

# Quantification of soy isoflavones and their conjugative metabolites in plasma and urine: an automated and validated UHPLC-MS/MS method for use in large-scale studies

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**Abstract** The biotransformation of isoflavones by gut microbiota and by drug metabolizing enzymes plays a crucial role in the understanding of their potential health-promoting effects. The purpose of our work was to develop a simultaneous, sensitive, and robust automated ultra high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method to quantify the soy isoflavones daidzein and genistein, their conjugative metabolites, as well as their major microbial degradation products in order to provide a method for use in large clinical trials or animal studies. An automated, 96-well solid-phase extraction method was used to extract the isoflavone analytes from plasma and urine. Separation of genistein, daidzein, and 19 of its metabolites, including five glucuronides, seven sulfates, and two sulfoglucuronides, as well as five microbial metabolites, was achieved in less than 25 min using a sub-2  $\mu\text{m}$  particle column and a gradient elution with acetonitrile/methanol/water as mobile phases. Analysis was performed under negative ionization electrospray MS via the multiple reaction monitoring (MRM). Validation was performed according to the analytical method validation guidelines of Food and Drug Administration (FDA) and International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) consisting of selectivity, accuracy, precision, linearity, limit of

detection, recovery, matrix effect, and robustness. All validated parameters essentially matched the FDA and ICH requirements. The application of this method to a pharmacokinetic study in postmenopausal women showed that isoflavones are extensively metabolized in vivo. A robust automated analytical approach was developed, which allows the handling of large sample sizes but nevertheless provides detailed information on the isoflavone metabolite profile leading to a better understanding and interpretation of clinical and animal studies.

**Keywords** Soy isoflavones · Sulfates · Sulfoglucuronides · Glucuronides · Clinical study · Automated sample preparation

## Introduction

The isoflavones (IF) genistein (GEN) and daidzein (DAI) occur in soy and soy food products. They are one of the best studied secondary plant metabolites of the last 20 years and are associated with various potential health benefits. These include lower risks of hormone-related cancers, such as breast and prostate cancer, and cardiovascular diseases, prevention of osteoporosis, and alleviation of menopausal symptoms (summarized in Mortensen et al. [1]).

Although a large number of clinical studies have been conducted, there is still a lack of convincing support for these health benefits, even though a part of the studies is promising. Among several reasons, human inter-individual variability in the bioavailability and metabolism of IF are believed to be most important [2]. In general, metabolism alters the chemical structure of a parent compound and can thereby tremendously modify its biological activity. A prominent example is the alteration of the estrogenicity of daidzein by microbial biotransformation to *S*-equol or, alternatively, to the ring-opened product *O*-desmethylangolensin. Whereas the estrogen agonist activity of *S*-equol was found to be higher compared with

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daidzein, *O*-desmethylangolensin showed reduced estrogenic properties [3]. It has been pointed out that the ability to metabolize DAI to *S*-equol might be crucial for the health effects of soy isoflavones [2]. Next to microbial metabolism, phase II metabolism to glucuronide and sulfate conjugates may substantially alter the biological properties of IF. In the past, this aspect has not always been considered important, assuming that conjugation necessarily leads to inactive compounds. Indeed, in the case of DAI, glucuronidation at the 4'- and 7-position resulted in a significant reduction in estrogenicity [4]. However, the 7-*O*-sulfate of DAI possesses a much higher activity than DAI itself and the same is true for the sulfoglucuronides [5, 6]. Therefore, comprehensive analyses of the metabolite profile of IF in biological matrices are essential for a meaningful interpretation of the health outcomes in human intervention studies. In addition, such analyses can help to identify differences in the IF metabolism in humans compared with experimental animals, which is important for the transferability of the results.

Current methods mostly use GC/MS, LC/DAD, or LC-MS/MS to determine IF in biological fluids, but only some of them allow the quantitative determination of the microbial metabolites or even the intact phase II metabolites in addition to the aglycones DAI and GEN (summarized in Ishii et al. [7]). This is mostly due to the fact that the metabolites, especially the conjugates, were not as easily available in the past. Moreover, only very few of the current methods are automated and designed for high-throughput analyses as would be required for clinical studies [8–10].

The aim of our study was to establish a method that combines all these qualities since, to the best of our knowledge, such a method is not yet available. A particular feature of the UHPLC-MS/MS method presented here is its validation according to the analytical method validation guidelines of the Food and Drug Administration (FDA) and the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [11, 12]. Authentic standard compounds were used for the 21 analytes included in the method. In addition, <sup>13</sup>C-labeled internal standards, namely [2,3,4-<sup>13</sup>C<sub>3</sub>]-DAI and [2,3,4-<sup>13</sup>C<sub>3</sub>]-GEN-7-β-D-glucuronide, were synthesized in our lab.

## Materials and methods

### Chemicals and reagents

Daidzein (DAI) and genistein (GEN) were purchased from LC Laboratories (Woburn, MA, USA) and exhibit purities of >99 %. Daidzein-4'-β-D-glucuronide (DAI-4'-G) (96 %), daidzein-7-β-D-glucuronide (DAI-7-G) (95 %), daidzein-7-β-D-glucuronide-4'-sulfate (DAI-7-G-4'-S) (98 %), genistein-

4'-β-D-glucuronide (GEN-4'-G) (98 %), genistein-7-β-D-glucuronide (GEN-7-G) (98 %), genistein-7-β-D-glucuronide-4'-sulfate (GEN-7-G-4'-S) (97 %), and dihydrodaidzein (DH-DAI) (>99 %) were purchased from Toronto Research Chemicals (North York, Canada). Dihydrogenistein (DH-GEN) (>99 %) and equol (>97 %) were purchased from APIN Chemicals Ltd. (Abingdon, UK). *O*-desmethylangolensin (ODMA) (>99 %) and 6'-hydroxy-*O*-desmethylangolensin (6' OH-ODMA) (>99 %) were purchased from Plantech UK (Reading, UK). (R,S)-equol-4'-sulfate (equol-4'-S) (>99 %) and (R,S)-equol-7-β-D-glucuronide (equol-7-G) (>99 %) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). [2,3,4-<sup>13</sup>C<sub>3</sub>]-daidzein (<sup>13</sup>C<sub>3</sub>-DAI) was synthesized as recently described [13].

Daidzein-4'-sulfate (DAI-4'-S), daidzein-7-sulfate (DAI-7-S), daidzein-7,4'-disulfate (DAI-7,4'-DS), genistein-4'-sulfate (GEN-4'-S), genistein-7-sulfate (GEN-7-S), and genistein-7,4'-disulfate (GEN-7,4'-DS) were synthesized according to the method of Fairley et al., with the following changes [14]: a total 5 mL dried pyridine, and the solution was cooled on ice; 165 μL of chlorosulfonic acid was carefully added and the resulting mixture was stirred overnight at room temperature. The solution was evaporated to dryness and the residue was resolved in 20 % aqueous methanol containing 0.05 g/mL NaHCO<sub>3</sub>. The mixtures of DAI mono- and disulfates, as well as of GEN mono- and disulfates present in these solutions were purified using a preparative LC system. The synthesized standard compounds were characterized by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (Electronic Supplementary Material S1) and had the following purities: DAI-4'-S, 98 %; DAI-7-S, 99 %; DAI-7,4'-DS, 99 %; GEN-4'-S, 98 %; GEN-7-S, 98 %; and GEN-7,4'-DS, 99 %.

