47	3.46 ± 0.11	1.05 ± 0.05 (763 ± 32)	77.45 ± 6.54	14.80 ± 0.90	4.53 ± 0.01 (3294 ± 9)
% Recovery	58%	35%	3%	55%	36%	6%	78%	15%	5%
Equol	0.71 ± 0.01	0.22 ± 0.01	0.08 ± 0.01 (58 ± 2)	6.94 ± 0.44	2.22 ± 0.16	0.82 ± 0.05 (596 ± 36)	62.65 ± 3.77	22.46 ± 1.25	14.89 ± 1.00 (10829 ± 707)
Eq-S	nd	nd	nd	nd	nd	nd	nd	nd	nd
Total	0.71 ± 0.01	0.22 ± 0.01	0.08 ± 0.01 (58 ± 2)	6.94 ± 0.44	2.22 ± 0.16	0.82 ± 0.05 (596 ± 36)	62.65 ± 3.77	22.46 ± 1.25	14.89 ± 1.00 (10829 ± 707)
% Recovery	75%	23%	8%	74%	23%	8%	67%	23%	16%

Abbreviations: Ge-Gluc, genistein-glucuronide; Ge-S, genistein-sulfate; M-Ge-Gluc, methoxy-genistein-glucuronide; M-Ge-S, methoxy-genistein-sulfate; De-Gluc, daidzein-glucuronide; M-De-Gluc, methoxy-daidzein-glucuronide; De-S, daidzein-sulfate; Eq-S, equol-sulfate; nd, not detected.

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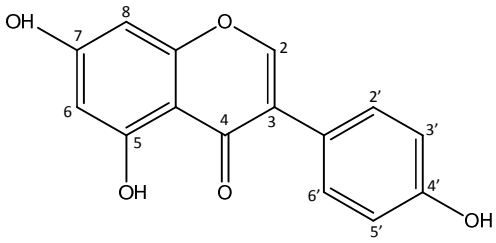
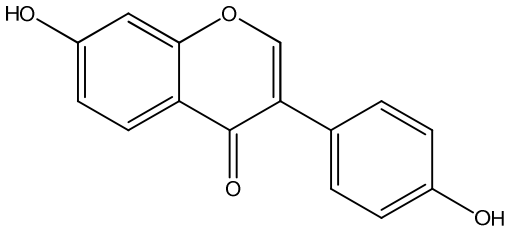
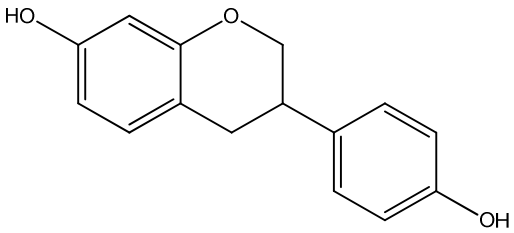
Figure 1



