Research Article

Simultaneous determination of daidzein, equol, genistein and bisphenol A in human urine by a fast and simple method using SPE and GC-MS

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Human diet contains weakly estrogenic compounds such as daidzein (DAI) and genistein (GEN), phytoestrogens present in soy and many vegetables as well as bisphenol A (BPA), a contaminant from packing materials and plastic containers for foods and beverages. In light of concerns about hormonally active agents, biomonitoring methods are needed to assess human exposure to such compounds. A method for simultaneous determination of DAI, its metabolite equol (EQ), GEN, and BPA by GC-MS analysis was established, validated and applied to measure concentrations in human urine. Sample preparation involves enzymatic conjugate cleavage, SPE and derivatization by silylation. For GC/MS analysis, deuterated DAI and GEN and ^{13}C-BPA are used as internal standards. LOD are 4, 4, 5 and 3 ng/mL urine for DAI, EQ, GEN and BPA, respectively. Interassay variations were 9% for DAI, 15% for EQ, 18% for GEN and 10% for BPA. Simple workup and accuracy of the method are suited for biomonitoring. An analysis of urine samples from 15 adults consuming typical German food revealed dietary exposure to phytoestrogens in all samples: GEN concentrations ranged between 13 and 238 ng/mL, those for DAI ranged from 12 to 356 ng/mL. More than half of the individuals excreted also the more estrogenic metabolite EQ, at levels of 8 – 128 ng/mL. Higher concentrations (GEN: 820, DAI: 960 and EQ: 1740 ng/mL) were measured in a 24 h urine sample upon ingestion of soy protein (50 g with 12.9 mg DAI and 25.2 mg GEN). Only urine collected after some days on strict phytoestrogen-free diet had undetectable isoflavone levels. BPA was detected in 9 of 15 urine samples ranging from 3 to 11 ng/mL, and at 55 ng/mL in one sample. In conclusion, a reliable method to determine BPA and isoflavones in urine was established and applied in a pilot study: Biomonitoring results show much higher dietary exposure to phytoestrogens than to BPA in German adults.

Keywords: Biomonitoring / Bisphenol A / Isoflavones / Phytoestrogens / Urine analysis

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1 Introduction

Hormonally active agents or so-called 'endocrine disruptors' are a widely debated issue due to concerns that exposure, especially early in life, may lead to developmental and reproductive toxicities [1, 2]. Human exposure to hormonally active compounds of natural or anthropogenic origin occurs mostly with foods [3, 4]. The phytoestrogens daidzein (DAI) or genistein (GEN) are present in many plants, with soybeans and soy products being the major source of dietary isoflavone uptake [5]. Intake of DAI and GEN depends largely on dietary habits, and estimated isoflavone amounts ingested daily range from about 0.8 mg for a Western-style diet up to 47 mg for a traditional Asian diet [6 – 9].

Another environmental estrogen is bisphenol A (BPA), an important monomer in the production of epoxy resins, polycarbonate and other plastics. BPA can leach from polycarbonate bottles and coatings in beverage or food cans, and has been found as contaminant in foods and beverages [10 – 12]. It was also detected in saliva shortly after patients had received dental composite fillings with BPA containing monomer. Exposure estimates by the European Commission [12] for BPA from various sources amount to less than 30 μg *per* day for an adult (or 0.5 μg/kg body weight (b.w.)).

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Abbreviations: BPA, bisphenol A; **DAI,** daidzein; **EQ,** equol; **GEN,** genistein; **MtBSTFA,** *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide; **TIC,** total ion chromatogram

Such information provides a frame of reference for estimating intakes of BPA and estrogenic isoflavones, yet indicates also rather different and variable exposures. Whilst exposure data are a key element in risk assessments, a toxicological evaluation of environmental estrogens has to consider also toxicodynamics (mode of action, hormonal potency) and toxicokinetics (absorption, distribution, metabolism, elimination) as well as special aspects related to sensitivity of the endocrine system [1, 13, 14]. The estrogenic potency of DAI, GEN, BPA and some combinations was assessed in numerous bioassays (*e.g.* [15–18]) both *in vitro* and *in vivo*, and characterized as 'weak' for the phytoestrogens and for BPA in relation to that of steroid estrogens. Studies on kinetics of isoflavones and BPA in rodents revealed extensive conjugation by phase II enzymes to polar metabolites [19–23]. Studies (reviewed in ref. [14, 24]) show a similar metabolic fate in humans and efficient clearance with elimination half-lives of 5–8 h for DAI and GEN, and less than 6 h for BPA [25]. The compounds or more precisely, their polar metabolites, are excreted mainly with urine: This facilitates exposure assessment by measuring urinary concentrations.

Urine samples are easy to collect, and excreted amounts will not only reflect external exposure, but also differences in the absorption and metabolism of ingested compounds and thus internal exposure [24]. Whilst lipophilic BPA is directly absorbed in the small intestine, the absorption of isoflavones appears to vary with the form (aglycone or conjugate) present in the diet [26, 27]. Soybeans contain DAI and GEN mainly as glucosides, acetylglucosides or manoylglucosides which need to be deconjugated prior to absorption. The sugar moieties are hydrolyzed by gastric acid and intestinal β-glucosidases or gut bacterial enzyme [28]. A source for DAI and GEN are also the plant precursors formononetin and biochanin A that are demethylated by enzymes in gut bacteria and liver microsomes [29, 30]. Intestinal bacteria in rodents and humans can reduce DAI to equol (EQ) and convert GEN to *p*-ethylphenol [31]. Figure 1 depicts this and also the major routes of isoflavone metabolism by phase II enzymes to glucuronides, sulfates or mixed sulfoglucuronides which are readily excreted with urine.

With regard to hormonal activity, it is of interest that conjugates of BPA and isoflavones do not bind efficiently to intracellular estrogen receptors and thus lack estrogenicity [22, 32–34]. In contrast, the metabolite EQ shows a similar or higher estrogenic activity than DAI *in vitro* [35, 36]. Interestingly, EQ (formed by the gut microflora), is excreted by about one-third to one-half of the human population, and can account for a considerable part of ingested DAI in so-called 'EQ producers' [9, 27, 37, 38]. Hence, analysis of EQ should be included in investigations on urinary isoflavone levels as biomarkers of dietary phytoestrogen intake and other biomonitoring studies.