[2,3,4-<sup>13</sup>C<sub>3</sub>]-genistein-7-β-D-glucuronide (<sup>13</sup>C<sub>3</sub>-GEN-7-G) was synthesized based on the procedure as recently described [15]. Acylation of [2,3,4-<sup>13</sup>C<sub>3</sub>]-GEN with excess of hexanoyl chloride in pyridine at 0 °C afforded 5,7,4'-tri-*O*-acyl [2,3,4-<sup>13</sup>C<sub>3</sub>]-genistein in 93 % yield, which on treatment with the nucleophile thiophenol in presence of imidazole in *N*-methyl 2-pyrrolidinone (NMP) gave selectively the targeted 4',5-*O*-dihexanoylated [2,3,4-<sup>13</sup>C<sub>3</sub>]-GEN in 78 % yield. To a mixture of the 4',5-*O*-dihexanoylated [2,3,4-<sup>13</sup>C<sub>3</sub>]-GEN (0.94 g, 2 mmol), glucuronyl (*N*-*p*-methoxyphenyl)-trifluoroacetamide (1.61 g, 3 mmol), and freshly dried molecular sieves 4 Å (2 g) in dry dichloromethane (20 mL), boron trifluoride diethyl etherate (0.037 mL, 0.3 mmol) was added under nitrogen. After stirring for 5 h at room temperature, dry triethylamine was added and stirring was continued for 15 min. The solids were removed by filtration through a pad of Celite and the filtrate was evaporated. The residue was purified by flash chromatography in petrolether-ethyl acetate 20 %→40 % to give the compound methyl (5,4'-*O*-

dihexanoyl-[2,3,4- $^{13}\text{C}_3$ ]-GEN-7-yl-b-D-2'',3'',4''-triacylglucopyranoside urinate (1.38 g, 88 %) as a white solid. A mixture of methyl (5,4'-O-dihexanoyl-[2,3,4- $^{13}\text{C}_3$ ]-GEN-7-yl-b-D-2'',3'',4''-triacyl-glucopyranoside urinate (1.3 g, 1.66 mmol) and anhydrous  $\text{K}_2\text{CO}_3$  (0.46 g, 3.32 mmol) was dissolved in a solution of MeOH/THF/ $\text{H}_2\text{O}$  (5 mL:5 mL:1 mL) at room temperature under nitrogen atmosphere. After stirring for 5 h at 40 °C, the mixture was cooled to room temperature, neutralized with Dowex-50 H<sup>+</sup>, and then filtered and concentrated. The pale yellow solid was purified by preparative HPLC ( $\text{H}_2\text{O}:\text{CH}_3\text{CN}$  6:4) furnishing  $^{13}\text{C}_3$ -GEN-7-G as a white crystalline solid (0.69 g, 93 %) as white solid.  $^{13}\text{C}_3$ -GEN-7-G was characterized by NMR spectroscopy (Electronic Supplementary Material S1). All other chemicals and solvents used were of analytical grade. The chemical structures of the IF analytes are shown in Fig. 1.

#### Human sample material

To prepare an IF-free plasma and urine pool, eleven healthy volunteers (4 women, 7 men) were instructed to avoid IF-containing food products for at least 48 h before blood donation. Venous fasting and non-fasting blood samples were collected in tubes containing potassium-EDTA as anticoagulant

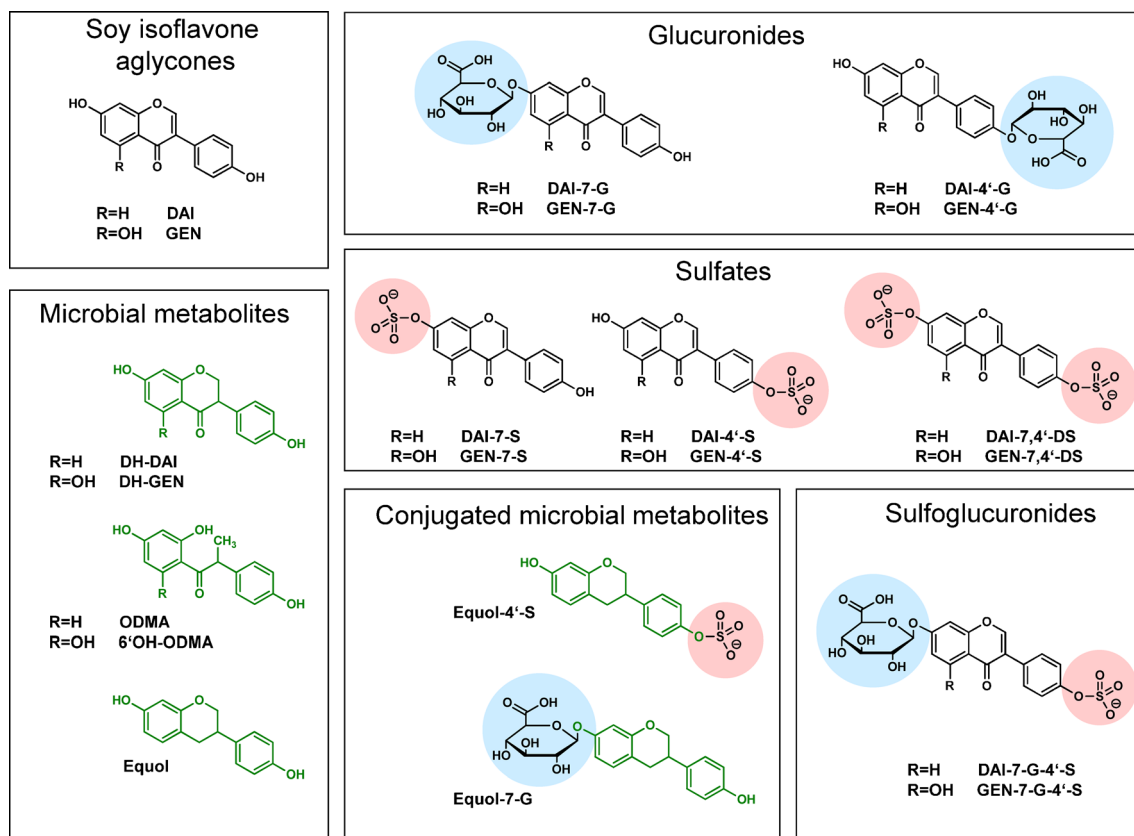
(S-Monovettes 9.0 mL; Sarstedt AG & Co., Nümbrecht, Germany) and were immediately centrifuged (1500 rpm, 10 min, and 4 °C). A blank plasma pool was generated by combining aliquots of these single plasma samples. First void urine samples were collected from six volunteers (1 woman and 5 men). A blank urine pool was prepared by combining aliquots from the single urine samples. Both pooled blank matrices, plasma and urine, were checked for the absence of IF.

Eleven postmenopausal women participated in the intervention study. They ingested a single-dose of an IF-rich soy extract (1.0 mg/kg BW calculated as IF aglycone equivalents of DAI and GEN) in a hard gelatin capsule after a standardized breakfast. Plasma samples were collected 6 h after ingestion. Urine was collection between 0 and 6 h after ingestion of the soy extract.

Ethical approval was obtained from the State Medical Chamber Baden-Württemberg, Stuttgart, Germany, under accession number #F-2011-004. All samples were stored at -80 °C until further use.