Genistein



Daidzein



Equol

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Figure 2

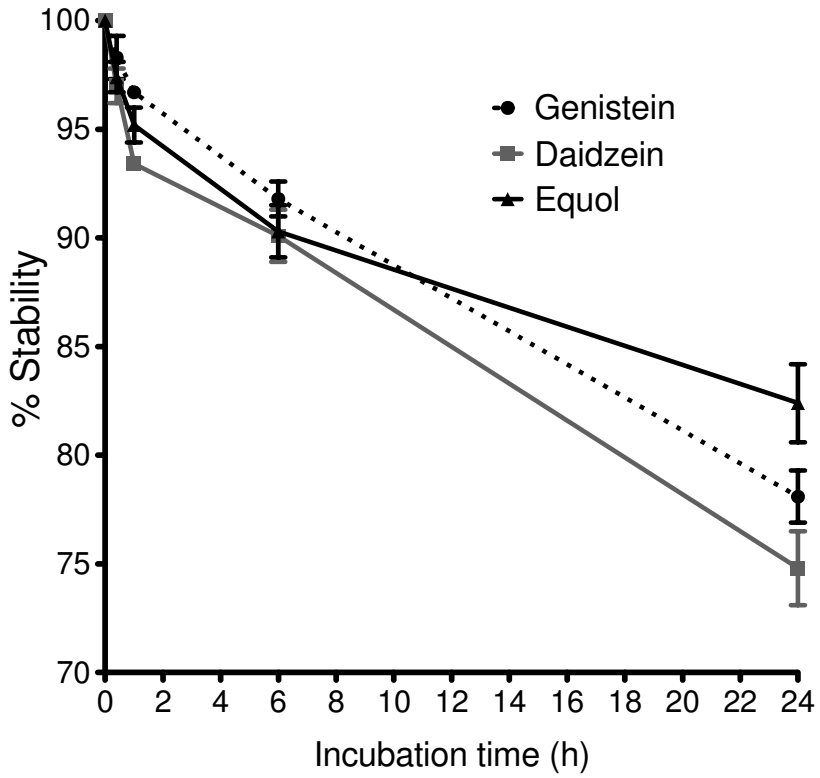

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Figure 3.