The present method has been developed with the aim to analyse urinary levels of isoflavones (DAI and GEN), the isoflavan EQ, and measure concurrently also BPA background exposure. Here we report the details of the method that has been validated for small urine samples: It involves enzymatic cleavage of conjugates, use of internal standards, and a simple SPE clean-up step prior to derivatization for GC-MS analysis. Subsequently, we applied the method to urine samples collected from a group of German adults on a typical Western diet and samples obtained from a volunteer after strict isoflavone-free diet and after soy challenge.

2 Materials and methods

2.1 Chemicals and reagents

All chemicals, reagents and solvents were of analytical grade or higher quality. BPA [2,2-bis(4-hydroxyphenyl)-propane], purity 99.94%, was from Bayer (Wuppertal, Germany). DAI (7,4′-dihydroxyisoflavone) ≥98%, EQ [3,4-Dihydro-3-(4-hydroxyphenyl-2H-1-benzopyran-7-ol] ≥98%, and GEN (5,7,4′-trihydroxyisoflavone) ≥98% were obtained from Fluka (Seelze, Germany). BPA (ring-$^{13}C_{12}$), 99%, DAI (3′,5′,8-D3), 97%, and GEN (3′,5′,6,8-D4), 95% were purchased from Cambridge Isotope Laboratories (Andover, USA). ACN, acetic acid, ascorbic acid, EDTA, ethyl acetate, methanol (suprasolv) and *n*-hexane were from Merck (Darmstadt, Germany). Acetic anhydride and PFB (2,3,4,5,6-pentafluorobenzoylchloride) were from Fluka, *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MtBSTFA) with 1% tBDMCS was supplied by Alltech (Unterhaching, Germany) and sodium acetate trihydrate by Sigma-Aldrich (Seelze, Germany). A commercial soy protein (``Soja 80' from DECATHLON Sportartikel, Herne, Germany) was analysed for its isoflavone content by established methods [39], and 50 g were found to contain 12.9 mg of DAI and 25.2 mg of GEN. For analytical purposes, BPA-free water was prepared by glass distillation and additional purification with a C_{18} SPE column (500 mg of Lichrolut; Merck) pretreated with 5 mL of methanol.

2.2 Stock solutions and calibration samples

Stock solutions of the analytes were prepared in methanol at final concentrations of 50 μg/mL for DAI, EQ and GEN, and 22 μg/mL BPA. For use in calibration standards, 1 mL each of DAI, EQ and GEN stock solution was combined with 500 μL of BPA stock solution and diluted with BPA-free water to 10 mL for mixture V1 (with c = 5 and 1.1 μg/mL, respectively). Mixture V2 (c = 0.5 and 0.11 μg/mL, respectively) was obtained by the dilution of 1–10 mL of V1 with BPA-free water.

The internal standard mixture was prepared from commercially available isotopically labelled (^{2}H (D) or ^{13}C) standards: First, 200 μL of D3-DAI (c = 60 μg/mL), 120 μL of D4-GEN (c = 100 μg/mL) and 60 μL $^{13}C_{12}$-BPA (c = 100 μg/mL) in ACN (as delivered) were mixed with