#### Preparation of standard stock solutions

Each IF analyte, as well as the two  $^{13}\text{C}$ -labeled standard compounds were dissolved in an appropriate amount of



**Fig. 1** Chemical structures of daidzein (DAI), genistein (GEN), and their metabolites determined with the present method. G: glucuronide; S: sulfate; DH: dihydro; ODMA: *O*-desmethylangolensin

dimethylsulfoxide (DMSO) to prepare the respective 5 mM standard stock solutions. A master-mix standard solution was generated by mixing an equivalent volume of each standard stock solution (with the exception of  $^{13}\text{C}_3$ -DAI and  $^{13}\text{C}_3$ -GEN-7-G) to obtain a concentration of 2312.5  $\mu\text{M}$  for equol, and of 125  $\mu\text{M}$  for all other analytes. The master-mix standard solution was diluted with DMSO to obtain the following additional calibration point solutions: 25  $\mu\text{M}$  (462.5  $\mu\text{M}$  for equol), 5  $\mu\text{M}$  (92.5  $\mu\text{M}$  for equol), 1  $\mu\text{M}$  (18.5  $\mu\text{M}$  for equol), 0.2  $\mu\text{M}$  (3.7  $\mu\text{M}$  for equol), and 0.04  $\mu\text{M}$  (0.74  $\mu\text{M}$  for equol). A 5  $\mu\text{M}$  internal standard mixture was prepared by combining an appropriate volume of the  $^{13}\text{C}_3$ -DAI and  $^{13}\text{C}_3$ -GEN-7-G standard stock solutions.

## Sample preparation

### *Automated extraction of IF from plasma and urine*

An automated solid-phase extraction (SPE) using a robotic workstation (Microlab Star; Hamilton Robotics GmbH, Martinsried, Germany) was used to purify plasma and urine samples. The workstation was equipped with eight pipetting channels (1000  $\mu\text{L}$ ) using 1000  $\mu\text{L}$  and 300  $\mu\text{L}$  tips, one plate carrier for microtiter and deepwell plates (purified sample storage), three sample carriers for 96 tubes (12 $\times$ 75 mm for raw sample storage), one tip carrier containing three 1000  $\mu\text{L}$ -tip-racks and two 300  $\mu\text{L}$ -tip-racks, two reagent carriers for six 125 mL reagent containers (solvent storage), a CORE grip tool for plate movement, and a crystal vacuum system containing a pump, chamber, and manifold (SPE processing). The robotic workstation was controlled by the software VENUS 2 (version 4.3). To ensure an easy and error-free operation by the laboratory staff, an interactive program was developed, which guides the operator step by step through the workflow by using graphically assisted input options for sample quantity, sample volume, and well allocation of plates. To optimize the pipetting conditions, different liquid classes provided by the software program were selected. Because of similar pipetting properties, the liquid class “water” was adjusted for all aqueous solutions and the liquid class “ethanol” for all methanolic solutions. The extraction was done as follows: 500  $\mu\text{L}$  of each thawed sample (plasma or urine) was mixed with 5  $\mu\text{L}$  of each internal standard solution (5  $\mu\text{M}$   $^{13}\text{C}_3$ -DAI and 5  $\mu\text{M}$   $^{13}\text{C}_3$ -GEN-7-G stock solution in DMSO). The robotic workstation was loaded with the spiked samples and the automated SPE was started. First, the samples were acidified with 1 mL of 50 mM  $\text{H}_3\text{PO}_4$  solution. Each well of the SPE plate (Strata-X AW, 96-well, 60 mg; Phenomenex LTD, Aschaffenburg, Germany) was then conditioned with 2 mL of methanol and equilibrated with 2 mL of water. After loading with the sample solutions, the wells were washed with 2 mL of 2 % (v/v) formic acid in water, followed by 2 mL of 50 % (v/v) methanolic solution. The analytes were eluted in a 96-deepwell plate with 1 mL of

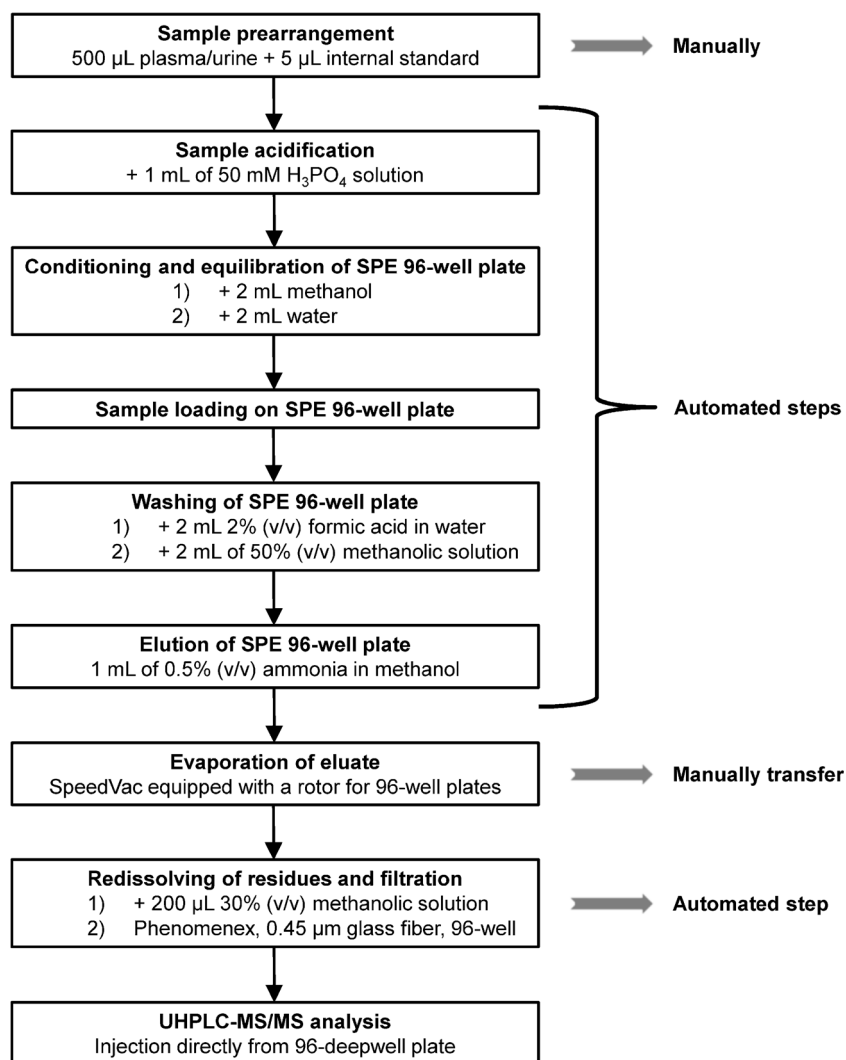
0.5 % (v/v) ammonia in methanol, and evaporated to dryness using a SpeedVac (SPD131; Thermo Electron LED GmbH, Langensfeld, Germany) equipped with a rotor for 96-well plates. After this, the robotic processing was continued by dissolving the residues in 200  $\mu\text{L}$  of 30 % (v/v) methanolic solution. Then a clean-up step was performed by using a flow-through filtration plate (Phenomenex, 0.45  $\mu\text{m}$  glass fiber, 96-well). Aliquots of the filtrates were injected directly from the 96-deepwell plates to the UHPLC-MS/MS system and analyzed as described below. An illustration of the automated extraction procedure is given in Fig. 2.

## UHPLC-MS/MS method

The concentrations of the IF metabolites in the prepared samples were determined by UHPLC-MS/MS. The analyses were performed on an QTrap 5500 mass spectrometer (AB Sciex Germany GmbH, Darmstadt, Germany) equipped with a Nexera LC system (Shimadzu Europa GmbH, Duisburg, Germany), which consisted of a controller (CBM-20A), a degasser (DGU-20A5), two pumps (LC-30 AD), an autosampler (SIL-30 AC), a column oven (CTO-20 AC), and a DAD (SPD-M20A). The system was controlled by the software Analyst 1.5.2. Separation of the analytes was achieved on a Acquity HSS T3 (2.1 mm internal diameter, 100 mm length, 1.8  $\mu\text{m}$  particle size; Waters GmbH, Eschborn, Germany) equipped with a pre-column (Waters Acquity HSS T3, 2.1 mm diameter, 5 mm length, 1.8  $\mu\text{m}$  particle size) and a pre-in-line filter (Phenomenex Krudkatcher, 0.5  $\mu\text{m}$ ). The column oven temperature was adjusted to 40  $^\circ\text{C}$ . Solvent A was a 40 mM ammonium formate buffer (pH 3) and solvent B was an acetonitrile/methanol mixture (1.0/2.5, v/v). The flow rate was set to 0.5 mL/min. The injected sample volume was 20  $\mu\text{L}$ . The gradient elution profile was as follows: 0–2.6 min isocratic with 3 % B, 2.6–16.7 min from 3 % to 56 % B, 16.7–17.3 min from 56 % to 95 % B, 17.3–19.9 min isocratic with 95 % B, 19.9–20.5 min from 95 % to 3 % B, and 20.5–24.7 min isocratic at the initial conditions. To minimize contamination of the ion source, the eluate was only transferred to the MS at the time when the analytes of interest eluted from the column.