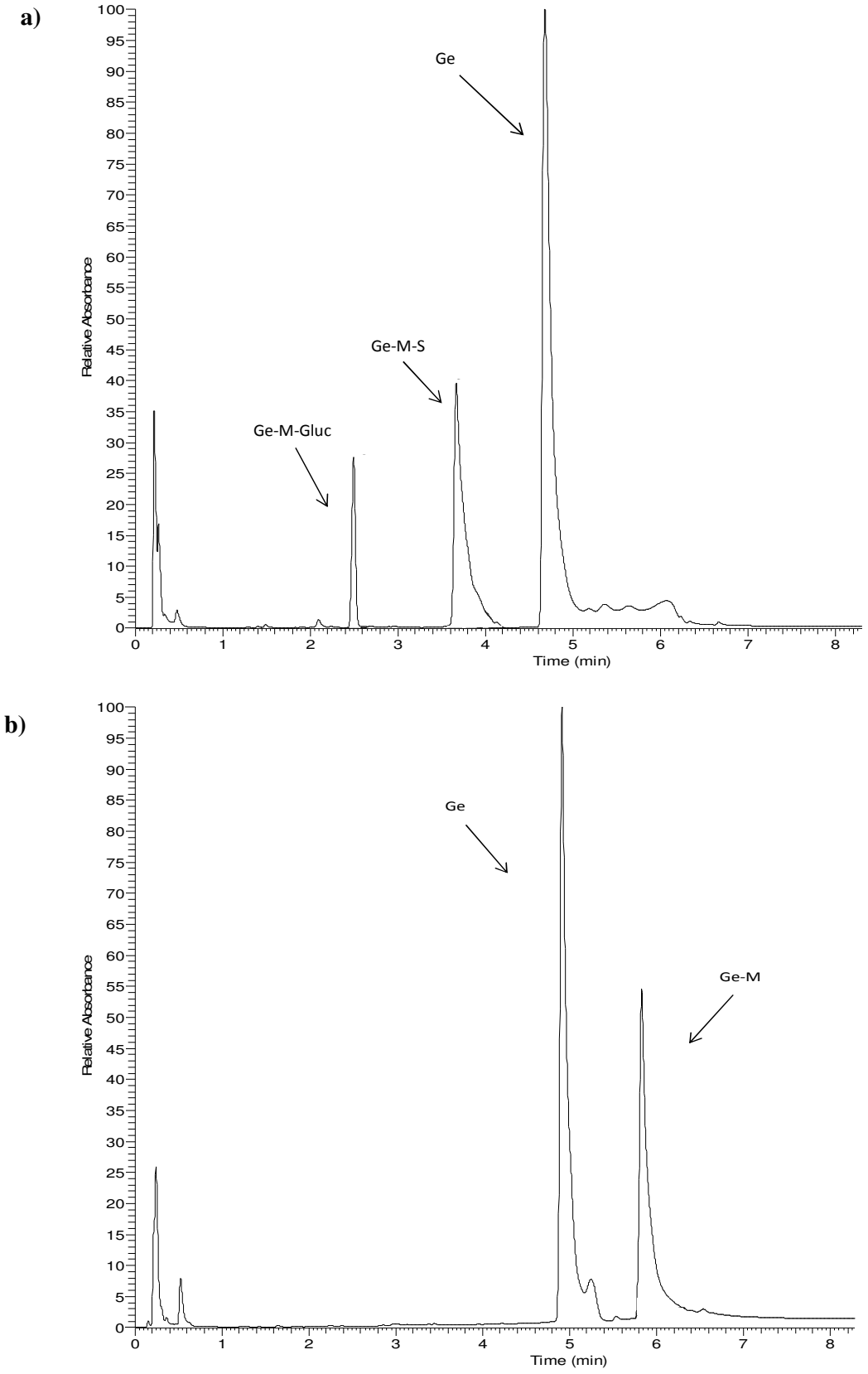

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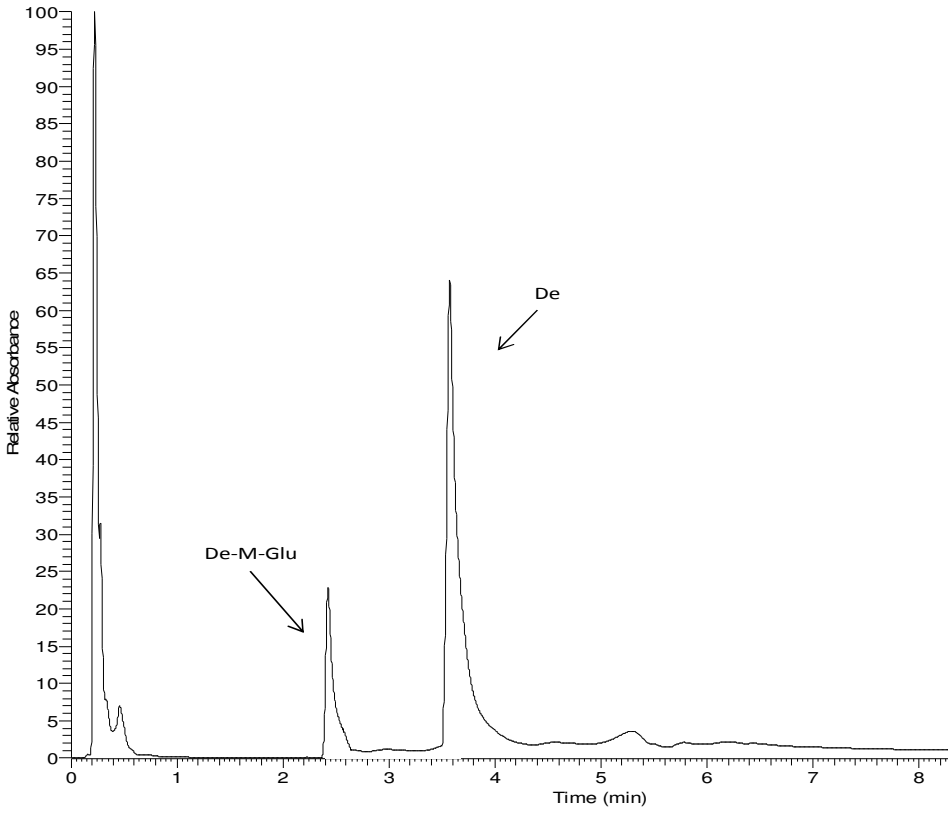
Figure 4.

a)



b)