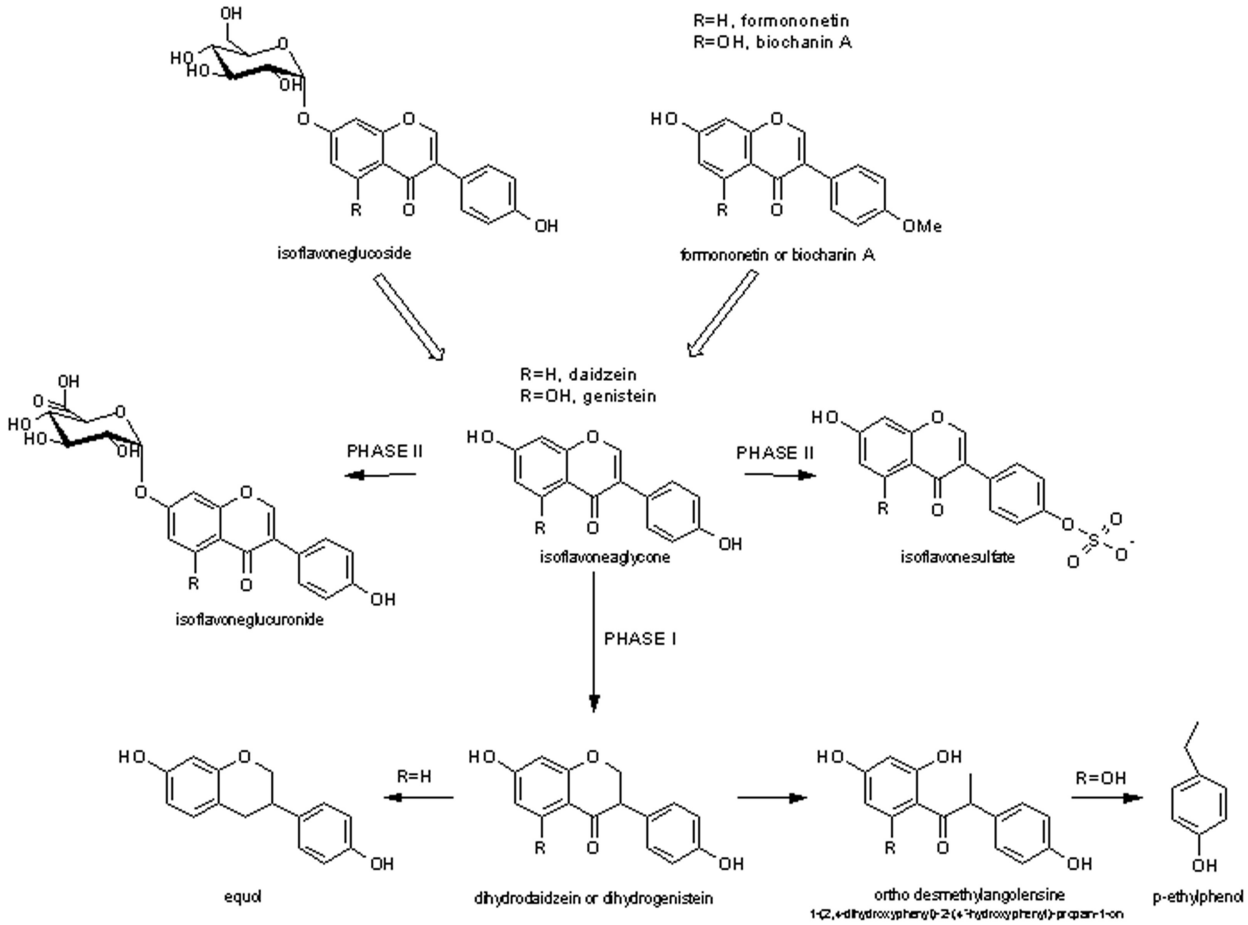

Figure 1. Metabolism of isoflavones involves the action of intestinal microflora (hydrolysis; demethylation) as well as modification by conjugating enzymes (phase II) and/or phase I enzymes (reduction, hydroxylation). See text for details.

220 µL of ACN (suprasolv). Then 200 µL of this solution was diluted to 2 mL with BPA-free water (IS mixture with final concentrations of 1 µg/mL $^{13}C_{12}$-BPA as well as c = 2 µg/mL D3-DAI and D4-GEN each).

All standard solutions were stored at 4°C and allowed to equilibrate at ambient temperature for at least 1 h before use. Calibration samples were prepared by adding different volumes of stock mixture (4 – 20 µL of V1 or 5 – 20 µL of V2) and IS mixture (8 µL) to 200 µL of a (isoflavone-free) reference human urine.

2.3 Samples and sample preparation

Since BPA can leach from laboratory articles made of plastic and from water purification cartridges, great care must be taken in analytical studies to avoid contamination of water and samples. Urine samples (24 h urine samples and spot urine samples) were obtained from healthy German volunteers, aliquoted at the laboratory in well washed 500 µL polyethylene bottles, and stored refrigerated until analysis. Urine was allowed to thaw to RT, then 200 µL of aliquots were transferred to a reaction vial, and 8 µL internal standard mixture (c = 1 µg/mL $^{13}C_{12}$-BPA and c = 2 µg/mL D3-DAI and D4-GEN each) was added. After gentle mixing, samples were prepared for the enzymatic hydrolysis of BPA conjugates and isoflavone conjugates by adding 10 µL concentrated hydrolysis buffer (prepared from 13.6 g sodium acetate trihydrate, 1 g ascorbic acid and 0.1 g EDTA in 100 mL BPA-free water, adjusted with acetic acid to pH 5). Then 8 µL β-glucuronidase (140 U/mL glycerine, as purchased from Roche, Mannheim, Germany) and 4 µL sulfatase (100 U/mL from Sigma-Aldrich were added and hydrolysis was allowed to proceed overnight at 37°C with shaking. After cooling to RT, the samples were centrifuged for 1 min at 13 000 rpm, then the aglycones were extracted from the hydrolysate *via* SPE.

2.4 SPE and derivatization

SPE cartridges (1 mL, type Emporedisk® SD from 3 M) were purchased from Phenomenex (Aschaffenburg, Germany) and conditioned with 300 µL of methanol and

300 μL of BPA-free water rinses, prior to use. Urine calibration standards (see 2.2) or hydrolyzed urine samples were transferred completely to conditioned cartridges, and drawn through at partial vacuum. The cartridge was then washed with 300 μL 5% methanol in water before the analytes were eluted with 300 μL ACN/ethyl acetate (1:1). The eluates were evaporated to dryness at 40°C in a SpeedVac® (Thermo Electron, Dreieich, Germany). The residues were redissolved in 50 μL derivatization reagent (MtBSTFA with 1% tBDMCS) with sonication (1 min). Samples were kept at 75°C for 30 min under shaking (at 1400 rpm). After cooling, the surplus reagent was removed at RT under a stream of nitrogen. The dried residues were dissolved in 70 μL *n*-hexane, sonicated for 1 min, centrifuged (1 min at 13 200 rpm) and transferred to autosampler vials. The derivatized samples dissolved in *n*-hexane proved to be best for a large volume injection and evaporation in the CIS, as other solvents tested like acetone, ACN, MtBSTFA or methanol resulted in signal losses.

2.5 GC-MS analysis

Analysis was carried out on a GC/MS instrument (GC 6890N coupled to MS 5973, Agilent Technologies, Waldbronn, Germany) in total ion mode. The instrument was equipped with a cooled injection system (CIS 4 plus, Gerstel Mühlheim, Germany) to allow a high volume sample injection of 20 μL in solvent vent modus (start temperature 25°C, 0.08 min; 200 mL/min helium; injection speed 4 μL/s; solvent vent; 12°C/s up to 320°C, maintained at 320°C/min for 2 min and finally, 12°C/s up to 400°C). Separation was achieved on a DB-1ms fused silica capillary column (15 m × 0.25 mm id, 0.25 μm film; J&W Scientific, Folsom, USA) with carrier gas helium (helium 99 996% from Messer Griesheim, Krefeld, Germany), additionally purified by passage through an oxygen and moisture trap (from Alltech) at a constant flow of 2 mL/min, and an oven temperature program starting with constant 160°C for 2 min followed by a fast increase at 30°C/min to 310°C holding for 5 min. Temperatures of the GC/MS source, quadrupol and transferline were 230, 150 and 280°C, respectively. Total ion chromatograms (TIC) from $m/z = 200-600$ amu were obtained with electron impact ionization (70 eV). Data acquisition and processing were carried out using ChemStation G1701 DA V.1.27 software (Agilent Technologies).

The ions monitored for identification were the following: $m/z = 425$, 482 for DAI, $m/z = 428$, 485 for D3-DAI, $m/z = 470$, 234 for EQ, $m/z = 555$ for GEN, $m/z = 558$ for D4-GEN, $m/z = 213$, 441, 456 for BPA and $m/z = 225$, 453, 468 for $^{13}C_{12}$ BPA. The ions used for quantification were: $m/z = 425$ (DAI), 428 (D3-DAI), 470 (EQ), 555 (GEN) and $m/z = 558$ (the most sensitive ion from D4-GEN which loses one D in position 6 under acidic conditions; [40]). The amounts of DAI, EQ, GEN and BPA in processed urine samples were quantified by means of calibration curves formed from known concentrations of a mixture of analyte standards in reference urine. The reference urine was obtained from a volunteer after 54 h on a strict isoflavone-free diet. All signal area values were corrected by division with the area of the internal standard. We used D3-DAI for correction of DAI, and D4-GEN for GEN; BPA was corrected with $^{13}C_{12}$-BPA and EQ was also corrected with $^{13}C_{12}$-BPA because pretests showed the best results with ^{13}C-BPA. All mathematical operations were done using Microsoft Excel™.