The turbo spray ESI source of the MS was operated in the negative mode. The source parameters were as follows: curtain gas (CUR) 40 psi, ion spray voltage (IS) –4500 V, ion source gas-1 (GS 1) 80 psi, ion source gas-2 (GS 2) 70 psi, ion source gas-2 temperature (TEM) 600  $^\circ\text{C}$ . The quadrupole detectors Q1 and Q3 were operated at unit resolution in the scheduled multiple-reaction monitoring (sMRM) mode using two transitions (a quantifier and a qualifier ion transition for each compound) for unambiguous identification of each analyte. The sMRM experiments with the associated settings are summarized in the Electronic Supplementary Material S2. Nitrogen was used as collision gas.

**Fig. 2** Illustration of the automated extraction procedure of IF metabolites from plasma and urine samples



### Method validation

The validation of the analytical method was performed according to the guidelines of the US FDA and of the ICH [11, 12].

### Calibration curves and linearity

For quantification, an external calibration curve with six calibration points was prepared for each blank matrix. Therefore, 5 µL of the specific diluted master-mix standard solution (in an end concentration range between 18.5 and 57812.5 nM for equol and 1 and 3125 nM for all other analytes) and 5 µL of internal standard mix were added to 500 µL blank plasma or urine and these spiked samples were worked up as described above. To obtain the calibration curves, the ratios of the analyte peak areas to the internal standard peak area were calculated and plotted against the analyte concentration. The aglycones and conjugates referred to  $^{13}\text{C}_3$ -DAI and  $^{13}\text{C}_3$ -

GEN-7-G, respectively. A best fit line was obtained by linear regression using a weighting of  $1/x^2$ . The calculated correlation coefficient was used as an indicator for the quality of the calibration curves and the linearity. Because of adding the analytes before extraction and the use of an internal standard, no recovery correction was performed for the measured IF values.

### Limit of quantification and limit of detection

The limit of quantification (LOQ) and limit of detection (LOD) were defined as the concentrations with a signal-to-noise (S/N) ratio of 10 and of 3, respectively. To calculate the LOQ and the LOD, the S/N ratio was determined for each analyte and matrix at the four lowest calibration points, using six replicate samples at each level. For each analyte, the calibration point with the lowest S/N ratio (but greater than 10 for LOQ and greater than 3 for LOD) was chosen to calculate the respective LOQ and LOD values.

### Accuracy and precision

To determine the accuracy, intra-day precision and inter-day precision of the method, the three IF concentration levels, i.e. low (37 nM for equol and 2 nM for all other analytes), medium (925 nM for equol and 50 nM for all other analytes), and high (23125 nM for equol and 1250 nM for all other analytes), were chosen for plasma samples. In the case of urine samples, the concentration levels medium and high (see plasma samples) were used. The preparation of these samples was done in the same way as described for the calibration points. Six samples for each concentration level were prepared within a day as described above and the concentrations of the analytes were measured. The accuracy was calculated as the ratio of the measured to the nominal concentration. The intra-day precision was expressed as the coefficient of variation (CV) for the six values. For determination of the inter-day precision, the same procedure was performed on a different day and by another person, and the CV for the both days was calculated.

### Recovery

The recovery of the analytes in plasma and urine was investigated using the same concentration levels for each matrix as described in section “Accuracy and precision”. For each concentration, 12 samples were prepared. Six of these were spiked before extraction and the other six samples after the extraction and evaporation step. All samples were measured by UHPLC-MS/MS analysis. The recoveries were calculated as the ratio of the resulting peak areas for each sample spiked before and after the extraction procedure.

### Matrix effects

The matrix effect was determined as described by Matuszewski et al. [16]. Briefly, two sets of samples were prepared. For the first set (“after extraction”) the blank matrix samples were processed to the end of the evaporation step. The samples were spiked with an appropriate amount of the analytes and the sample preparation was continued. For the second set (“standard”), six samples were prepared in 30 % (v/v) methanolic solution (without any matrix) for each concentration point. To calculate the matrix effect, the ratio between the peak areas of the analytes in the set “after extraction” and the peak areas of the analytes in the set “standard” was determined. The matrix effect for plasma was determined using eleven blood plasma samples of different origin (4 women and 7 men), as well as the pooled plasma sample. In the case of urine, the matrix effects were determined for six urine samples of different origin (1 woman and 5 men), as well as for the pooled urine sample (see “Human sample material”).

### Stability

The stability of the DMSO stock solutions towards multiple thawing and freezing, the stability of the analytes in purified samples, and the stability of the MS signal as related to measurements of a large sample contingency were determined.

For assessing the stability of the DMSO stock solutions, a 5  $\mu$ M master-mix standard solution was thawed and frozen 15 times. At the beginning and at every fifth thawing event, six aliquots of 10  $\mu$ L were diluted in 190  $\mu$ L of 30 % (v/v) methanolic solution (technical replicates). These samples were measured with the UHPLC-MS/MS method described above and the decrease of the analyte MS signal (peak area) was determined.

To determine the stability of the analytes in samples of each matrix (plasma and urine), six samples with the concentration “medium” were prepared as described above. The samples were stored in the autosampler of the UHPLC-MS/MS system at 4 °C. After 0, 24, and 48 h, the samples were measured as described above and the analyte MS signal (peak area) over time was determined. Furthermore, the stability of the MS signal was assessed by repeat injections of 30 identical plasma as well as urine samples using the concentration medium.

### Statistics

Statistical evaluations were performed using the SigmaPlot 11.0 software.

## Results and discussion

### Analysis method optimization

Depending on their different lipophilicity and acidity characteristics, we divided the IF analytes in four different groups (Electronic Supplementary Material S3). The aglycones exhibit relative high lipophilicity and are mainly unionized at pH values below 6. In contrast, the IF glucuronides are weak acids and exhibit a relative low lipophilicity. Changes in the pH between 1 and 7 affect the ionization state of the carboxylic acid group. Sulfates as the third group possess lipophilicity characteristics in-between the aglycones and the glucuronides. As strong acids, sulfates are completely ionized when dissolved in water. The sulfoglucuronides combine the chemical properties of glucuronides and sulfates. They show a relatively low lipophilicity and possess a permanent ionized sulfate group. These different chemical properties must be considered when optimizing the conditions for sample extraction, chromatography, and mass spectrometric detection. For this reason, a liquid–liquid extraction (e.g., with ethyl acetate or diethyl ether) is not suitable because of the low solubility of

the conjugates in the organic layer phase. We chose a SPE cleanup using a polymer with weak anion exchange features (Strata-X AW, a styrene-divinylbenzene polymer with primary and secondary amine functionalities) for the following reasons: First, the polymer phase is not affected by drying out, making it suitable for the automated extraction in a 96-well format by a robotic workstation, and second, the weak anion exchange exhibits also good extraction efficiency for the ionized analytes.