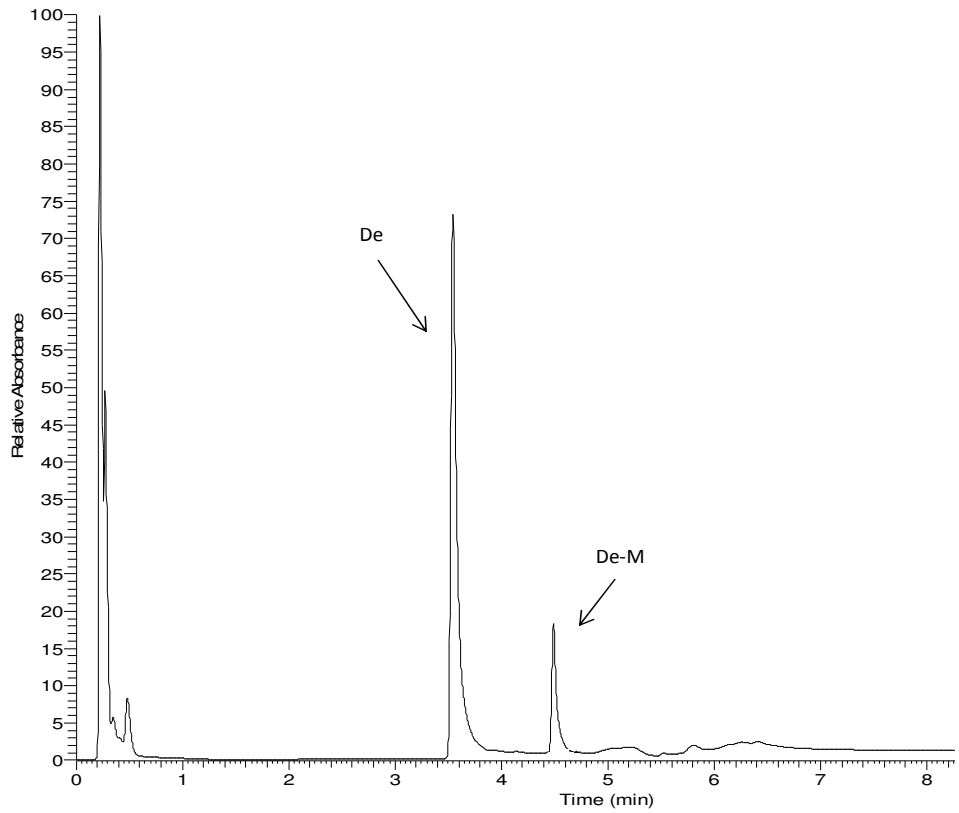


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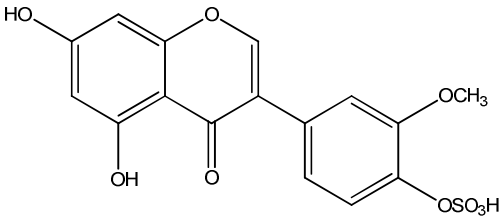
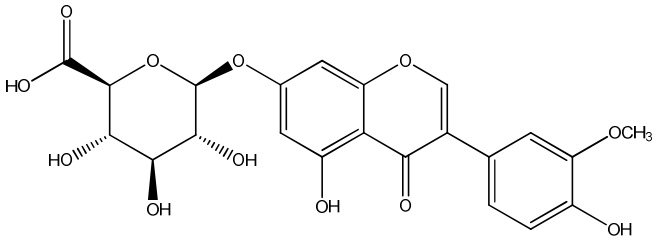
Figure 5

a)



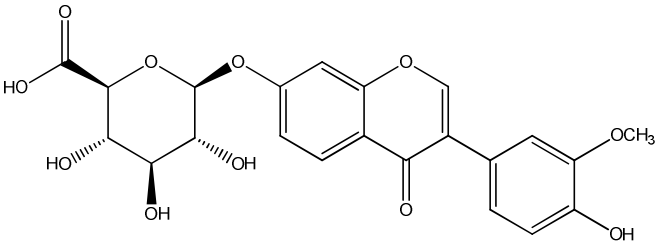
3'-*O*-methyl-genistein-4'-sulfate

b)



3'-*O*-methyl-genistein-7-glucuronide

c)