3 Results

A GC-MS method for analysis of DAI, EQ, GEN and BPA suitable for small volumes of human urine was established and optimized. Sample workup consists of three steps, conjugate hydrolysis, SPE and derivatization. Upon validation the method was applied to assess isoflavone and BPA levels in urine samples from adults on a typical German diet.

3.1 Analyte deconjugation by enzymatic hydrolysis

For this step, we chose enzymatic hydrolysis with pure β-glucuronidase and sulfatase (preparations from *E. coli*) in acetate buffer (pH 5) as used previously for biological samples containing isoflavone and BPA conjugates [19, 21, 23]. The crude solution of *H. pomatia* with β-glucuronidase/arylsulfatase enzyme is not recommended here, since the gastric juice of the snails can contain isoflavones and may thus contaminate samples with DAI and GEN [41].

3.2 Analyte purification

A factor to be considered in method development is sample volume. We decided to develop the method for 200 μL of urine, since in planned applications only 1 mL aliquots of urine samples will be available for DAI, EQ, GEN and BPA analysis in duplicate. For extraction of the analytes there are two options: As liquid/liquid extraction with ethyl acetate offers poor chances for automation, we chose SPE. For small volumes, (≤300 μL) only a limited number of SPE cartridges are available. We conducted pretests of some SPE cartridges with C18 material; the one which worked best in our hands was the Emporedisk SD from 3 M. Figure 2 depicts results from tests with repeated 100 μL steps to define suitable washing and eluent volumes. The majority of analytes (>95%) eluted in the first 300 μL of ACN/ethyl acetate (1:1), whilst the amounts lost in washing steps with 5% methanol remained very low up to 400 μL. Based on this result we used subsequently 300 μL for all steps (conditioning, washing and elution).

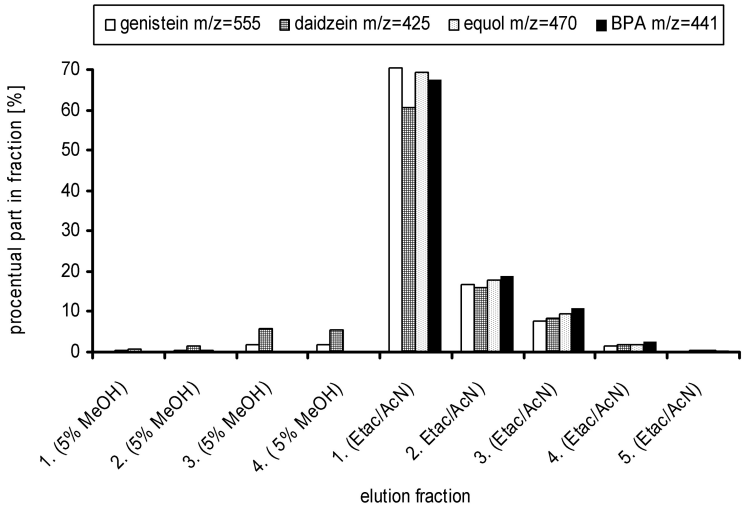

Figure 2. Optimization of SPE conditions. Percentage of applied analyte found in washing fluid ($4 \times 100\ \mu L$ fractions of 5% methanol) and in eluate ($5 \times 100\ \mu L$ fractions of ACN/ethyl acetate mix). Each bar depicts the mean value of $n = 6$ cartridges.

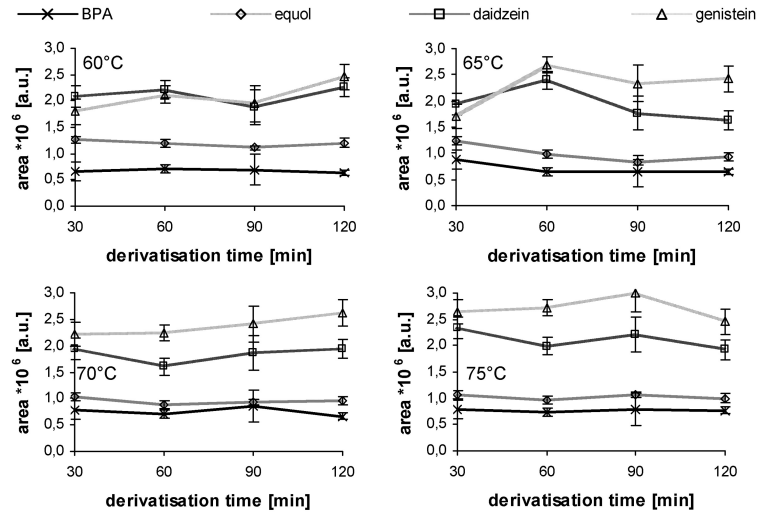

Figure 3. Optimization of derivatization conditions. Areas for analytes at different temperatures and times of derivatization. Data points depict the mean value of $n = 4$ measurements ± SD).

3.3 Analyte derivatization

Pretests with several derivatization reagents for acylation and silylation were carried out: With PFB or acetic anhydride we obtained stable signals for BPA, but no signals for DAI, EQ or GEN were detected (data not shown). Silylation with BSTFA resulted in less reproducible signals for the TMS-ethers, whilst tBDMS-ethers formed with MtBSTFA resulted in more reproducible signals, especially at temperature programmed injection with the CIS 4 plus. To further optimize derivatization conditions, we used identical analyte samples (without matrix and internal standard; internal standard correction is not indicated at this point for 'correction' of derivatization yield.), and evaporated the solvent at 40°C in a SpeedVac and dissolved the samples in MtBSTFA. After distinct temperatures and times of derivatization, the samples were injected directly after cooling. Figure 3 depicts the recorded areas (mean of $n = 4$) at each condition. By and large, for most of the conditions area values were in the range of the quadruplicate-measurement variation. So we chose 75°C, the temperature with the highest area values after 30 min reaction time.