To accelerate the chromatography, an UHPLC method using a sub-2  $\mu\text{m}$  particle column was developed. To obtain a high resolution in an acceptable analysis time, we used a column with a length of 100 mm, an internal diameter of 2.1 mm, and a particle size of 1.8  $\mu\text{m}$  operating with a flow rate of 0.5 mL/min. Because of the existence of isomeric and isobaric IF metabolites (e.g., DAI-4'-S and DAI-7-S), which cannot be distinguished in the mass spectrometer due to the same molecule ion mass and similar fragmentation patterns, we used a C18 column especially developed for polar compounds (Waters Acquity HSS T3), which shows a high retention also for analytes like the sulfoglucuronides. To optimize separation of the IF analytes, different pH values (pH 3, 5, and 7), organic eluents (acetonitrile, methanol, mixture of both), column temperatures (20, 30, 40, and 50  $^{\circ}\text{C}$ ), gradients, as well as eluent modifiers (formic acid, ammonium acetate, ammonium formate), were investigated. The best result was obtained with a 40 mM ammonium formate buffer (pH 3) and an acetonitrile/methanol mixture (1/2.5, v/v) as eluents, using a column temperature of 40  $^{\circ}\text{C}$ . A representative standard chromatogram is illustrated in Fig. 3. The relatively high concentration of the ammonium formate buffer was necessary

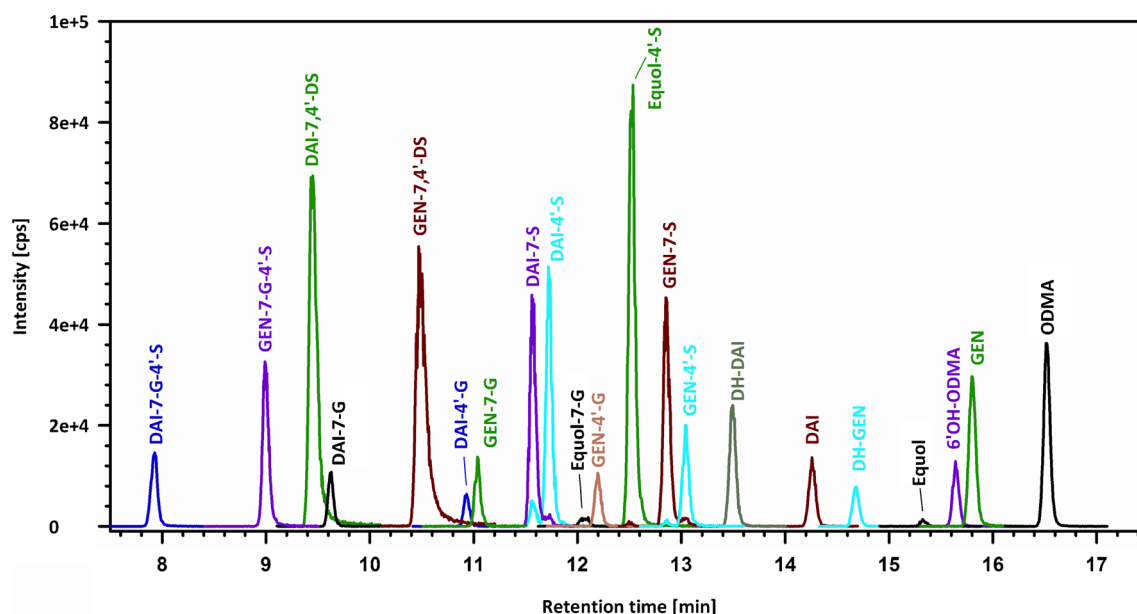
to achieve an optimal peak shape for the sulfate conjugates, especially for the disulfates. Lower buffer concentrations caused broad tailing peaks.

For the mass spectrometric detection, an ESI source operating in the negative mode gave the best sensitivity for the conjugates (data not shown), and was also suitable for aglycones as already previously observed [17]. The UHPLC conditions were optimized with the primary aim to achieve a high chromatographic resolution of the analytes. Because the LOQ values were satisfying (see section “*Selectivity, linearity, and sensitivity*”), we accepted that the UHPLC conditions selected (low pH and high buffer concentration) were, on the other hand, unfavorable for the detection of the analytes with ESI-MS in the negative mode [18]. The MRM mode was chosen because it has been described as the most sensitive mode for triple quadrupole for quantification of small molecules [19]. Since 46 MRM transitions (two transitions for each analyte) were monitored, data acquisition was performed in the scheduled MRM (sMRM) mode, in which transitions were only measured around the expected retention time of the analytes. This approach enables the use of higher dwell times for each transition, which in turn leads to lower noise signals and to a higher sensitivity.

#### Method validation

##### *Selectivity, linearity, and sensitivity*

The FDA defines selectivity as “the ability of an analytical method to differentiate and quantify the analyte in the



**Fig. 3** UHPLC-MS/MS chromatogram of a standard mixture of IF analytes (50 nM) in plasma; the extracted ion chromatograms (XIC) of the single sMRM traces are displayed

presence of other components in the sample” [11]. For unambiguous identification of analytes, the retention time and two MRM transitions for each analyte were used. The quotient of MRM transition-1 and -2 for each analyte was calculated and compared with the quotient of the respective standard. This procedure meets the criteria of different regulatory authorities such as e.g., the European Union [20].

For quantification, an external calibration curve with six calibration points was prepared in blank plasma and in blank urine. The standard curves in plasma were linear within the used concentration range (DAI, GEN, DH-DAI, DH-GEN, ODMA, 6'OH-ODMA, disulfates, and sulfoglucuronide: 1–3125 nM; glucuronides: 5–3125 nM; equol: 18.5–57812.5 nM) with correlation coefficients >0.9900. The standard curves in urine were also linear in the concentration range used (DAI, GEN, DH-DAI, DH-GEN, ODMA, and 6'OH-ODMA: 1–3125 nM; glucuronides: 25–3125 nM; disulfates and sulfoglucuronides: 5–3125 nM; equol: 92.5–57812.5 nM), with correlation coefficients >0.9900. In the case of the monosulfates, slight saturation occurred at the highest calibration point (3125 nM), leading to correlation coefficients 0.9839–0.9900. Therefore, sulfate concentrations above 625 nM should be diluted, as discussed below.

LOQ and LOD were determined as described in section “Limit of quantification and limit of detection” and the values are listed in Table 1. For analysis of IF in plasma, the LOD exhibited values <1.67 nM, except for equol (39.7 nM). A comparison of selected LOD values with those reported in the literature is shown in the Electronic Supplementary Material S4. The method presented here in most cases exhibits lower LOD values, even though a compromise was made between the chromatographic and the mass detection conditions as discussed above. Therefore, the optimal sensitivity could not be reached for each single analyte measured. The low sensitivity for equol using MS detection compared with all the other IF analytes is conspicuous and has also been described by others [21, 22].

#### Accuracy and precision

For IF analysis, blank plasma samples were spiked with three concentrations (low, medium, and high) and blank urine samples with two analyte concentrations (medium and high), so as to determine the accuracy, intra-day precision and inter-day precision. The results for accuracy and precision are shown in Table 2. The FDA accepts values for accuracy and for precision of  $\pm 15\%$  in general, and  $\pm 20\%$  near the LOQ [11].