3'-*O*-methyl-daidzein-7-glucuronide

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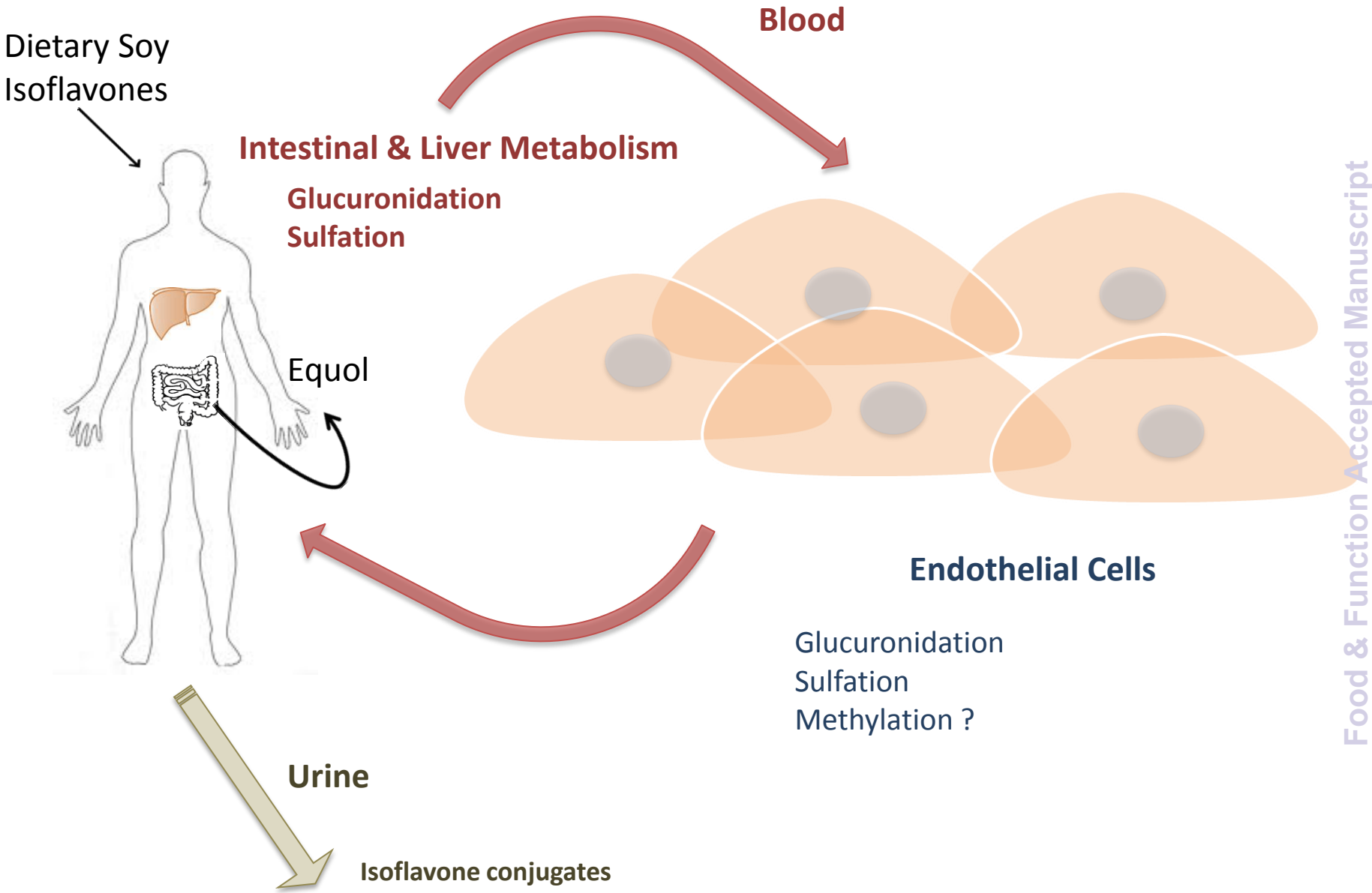

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