3.4 GC-MS conditions

Analysis was carried out on a GC-MS unit with a cooled injection system (CIS) in solvent vent mode with 20 μL injection volume (see Section 2.5). Chromatographic separation with baseline resolution of the analytes was achieved on a 100% dimethylpolysiloxane column (DB-1ms). The shape of the signals was better on the DB-1ms column compared to one with DB-5ms, another often used GC stationary phase. Figure 4 depicts the chromatogram with the quantifier isolated from TIC of calibration standard. On the MS, total ions in the range from 200 – 600 amu were recorded after solvent delay. Data acquisition with SIM did not result in a lower variation (LOQ) compared to mathematical extraction from TIC. Thus, and because of better recognition of problematic background in some urine samples, TIC rather than SIM was applied.

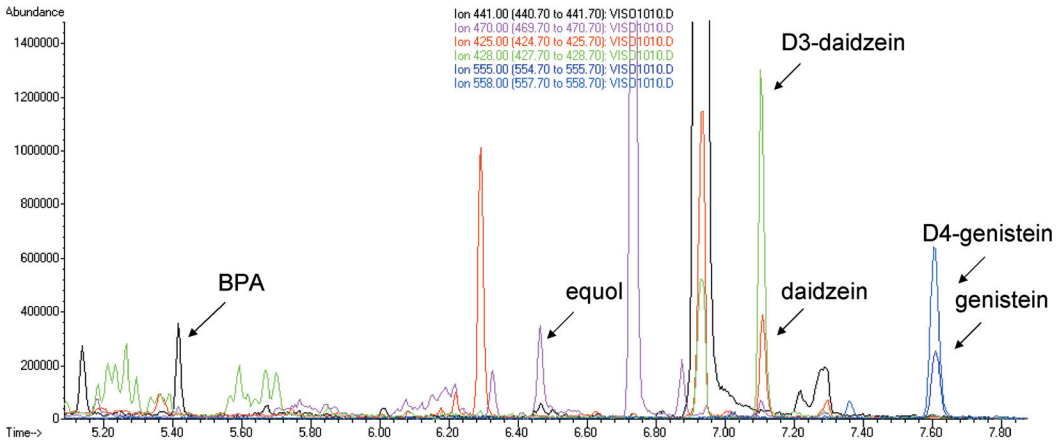

Figure 4. Chromatogram of a calibration standard (23 ng/mL isoflavones and 5 ng/mL BPA). Ions for quantification were mathematically extracted from TIC.

3.5 Calibration and validation

Internal standards were used to correct for variable losses of analytes which can occur during sample preparation and processing. Commercially available isotopically labelled standards were used here. As D4-GEN contains a proton in an acidic-labile position (prone to a possible deuterium-hydrogen-exchange), pretests were carried out to confirm stable fragmentation patterns of the standards under workup conditions. Mean calibration functions were calculated from three independent calibrations for each analyte (Fig. 5). They were used to calculate the LOD, limit of decision, and LOQ, according to DIN 32645 calibration function method. It uses the variation of independent calibrations to calculate the LOD and LOQ, reflecting a more detailed picture on the reliability of values near the detection limit. We used 11 calibration points ranging from 0.165 to 55 ng/mL for BPA and from 0.75 to 250 ng/mL for the isoflavones to calculate the LODs given in Table 1.

Several quality parameters were assessed for the method. By ten-fold injection of one sample a 1% precision of measurement was found. Recovery rates were calculated from the home-made reference urine (practically isoflavone-free) spiked with three different isoflavone and two BPA concentrations (Table 2). Dependent on concentration and compound recovery rates range from 80–104% (intra-assay) and 90–126% (interassay). For samples at the middle concentration we calculated an intra-assay variance of 6% for DAI, 8% for EQ, 7% for GEN and 3% for BPA (Table 3). The interassay variance was calculated from six spiked urine samples measured in six independent series (new stock solution, different operator) with 9% for DAI, 15% for EQ and 18% for GEN at 75 ng/mL, and 10% for BPA at 16.5 ng/mL.

The accuracy of the method was confirmed using Student's *t*-test. We created six independent calibrations (new stock solution and different operators) and used each calibration to analyse the content of four independent samples with identical concentration from an independent series. The variance s of this six mean values were calculated and used to calculate *t*-values according to $t = |\bar{x} - \mu| / s \cdot n$.

Table 1. LOD, limit of decision and LOQ calculated according to DIN 32645 calibration function method for DAI, EQ, GEN and BPA

Parameter (ng/mL)	BPA	EQ	DAI	GEN
LOD	3	4	4	5
Limit of decision	6	8	7	13
LOQ	7	11	9	18

μ is the expected value and n the number of measurements and $|\bar{x} - \mu|$ describes the systematic error (bias). For a mean value of 16.5 ng/mL for BPA and 75 ng/mL for the isoflavones, the following *t*-values were calculated: 1.2 for DAI, 0.9 for EQ, 0.7 for GEN and 0.3 for BPA, with mean variance coefficients ranging from 8 to 18%. Student's *t*-test is passed when *t*-values are below 2.571 ($n = 6$; $P = 95\%$, $f = n-1$). Thus, the trueness of the method has been demonstrated.

The linearity of the calibration function was tested between LOD 500 and 110 ng/mL for isoflavones and BPA, respectively. The calibration function for BPA was linear over the range, but calibration points of the isoflavones above 250 ng/mL did not correlate very well. Upon analysis of concentrations between 250 and 500 ng/mL we found a decrease of correlation, depending on substance and matrix, between 350 and 400 ng/mL as a result of a detector overload. Thus, we omitted the highest calibration standards and defined the linear working range as LOD to 250 and 110 ng/mL for for isoflavones and BPA, respectively. All data from the precision and accuracy measurement are listed in Table 3.