The values determined for accuracy in plasma fulfill the criteria of the FDA with one exception (i.e., that concerning the sulfates of DAI and GEN). In this case, the deviations of accuracy lie between  $-31\%$  and  $-5\%$  for the highest concentration because of a slight saturation as discussed above. For the low and medium concentrations, the accuracy for sulfates

**Table 1** Limits of quantification (LOQ) and limits of detection (LOD) for IF and metabolites in plasma and undiluted urine

Analytes	Plasma (500 $\mu$ L sample volume)		Urine (500 $\mu$ L sample volume)	
	LOQ (nM)	LOD (nM)	LOQ (nM)	LOD (nM)
DAI	1.00	0.30	2.67	0.80
DAI-4'-G	2.73	0.82	43.5	13.0
DAI-7-G	1.42	0.43	10.3	3.08
DAI-4'-S	0.40	0.12	4.30	1.29
DAI-7-S	0.26	0.08	0.24	0.07
DAI-7,4'-DS	0.29	0.09	1.32	0.40
DAI-7-G-4'-S	0.42	0.13	1.23	0.37
GEN	0.38	0.11	1.41	0.42
GEN-4'-G	4.66	1.40	76.1	22.8
GEN-7-G	3.51	1.05	21.5	6.44
GEN-4'-S	0.69	0.21	1.14	0.34
GEN-7-S	0.51	0.15	1.11	0.33
GEN-7,4'-DS	0.92	0.28	1.83	0.55
GEN-7-G-4'-S	0.23	0.07	0.71	0.21
DH-DAI	0.51	0.15	1.16	0.35
ODMA	0.20	0.06	0.48	0.14
Equol	132	39.7	251	75.2
Equol-7-G	5.57	1.67	40.3	12.1
Equol-4'-S	0.30	0.09	0.74	0.22
DH-GEN	0.50	0.15	1.33	0.40
6'OH-ODMA	1.29	0.39	1.61	0.48

DAI: daidzein; GEN: genistein; G: glucuronide; S: sulfate; DH: dihydro; ODMA: *O*-desmethylangolensin

showed values ranging from 96 % to 114 %. The intra-day precision and inter-day precision in plasma also meets the criteria of FDA, except for the inter-day precision of DAI-7,4'-DS for the ‘low’ concentration with 22.3 %. For the analysis of urine, the accuracies showed values between 86 % and 113 % with the exception for DAI-7-S with an accuracy of 83 % for the ‘high’ concentration. Precision values of between 0.9 % and 9.4 % were determined, which again meets the criteria of FDA.

#### Recovery and matrix effect

The mean extraction recoveries in plasma and urine using six replicates at the same concentration levels as accuracy determination are shown in Table 3.

The recoveries were not lower than 80 % for all analytes at low, medium, and high concentrations in plasma, except for 6'OH-ODMA and the disulfates with values between 49 % and 77 %. For urine, the recoveries of all analytes exhibited values >79 %. The lower recovery for 6'OH-ODMA and for the disulfates in plasma does not seem to be caused by a lower



**Table 2** Mean accuracy, intra-day precision, and inter-day precision for the quantification of IF and metabolites in plasma and undiluted urine samples using the present method. Accuracy, intra-day precision, and inter-day precision were determined for three plasma and two urineconcentrations. For accuracy and intra-day precision, six replicates were analyzed ( $n=6$ ). Inter-day precision was determined over 2 d with six replicates for each day

Analyt	Concentration 2 nM <sup>b</sup> Plasma			Concentration 50 nM <sup>b</sup> Plasma Urine*			Concentration 1250 nM <sup>b</sup> Plasma Urine*		
	Accuracy [%]	Intra-day precision [%]	Inter-day precision [%]	Accuracy [%]	Intra-day precision [%]	Inter-day precision [%]	Accuracy [%]	Intra-day precision [%]	Inter-day precision [%]
DAI	100	4.4	8.1	99 101*	2.6 2.2*	5.2 2.3*	94 95*	3.4 1.5*	4.9 1.7*
DAI-4'-G	91 <sup>a</sup>	7.7 <sup>a</sup>	19.1 <sup>a</sup>	102 100*	3.4 5.0*	3.5 5.7*	95 95*	4.0 2.9*	3.1 5.6*
DAI-7-G	85	8.5	17.5	103 105*	3.0 4.0*	2.8 4.4*	98 96*	4.4 3.2*	3.4 3.5*
DAI-4'-S	100	4.6	7.7	102 109*	5.9 2.8*	6.9 4.6*	82 91*	3.3 5.0*	4.1 4.6*
DAI-7-S	109	3.8	4.7	105 120*	2.1 4.2*	5.6 5.5*	69 83*	3.9 4.2*	4.0 4.1*
DAI-7,4'-DS	108	10.6	22.3	114 105*	7.5 5.1*	8.8 5.5*	79 99*	6.9 9.4*	11.8 6.9*
DAI-7-G-4'-S	103	8.2	6.3	102 103*	3.0 3.8*	3.8 3.1*	90 98*	3.4 3.0*	2.9 2.8*
GEN	101	4.5	4.9	97 101*	3.4 1.8*	6.1 2.9*	93 96*	6.3 1.8*	6.9 2.5*
GEN-4'-G	95 <sup>a</sup>	7.6 <sup>a</sup>	8.4 <sup>a</sup>	101 118* <sup>a</sup>	3.6 5.6* <sup>a</sup>	4.7 7.2* <sup>a</sup>	95 101*	2.6 3.6*	3.7 5.9*
GEN-7-G	114 <sup>a</sup>	16.4 <sup>a</sup>	14.6 <sup>a</sup>	102 104*	3.5 3.8*	4.9 3.8*	94 98*	2.6 3.5*	3.5 4.3*
GEN-4'-S	96	9.5	9.7	101 107*	7.3 4.0*	8.0 4.5*	95 96*	6.8 3.1*	6.9 5.0*
GEN-7-S	101	9.6	10.0	100 111*	6.0 5.0*	6.9 4.1*	77 86*	6.2 4.2*	6.6 4.5*
GEN-7,4'-DS	108	12.6	20.2	115 100*	11.3 3.6*	11.2 3.5*	89 105*	7.6 6.6*	13.8 5.4*
GEN-7-G-4'-S	102	6.9	6.8	103 100*	5.4 2.5*	5.7 2.9*	83 99*	3.2 4.1*	5.0 3.5*
DH-DAI	96	4.4	6.0	97 100*	3.6 1.8*	5.4 2.0*	92 94*	3.8 0.9*	5.9 2.2*
ODMA	99	5.5	4.2	98 101*	3.3 1.4*	6.2 2.0*	95 95*	4.3 1.7*	5.9 2.2*
Equol	87 <sup>a</sup>	27.1 <sup>a</sup>	24.7 <sup>a</sup>	107 106*	4.3 3.4*	5.1 3.8*	106 88*	2.8 2.7*	3.0 2.6*
Equol-7-G	88 <sup>a</sup>	19.5 <sup>a</sup>	21.1 <sup>a</sup>	98 94*	4.1 4.1*	6.4 6.0*	92 98*	3.6 3.2*	5.2 3.4*
Equol-4'-S	101	9.1	8.0	98 107*	8.3 4.0*	7.3 3.4*	86 93*	6.6 4.1*	5.4 3.7*
DH-GEN	105	3.4	5.2	101 97*	4.0 1.7*	4.4 3.4*	97 92*	4.0 3.1*	3.9 4.1*
6'OH-ODMA	91	13.8	13.4	99 113*	14.5 4.4*	11.9 8.2*	86 89*	12.1 4.1*	11.9 4.5*

DAI: daidzein; GEN: genistein; G: glucuronide; S: sulfate; DH: dihydro; ODMA: *O*-desmethylangolensin<sup>a</sup> Tested concentration <LOQ<sup>b</sup> Concentration of equol was 18.5-times higher

**Table 3** Mean extraction recoveries of IF and metabolites in plasma and undiluted urine at different concentrations ( $n=6$ )