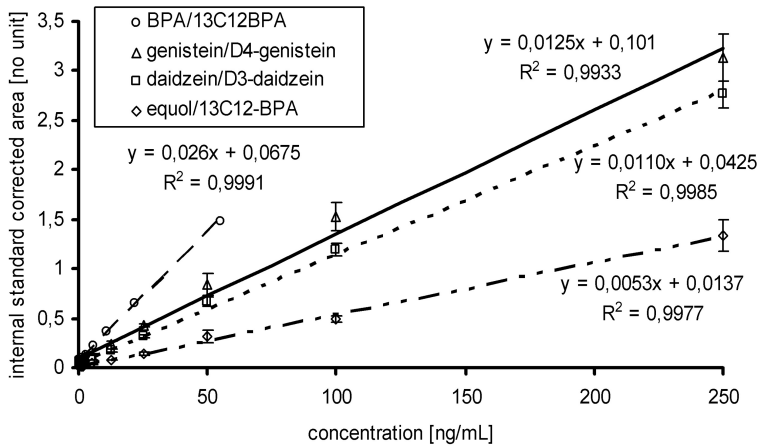

Figure 5. Calibration functions as mean of three independent calibrations. Upper chart: Calibration functions for DAI, EQ and GEN (11 data points, ranging from 0.75–250 ng/mL); bottom chart: Calibration for BPA (11 data points, ranging from 0.165–55 ng/mL BPA). All data points depict mean values ± SD. These data points are also used for the calculation of LOD.

Table 2. Recovery rates of low, middle and high spiked samples intra- and interassay

Sample	Expected concentration (ng/Ml)		Recovery rates [%]							
			Intra-assay				Interassay			
(n = 4)	BPA	Isoflavone	BPA	EQ	DAI	GEN	BPA	EQ	DAI	GEN
Low	2.2	10	-	100	92	80	–	–	–	–
Middle	16.5	75	101	95	104	100	119	90	127	126
High	27.5	125	97	102	104	104	90	112	104	105

Table 3. Precision, accuracy and linear working range for DAI, EQ, GEN and BPA analysis with SPE clean-up and GC-MS determination

	BPA	EQ	DAI	GEN
Precision (intra-assay) (n = 4; c = 16.5–22 ng/mL BPA; c = 10–125 ng/mL isoflavones)	4–6%	5–6%	1–7%	3–15%
Precision (interassay) (n = 6; c = 16.5 ng/mL BPA; c = 75 ng/mL isoflavones)	10%	18%	8%	15%
Student's t-test (n = 6) (should be below 2.571)	0.3	0.9	1.2	0.7
Linear range from LOD (ng/mL)	110	250	250	250

3.6 Urinary phytoestrogen levels after isoflavone-free diet and soy challenge

The method described above was applied to evaluate phytoestrogen levels in urine samples from a volunteer (i) on his usual diet, (ii) under a strict isoflavone-free diet (for up to 56 h) and (iii) after ingestion of a known amount of isoflavone with soy protein. The results are depicted in Fig. 6: Samples 1 and 2 refer to spot urine samples of the volunteer (S.M.) on his usual (Western-type) diet. The next five samples, taken at different times on isoflavone-free food, show a decrease in DAI and GEN below the LOD after 50 h and more, whilst EQ was still detectable. This observation is in accord with the delay in EQ excretion compared to half-lives reported for DAI and GEN [14, 24]. Only samples collected under isoflavone-free diet are suitable as reference urine for a method validation, since urine samples of volunteers consuming their usual food show considerable 'background' levels resulting from dietary phytoestrogen exposure.

Much higher GEN, DAI and EQ levels are found in 24 h urine (Fig. 6, last column) after ingestion of 12.9 mg DAI and 25.2 mg GEN with 50 g soy protein. This dose is similar to that taken up with an Asian-style diet [9]. From concentrations measured in 1.7 L total urine, we calculated the amounts excreted within the first 24 h after soy challenge as 6% for GEN and 13% for DAI or 37% when EQ is included. Thus, in the volunteer's urine about 1/5 of the ingested isoflavones were recovered within a day.

3.7 Urinary phytoestrogen and BPA levels resulting from dietary exposure

Spot urine sample were obtained during the day (10 am to 2 pm) from seven German volunteers (A–G, all males, age

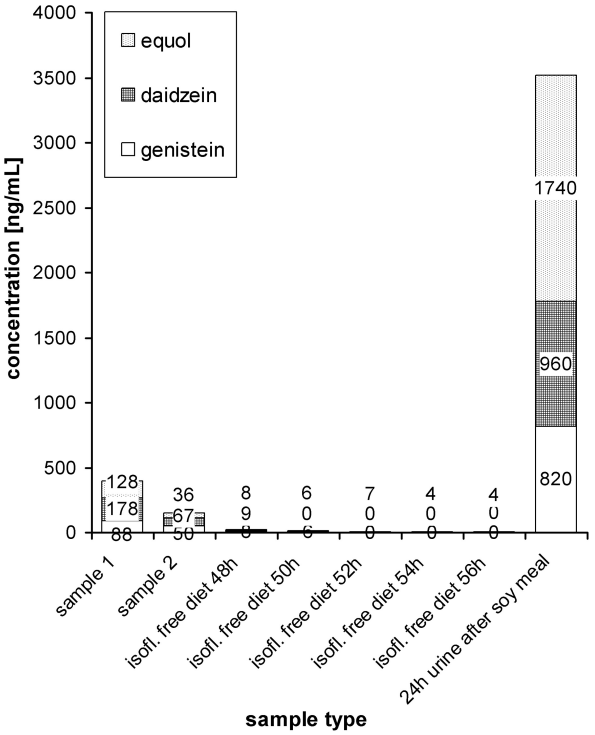

Figure 6. Isoflavone content in spot urine samples of one volunteer at normal diet (sample 1 and 2), after isoflavone-free diet (0 = below LOD) and in a 24 h urine sample collected after ingestion of 50 g soy protein. See text for details.

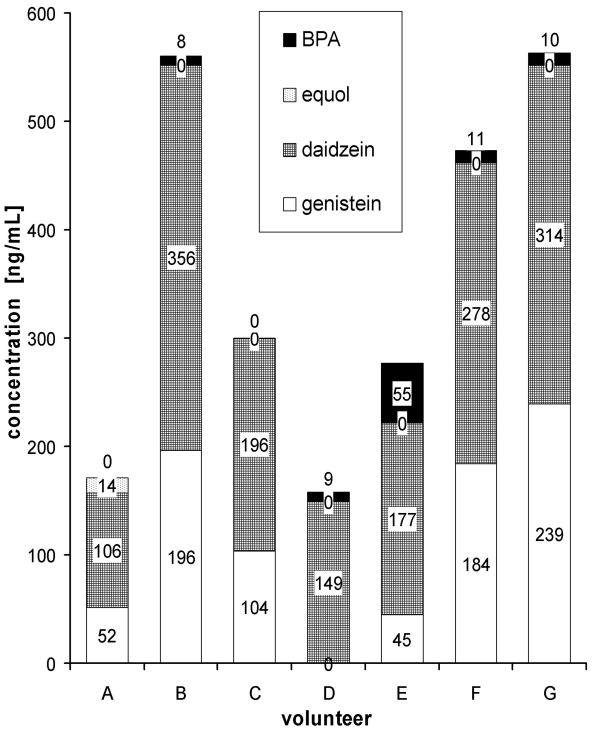

Figure 7. Isoflavone and BPA levels in spot urine samples of seven male German volunteers (18–32 years old) on their usual diet. The bar chart depicts values of single measurements. Samples below the LOD (3 ng/mL for BPA, 4 ng/mL for EQ) are marked with 0. See text for details.

18–32) and analysed to gain some insight into the spectrum of urinary levels in people on a normal diet. The results, depicted in Fig. 7, show variable urinary concentrations of DAI (106–356 ng/mL) and GEN (45–239 ng/mL). EQ was only detected in one of the seven urine samples from this group. BPA was detected in five of seven urine samples, at levels between 8 and 55 ng/mL, *i.e.* concentrations are lower than those measured for isoflavones.

Another group of eight volunteers (five males and three females employed at IfADo) agreed to collect 24 h urine samples for further analysis under their usual diet conditions. Whilst spot urine samples do provide, already, insight into dietary exposure, concentrations measured in 24 h urine samples allow more reliable calculation of urinary excretion. The mean values of two measurements were used to calculate the excretion of GEN, DAI, EQ and BPA for this group: As depicted in Fig. 8, all individuals excreted the isoflavones DAI and GEN and seven volunteers excreted EQ. In four urine samples, BPA was below the LOD, in the other four BPA concentrations were measurable, but near the limit of decision (mean value 4.8 ng/mL). On average, the small collective of volunteers on a typical German diet excreted 5 μg/day of BPA, 62 μg/day of DAI, 24 μg/day of EQ and 65 μg/day of GEN. This indicates that BPA exposure is lower than that to dietary isoflavones. Moreover, 9 of 16 individuals in this small German collective, *i.e.* more than 50% were EQ producers.

4 Discussion

For reasons given in Section 1, there is much interest in phyto- and xenoestrogens, and a need to assess human exposure to dietary isoflavones and to BPA. A number of analytical techniques have been used to quantify phytoestrogens and their metabolites in urine, including HPLC, GC, GC-MS and LC-MS [24, 38, 42, 43; and references cited therein]. All have some limitations, *e.g.* low sensitivity to measure certain analytes (EQ; for instance, the detection limit for EQ using negative single-ion monitoring is ~40 times higher than for the other isoflavonoids [43].), the need for large volumes of urine, laborious complex clean-up procedures and derivatization steps, expensive equipment or use of isotope standards that are not commercially available. We focused on GC-MS methods also since previous analysis of BPA in urine had used GC-MS [25, 44].

The disadvantage of some methods developed for GC-MS analysis of phytoestrogens is their complex, multistage purification procedures prior to derivatization and analysis [45–47]. Sample preparation should ideally be kept as sim-

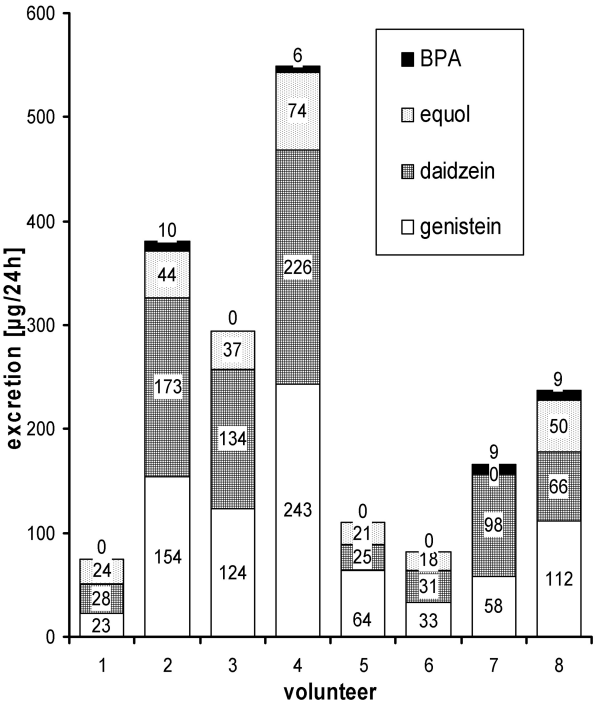

Figure 8. Isoflavone and BPA excretion in 24 h urine samples of eight German volunteers (18 – 55 years old, 5 males, 3 females) on their usual diet. The bar chart depicts mean values of duplicate measurements; see text for details.

ple as possible, since the treatment of samples prior to analysis is often the cause of the great variability in the results [38], and a simple sample preparation procedure is less labour intensive and later on more amenable to automation for higher throughput of samples. The method of Grace *et al.* [38] served as a starting point for our work: They used a similar sample clean-up step (SPE), yet applied $^{13}C_3$-labelled isoflavone and isoflavan standards that are not commercially available. Also derivatization and chromatographic separation required adaptations and optimization, since we intended to measure BPA concurrently with DAI, EQ and GEN. We are only aware of one published GC-MS method for simultaneous determination of BPA and isoflavones, but it was developed for the analysis of infant formula powders, and thus did not include the metabolite EQ [48]. Their sample clean-up was done with C_{18} SPE cartridges, and trimethylsilylation was used for derivatization. In our tests with C_{18} SPE cartridges, the 1 mL Emporedisk SD from 3 M proved to be the best choice for clean-up of urine samples and gave good recovery of all analytes in a total elution volume of 300 µL ACN/ethyl acetate mixture (Fig. 2 and Section 3.2.).