Analyt	Concentration 2 nM <sup>b</sup> Plasma		Concentration 50 nM <sup>b</sup> Plasma Urine*		Concentration 1250 nM <sup>b</sup> Plasma Urine*	
	Recovery [%]	SD [%]	Recovery [%]	SD [%]	Recovery [%]	SD [%]
DAI	91	6.9	91 93*	1.4 1.7*	95 93*	2.4 2.5*
DAI-4'-G	98 <sup>a</sup>	7.4 <sup>a</sup>	93 85*	3.6 9.2*	95 88*	1.6 4.6*
DAI-7-G	99	5.5	93 92*	1.7 3.8*	95 94*	1.8 2.4*
DAI-4'-S	89	4.0	90 89*	7.6 3.9*	91 95*	2.3 1.6*
DAI-7-S	89	5.1	92 92*	2.6 3.2*	93 93*	1.6 2.8*
DAI-7,4'-DS	84	3.6	57 88*	5.0 2.6*	62 93*	4.7 3.2*
DAI-7-G-4'-S	91	8.0	91 76*	1.1 6.6*	94 80*	1.6 4.2*
GEN	90	5.3	87 89*	1.9 2.1*	92 91*	1.9 1.7*
GEN-4'-G	89 <sup>a</sup>	5.8 <sup>a</sup>	90 79* <sup>a</sup>	1.9 12.5* <sup>a</sup>	90 81*	2.3 3.8*
GEN-7-G	86 <sup>a</sup>	10.4 <sup>a</sup>	93 84*	4.0 4.9*	94 87*	1.2 3.6*
GEN-4'-S	86	5.6	80 89*	5.4 1.5*	81 89*	3.6 2.6*
GEN-7-S	81	9.6	86 89*	3.0 2.1*	88 93*	2.6 2.4*
GEN-7,4'-DS	77	6.5	49 79*	6.5 3.0*	54 84*	4.5 2.1*
GEN-7-G-4'-S	84	3.7	88 60*	2.6 7.7*	89 63*	1.1 4.6*
DH-DAI	94	2.3	89 92*	1.4 2.1*	94 94*	2.0 1.6*
ODMA	90	2.9	88 91*	1.2 2.1*	91 92*	1.9 1.8*
Equol	99 <sup>a</sup>	29.3 <sup>a</sup>	88 91*	5.9 5.0*	92 93*	3.7 3.3*
Equol-7-G	103 <sup>a</sup>	22.1 <sup>a</sup>	96 87*	5.5 9.3*	93 85*	1.6 4.5*
Equol-4'-S	91	7.0	91 93*	2.3 3.7*	94 93*	1.3 4.4*
DH-GEN	84	7.9	84 93*	2.6 3.1*	90 92*	2.4 2.4*
6'-OH-ODMA	45	7.4	51 82*	4.1 2.9*	51 81*	4.6 2.0*
<sup>13</sup> C <sub>3</sub> -DAI			95 92*	1.7 1.5*		
<sup>13</sup> C <sub>3</sub> -GEN-7-G			94 82*	3.1 3.0*		

SD: standard deviation; DAI: daidzein; GEN: genistein; G: glucuronide; S: sulfate; DH: dihydro; ODMA: *O*-desmethylangolensin<sup>a</sup> tested concentration <LOQ<sup>b</sup> Concentration of equol was 18.5-times higher

chemical stability. Instead, we assume an unspecific protein binding, which might not be fully broken by the SPE conditions used. Csanády et al. described such unspecific protein binding for DAI [23]. The lower recoveries for 6'OH-ODMA and the disulfates in plasma were acceptable because the accuracy, precision, and LOD for these analytes were suitable as discussed above.

The matrix effects were investigated for plasma and urine as described above. Eleven plasma samples of different origin as well as a pooled plasma sample were used. In the case of urine, six urine samples of different origin as well as a pool of urine samples were also used. The matrix effects are listed in the Electronic Supplementary Material S5. For plasma samples, the mean matrix effects exhibited values between 91 % and 111 %. The correlating standard deviations were from 2.1 % to 6.7 %, which represent the biological variation. Therefore, the use of different blank plasma samples for the

preparation of a calibration curve does not have a significant influence on the matrix effect.

For undiluted urine samples, the matrix effects were between 8 % and 177 %. Especially for the disulfates, high ion suppression (8 %–29 %) was observed. However, in pharmacokinetic studies, IF concentrations in urine are usually very high. Therefore, urine samples have to be diluted [24–26]. We investigated the matrix effect in the same blank urine samples after diluting these 10 times with water. The matrix effect was dramatically reduced, with mean values ranging from 94 % to 127 % for all analytes, and corresponding standard deviation between 1.2 % and 13.2 %, except for equol-4'-S (145 % ±15.5 %). Consequently, the matrix effect can be efficiently reduced by dilution of the samples and has, therefore, in most of the cases only a minor impact on the quantification of the analytes in study samples. This has also been reported by Dams et al. [27].

**Table 4** Unacceptable accuracies are obtained for most IF and metabolites in plasma and urine when calibration curves of surrogate compounds were used for quantification. The values in bold represent the accuracies

of the method when the appropriate identical standard compounds are used as done in this method

Analyt	Accuracy [%] for plasma and urine* ( <i>n</i> =6; 50 nM) calculated by using the calibration curve of...					
	DAI ( <sup>13</sup> C <sub>3</sub> -DAI)	DAI-7-G ( <sup>13</sup> C <sub>3</sub> -GEN-7-G)	DAI-4'-S ( <sup>13</sup> C <sub>3</sub> -GEN-7-G)	GEN ( <sup>13</sup> C <sub>3</sub> -DAI)	GEN-7-G ( <sup>13</sup> C <sub>3</sub> -GEN-7-G)	GEN-4'-S ( <sup>13</sup> C <sub>3</sub> -GEN-7-G)
DAI	<b>99</b> <b>101*</b>	153 180*	5 <0*	42 36*	121 140*	52 42*
DAI-4'-G	44 33*	66 60*	2 <0*	19 12*	53 40*	23 12*
DAI-7-G	67 59*	<b>103</b> <b>105*</b>	3 <0*	29 21*	82 77*	35 23*
DAI-4'-S	2015 1794*	3111 3185*	<b>102</b> <b>109*</b>	857 652*	2437 2618*	1048 779*
DAI-7-S	369 450*	569 800*	19 20*	157 163*	446 650*	192 194*
DAI-7,4'-DS	678 209*	1046 371*	34 4*	289 75*	820 297*	353 89*
DAI-7-G-4'-S	112 67*	172 119*	5 <0*	48 24*	136 89*	58 27*
GEN	228 279*	352 496*	11 9*	<b>97</b> <b>101*</b>	277 400*	119 119*
GEN-4'-G	70 64*	107 114*	3 <0*	30 23*	85 85*	36 25*
GEN-7-G	84 77*	129 136*	4 <0*	36 27*	<b>102</b> <b>104*</b>	44 31*
GEN-4'-S	193 252*	297 447*	10 7*	82 91*	234 360*	<b>101</b> <b>107*</b>
GEN-7-S	156 212*	240 377*	8 5*	66 77*	189 302*	81 90*
GEN-7,4'-DS	666 249*	1027 443*	34 7*	283 90*	805 356*	346 106*
GEN-7-G-4'-S	246 229*	379 408*	12 6*	105 83*	298 327*	128 98*

DAI: daidzein; GEN: genistein; G: glucuronide; S: sulfate; DH: dihydro; ODMA: *O*-desmethylangolensin. The used internal standard is displayed in parentheses

### Stability and robustness

The stability of the DMSO stock solutions towards multiple thawing and freezing, the stability of the analytes in purified samples, and the stability of the MS signal during prolonged analyses were determined as described above. The detailed results are shown in the Electronic Supplementary Material S6, S7, and S8.