Tests of several derivatization reagents led to MtBSTFA with the most reproducible GC/MS signals for all analytes (Section 3.3 and Fig. 3). An increased stability of the isoflavone tBDMS-ethers compared to, *e.g.* TMS-ethers was also reported by others [49]. Another type of derivatization, namely acetylation has been widely used for analysis of BPA, with good results for LOD and low variation [23]. But our tests showed that acetylation is not suitable for derivatization of isoflavones. Pentafluorobenzoate derivatization was also tested with good results for BPA and less favourable results for the isoflavones. Another reagent, namely PFBBr, was used for BPA analysis in urine by Brock *et al.* [44] who reported a very low LOD for their GC-MS method.

Chromatographic separation with baseline resolution of all analytes was achieved on a 100% dimethylpolysiloxane column (DB-1) within 10 min. For better analyte identification, a temperature programmed injection of the sample was chosen to increase the signal intensity. MS detection was done in total ion mode from which the quantifiers were mathematically extracted (Fig. 4 and Section 3.4.). Others have used SIM for detection to improve the LOD. Our calculation for the LOD in urine is based on the variation of the calibration function. We could not find a lower variation when changing to SIM; therefore and because of better recognition of interfering background in some urine samples, SIM was not applied here. No major advantage of SIM in the LOD was stated also by others [49].

The validity of our method was checked, and quality parameters are given in Table 3. LOD were calculated from the variance of the calibration functions, with 4 ng/mL for EQ and for DAI, 5 ng/mL for GEN and 3 ng/mL for BPA. Intra-assay variation ranged from 3 to 8% and interassay variation from 8 to 18%. These values are comparable to those reported for other GC-MS methods for analysis of isoflavones and BPA in urine [25, 38, 44 – 47]. Lower LOD reported for BPA [25, 44] are probably due to other derivatization reagents (not suitable for isoflavones); other internal standards for DAI, EQ and GEN, *e.g.* ^{13}C standards, can possibly further decrease the variance and the limits of detection. Yet, overall performance (robustness and sensitivity) of this method for simultaneous determination of all analytes of interest proved to be good in our studies with human urine samples from a small German cohort consuming their usual food or an isoflavone-free and isoflavone-rich diet.

To examine the high and low end of isoflavone excretion, we measured DAI, EQ and GEN in urine samples from a volunteer after isoflavone-free diet and after a defined soy protein intake. This individual excreted already measurable isoflavone levels on his normal, Western-style diet, and he also showed the capacity to convert DAI to EQ (Fig. 6). Only after strict avoidance of a number of foods (listed in the VENUS database to contain DAI, GEN or its precursors; www.venus-ca.org) for 48 h or more, *i.e.* 6 – 7 times the known half-lives of 5 – 9 h for DAI and GEN [9, 24, 26], urine levels had decreased two orders of magnitude and were at or below the LOD, demonstrating sufficient sensitivity of the method for isoflavones. To simulate isoflavone uptake with a normal Asian-type diet the volunteer ingested

12.9 mg DAI and 25.2 mg GEN with 50 g soy protein. In the urine collected after soy challenge, he excreted 960 µg/L DAI, 1740 µg/L EQ and 820 µg/L GEN, *i. e.* considerably higher levels than on his usual diet (Fig. 6). Taking the volume of the 24 h urine into account, this amounts to 13% of the DAI dose or 37% of DAI when EQ is included in the calculation, and 6% of the GEN dose, or a total of less than 1/5 of the ingested soy isoflavone dose.

That EQ excretion is highly variable among people [9, 26, 27, 37, 38] was confirmed in two groups of German volunteers who participated in our pilot study (Figs. 7 and 8). As there is no overlap between the groups and the other volunteer, the percentage being EQ-excretors in the pooled sample is calculated to be 56%. The percentage of equol excretors observed in the relatively small cohort of German volunteers is higher than the proportion (up to 40%) reported in populations of other Western countries [24, 37]. The proportion in our cohort is based on a small number and may change (decrease) as the number of individuals analyzed increases. The absolute numbers are small (9 of 16), but reveal nonetheless considerable differences in of these individuals' capacity to convert DAI to EQ.

Applying our method to seven spot urine samples (Fig. 7) and to eight 24 h urine samples (Fig. 8) of German volunteers on a normal Western diet, we found that all individuals excreted isoflavones far above the LOD, and at clearly higher levels than BPA. For the 24 h urine samples, mean values of 98 µg/day DAI, 38 µg/day EQ, 102 µg/day GEN and 9 µg/day BPA (only those > LOD) were calculated for urinary excretion. To the best of our knowledge, this is the first data on urinary isoflavone excretion in a group of Germans consuming their usual food and on BPA levels in these individuals.

Taking into account also the spot urine samples, BPA was detected in 9 of 15 urine samples, ranging from 3 to 55 ng/mL whilst 6 persons excreted BPA below the LOD (<3 ng/mL). As shown in kinetic studies [25], humans excrete more than 95% of BPA with urine, which facilitates estimates of exposure: Based on spot urine measurements (with the high, worst case BPA value) urinary excretion of BPA was on average 32 µg BPA *per* day or about 0.5 µg/kg b.w. for a 60 kg adult, a value far below the current tolerable daily intake (TDI) of 10 µg/kg b.w. set in the European Union [12]. Based on the 24 h urine samples only, a mean BPA value of 9 µg/day or <0.2 µg/kg b.w. for a 60 kg adult is calculated. Calafat *et al.* [50] reported recently similar, low urinary BPA levels in a large North American cohort. Although our method has a higher LOD than the one they used, it is sensitive enough to identify BPA ingestion about two orders of magnitude below the TDI.

In comparison, calculations of dietary isoflavone exposure based on urinary levels have to consider that the excreted amounts account only for a part of the ingested dose: In the case of our volunteer this was approximately 1/5 of the dose within 24 h. Even without further corrections for excreted fraction, our data readily indicate considerably higher dietary exposure to DAI and GEN than to BPA. The isoflavone levels found in urine samples of this sample of omnivorous German adults result from normal Western diet, and it is worth noting that isoflavone intake in Western countries is usually much lower (<1 mg) than in Asian countries (up to 47 mg) [6 – 9] and is reflected in biomonitoring results for isoflavones [38, 42, 45, 51]. As there is special concern regarding exposure to hormonally active agents early in life [1, 13, 52], further studies on urine samples of children are indicated.

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