The stability of analytes is shown for 10 freezing and thawing procedures of DMSO stock solutions. The analytes in the samples were stable for 24 h at 4 °C in the autosampler, except for 6'OH-ODMA, with a significant degradation of 9 % in plasma ( $p=0.024$ , unpaired  $t$ -test). For urine, most of the analytes degraded significantly after 48 h storage in the autosampler. Therefore, samples should be prepared and measured within 24 h.

To determine the inter-day precision as an important parameter for the robustness of the method, sample preparation and analysis were done by two different persons. The obtained values meet the criteria of FDA as discussed in the result section "Accuracy and precision", showing that the method is reliable. In addition, over 600 plasma samples and 700 urine samples were analyzed in the complete validation procedure and during analyses of the study samples. In these studies, many quality control samples were carried along, which show accuracies and precisions that are mainly in accordance with the FDA criteria (data not shown). Thus, the method presented here exhibits robustness over a long time period. As the measurement of plasma and urine samples can contaminate the MS source, we tested the stability of the MS signal during the measurement of 30 identical plasma as well as urine samples, respectively. The CV lie between 1.9 % and 7.8 %, showing that the SPE cleanup method that was developed leads to sufficiently purified samples to prevent unnecessary contamination of the MS source.

Errors in the quantification of analytes using the calibration curve of surrogate compounds

Several conjugative IF metabolites are not available commercially. Therefore, such analytes are often quantified by using a surrogate compound. Since almost all conjugative metabolites of DAI and GEN were available in our lab, we determined the impact of using such surrogate compounds on the accuracy of the LC-MS/MS method. For this purpose, we used the method validation data and calculated the concentration of selected analytes by applying the respective calibration curve to various other analytes. The results are summarized in Table 4 and indicate that such a procedure in most of the cases leads to an unacceptable accuracy. This is especially true when a calibration curve of another IF conjugate class is used. To give an example, the use of the calibration curve of DAI-7-G for the quantification of the

monosulfates, the disulfate, or the sulfoglucuronide of DAI led to a 1.7-fold to 31-fold overestimation of the respective analyte concentration. However, also within the same conjugate class, large errors can occur. For example, the accuracy for the quantification of DAI-7-S in plasma on the basis of the calibration curve of the isomer DAI-4'-S exhibits a value of 19 %, which means that the calculated concentrations would be 5-times lower as the true values. In contrast, the accuracy of GEN-7-S in plasma using the calibration curve of GEN-4'-S led to a value of 81 %. The accuracy for DAI-4'-G in plasma using the calibration curve of DAI-7-G is 66 %. For urine as matrix, a similar impact on the accuracy was detected.

Our data demonstrate that quantification of metabolites by using such an approach can lead to unacceptable errors. According to our assessment, a reliable prediction concerning the

**Table 5** Plasma concentrations and urine excretions of IF and metabolites in postmenopausal women ( $n=11$ ) after ingestion of an IF-rich soy extract (dose 1.0 mg/kg BW calculated as IF aglycone equivalents of DAI and GEN; plasma collection 6 h after ingestion; urine collection between 0 and 6 h after ingestion)

Analyte	Plasma [nM]			Urine [ $\mu$ mol]		
	Mean	SD	CV	Mean	SD	CV
DAI	17.4	12.8	74	0.05	0.04	80
DAI-4'-G	164.7	81.3	49	3.96	1.64	41
DAI-7-G	149.5	82.6	55	9.68	4.32	45
DAI-4'-S	64.3	38.7	60	0.21	0.15	70
DAI-7-S	144.2	63.0	44	1.09	0.85	79
DAI-7,4'-DS	7.9	6.3	80	n.d.		
DAI-7-G-4'-S	613.1	249.4	41	2.07	0.99	48
Percentage of free DAI [%]	1.4	0.6	43	0.29	0.19	66
GEN	10.1	9.7	96	0.01	0.01	100
GEN-4'-G	146.5	96.4	66	2.25	0.99	44
GEN-7-G	73.8	55.9	76	1.96	1.00	51
GEN-4'-S	6.1	3.5	57	0.01	0.02	176
GEN-7-S	142.2	71.4	50	0.11	0.13	121
GEN-7,4'-DS	0.7	1.3	183	n.d.		
GEN-7-G-4'-S	766.0	486.7	64	0.73	0.33	45
Percentage of free GEN [%]	0.8	0.4	50	0.18	0.16	89
DH-DAI	1.3	1.9	146	n.d.		
ODMA	0.1	0.2	200	n.d.		
Equol	n.d.			n.d.		
Equol-7-G	3.5	11.7	335	n.d.		
Equol-4'-S	0.5	1.6	329	n.d.		
DH-GEN	0.3	0.9	300	n.d.		
6'OH-ODMA	0.3	1.1	367	n.d.		

SD: standard deviation; CV: coefficient of variation in %; n.d.: not detected (<LOQ); DAI: daidzein; GEN: genistein; G: glucuronide; S: sulfate; DH: dihydro; ODMA: *O*-desmethylangolensin.

magnitude of the resulting error is not possible. Therefore, results based on such an approach in LC-MS/MS analysis should be interpreted carefully.

#### Application to human urine and plasma samples

To demonstrate the applicability and efficiency of the method presented here, we measured plasma and urine samples from a study with postmenopausal women ( $n=11$ ) after ingestion of an IF-rich soy extract (dose 1.0 mg/kg BW calculated as IF aglycone equivalents of DAI and GEN). Plasma and urine collection were done 6 h and 0–6 h after IF ingestion, respectively. The designated time points were chosen because in many pharmacokinetic studies plasma  $C_{\max}$  of IF were reported to be between 5 and 9 h after ingestion as summarized by Mortensen et al. [1]. The determined mean plasma concentrations and urine excretion of IF and metabolites are shown in Table 5. The sulfoglucuronides of DAI and GEN were the main metabolites in plasma, whereas in urine the glucuronides predominated. In both plasma and urine, less than 1.4 % of DAI and GEN were present as aglycones. A representative UHPLC-MS/MS chromatogram of a study sample is illustrated in the Electronic Supplementary Material S9.

#### Limitations of the method

The analysis of microbial-derived metabolites of IF is important for the interpretation of biological effects of IF. The method presented here only quantifies the free aglycones of these microbial metabolites, except for equol. It is well known that all microbially-derived metabolites (DH-DAI, DH-GEN, ODMA, 6'OH-ODMA, equol) also occur in biological fluids mainly in the conjugated form, as shown for GEN and DAI. However, a preceding enzymatic treatment of the samples using  $\beta$ -glucuronidase and arylsulfatase easily enables the determination of the total concentration of all microbial metabolites with the UHPLC-MS/MS method described (data not shown).

#### Conclusion

A robust, automated, and specific analytical approach has been developed and validated for the quantification of the soy IF DAI and GEN, their conjugative metabolites, as well as their major microbial degradation products in plasma and urine. Stable  $^{13}\text{C}$ -labeled standards including a labeled glucuronide were used as internal standards, and all analytes were available as further standard compounds. The main advantage of this method is the simultaneous analysis of the phase II metabolite profile of DAI and GEN despite the diverse chemical properties of the analytes. Furthermore, the approach

allows detecting individual differences in the equol-producing capability, since equol-7-G and equol-4'-S are also quantified. This is crucial because equol is an important metabolite of DAI with respect to the biological activity of IF.

A further advantage is that the method combines an automated sample preparation procedure with a highly selective (two MRM transitions per analyte) and rapid analysis (<25 min). This allows the application of the approach in clinical studies with large sample sizes as the practical involvement of laboratory staff is low.

The application of this analytical method to a pharmacokinetic study in postmenopausal women showed that the IF DAI and GEN are extensively metabolized in vivo. We assume that the application of our method may contribute to a better understanding and interpretation of in vivo studies and, in particular, of clinical trials evaluating the biological effects of soy IF in humans.

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